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ENZYMATIC REGULATION OF GLYCEROL METABOLISM
IN THE OVERWINTERING GALL MOTH
EPIBLEMA SCUDDERIANA

BY

ALEIXO MICHAEL MUISE, B.Sc. (St.F.X.U)

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

MASTER OF SCIENCE

Ottawa-Carleton Chemistry Institute
Carleton University
Ottawa, Ontario

August, 1993

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**"ENZYMATIC REGULATION OF GLYCEROL METABOLISM
IN THE OVERWINTERING GALL MOTH
EPIBLEMA SCUDDERIANA"**

Submitted by Aleixo M. Muise B.Sc.

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

Thesis Supervisor

Chair, Department of Chemistry

Carleton University
1993

ABSTRACT

Larvae of the gall moth *Epiblema scudderiana* are able to survive ambient winter temperatures as low as -40°C . These animals survive prolonged exposures to low temperature by a strategy known as freeze avoidance. The insects maintain a liquid state by depression of freezing and supercooling points, and utilizing glycerol as their sole cryoprotectant. The regulation of four critical enzymes involved in glycerol metabolism are studied.

Glycogen synthetase is found to exist in its less active, phosphorylated form (called the D form) throughout the winter time course and is further inactivated by low temperatures. The enzyme has lower affinities for the substrates, UDPG and glycogen, and the allosteric activator G6P at lower temperature. The inactivation of glycogen synthetase at cold temperatures ensures that carbon is not futilely recycled during glycerol production.

Hexokinase kinetics are also affected at low temperatures. The enzyme displays a stronger affinity for its substrates glucose and ATP. The enzyme is also less sensitive to inhibition by the reaction end products G6P and ADP. G6P inhibits hexokinase mixed competitively with respect to glucose, and competitively with respect to ATP; and ADP inhibits hexokinase non-competitively with respect to both substrates. The low activity of the enzyme and the decreased sensitivity to the inhibitors allow hexokinase to convert glucose (a strong inhibitor of glycogen phosphorylase and glycogenolysis) to G6P thus assisting in the production of glycerol.

Pyruvate kinase and Fructose-1,6-bisphosphatase are both covalently modified with cold overwintering in *Epiblema scudderiana*. Kinetic studies show that the dephosphorylated form of pyruvate kinase and the phosphorylated form of FBPase are the active forms with stronger affinities for substrates and activators, and less sensitivity for inhibitors. In response to cold temperatures and during the winter time

course the enzymes are maintained in their less active form to assist in glycerol production. These effects are reversed with the warmer spring temperatures.

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LIST OF ABBREVIATIONS

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

cAMP - cyclic adenosine monophosphate

DHAP - dihydroxyacetone phosphate

EDTA - ethylenediamine tetraacetate

EGTA - ethyleneglycol bis(B-aminoethyl ether) tetraacetate

FBPase - fructose 1,6-bisphosphatase

F1,6P₂ - fructose 1,6-bisphosphate

F2,6P₂ - fructose 2,6-bisphosphate

F6P - fructose 6-phosphate

GAP - glucose 6-phosphate

G1P - glucose 1-phosphate

G3P - glyceraldehyde 3-phosphate

G6P - glucose 6-phosphate

HMS - hexose monophosphate shunt

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

P_i - phosphate

PEP - phosphoenolpyruvate

PK - pyruvate kinase

PFK - phosphofructokinase

PMSF - phenylmethanesulphonyl fluoride

SDS-PAGE - sodium dodecyl sulphate polyacrylamide gel electrophoresis

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INTRODUCTION

Many animals are capable of surviving extreme environmental conditions and severe physiological stresses. Life exists in habitats that range in temperatures from -50 to 90°C. In order to survive in these harsh conditions, some species have developed strategies such as; freeze tolerance, freeze avoidance, and desiccation to a point where almost no free water is apparent. Biochemical adaptations have allowed animals to survive in virtually any environment on this planet. Animals that avoid freezing have developed the ability to withstand high levels of cryoprotectants, such as 2M glycerol, and shut down vital pathways to preserve energy.

Insects that survive in harsh winter conditions have developed a number of strategies to survive the cold. Freeze tolerant animals control the formation of extracellular ice while rendering cytoplasmic water unfreezable. These animals employ the following mechanisms to ensure intracellular ice does not form, and to control the growth of ice in extracellular spaces: (1) ice-nucleating proteins induce and control the formation of extracellular ice; (2) thermal-hysteresis proteins inhibit the recrystallization of ice particles which over time could form large damaging crystals; (3) reduction of the amount of extracellular ice, extent of cell water loss, and a cell volume decrease; (4) the use of low molecular weight protectants that stabilize the membrane bilayer structure when the cell volume is reduced; (5) and metabolic depression during freezing.

The second survival strategy is freeze avoiding. These animals are able to supercool body fluids to temperatures significantly lower than those of the normal equilibrium melting points. The animals can accomplish this by the following two mechanisms: (1) inhibition of ice nucleation, and (2) depression of the freezing point and supercooling of the body fluids. The first mechanism is accomplished through the addition of antifreeze proteins to extracellular body fluids, the removal of potential ice

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nucleating sites within the body, and the shielding from environmental ice. The second mechanism is accomplished by the addition of low molecular weight cryoprotectants to body fluids and by a decrease in total body water content (Storey & Storey, 1992).

The larvae of the insect *E. scudderiana* (Clemens) [Diptera, Tephritidea] were chosen to study carbon regulation because of their unique ability to produce high levels of glycerol as a cryoprotectant and to supercool. The insects utilize the cryoprotectant glycerol (at levels up to 2M, 18.7% of body weight) to lower their supercooling point to -38°C (Rickards et al, 1987). Although various aspects of cryoprotectant synthesis and enzymatic control have been studied by numerous authors, there are still key elements needed to completely understand how the animal survives such extreme environmental conditions. The life history of the insect has been described in detail by Miller (1976).

More specifically for *E. scudderiana*, the first mechanism for survival of freeze avoiding animals is achieved through: the use of thermal hysteresis proteins; the emptying of the gut to eliminate non-specific potential ice nucleators from the body; and the shielding of the larvae from environmental ice through the formation of a gall in which the larvae overwinters, and the formation of a silken cocoon that protects the larvae inside the gall. The second mechanism is accomplished through the use of the low molecular weight cryoprotectant glycerol, and the partial dehydration of water (Rickards *et al*, 1987). The enzyme regulation of the production and catabolism of glycerol is explored in this thesis.

Glycerol as a Cryoprotectant

The larvae of *E. scudderiana* use glycerol as their sole cryoprotectant. Not only does glycerol facilitate a decrease of the supercooling point to -38°C from -12°C without glycerol (Rickards et al, 1987), but it is highly soluble in water, is non-toxic

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with few effects on metabolism, and is able to penetrate the cellular membranes easily thus enabling it to surround and protect cellular organelles from damage due to quick decreases in temperatures (Storey & Storey, 1988). Figure 1.1 shows the accumulation of glycerol and the depletion of glycogen over the winter time course (Rickards et al, 1987). This figure illustrates the accumulation of glycerol in the early autumn months, and the rapid conversion of glycogen to glycerol with the colder winter months.

The four enzymes studied in this thesis, glycogen synthetase, hexokinase, fructose-6-bisphosphatase, and pyruvate kinase, all play a critical role in the overwintering survival of the larvae of *E. scudderiana*. As the insect prepares for freezing it must accumulate large quantities of glycerol that can be converted back to glycogen with the return of warm temperatures. The aforementioned enzymes are located at critical points in the metabolic pathway of glycerol synthesis and catabolism, and as such elucidation of their regulation may lead to further understanding of the complex mechanism of survival of these insects.

Enzyme Regulation

One of the keys to survival for any species is the ability to control metabolic pathways through enzyme adaptation. Enzymes are capable of regulating metabolism, for example channeling sugars for the production of cyroprotectants, and reversing these controls when the stresses are alleviated. Enzymes can be controlled by a number of mechanisms. Allosteric modifiers, including activators and inhibitors, can modify enzyme function by binding to the enzyme sites in an area other than the active site, thus changing the enzyme conformation and its relative effectiveness in catalyzing its reaction. Some allosteric inhibitors also compete with the substrate for binding at the active site of the enzyme. Allosteric modification can vary at different temperatures.

FIGURE 1.1 OVERWINTERING PROFILE OF GLYCEROL AND GLYCOGEN CONTENTS IN *E. SCUDDERIANA*.

The diagram shows the levels of glycerol and glycogen compared to the average ambient temperatures from September to April in *E. scudderiana* (Rickards et al, 1987).

Conformational changes in the protein caused by a change in temperature may alter the affinities of substrates and may affect the efficiency of activators and inhibitors (Storey, 1992).

Posttranslational modification of the enzyme through reversible protein phosphorylation/dephosphorylation is another strategy used to regulate enzymes. This type of modification typically affects enzymes at critical points in a metabolic pathway including entry into major pathways, important branch points, and control points in bidirectional pathways. Reversible phosphorylation can provide a virtual on-off regulation of pathway flux. Protein kinases and phosphatases also may have many different enzyme proteins as substrates, hence reversible protein phosphorylation may coordinate control over multiple enzymes and/or pathways to allow for an integrated response to metabolic needs. Phosphorylation/dephosphorylation of the enzyme may produce a conformational change in the enzyme that allows one form of the enzyme to be more/less active than the other. The two forms of the enzyme may have very different kinetic properties that allow for control under various stresses (Storey, 1992).

The present study looks at four enzymes involved in regulating carbon flow in the larvae of the supercooling insect *Epiblema scudderiana*. The enzymes, glycogen synthetase, hexokinase, fructose-1,6-bisphosphatase (FBPase), and pyruvate kinase, all play key roles in the winter survival of the species. The enzymes hexokinase and pyruvate kinase are involved in glycolysis; whereas, glycogen synthetase and FBPase are involved in glycogenesis and gluconeogenesis respectively. These pathways, their enzymatic control, and the characteristics of the supercooling larvae of *E. scudderiana* that make it an ideal model to investigate enzymatic control will be explored further.

Glycerol Production and Catabolism

Figure 1.2 shows the metabolic pathway of glycerol production and catabolism in cold hardy insects. The flow of carbon for the production of glycerol begins at glycogen. The conversion of glycogen to glucose-1-phosphate (G1P) is termed glycogenolysis, with the reverse being termed glycogenesis. Carbon is also directed into the pathway by the conversion of glucose to glucose-1-phosphate (G6P). This reaction, along with the accompanying 9 reactions, is traditionally known as glycolysis with the reverse being known as gluconeogenesis. In this thesis the breakdown/ synthesis of glycogen will be termed glycogenolysis/ glycogenesis. The flow of carbon from other sources or the interaction of intermediates further down the pathway will be termed glycolysis/gluconeogenesis.

GLYCOGEN SYNTHETASE/GLYCOGEN PHOSPHORYLASE: Glycogen phosphorylase catalyzes the rate limiting step in glycogen degradation (Werve & Jeanrenaud, 1987). In insects, a primary response to low temperatures is the activation of glycogen phosphorylase in fat body to initiate the conversion of glycogen to glycerol. Churchill & Storey (1989b) have shown that in the larvae of *E. scudderiana* the percentage of phosphorylase in the active α phosphorylated form rapidly increases in response to cold temperatures. Figure 1.3 shows the control of glycogen metabolism highlighting the control of glycogen synthetase and phosphorylase (Storey and Storey, 1992). The control of glycogen synthetase and phosphorylase is associated with the effects of low temperature on phosphorylase kinase and phosphorylase phosphatases. Over the range of 30 to 0°C phosphorylase kinase undergoes a normal temperature reduction in activity; however, the phosphatase activity is greatly reduced at low temperatures.

FIGURE 1.2 METABOLIC PATHWAY FOR GLYCEROL IN COLD HARDY INSECTS

This figure illustrates the enzymes involved in the conversion of glycogen to glycerol and pyruvate. The enzymes of importance to this thesis are: (1) glycogen phosphorylase, (2) glycogen synthetase, (3) hexokinase, (4) phosphofructokinase, (5) fructose-1,6-bisphosphatase, and (6) pyruvate kinase.

FIGURE 1.3 CONTROL OF GLYCOGEN METABOLISM

The figure illustrates the control of glycogen metabolism by the reciprocal regulation of the enzymes glycogen phosphorylase and glycogen synthetase (Storey & Storey 1992).

The upper portion shows the activation and inactivation of the enzymes glycogen phosphorylase and glycogen synthetase. The lower portion shows the regulation of phosphorylase and synthetase in the following situations: low temperature activation of polyol synthesis, inhibition of polyol synthesis when complete at low temperatures, and reconversion of glycerol to glycogen at warm temperatures.

The net result at low temperatures is that the kinase activity greatly exceeds the phosphatase. Hence, at low temperatures phosphorylase is rapidly converted from the *b* to the *a* form with glycogenolysis being activated. The phosphorylase *a* form inhibits glycogen synthetase therefore ensuring carbon is not recycled. At higher temperatures the activities of phosphorylase kinase and phosphatases readjusts leading to an activation of synthetase and inactivation of phosphorylase. This leads to the reconversion of cryoprotectants to glycogen (Storey & Storey, 1992).

Glycogen synthetase (Figure 1.2 enzyme #2) catalyzes the rate limiting step in glycogen synthesis. In contrast to glycogen phosphorylase, synthetase is more active in the dephosphorylated I form. The phosphorylated form, glycogen synthetase D, is more dependent on the activator glucose-6-phosphate (G6P) than the dephosphorylated form (Srivastava et al, 1980). As shown in Figure 1.3, phosphorylase kinase also phosphorylates glycogen synthetase to its less active form. Thus at lower temperatures, glycogen synthetase will be predominantly in its less active form while glycogen phosphorylase will be activated. Glucose is also known to be a strong activator of glycogen synthetase, as well as an important inhibitor of glycogen phosphorylase. Glycogen synthetase will be further investigated in Chapter 2.

HEXOKINASE: Glycolysis is a ten step reaction sequence that converts glucose to pyruvate with the production of adenosine triphosphate (ATP). Hexokinase (Figure 1.2, enzyme #3) catalyzes the conversion of glucose to G6P and is key in regulating the amount of glucose that is channeled through glycolysis. Hexokinase is feed-back inhibited by its end product G6P (van Holde, 1990). Maximal levels of hexokinase must be present when polyol synthesis takes place in the fat bodies of insects in order to ensure that high levels of glucose and other intermediates are not accumulated. High levels of glucose and intermediates may facilitate the inhibition of glycogenolysis and thereby interfere with the production of the cryoprotectant. Hexokinase must be able to

assist in the accumulation of 2M glycerol to prepare *E. scudderiana* for overwintering. Hexokinase will be looked at in detail in Chapter 3.

FRUCTOSE 1,6-BISPHOSPHATASE/PHOSPHOFRUCTOKINASE: Fructose

1,6-bisphosphatase-1 (Figure 1.2, enzyme #5) (FBPase-1) and phosphofructokinase-1 (Figure 1.2, enzyme #4) (PFK-1) catalyze the rate limiting steps in gluconeogenesis and glycolysis, respectively (Rawn 1989). Figure 1.4 illustrates the relationship between both enzymes. PFK-1 catalyzes the committing step of sugar phosphates derived from glycogen or glucose into the triose phosphate portion of the glycolysis. The insects that utilize glycerol as a cryoprotectant need to regulate PFK-1 in the cold. Insects that require trehalose as a cryoprotectant need to inhibit PFK-1 (Churchill & Storey, 1989). Insects that utilize two polyols as cryoprotectants show a strong temperature dependent control of PFK-1 that readily allows for glycerol production at temperatures between 10-15°C but at lower temperatures diverts carbon flux into the production of sorbitol (Storey & Storey, 1988).

As Figure 1.4 a) illustrates, both PFK-1 and FBPase-1 are allosterically regulated by the effector fructose 2,6-bisphosphate (F2,6P₂). F2,6P₂ is synthesized by PFK-2 (the 2 indicates that the enzyme catalyzes phosphoryl-group transfer from ATP to the C-2 hydroxyl group of F6P). This reaction is reversed by the enzyme fructose 2,6-bisphosphatase (FBPase-2). The dephosphorylated form of PFK-2 catalyzes the conversion of fructose-6-phosphate (F6P) to F2,6P₂. F2,6P₂, in turn, activates PFK-1 which catalyzes the glycolytic conversion of F6P to fructose 1,6-bisphosphate (F1,6P₂). In reverse, the phosphorylation of PFK-2 inhibits the formation of F2,6P₂ thereby activating Fructose 2,6-bisphosphatase. This causes the inhibition of PFK-1 and the activation of FBPase-1 which in turn inhibits the conversion of F6P to F1,6P₂.

FIGURE 1.4 REGULATION OF PHOSPHOFRUCTOKINASE AND FRUCTOSE-1,6-BISPHOSPHATASE

Figure 1.4 A) shows the interaction of PFK-1 and FBPase-1 in mammals (Rawn, 1989). The diagram illustrates the activation and inactivation of PFK-2 and FBPase-2 by phosphorylation and dephosphorylation. PFK-2 and FBPase-2 regulate F2,6P₂ which is a critical regulator of PFK-1 and FBPase-1.

Figure 1.4 B) shows the interaction of PFK-1 and FBPase-1 in the larvae of *E. scudderiana* (Storey & Storey, 1992). The diagram illustrates the regulation of PFK-2 by G3P and reversible protein phosphorylation.

This model has been developed to include the interactions of the cryoprotectant glycerol in *E. scudderiana* (Figure 1.4b) (Storey & Storey, 1992). After two hours of cold exposure of the larvae to -4°C there is a 4.5 fold increase in G6P and a 60% increase in F1,6P₂ (Figure 1.4). This indicates an activation of the phosphorylase and PFK-1 that synthesize these compounds. There is also a 50% decrease in glycerol-3-phosphate (G3P), indicating the activation of glycerol-3-phosphatase. The activation of PFK-1 is predominantly due to the increase in F2,6P₂ which increased two fold in the first few hours of cold exposure and increased 40 fold after several days when compared to the levels in the 16°C control levels (Figure 1.5). The authors point out a number of important results of the rapid increase in F2,6P₂. The activation of PFK-1 allows for facilitation of the production of triose phosphates and, F2,6P₂ also inhibits FBPase-1 thus preventing the useless reconversion of triose phosphates to F6P. Overall, the high F2,6P₂ levels ensure the one-way flux of glycerol production. PFK-2 synthesizes F2,6P₂ and is allosterically inhibited by G3P and reversible phosphorylation. As Figure 1.5 indicates there is a sharp drop in the levels of G3P in the larvae of *E. scudderiana* during the first two hours of cold exposure. This decrease in G3P, which is one of the first responses to -4°C exposure, eliminates any inhibition of PFK-2 by G3P, thereby, produces F2,6P₂, activates PFK-1, and inhibits FBPase-1. These regulations ensure the constant flow of carbon for the production of glycerol.

PYRUVATE KINASE: The final enzyme, pyruvate kinase (6) (PK), is one of the key regulatory enzymes in glycolysis as it controls carbon flux into the citric acid cycle. The enzyme catalyzes the phosphoryl-group transfer from phospho- enolpyruvate (PEP) to adenosine diphosphate (ADP) to produce pyruvate and ATP. Reversible phosphorylation of PK is known to occur in the various forms of PK (Whitwam &

**FIGURE 1.5 LEVELS OF METABOLITES IN ASSOCIATION WITH
GLYCEROL SYNTHESIS DURING LOW TEMPERATURE ACCLIMATION
OF *E. SCUDDERIANA***

The figure shows the changes in metabolites during glycerol production when the insect, *E. scudderiana*, was acclimated to -4°C (Churchill & Storey, 1967). The time points were 0, 2, 6, 12 and 18h and 2, 4, 6, 10, and 14d.

Storey, 1990). The more active form of PK is found to be the dephosphorylated form, while the phosphorylated form the less active. PK can be controlled in an on-off manner with the need for glycolytic versus gluconeogenic carbon needs. PK is also allosterically regulated by a number of inhibitors and activator and will be explored further in Chapter 5.

CHAPTER TWO
GLYCOGEN SYNTHETASE
FROM THE LARVAE OF THE FREEZE AVOIDING GALL MOTH
EPIBLEMA SCUDDERIANA

INTRODUCTION TO GLYCOGEN SYNTHETASE

Glycogen synthetase (uridine diphosphate glucose: 1,4-glucosyl transferase, EC 2.4.1.11) catalyzes the rate limiting step in glycogen synthesis (Eichner & Arnold, 1979). Glycogen synthetase adds glucose residues to the nonreducing ends of glycogen polymers. The enzyme is known to exist in at least two interconvertible forms. The two forms differ in their activation by G6P and content of covalently bound phosphate. The I form (independent of G6P) can be phosphorylated by protein kinases and converted into the D form (dependent on G6P). Protein phosphatases can readily convert the D form back to the I form (Castineiras et al, 1978).

Glycogen synthetase is believed to be phosphorylated at multiple sites with the phosphorylation generally leading to a decreased activity. Casein kinase I and other protein kinases work in concert to inactivate the enzyme. Phosphorylation, at sites on rabbit muscle glycogen synthetase, by cAMP-dependent protein kinase leads to inactivation of the enzyme; whereas phosphorylation by the same protein kinase does not result in any inactivation in the liver. However, introducing casein kinase I after the cAMP-dependent protein kinase allows for total inactivation of the enzyme. Secondary phosphorylation by glycogen synthetase kinase-3 also permits full inactivation of the enzyme (Roach, 1990).

Studies on adipose tissue from diabetic rats have shown that glycogen synthetase, in the presence of high levels of glucose, is in a highly phosphorylated less active state. The authors illustrate that the conversion of glycogen synthetase to the more active I (dephosphorylated) form by phosphatases becomes increasingly difficult with the amount of phosphorylation of glycogen synthetase and the presence of glucose (Woodford et al, 1992). These results may be of importance as the larvae of *E. scudderiana* are exposed to high levels of glucose during glycogen production (Zachariassen et al, 1985).

Glucose and other monosaccharides activate glycogen synthetase in the liver.

The method of activation is thought to be a sequential mechanism whereby glucose binds to glycogen phosphorylase rendering it a better substrate for phosphorylase phosphatase. The glucose-phosphorylase complex is thus dephosphorylated and inactivated. This inactivation of glycogen phosphorylase releases the inhibitory effects on glycogen synthetase phosphatases, thereby allowing glycogen synthetase to be dephosphorylated and activated.

Recently, studies of rat hepatocytes have indicated that G6P rather than glucose could initially activate glycogen synthetase (Carabaza et al, 1992). The authors have shown that glucose facilitates the inactivation of glycogen phosphorylase but does not necessarily activate glycogen synthetase. The authors believe that levels of G6P may trigger the activation of synthetase.

Furthermore, studies of the oyster mantle of *M. edulis* have shown that the D form of glycogen synthetase may be the predominant form of the enzyme in the fall and winter months of some species (Gabbot & Whittle, 1986; Swift et al, 1988). Cook and Gabbot (1978) have also shown that the percentage of I vs D form of glycogen synthetase from oyster adductor muscle is extremely low in the fall, winter, and spring months when glycogen is broken down for energy; but, the percentage increases dramatically in the summer as glycogen is being actively stored.

This study is performed in order to discover the role of glycogen synthetase in the regulation of winter carbohydrate metabolism. Holden and Storey (in press d) have shown that glycogen phosphorylase α is activated at low temperatures to assist in the production of glycerol. It can be predicted that glycogen synthetase would be inactive at low temperatures, thereby ensuring that substrate recycling would not take place when glycogen phosphorylase α is activated.

A study of kinetic parameters at different temperatures will be examined to determine the role of glycogen synthetase in regulation of winter carbohydrate metabolism.

MATERIALS AND METHODS

Animals and Chemicals

Galls containing the larvae of the moth *Epiblema scudderiana* were gathered throughout the Ottawa valley region in the fall of 1989. Animals were left in their galls and stored outdoors where they experienced the natural winter temperatures. At various time points throughout 1989-90 (Sept. 10, Sept. 15, Nov. 1, Jan. 4, March 3, March 31, April 12, and April 21, see Table 2.1), groups of galls were brought into the lab and placed in incubators set at a temperature synchronized with the mean outdoor temperature. The larvae were removed from the galls, quickly frozen in liquid nitrogen, and stored at -80°C until further use. In October of 1989, other groups of galls were acclimated to constant temperatures in incubators set to -4° and 15°C for three weeks. The -4°C temperature was chosen to stimulate rapid glycerol synthesis while at 15°C glycerol levels should remain low as glycerol was not being produced. The larvae acclimated in the laboratory to 15°C were used in studies of glycogen synthetase and hexokinase; the larvae from the seasonal time course and laboratory acclimated larvae were used in studies of pyruvate kinase and fructose,6-bisphosphatase.

Chemicals for all studies were purchased from Boehringer-Mannheim Corp. Montreal, P.Q. or Sigma Chemical Company St. Louis, MO. unless otherwise stated. Chemicals were of the highest quality and used without further purification unless otherwise stated.

TABLE 2.1 AVERAGE OTTAWA MONTHLY TEMPERATURES

COLLECTION DATE	PRECEDING MONTH (°C ± SD)		PRECEDING WEEK (°C ± SD)	
	MAX	MIN	MAX	MIN
SEPT.15	23.5 ± 3.1	13.1 ± 3.9	22.7 ± 4.6	14 ± 4.1
NOV.1	14.4 ± 6.2	4.1 ± 3.4	21.1 ± 0.6	6.0 ± 3.1
JAN.4	-10.8 ± 6.2	-20.0 ± 6.3	-6.0 ± 9.2	-17.4 ± 9.3
MAR.3	-1.9 ± 6.2	-11.6 ± 6.5	-4.4 ± 6.7	-14.3 ± 7.1
MAR.19	-0.1 ± 7.4	-9.7 ± 8.5	7.4 ± 2.4	1.4 ± 1.2
MAR.31	2.2 ± 6.1	-6.4 ± 7.1	0.3 ± 3.1	-8.0 ± 1.2
APR.12	4.8 ± 3.7	-1.9 ± 4.0	5.2 ± 2.3	-1.9 ± 2.3
APR.21	5.3 ± 4.3	-2.3 ± 4.2	9.5 ± 3.3	0.6 ± 3.6
APR.23	9.9 ± 8.2	0.7 ± 5.3	22.9 ± 6.0	7.6 ± 4.1

AVERAGE OTTAWA MONTHLY (30-31 DAY PERIOD) AND WEEKLY (7 DAY PERIOD) TEMPERATURES IMMEDIATELY PRECEDING *E. scudderiana* DATA COLLECTION DATES. NUMBERS WERE CALCULATED FROM WEATHER SUMMARIES OBTAINED FROM THE LAND RESOURCES CENTER (AGRICULTURE CANADA).

Purification and Preparation of Homogenates

Samples of 15°C acclimated frozen larvae (0.07 g = 1 larva) were quickly weighed and immediately homogenized 1:10 w/v in ice cold buffer A: 15 mM imidazole-HCl, 15 mM 2-mercaptoethanol, 50 mM NaF, 5 mM EGTA, 5 mM EDTA, and 20% (v/v) glycerol, pH 6.5. This buffer was designed to inhibit both protein kinases and protein phosphatases. A few crystals of phenylmethylsulfonyl fluoride (PMSF) were added immediately before homogenization of the sample with a Polytron PT10 homogenizer. After homogenization the extracts were allowed to settle, with the supernatant being removed from the pellet and fat layer, and stored on ice.

The G6P dependent glycogen synthetase (D) was assayed for all purifications and kinetics.

Glycogen synthetase was purified by utilizing ionic and affinity chromatography columns in sequence. The supernatant was layered onto a DEAE⁺ Sephadex column (7 cm x 1.5 cm) equilibrated in buffer A. The column was washed twice with an equivalent volume of buffer A. The enzyme was eluted with a linear gradient of 0-1 M KCl (20 ml) in buffer A and 1 ml fractions were collected with a Gilson fraction collector. The fractions were assayed for enzyme activity with the 5 most active fractions being pooled and used in the next purification step.

The pooled fractions were layered onto a Blue Dextran column (7 cm x 1.5 cm) which was equilibrated in buffer B (buffer A plus 150 mM KCl, pH 7.5). The column was washed with two 10 ml volumes of buffer A. The enzyme was eluted with a volume of buffer B equal to the original volume of enzyme preparation placed on the column. The new eluant was assayed for activity. At this point the filtrate was separated for two distinct purification steps.

One aliquot of filtrate from the Blue Dextran column was placed on a Matrix gel Red A column (crosslinked 5% agarose with covalently coupled procion) (7 cm x

1.5 cm) equilibrated in buffer A, pH 7. The enzyme was eluted with a linear salt gradient of 0-500 mM KCl in buffer A. The fractions were collected and assayed.

Alternatively, a Concanavalin A Sepharose column (7 cm x 1.5 cm) (Kaslow & Lesikar, 1984) was equilibrated in Buffer C: 15 mM imidazole, 15 mM 2-Mercaptoethanol, and 50 mM NaF. Eluant from the Blue Dextran column was desalted by centrifugation (1 min at 13,000 g) through a G-25 Sephadex column in buffer C. The filtrate was placed on the Con A column, washed with buffer C, and eluted with 200 mM glucose in buffer C. The eluant was collected in fractions and assayed.

Protein Determination

Protein concentration was measured using the Coomassie blue dye-binding method and Bio-Rad Laboratories prepared reagent with a standard of bovine gamma globulin (Bradford, 1976).

SDS Polyacrylamide Gel Electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1977) was performed using a 10 % w/v polyacrylamide separating gel (16 x 18 cm x 2 mm) and 5 % w/v stacking gel. Protein samples were mixed 1:1 v/v with sample loading buffer containing 100 mM Tris-HCl pH 6.8, 4% w/v SDS, 0.2% w/v bromophenol blue, and 20% v/v glycerol. The samples were heated to denature the proteins and 2-mercaptoethanol was added before loading samples onto the gel. The samples were run parallel to prestained SDS-PAGE standards with the following molecular weights: β -galactosidase (116,000), fructose-6-phosphate kinase (96,000), pyruvate kinase (80,400), ovalbumin (55,700), lactate dehydrogenase (43,700), and

triosephosphate isomerase (38,800). The gel was run at 25 mA in Laemmli buffer (25 mM Tris, 250 mM glycine, and 0.1% w/v SDS) for 8 h at 22°C, pH 7. It was then fixed overnight in 10% v/v acetic acid and 30% v/v methanol and then stained for 2 h in 0.25% w/v Coomassie brilliant blue R in the above acetic acid/methanol solution. The gel was destained in 10% v/v acetic acid and 35% v/v methanol solution for 6h.

Enzyme Kinetics and Assay

Glycogen synthetase activity was measured by a coupled enzyme assay that measured UDP release and resulted in the conversion of NADH to NAD⁺ which was monitored at 340 nm with a Gilford 260 spectrophotometer and recorded with a Kipp and Zonen BD-111 chart recorder. Low temperature kinetics and Arrhenius plots were obtained by cooling the spectrophotometer cell block with an attached Brinkmann Lauda K-2/R water bath.

Scheme 1. GLYCOGEN SYNTHETASE ANALYSIS COUPLED TO PYRUVATE KINASE AND LACTATE DEHYDROGENASE

Optimal assay conditions, pH 7.2, for G6P dependent glycogen synthetase D were found to be 1 mM PEP, 10 mM Glucose-6-phosphate (G6P), 2 mM uridine diphosphoglucose (UDPG), 2 mg/ml glycogen, 0.15 mM NADH, 5 mM MgCl₂, 50

mM KCl, with 1 unit of each of the coupling enzymes pyruvate kinase (PK) and lactate dehydrogenase (LDH) in 50 mM imidazole-HCl buffer. To measure the G6P independent form of the enzyme (glycogen synthetase I) the above optimal conditions were applied with the absence of G6P. One unit of glycogen synthetase activity was defined as the amount of enzyme that produces 1 μ mol of UDP per min at 22°C. The specific activity of the enzyme was described as the activity in Units per mg protein.

The kinetic parameters used were defined as follows: K_m , (the Michaelis-Menten substrate affinity constant) the substrate concentration that gives half maximal reaction velocity; K_a , the activator concentration which produces 50% of maximal activation.

The values for K_m and K_a were determined by a computer program which fitted data through a nonlinear least squares regression. A modified Hill equation, which introduces a V_o term (rate at zero activator concentration), was used to determine K_a values (Brooks, 1992). All kinetic values were determined at varied substrate concentrations. Results were presented as means $\pm$ S.E.M. with at least $n=3$ of different enzyme preparations. Statistical differences were determined through the Student's t-test (two-tailed) and reported as $P < 0.005$ or $P < 0.001$.

RESULTS

Purification

Glycogen synthetase D was studied in *E. scudderiana*. Figures 2.1 and 2.2 show the elution profiles of glycogen synthetase from DEAE⁺ Sephadex and Matrix gel Red A, chromatography columns respectively. The DEAE⁺ column retained 45% of glycogen synthetase and eliminated 58 % of the total protein. Figure 2.1 shows that the enzyme was eluted from the DEAE⁺ column at 200-250 mM KCl. Next, the enzyme was applied to a Blue Dextran column and eluted in buffer A containing 150 mM KCl. This step removed much of the remaining proteins while giving a 3 fold purification. The final purification step, Matrix Gel Red A column, gave a final specific activity of 16.1 U/mg when the enzyme was assayed at 22°C at the suboptimal pH of 7.5. The final steps gave a 130 fold purification with a 20% yield.

Alternatively, Concanavalin A affinity chromatography was used instead of Matrix Gel Red A as a final step in purification. However, this proved to be a poor step as the fold purification was only 6-fold.

The purification scheme is summarized in Table 2.2. The Matrix Gel Red A purification step was used for all kinetic and enzyme studies.

SDS PAGE

Both purification strategies showed a double band on a 10% SDS-polyacrylamide gel (Figure 2.3). The apparent sub-unit molecular weight was determined by comparison with the migration of known molecular weight markers (Figure 2.4).

TABLE 2.2 PURIFICATION TABLE OF GLYCOGEN SYNTHETASE D FROM *E. scudderiana*

STEP	TOTAL ACTIVITY UNITS	TOTAL PROTEIN mg PROTEIN	YIELD %	FOLD PURIFICATION	SPECIFIC ACTIVITY U/mg
CRUDE SUPERNATANT	2.25	18.7	-	-	0.123
DEAE SEPHADEX	1.12	7.60	50	1.1	0.147
BLUE DEXTRAN	0.481	1.26	21	3.1	0.381
MATRIX GEL RED A	0.450	0.028	20	130.9	16.1

**Figure 2.1 ELUTION PROFILE OF GLYCOGEN SYNTHETASE
D ON A DEAE⁺ SEPHADEX COLUMN**

The enzyme, from the 15⁰C acclimated larvae, was eluted with a linear gradient of 0-1 M KCl. One ml fractions were collected and 100 ul of each fraction was assayed. Activities are given relative to the enzyme velocity in the peak tube.

**Figure 2.2 ELUTION PROFILE OF GLYCOGEN SYNTHETASE
D ON A MATRIX GEL RED A COLUMN**

The enzyme, from 15°C acclimated larvae and purified on DEAE Sephadex and Blue Dextran chromatography columns, was eluted with a linear gradient of 0-300 mM KCl. One ml fractions were collected and 100 ul of each fraction was assayed. Activities are given relative to the enzyme velocity in the peak tube.

Figure 2.3 STANDARD CURVE OF LOG MOLECULAR WEIGHT VS RELATIVE MOBILITY OF PROTEIN STANDARDS RUN ON SDS-PAGE GEL

The log of the following molecular weight markers were plotted against the relative mobility to determine the molecular weight of glycogen synthetase. The molecular weights were: 1) galactosidase (116,000), 2) fructose-6-phosphate kinase (96,000), 3) pyruvate kinase (80,400), 4) ovalbumin (55,700), 5) lactic dehydrogenase (43,700), and 6) triosephosphate isomerase (38,800). The black circle shows the subunit molecular weight for glycogen synthetase D.

Figure 2.4 SDS-PAGE GEL OF THE TWO PURIFICATION SCHEMES FOR GLYCOGEN SYNTHETASE D

The SDS-PAGE gel shows the complete purification of glycogen synthetase D along with the location of standards. Lane 2 is the crude homogenate, Lane 1 purification after the DEAE⁺ Sephadex, Lane 3 purification after Blue Dextran, Lane 4 purification after Concavalin Red A column, and Lane 5 is the purification after Matrix Gel Red column. The furthest right column shows the standards. The arrow shows the apparent subunit molecular weight of glycogen synthetase D.

The subunit molecular weight of glycogen synthetase was determined to be 84,000 daltons.

pH Profile

Figure 2.5 shows that the enzyme exhibited optimal activity at pH 8.0 at 22^o C, and 7.8 at 5^oC. Therefore, it appears the pH optimum remains constant with temperature.

Arrhenius Plot

The effects of temperatures on enzyme activity, illustrated by an Arrhenius plot (Figure 2.6.), shows a break at 12^o C. The activation energy (E_a) of the lower temperature (3-12^oC) portion was 102.3 ± 6.1 kJ/mol and the upper portion (15-30^o C) E_a being 36.9 ± 2.9 kJ/mol. This break may indicate a conformational change at lower temperatures that may effect the activity of the enzyme. During cold temperatures the animal accumulates large amounts of glycerol. To determine if the glycerol at high concentrations protected the enzyme at all experimental temperatures, an Arrhenius plot was repeated in the presence of 20 % v/v glycerol. The plot showed the enzyme was protected by 20 % v/v glycerol with no breaks and a E_a of 47.9 ± 3.3 kJ/mol.

Kinetic Analysis

Kinetic constants were determined at assay temperatures of 22 and 4^oC with the results summarized in Table 2.3. All enzyme kinetics were performed on the G6P dependent glycogen synthetase D form purified by the following chromatography

Figure 2.5 pH PROFILE FOR GLYCOGEN SYNTHETASE D AT 5 AND 22⁰ C

A pH profile for glycogen synthetase was determined at 5 and 22⁰C with optimal assay conditions. The profile shows the enzyme to have a optimal pH of 8 at 22⁰C and a pH optimal of 7.8 at 4⁰C. All values are reported as means $\pm$ S.E.M. Where S.E.M. value are not visible in the form an of error bar they are contained within the dimensions of the symbols.

**Figure 2.6 ARRHENIUS PLOT FOR GLYCOGEN SYNTHETASE
D**

An Arrhenius plot was obtained to determine if a conformational change in the enzyme occurred as a result of temperature change. All measurements were taken at optimal assay conditions. Glycogen synthetase activity was measured from 3-32°C. In the presence of added 20% v/v glycerol the plot showed no break (E_a of 47.9 ± 3.5 kJ/mol). Without glycerol there was a break at 15°C with a E_a changing from 36.9 ± 2.9 kJ/mol (upper portion 15-30°C) to 102.3 ± 6.1 kJ/mol (lower portion 3-12°C). All values are reported as means $\pm$ S.E.M., $n=3$. Where S.E.M. values are not visible in the form of an error bar they are contained within the dimensions of the symbol.

TABLE 2.3 KINETIC PROPERTIES OF GLYCOGEN SYNTHETASE D
PURIFIED FROM *E. scudderiana*

	22°C	4°C
Km UDPG (mM)	0.048 ± 0.004	0.088 ± 0.003 *
Km Mg ²⁺ (mM)	2.31 ± 0.33	2.54 ± 0.25
Km GLYCOGEN (mg/ml)	1.17 ± 0.35	5.16 ± 0.16 **
Ka G6P (mM)	2.10 ± 0.16	6.16 ± 0.15 **

DATA ARE MEANS ± S.E.M. FOR ALL WITH AT LEAST n = 3

DIFFERENT PREPARATIONS OF THE PURIFIED ENZYME. *

SIGNIFICANTLY DIFFERENT FROM THE CORRESPONDING VALUES AT
22°C BY THE STUDENT'S t-TEST (TWO-TAILED), P < 0.005;

** P < 0.001.

columns in sequence: DEAE⁺ Sephadex, Blue Dextran, and Matrix Gel Red A. The data showed that the enzyme had a higher affinity for both glycogen and UDPG at high temperatures, with K_m values almost five and two fold lower at 22°C, respectively. Both [UDPG] and [glycogen] were plotted against relative velocity in Figure 2.7. The K_a values for glucose-6-phosphate were determined at optimal glycogen levels (5 mg/ml) and suboptimal UDPG levels (0.4 mM). The K_a for G6P at 4°C was almost three fold greater than at 22°C. The [G6P] is plotted against relative velocity in Figure 2.8. K_m values for the cation Mg^{2+} were not significantly different between 22°C and 4°C.

**Figure 2.7 PLOTS OF VELOCITY VS SUBSTRATE
CONCENTRATION FOR GLYCOGEN SYNTHETASE D**

The representative plots illustrate the change in enzyme activity when the substrate concentrations (UDPG and Glycogen, respectively) were varied at 4 and 22°C. Hollow circles give glycogen synthetase activity at 22°C and filled circles give activity at 4°C. The velocity was relative and determined with purified enzyme.

Figure 2.8 G6P ACTIVATION OF GLYCOGEN SYNTHETASE D

The representative plot illustrates the change of activity when the optimal assay conditions remain the same (except 0.4 mM UDPG) and the concentration of G6P was varied at 4 and 22°C. Hollow circles give glycogen synthetase activity at 22°C and filled circles give activity at 4°C. The velocity was relative and determined with purified enzyme.

DISCUSSION OF GLYCOGEN SYNTHETASE

Larvae from the insect *Epiblema scudderiana* have developed strategies to survive cold winter temperatures through the production of high concentrations of the cryoprotectant glycerol. Regulation of glycogen synthetase could prevent the futile recycling of carbon derived from glycerol to glycogen. The insect appears to have regulated this locus of control through phosphorylation of the enzyme and by a conformational change in the protein which facilitates a kinetically less active form at lower temperatures.

Glycogen Synthetase D

Studies of other invertebrates have shown that the less active phosphorylated D form of glycogen synthetase predominates during low seasonal temperatures (Swift et al, 1987; Cook & Gabbot, 1978). The only measurable form of glycogen synthetase found in the larvae of *E. scudderiana* throughout the seasonal time course is the less active D form. This result should be expected as throughout the fall and winter months the larvae experiences low minimum mean temperatures ranging from 4°C in September to -2°C in March (see table 2.1). These temperatures correlate to the nutritional needs of the insect at the time. During the fall and winter months, the insect is finished storing glycogen and is prepared for overwintering. Thus, carbon would only be used for the production of the cryoprotectant glycerol or for the everyday metabolic needs of the insect. Therefore, the need for the larvae to convert G1P to glycogen or maintain a supply of the active form of glycogen synthetase would be eliminated. Figure 1.1 shows that once glycogen stores drop to minimum levels it remains constant for the entire time course. There is no evidence of the reconversion of G1P to glycogen and therefore little need for a more active form of glycogen synthetase

during the late autumn and winter months. The presence of the more active I form, during the time course, could cause the futile recycling of glycogen. The lack of the enzyme in the more active form does not exclude the possibility that glycogen synthetase exists in the I form during the summer months, when glycogen is being actively stored.

Purification

Glycogen synthetase D from *E. scudderiana* is purified 131-fold with a final specific activity of 16.1 U/mg. This specific activity corresponds to the specific activities of glycogen synthetase from human liver 40 U/mg, rat liver 20 U/mg, and whelk mantle 7-30 U/mg (Westphal & Nuttall, 1992; Swift et al, 1988).

Glycogen synthetase is purified with three chromatography columns in sequence. The first column, a DEAE⁺ ion exchange column, removes 60% of the total protein. As figure 2.1 illustrates, the enzyme is eluted in a single peak with a 1 M KCl wash. The second column, the Blue Dextran affinity column, removes much of the remaining protein (93% of total protein) and gave a 3 fold purification. The final column, Red A matrix gel affinity column, is specific for glycogen synthetase as it gave a final fold purification of 131.9 and a final specific activity of 16.1 U/mg.

SDS-PAGE

SDS-PAGE gels gives a subunit molecular weight of 84,000. This value is in agreement with human liver glycogen synthetase 80-85,000, rat liver 86,000, and rabbit muscle 85,000 (Westphal & Nuttall, 1992; Hiken & Lawrence, 1984; Roach, 1990). All of the above forms of glycogen synthetase are monomers with the same apparent native molecular weight. Figure 2.4 shows the SDS-PAGE gel and outlines

the various purification steps involved. Two bands can be seen on the gel. Both purification schemes (final column Matrix Gel Red A and Con A Sepharose) show two bands. The first band corresponds to glycogen synthetase. Certainly, the Matrix Red A purification scheme gives an enzyme pure enough to perform kinetic studies on.

Arrhenius Plot

The Arrhenius plot (Figure 2.6) shows the effects of temperature on enzyme activity of glycogen synthetase. The plot shows a break at 12°C, with the upper portion having an 2.8-fold lower E_a . This break may indicate a conformational change in the enzyme that may effect the activity at lower temperatures. As the kinetic studies show (Table 2.3), glycogen synthetase is less active at lower temperatures. The conformational change may alter the enzyme in a manner that would change the affinities for substrates and activators. The change in the enzyme conformation may inhibit the enzyme at lower temperatures when glycerol is being produced in the insect. This would allow the flux of glycogen through this locus without futile recycling of carbon. The Arrhenius plot also shows that glycerol protects the conformation of the enzyme as no break in the plot in the presence of glycerol is observed. Once glycerol levels reach 2M, the enzyme would be protected. This resumption of the enzyme conformation may help regulate the net amount of glycogen that is converted to G1P and thereby ensure that a minimum level glycogen would be available after glycerol production is complete.

Kinetic Analysis

The kinetic properties (Table 2.3) of glycogen synthetase D show that the glycogen synthesis is regulated at low temperatures in the following manner. First, the enzyme has a lower affinity for its substrates UDPG and glycogen at lower temperatures. Second, glycogen synthetase is less affected by allosteric activators at low temperatures. These results are opposite to those of glycogen phosphorylase in its active form in *E. scudderiana* (Holden & Storey, in press d). In the insect, phosphorylase is activated at lower temperatures with the initiation of cryoprotectant synthesis. The less active glycogen synthesis allows for undisturbed conversion of glycogen to glycerol at lower temperatures. However, at warm temperatures, the more active form of the glycogen synthetase may assist glycerol catabolism and the reconversion of GIP to glycogen.

Conclusion

The larvae of the insect *Epiblema scudderiana* produce up to 2 M of the cryoprotectant glycerol to survive overwintering at extremely cold temperatures. The regulation of glycogen synthetase enables the larvae to accumulate high levels of cryoprotectant. In order to produce glycerol, glycogen synthetase must be shut off. The less active D form of the enzyme is the only form found throughout the seasonal time course and at lower temperatures. The prominence of the less active form helps to ensure that futile recycling of carbon will not take place throughout the winter months. This facilitates the unidirectional conversion of glycogen to GIP and its use for glycerol production. The second form of regulation occurs through a conformational change in the enzyme to a less active form, at lower temperatures. The Arrhenius plot shows that glycogen synthetase D may undergoes a conformational change at low

temperatures. Glycogen synthetase is less active at lower temperatures which is reflected as a decreased affinity for its substrates UDPG and glycogen, as well as its allosteric activator G6P. This decreased affinity of glycogen synthetase prevents the futile recycling of glycogen throughout cryoprotectant synthesis. The above factors illustrate that glycogen synthetase is tightly regulated to assist in the production of the cryoprotectant glycerol in *Epiblema scudderiana*.

CHAPTER THREE
HEXOKINASE
FROM THE LARVAE OF THE FREEZE AVOIDING GALL MOTH
EPIBLEMA SCUDDERIANA

INTRODUCTION TO HEXOKINASE

Hexokinase (ATP: D-hexose-6-phosphotransferase, EC 2.7.1.1) has been described as the pacemaker of glycolysis (Solheim & Fromm, 1981). The enzyme catalyzes the phosphoryl group transfer from ATP to the C-hydroxyl group of glucopyranose, forming glucopyranose 6-phosphate. The enzyme occurs in a single form (multiple isozymes are found in different tissues in some mammalian species) and exists as a monomer with a molecular weight of 96-99,500 (Wilson, 1973; Magnani et al, 1982; & Purich et al, 1973). The two substrates for hexokinase are ATP and Glucose. These substrates have catalytic binding sites on hexokinase, and it is believed that a third binding site for G6P (the end product and inhibitor) exists. It is argued that the binding of G6P occurs at a catalytic site (Colonic, 1973; Purich & Fromm, 1971) while others suggest the inhibition occurs at an allosteric site (Palings & Fromm, 1977; Rude et al, 1985). Storey (1980) adds that G6P may interact with both allosteric and catalytic sites. The inhibition at the allosteric site can be reversed by P_i while the inhibition at the catalytic site can be reversed by alanine. Regardless, the binding G6P allows for major feedback control of hexokinase.

Hexokinase is strongly regulated by both reaction products, ADP and G6P. The enzyme is found to be competitively inhibited by G6P with respect to ATP and glucose, and to be non-competitively inhibited by ADP with respect to ATP and glucose (Purich et al, 1973; Storey, 1980).

Hexokinase has been shown to be regulated in response to physiological stress in a number of invertebrates. The marine invertebrate *M. edulis* regulates hexokinase in response to nutritional needs caused by seasonal changes in the environment. The affinities for substrates of hexokinase vary depending on the time of the year, and the amount of food available (Livingstone & Clark, 1982). Hexokinase has been found to decrease in activity in the larval-pupal transformation of the silkworm *H. cecropia*. In

this invertebrate, there is a loss of activity during pupation. The reduction of enzyme activity is expected as there is a decrease in the amount of free glucose available at the time of pupation (Jungries, 1974). Another example of regulation of hexokinase can be seen in the flight muscles of locusts. Hexokinase is found to be activated during flight. The inhibition by high levels of G6P that would be expected are counteracted by alanine and P_i . Therefore, hexokinase remains active during flight (Storey, 1980).

This study looks at the allosteric properties of hexokinase in the larvae of *E. scudderiana* at 22 and 4°C. The larvae are able to accumulate up to 2M glycerol as a cryoprotectant to aid in their survival of harsh winter temperatures. During the production of glycerol, there is an increase in glucose levels. Glucose is a powerful inhibitor of glycolysis. The regulation of hexokinase may prevent this inhibition by ensuring glucose levels remain low by converting glucose to G6P. The G6P that is produced from this reaction may now be used in the production of glycerol.

Animals

Autumn collected insects acclimated to 15°C in the laboratory were used for studies of the enzyme hexokinase.

Preparation of Homogenates and Purification

Two grams of frozen insects (1 larva = 0.07 g) were weighed and homogenized 1:10 w/v, after the addition of a few crystals of PMSF, in the following buffer A: 7.5 mM imidazole-HCl, 15 mM 2-mercaptoethanol, 50 mM NaF, 2.5 mM EGTA, 2.5 mM EDTA, and 20 % (v/v) glycerol, pH 8. The homogenate was centrifuged for 20 min at 14,000 g in a Sorvall RC-5B refrigerated centrifuge. The pellet was discarded and the supernatant was removed and kept on ice for subsequent enzyme purification.

The supernatant was applied to a DE-52 Sephadex column (packed in a 5 ml syringe barrel) equilibrated in buffer A. The column was washed with buffer A until the A₂₈₀ of the eluant fell to near zero absorbance. Hexokinase was then eluted with a linear gradient of 0-1 M KCl in buffer A. The peak fractions were pooled and dialyzed against buffer B (Buffer A adjusted to pH 7) for 1 h.

The partially purified dialyzed preparation was applied to a phosphocellulose column (packed in a 5 ml syringe barrel) equilibrated in buffer B. The column was washed with buffer B and the enzyme was eluted with a linear gradient of 0-1 M KCl in buffer B. The fractions were collected and assayed.

The pooled peak fractions were applied to a Blue Dextran column (7 cm x 1.5 cm). The column was washed in buffer B and hexokinase was eluted with a wash of 300 mM KCl in buffer B. The final eluant was collected and the enzyme was tested for homogeneity by SDS-PAGE electrophoresis (as explained in Chapter 2).

Enzyme Kinetics and Assay

Hexokinase activity was assayed spectrophotometrically at 340 nm by one of two coupled enzyme assays. The assays measured either the conversion of A) NADP^+ to NADPH, or B) NADH to NAD^+ . Optimal assay conditions were: 15 mM imidazole - HCl, 2 mM glucose, 2 mM ATP, 10 mM MgCl_2 , and either A) 0.5 mM NADP^+ , and excess dialyzed glucose-6-P dehydrogenase; or B) 0.2 mM phosphoenolpyruvate, 0.1 mM NADH, and excess dialyzed pyruvate kinase and lactate dehydrogenase.

SCHEME 1. HEXOKINASE ANALYSIS COUPLED TO GLUCOSE-6-P DEHYDROGENASE

SCHEME 2. HEXOKINASE ANALYSIS COUPLED TO PYRUVATE KINASE AND LACTATE DEHYDROGENASE.

Kinetic studies of hexokinase were performed at 22 and 40°C. K_m values were determined by a computer program which fitted data through a nonlinear least squares regression (Brooks, 1992). The K_a for MgSO_4 was determined through plots of $V_{\max}$

vs [activator] at suboptimal K_m glucose concentrations. I_{50} (the concentration of inhibitor at which enzyme velocity is decreased by 50%) values were determined for ADP and G6P by plotting $V_{max}/V_{inhibitor}$ vs [inhibitor] at glucose levels slightly above the K_m value. K_i values were obtained for ADP with respect to glucose and ATP, and G6P with respect to ATP and Glucose. These values were obtained by plots of [inhibitor] vs $1/\text{velocity}$ at various substrate concentrations. Double reciprocal plots of substrate concentration vs velocity at varying inhibitor concentrations were also determined.

Arrhenius plots were obtained by assaying hexokinase under optimal assay conditions over a range of temperatures from 4 to 37°C in the presence vs absence of added glycerol.

All values were reported as means $\pm$ S.E.M. with an $n=3$ for separate purified preparations. Statistical differences were tested using the Student t-test (2-tailed) with $P < 0.05$ or $P < 0.005$ for 4 and 22°C.

RESULTS

Purification

Table 3.1 summarizes the purification of hexokinase. In the first step, the ion exchange DE-52 Sephadex column removed 86% of the total protein present in the crude homogenate with a 4-fold purification of hexokinase activity and a 57% yield. The elution profile of the DE-52 column purification is presented in Figure 3.1. The phospho-cellulose column gave a 15 fold purification while removing most of the remaining protein (Figure 3.2). The final Blue-Dextran step led to a final specific activity of 75.8 Units/mg when assayed at 22 °C. Overall, the chromatography columns in sequence gave a 20 fold purification with a 9% yield.

SDS PAGE

The purified hexokinase gave a single band when run on a 10% SDS-polyacrylamide gel (Figure 3.3). The apparent subunit molecular weight was determined by comparison with the migration of known molecular weight markers (Figure 3.4). The subunit molecular weight of hexokinase was determined to be 94,000 daltons.

TABLE 3.1 PURIFICATION TABLE OF HEXOKINASE FROM E. scudderiana

STEP	TOTAL ACTIVITY UNITS	TOTAL PROTEIN mg PROTEIN	YIELD %	FOLD PURIFICATION	SPECIFIC ACTIVITY U/mg
CRUDE SUPERNATANT	45.0	11.9	-	-	3.78
DE52 SEPHADEX	25.6	1.72	57	3.9	14.9
PHOSPHO- CELLULOSE	6.40	0.110	.14	15.4	58.2
BLUE DEXTRAN	4.02	0.053	9	20.1	75.8

Figure 3.1 ELUTION PROFILE OF HEXOKINASE ON A DE-52 SEPHADEX COLUMN

The enzyme, from 15°C acclimated larvae, was eluted with a linear gradient of 0-1 M KCl. One ml fractions were collected and 100 ul of each fraction was assayed. Activities are given relative to the enzyme velocity in the peak tube.

Figure 3.2 ELUTION PROFILE OF HEXOKINASE ON A PHOSPHOCELLULOSE COLUMN

The enzyme, from 15°C acclimated larvae and purified with a DE-52 Sephadex chromatography column, was eluted with a linear gradient of 0-1 M KCl. One ml fractions were collected and 100 ul of each fraction was assayed. Activities are given relative to the velocity in the peak tube.

Figure 3.3 STANDARD CURVE OF LOG MOLECULAR WEIGHT VS RELATIVE MOBILITY OF PROTEIN STANDARDS RUN ON A SDS-PAGE GEL

The log of the following molecular weight markers were plotted against the relative mobility to determine the molecular weight of hexokinase.

The molecular weights were: 1) galactosidase (116,000), 2) fructose-6-phosphate kinase (96,000), 3) pyruvate kinase (80,400), 4) ovalbumin (55,700), and 5) lactic dehydrogenase (43,700). The black circle shows the subunit molecular weight for hexokinase.

Figure 3.4 SDS-PAGE GEL OF THE PURIFICATION OF HEXOKINASE

The SDS-PAGE gel shows the complete purification of hexokinase along with the location of standards. Lane 1 is the crude homogenate, Lane 2 purification after the DE-52 Sephadex column, Lane 3 purification after Phosphocellulose column, Lane 4 purification after Blue Dextran column, and Lane 5 is the standards. The arrow shows the apparent subunit molecular weight of hexokinase.

Arrhenius Plot

The Arrhenius plot (Figure 3.5) shows the effect of assay temperatures on hexokinase activity. Assays were conducted with or without the addition of the cryoprotectant glycerol. In both situations the plot revealed a sharp break between 11-13°C indicating a conformational change may have occurred in the protein as the result of temperature change. In the absence of glycerol the Arrhenius plot showed a break at 11.5 °C with the upper portion (16.5-38 °C) having a calculated activation energy (E_a) of 28.8 ± 2.1 kJ/mol and the lower portion (4-11.5 °C) having an E_a of 116.9 ± 4.3 kJ/mol. With 10% v/v glycerol present the conformation of the protein hexokinase was slightly protected with a break at 13.3 °C. The upper portion of the curve (16-31 °C) gave a E_a of 26.2 ± 2.3 kJ/mol with glycerol and the lower portion (4-12) gave an E_a of 67.5 ± 5.2 kJ/mol.

Kinetic Analysis

Analysis of enzyme parameters were carried out at 4 and 22°C assay temperature. All kinetics were performed on enzyme purified from 15°C acclimated animals with the results summarized in Table 3.2. The data shows that hexokinase had a significantly higher affinity for all substrates at the lower assay temperature. The K_m values for ATP, glucose, each decreased about two fold and the K_a for Mg^{2+} decreased over 20-fold at the lower temperature. The affinity differences are apparent in Figures 3.6 and 3.7 which plot velocity differences against differing substrate/concentrations.

Figure 3.5 ARRHENIUS PLOT FOR HEXOKINASE

An Arrhenius plot was obtained to determine if a conformational change in the enzyme occurred as a result of temperature change. All measurements were taken at optimal assay conditions. Hexokinase activity was measured from 4-38°C. In the presence of added 10% v/v glycerol, the plot showed a break at 13.3°C with the E_a changing from 26.2 ± 2.1 kJ/mol (upper portion 16-31°C) to 67.5 ± 5.2 kJ/mol (lower portion 4-12°C). Without glycerol there was a break at 11.5°C with a E_a changing from 28.8 ± 2.1 kJ/mol (upper portion 16.5-38°C) to 115.0 ± 4.3 kJ/mol (lower portion 4-11.5°C). All values are reported as means $\pm$ S.E.M, $n=3$. Where S.E.M. values are not visible in the form of an error bar they are contained within the dimensions of the symbol.

TABLE 3.2 KINETIC PROPERTIES OF HEXOKINASE PURIFIED FROM *E. scudderiana*

	22°C	4°C
Km GLUCOSE (mM)	0.29 ± 0.02	0.16 ± 0.01 **
Km ATP (mM)	0.26 ± 0.02	0.13 ± 0.01 **
Ka Mg ²⁺ (mM)	1.50 ± 0.18	0.066 ± 0.01**
I ₅₀ G6P (mM)	0.21 ± 0.02	0.27 ± 0.02 *
I ₅₀ ADP (mM)	2.29 ± 0.29	3.07 ± 0.12 *

DATA ARE MEANS ± S.E.M. FOR ALL WITH AT LEAST n = 3

DIFFERENT PREPARATIONS OF THE PURIFIED ENZYME. *

SIGNIFICANTLY DIFFERENT FROM THE CORRESPONDING VALUES AT 22°C BY THE STUDENT'S t-TEST (TWO-TAILED), P< 0.05;

** P< 0.005.

Figure 3.6 PLOTS OF VELOCITY VS SUBSTRATE CONCENTRATION FOR HEXOKINASE

The representative plots illustrate the change in enzyme activity when substrate concentrations (glucose and ATP) were varied at 4 and 22°C. Hollow circles give hexokinase activity at 22°C and filled circles give activity at 4°C. The velocity was relative and determined with purified enzyme.

Figure 3.7 PLOTS OF VELOCITY VS SUBSTRATE CONCENTRATION FOR HEXOKINASE

The representative plot illustrates the change in enzyme activity when the substrate concentration [Mg^{2+}] was varied at 4 and 22°C. Hollow circles give hexokinase activity at 22°C and filled circles give activity at 4°C. The velocity is defined and determined with purified enzyme..

Hexokinase also exhibited inhibition from ADP and G6P. The I_{50} value for ADP was 25% higher at the lower temperature; and the I_{50} value for G6P, 22% higher.

K_i values were obtained for ADP and G6P. ADP showed non-competitive inhibition with respect to ATP and glucose; whereas, G6P showed mixed competitive inhibition with respect to ATP and competitive inhibition with respect to glucose. The K_i values are given in Table 3.3.

Double reciprocal plots of velocity vs substrate concentration also showed the above mentioned inhibition. Figures 3.8 -3.11 show the effects of ADP and G6P on the K_m of glucose and ATP respectively. Table 3.4 shows the effects of inhibitor concentration on the K_m of the two substrates. At 0.5 mM G6P, the K_m for glucose increased 2.5 fold at 22°C, and 10 fold at 4°C. At the same G6P concentration, the K_m for ATP increased 1.6 fold at 22°C and 4.6-fold at 4°C.

Figure 3.8 DOUBLE RECIPROCAL PLOTS SHOWING ADP EFFECTS ON THE AFFINITY OF HEXOKINASE FOR GLUCOSE AT 4 AND 22°C.

The representative plots illustrate the non-competitive inhibition of ADP with respect to glucose at 4 (bottom) and 22°C (top) respectively, as the K_m for glucose does not change. The assay conditions were optimal with varying concentrations of glucose and the following concentrations of ADP: hollow circles 2 mM, filled circles 1.5 mM, hollow triangles 0.75 mM, and filled triangles 0 mM. The velocity was relative and determined with purified enzyme.

TABLE 3.3 INHIBITION OF HEXOKINASE PURIFIED FROM *E. scudderiana* BY G6P AND ADP

	TEMP	INHIBITION
K _I G6P wrt ATP (mM)	4°C 0.045 ± 0.002	COMPETITIVE
	22°C 0.025 ± 0.001*	COMPETITIVE
K _I G6P wrt GLUCOSE (mM)	4°C 0.040 ± 0.004	COMPETITIVE
	22°C 0.023 ± 0.002*	COMPETITIVE
K _I ADP wrt ATP (mM)	4°C 1.287 ± 0.003	NONCOMPETITIVE
	22°C 1.268 ± 0.071	NONCOMPETITIVE
K _I ADP wrt GLUCOSE (mM)	4°C 0.61 ± 0.04	NONCOMPETITIVE
	22°C 0.60 ± 0.01	NONCOMPETITIVE

DATA ARE MEANS ± S.E.M. FOR ALL WITH AT LEAST n = 3

DIFFERENT PREPARATIONS OF THE PURIFIED ENZYME. *

SIGNIFICANTLY DIFFERENT FROM THE CORRESPONDING VALUES AT 4°C
BY THE STUDENT'S t-TEST (TWO-TAILED), P < 0.05

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

Figure 3.9 DOUBLE RECIPROCAL PLOTS SHOWING ADP EFFECTS ON THE AFFINITY OF HEXOKINASE FOR ATP AT 4 AND 22°C.

The representative plots illustrate the non-competitive inhibition of ADP with respect to ATP at 4 (bottom) and 22°C (top) respectively, as the K_m for ATP does not change. The assay conditions were optimal with varying concentrations of ATP and the following concentrations of ADP: hollow circles 2 mM, filled circles 1.5 mM, hollow triangles 0.75 mM, and filled triangles 0 mM. The velocity was relative and determined with purified enzyme.

Figure 3.10 DOUBLE RECIPROCAL PLOTS SHOWING G6P EFFECTS ON THE AFFINITY OF HEXOKINASE FOR GLUCOSE AT 4 AND 22°C.

The representative plots illustrate the competitive inhibition of G6P with respect to glucose at 4 (bottom) and 22°C (top) as the K_m for glucose increases 10 and 2.5 fold respectively. The assay conditions were optimal with varying concentrations of glucose and the following concentrations of G6P: hollow triangles 0.5 mM, and filled circles 0.1 mM, hollow circles 0.05 mM, and filled triangles 0 mM. The velocity was relative and determined with purified enzyme.

Figure 3.11 DOUBLE RECIPROCAL PLOTS SHOWING G6P EFFECTS ON THE AFFINITY OF HEXOKINASE FOR ATP AT 4 AND 22°C.

The representative plots illustrate the competitive inhibition of G6P with respect to ATP at 4 (bottom) and 22°C (top) as the K_m for ATP increases 4.7 and 1.6 fold respectively. The assay conditions were optimal with varying concentrations of ATP and the following concentrations of G6P: hollow triangles 0.5 mM, and filled circles 0.1 mM, hollow circles 0.05 mM, and filled triangles 0 mM. The velocity was relative and determined with purified enzyme.

enzyme phosphofructokinase (PFK). Two examples show that FBPase can play an integral role in the control of the influx of carbon in glycolysis or gluconeogenesis during physiological stresses. The Arctic king crab, *P. camtschatica*, periodically molts its old shell for a larger one. In order to produce chitin (material in which the shell was made from), the flow of carbon must be reversed into gluconeogenesis which leads towards glycogen synthesis and chitin production. Two forms of FBPase contribute to the regulation of the gluconeogenic pathway in hypodermic tissue. The two forms of FBPase aid in the controlling of carbon through this locus toward glycogen synthesis (FBPase I) or chitin (FBPase II). Both forms of FBPase are inhibited by AMP but tighter control of the enzyme is needed to ensure the production of both end products. When physiological conditions favor gluconeogenesis, AMP levels must be closely controlled in order to prevent inhibition of both forms of FBPase. In the crabs, the AMP levels are controlled by the pH dependence on K^+ . The inhibition by AMP of FBPase is most potent in the presence of high concentrations of K^+ . The crab can control the levels of K^+ in the cell by adjusting the pH. The crab also uses the de-inhibition effects of Mg^{2+} to ensure maximal levels of FBPase are present when needed. The two forms of FBPase are controlled by different affinities to inhibitors. FBPase I (used for glycogen synthesis) is inhibited by high UDP-GluNAc (precursor to chitin), while FBPase II is not (Hochachka, 1971). This example shows how tight regulation of FBPase can determine the outcome of carbon flow through gluconeogenesis.

The second example is from the flight muscle of the bumblebee *B. terrestris*. FBPase in this muscle is not inhibited by AMP and is present in a 1:1 ratio with PFK (catalyzes the reverse reaction). By having both enzymes, FBPase and PFK, in equal amounts, it appears that the bumblebee is futilely recycling F6P and F1,6BP. This is not the case. Ingeniously, this recycling enables the net hydrolysis of ATP for the production of metabolic heat to warm up the insect before flight when ambient

DISCUSSION OF HEXOKINASE

The insect *Epiblema scudderiana* is able to quickly accumulate up to 2M glycerol in response to cold temperatures. The insect channels carbon into glycogenolysis for the production of the cryoprotectant glycerol. Glucose, the substrate for hexokinase, is a strong inhibitor of glycogen phosphorylase, the enzyme that catalyzes the conversion of glycogen to G1P, one of the control points of cryoprotectant production. High glucose levels would inhibit glycogen phosphorylase, thus inhibiting the production of glycerol (Storey, 1990). As Figure 1.1 illustrates, during active glycerol synthesis, glucose levels remain relatively low with cold exposures until maximal glycerol levels are reached (after 4 days). Subsequently, glucose accumulates, possibly as a response to maximal cryoprotectant levels. Hexokinase, by converting glucose to G6P, may ensure that cryoprotectant production is not inhibited by glucose and that carbon is pushed back into glycolysis for the production of glycerol. Hexokinase appears to be regulated by a conformational change in the protein to a more active form at low temperatures.

Purification

The purification of hexokinase gives a 20-fold purification and a final specific activity of 75.8 U/mg. The final specific activity is a little lower than those reported for rat skeletal muscle 120 U/mg, rabbit brain 165 U/mg, rabbit red blood cells 145 U/mg, and locust flight muscle 125 U/mg (Qadri & Easterby, 1980; Magnani et al, 1982; Magnani *et al*, 1980; Storey, 1980).

The enzyme is purified with three ion exchange columns in sequence. The first column, the DE52⁺ ion exchange column is able to eliminate the majority of protein. The second column, a phosphocellulose column, is specific for hexokinase as it gives a specific activity of 58.2 U/mg. The final purification step (Blue Dextran affinity column), results in a final specific activity of 75.8 U/mg with a 20-fold final purification. The three columns give an enzyme that was pure enough for kinetic studies.

SDS-PAGE

SDS-PAGE gives a subunit molecular weight of 94,000. The value agrees with the reported values of 96,000 for rat brain hexokinase, 110,000 for rabbit red blood cells, 100,000 for silkworm, and 97,000 for locust flight muscle (Chou & Wilson, 1972; Magnini et al, 1982; Supowit & Harris, 1976; Storey, 1980). This purification scheme results in a purified enzyme, as the gel in Figure 3.4 shows a single band.

Arrhenius Plot

The Arrhenius plot (Figure 3.5) shows the effect of temperature on enzyme activity. The plot shows a break at 11.5°C indicating a conformational change in the enzyme may have occurred. The E_a of the upper portion is 4-fold less than the lower portion. With the addition of 10% v/v glycerol, there is a break in the plot at 13.4°C. The E_a of the upper portion is found to be 2-fold less than the lower portion. The results indicate that the proteins conformation changes in response to lower temperatures with glycerol slightly protecting the conformation as the break in the plot occurred at a higher temperature.

Kinetic Analysis

The kinetic properties (Table 3.2) show that hexokinase is activated at lower temperatures. The affinity for its substrates glucose and ATP, and allosteric activator Mg^{2+} were lower at 4°C. The more active hexokinase would convert glucose to G6P thereby facilitating the flux of carbon into glycolysis and assisting in production of the cryoprotectant glycerol.

The inhibitors, G6P and ADP, showed less inhibition at the lower temperature. The higher I_{50} value for G6P, at the lower temperature, are important as the levels of G6P are highest with the initiation of the production of glycerol.

Table 3.3 shows the inhibition of hexokinase by G6P and ADP. G6P is a mixed competitive inhibitor of hexokinase with respect to ATP and a competitive inhibitor with respect to glucose. On the other hand, ADP is a noncompetitive inhibitor of hexokinase with respect to both substrates. The K_i values for G6P with respect to glucose and ATP are almost two fold higher at 4°C. This also indicates that at low temperatures the enzyme is capable of withstanding higher concentrations of G6P before being inhibited.

Conclusion

Hexokinase from *Ebiblema scudderiana* is regulated at low temperatures in response to cryoprotectant synthesis. The Arrhenius plot shows that the enzyme may undergo a conformational change at low temperatures. The change in enzyme structure may facilitate the activation of the hexokinase at low temperatures. Kinetic studies have shown that hexokinase is more active at low temperatures. As the conversion of glycogen to G1P occurs during cryoprotectant synthesis, there is an increased carbon flux. This results in an increased accumulation of glucose. Glucose is a potent inhibitor

of glycogen phosphorylase and glycogenolysis. In order to prevent the inhibition of glycogenolysis, hexokinase must convert glucose to G6P. The inhibitory effects of G6P on hexokinase are overcome by the decreased sensitivity of the enzyme to the inhibitor at low temperature. Overall, this regulation results in the depletion of glucose and the facilitation of the flux of carbon back into the glycolysis. This is accomplished by a more active hexokinase at low temperatures. .

CHAPTER FOUR
FRUCTOSE 1,6-BISPHOSPHATASE
FROM THE LARVAE OF THE FREEZE AVOIDING GALL MOTH
EPIBLEMA SCUDDERIANA

INTRODUCTION TO FRUCTOSE 1,6-BISPHOSPHATASE

Fructose 1,6-bisphosphatase (FBPase) (D-fructose-1,6-bisphosphate 1-phosphohydrolase, EC 3.1.3.11) plays a key role in gluconeogenesis in a number of animals (Tejwani, 1983). The enzyme catalyzes the reversible conversion of fructose-1,6-bisphosphate (F1,6P₂) to fructose-6-phosphate (F6P). The regulation of the enzyme has been well documented. Fructose 2,6-bisphosphate (F2,6P₂), and AMP are strong inhibitors of FBPase, whereas bivalent metal cations are strong activators. The enzyme is also subject to reversible phosphorylation/dephosphorylation. It has been reported that the phosphorylated form of the enzyme is the more active one. In rat liver, the phosphorylated form of FBPase shows a higher affinity for F1,6P₂ and experiences less inhibition by F2,6P₂ and AMP than the dephosphorylated form (Vidal et al, 1986). Alternatively, Meek & Nimmo (1984) have reported that phosphorylation of FBPase by cAMP dependent protein kinases causes a 2-fold decrease in the K_m of F1,6P₂ but did not affect the allosteric responses of AMP, Mg²⁺, and F2,6P₂. Recently, it has been discovered that FBPase in rat liver could be phosphorylated at multiple sites. The phosphorylation at these different sites affects both the K_m for F1,6P₂, as well as the affinities of AMP and F2,6P₂. It has been determined that the first phosphorylation site could be used for binding of protein kinase A but the second site is an atypical protein kinase A binding site. The third phosphorylation site is only phosphorylated when FBPases crystal structure remains intact and all three phosphorylation sites are equally phosphorylated (Ekdahl, 1992). The complexity in the phosphorylation of the enzyme has made the study of its regulation difficult.

There are a number of studies that illustrate the regulation of gluconeogenesis by FBPase. Studies on *S. cerevisiae* have shown that FBPase exists in two interconvertible phosphorylation forms. (Gancedo et al, 1982). The regulation of this reversible reaction (F1,6P₂ to F6P) has been historically dominated by the reciprocal

enzyme phosphofructokinase (PFK). Two examples show that FBPase can play an integral role in the control of the influx of carbon in glycolysis or gluconeogenesis during physiological stresses. The Arctic king crab, *P. camtschatica*, periodically molts its old shell for a larger one. In order to produce chitin (material in which the shell was made from), the flow of carbon must be reversed into gluconeogenesis which leads towards glycogen synthesis and chitin production. Two forms of FBPase contribute to the regulation of the gluconeogenic pathway in hypodermic tissue. The two forms of FBPase aid in the controlling of carbon through this locus toward glycogen synthesis (FBPase I) or chitin (FBPase II). Both forms of FBPase are inhibited by AMP but tighter control of the enzyme is needed to ensure the production of both end products. When physiological conditions favor gluconeogenesis, AMP levels must be closely controlled in order to prevent inhibition of both forms of FBPase. In the crabs, the AMP levels are controlled by the pH dependence on K^+ . The inhibition by AMP of FBPase is most potent in the presence of high concentrations of K^+ . The crab can control the levels of K^+ in the cell by adjusting the pH. The crab also uses the de-inhibition effects of Mg^{2+} to ensure maximal levels of FBPase are present when needed. The two forms of FBPase are controlled by different affinities to inhibitors. FBPase I (used for glycogen synthesis) is inhibited by high UDP-GluNAc (precursor to chitin), while FBPase II is not (Hochachka, 1971). This example shows how tight regulation of FBPase can determine the outcome of carbon flow through gluconeogenesis.

The second example is from the flight muscle of the bumblebee *B. terrestris*. FBPase in this muscle is not inhibited by AMP and is present in a 1:1 ratio with PFK (catalyzes the reverse reaction). By having both enzymes, FBPase and PFK, in equal amounts, it appears that the bumblebee is futilely recycling F6P and F1,6BP. This is not the case. Ingeniously, this recycling enables the net hydrolysis of ATP for the production of metabolic heat to warm up the insect before flight when ambient

temperatures are low (Storey, 1982). Both these examples show that FBPase can play a major role in the control of carbon flux.

Potentially, FBPase can be an important regulator of carbon flow in the larvae of the gall moth *Epiblema scudderiana*. The enzyme may be able to assist in turning off gluconeogenesis during glycerol production, and then help to return the flow of carbon through gluconeogenesis during glycerol catabolism. With the huge amounts of carbon flowing to and from glycerol, tight regulation at this locus is essential.

MATERIALS AND METHODS

Animals

Insects collected throughout the time period of 1989-90, as well as the animal acclimated in the laboratory to 15 and -4°C, were used for experiments with fructose-1,6-bisphosphatase (FBPase).

Enzyme Preparation and Purification

Samples of frozen larvae (1 larva = 0.07 g) were homogenized, with a few crystals of PMSF added, 1:10 w/v in buffer A: 15 mM imidazole-HCl, 75 mM NaF, 3.5 mM EDTA, 3.5 mM EGTA, 15 mM 2-mercaptoethanol, and 20% v/v glycerol, pH 7.5. After homogenization, the crude preparation was quickly centrifuged for 20 min at 13,000 r.p.m. in a Sorvall RC-5B refrigerated centrifuge set at 5°C. After centrifugation, the supernatant was removed from the pellet and fat layer, and placed on ice.

The supernatant was then placed on a phosphocellulose column equilibrated in buffer A. The column was washed with 20 ml of buffer A and then FBPase was eluted with a gradient of 0-1 M KCl in buffer A. One ml fractions were collected and assayed. The 5 peak fractions were pooled and desalted by centrifugation (1 min at top speed on a desk top centrifuge) through a G-25 Sephadex column equilibrated in buffer B. Buffer B contained 10 mM imidazole-HCl, 50 mM NaF, 2.5 mM EGTA, 2.5 mM EDTA, 15 mM 2-mercaptoethanol, and 20 % v/v glycerol, pH 7.9.

The eluant from the G-25 column was layered onto a CM-cellulose column equilibrated in buffer B. The column was washed with 20 ml of buffer B and eluted

with a linear gradient of 0-300 mM KCl in buffer B. The peak fractions were collected, pooled, and desalted by centrifugation through a G-25 column equilibrated in buffer B.

In vitro Phosphorylation and Dephosphorylation Incubations

The CM-cellulose column showed that FBPase eluted as two peaks. To determine if these peaks corresponded to phosphorylated and dephosphorylated forms of FBPase, enzyme samples were incubated *in vitro* under conditions that would promote either phosphorylation or dephosphorylation of the enzyme.

Samples of the frozen larvae were homogenated in buffer B and centrifuged as previously described. The supernatant was removed, divided into two equal portions, and then stored on ice. The first portion was desalted by centrifugation through a G-25 column equilibrated in buffer C (buffer B minus EGTA and EDTA). A 0.5 ml fraction of the eluant was incubated in 0.5 ml of buffer C plus 1 mM cAMP, 10 mM MgCl₂, and 10 mM ATP, pH 7, in order to promote phosphorylation of the enzyme. The second portion was desalted by centrifugation through a G-25 column in buffer D (buffer B minus NaF, EGTA, and EDTA). A 0.5 ml fraction of the eluant was incubated in 0.5 ml of buffer D plus 10 mM MgCl₂, pH 7, in order to promote dephosphorylation of the enzyme.

Enzymes were incubated at 4°C for (24 h) and then desalted by centrifugation through a G-25 column equilibrated in buffer B. The preparations were then chromatographed on phosphocellulose and CM-cellulose columns as described earlier.

Enzyme Assay and Kinetics

FBPase was measured by monitoring F1,6P₂ use in a coupled enzyme reaction that resulted in the conversion of NADP⁺ to NADPH. The reduction was monitored at 340 nm on a Gilford 260 spectrophotometer.

SCHEME 1 ANALYSIS OF FBPase COUPLED TO PHOSPHOGLUCOSOMERASE

AND GLUCOSE-6-PHOSPHATE DEHYDROGENASE

The optimal assay conditions were found to be 0.1 mM F1,6P₂, 5 mM MgSO₄, 0.2 mM NADP⁺, and 1 unit each of the following coupling enzymes phosphoglucosomerase, and glucose-6-phosphate dehydrogenase.

Kinetic studies were performed on samples of the larvae that were acclimated in the laboratory to -4°C. FBPase was partially purified by chromatography on phosphocellulose and CM-cellulose columns. The K_m for the substrate F1,6P₂ was determined with Michaelis-Menten plots. The K_a for MgSO₄ was determined through plots of V_{max} vs [activator] at suboptimal F1,6P₂ concentrations, and I₅₀ values for AMP and F2,6P₂ were determined from plots of V/V_{max} vs [inhibitor] at above optimal concentrations for F1,6P₂.

All values were reported as means $\pm$ S.E.M. with at least n = 3. Statistical differences were determined using the Student's t-test (two-tailed) with P < 0.025 and P < 0.005.

RESULTS

Enzyme Preparation & In vitro Incubations

After initial preparation with a phosphocellulose column (Figure 4.1), the CM-Cellulose column chromatography showed that there were two forms of FBPase in -4°C acclimated larvae of *E. scudderiana* (Figure 4.2). To determine whether these forms were isozymic forms or phosphorylation forms of the enzyme, *in vitro* incubations of the enzyme preparation were performed with conditions that promoted either the dephosphorylation or phosphorylation of the enzyme by endogenous protein phosphatases or protein kinases. The results shown in Figure 4.3 indicated that the two peaks represent phosphorylated and dephosphorylated forms of FBPase. Incubations that promoted phosphorylation shifted virtually all the enzyme activity into a single peak eluting at 63 ± 2 mM KCl whereas incubations under conditions that promoted dephosphorylation shifted activity into a peak that eluted later at 110 ± 7 mM KCl. These two peaks each corresponded with one of the peaks identified in Figure 4.2 and allowed for identification of these peaks as the phosphorylated (eluting first) and the dephosphorylated (eluting second) forms of FBPase.

Seasonal Changes in the Percentage of Phosphorylated and Dephosphorylated forms of FBPase

Figure 4.4 shows the seasonal changes in the percentage of FBPase in the phosphorylated vs dephosphorylated forms *in vivo* in the larvae at different sampling times between September and April. Between

Figure 4.1 ELUTION PROFILE OF FBPase ON A PHOSPHOCELLULOSE COLUMN

The enzyme, from -4°C acclimated larvae, was eluted with a linear gradient of 0-1 M KCl. One ml fractions were collected and 100 ul of each fraction was assayed. Activities are given relative to the velocity in the peak tube.

Figure 4.2 ELUTION PROFILE OF FBPase ON A CM-CELLULOSE COLUMN

The enzyme, from -4°C acclimated larvae and purified on a phosphocellulose chromatography column, was eluted with a linear gradient of 0-300 mM KCl. One ml fractions were collected and 100 ul of each fraction was assayed. Activities are given relative to the velocity in the peak tube.

Figure 4.3 EFFECTS OF in vitro INCUBATIONS UNDER CONDITIONS PROMOTING PHOSPHORYLATION OR DEPHOSPHORYLATION ON THE SUBSEQUENT ELUTION PROFILE OF FBPase ON A CM-CELLULOSE COLUMN.

The representative plot show the results of two separate incubations. The filled circles represent the forced phosphorylation activity by promoting endogenous cAMP-dependent protein kinase activity. The hollow triangles represent the forced dephosphorylation by promoting endogenous phosphatase activity. The enzyme forms were eluted with a linear gradient of 0-300 mM KCl. One ml fractions were collected and 100 ul of each fraction was assayed. Activities are given relative to the velocity in the peak tube.

**Figure 4.4 SEASONAL CHANGES IN THE PERCENTAGE OF
FBPase IN THE PHOSPHORYLATED VS
DEPHOSPHORYLATED FORMS IN EPIBLEMA
SCUDDERIANA LARVAE**

The forms of the enzyme were eluted from a CM-cellulose column with a 0-300 mM KCl linear gradient. The percentages were calculated through determination of areas of phosphorylated and dephosphorylated peaks. The filled bars represent the phosphorylated form while the hollow bars, the dephosphorylated. All values are means $\pm$ S.E.M. * Significantly different from the corresponding value of the phosphorylated enzyme form by the Student's t-test (2-tailed) $P < 0.01$; ** $P < 0.001$.

September 10 and 15 there was a rapid increase in the percentage of the enzyme in the dephosphorylated form. The percentage of phosphorylated and dephosphorylated forms of the enzyme became equal near November 1. Throughout most of the winter months the percentage of phosphorylated to dephosphorylated remained constant at approximately 60 and 40% respectively. As spring returned (April 21), there was a return to the prominence of the phosphorylated form. The graph also shows that at 15°C FBPase was almost 70% in the phosphorylated form. The percentage in the phosphorylated form dropped to 50% at -4°C. To understand these results it is crucial that any kinetic properties of the phosphorylated and dephosphorylated forms are explored.

Kinetic Analysis

Frozen larvae of the -4°C acclimated laboratory animals were partially purified and chromatographed on a CM-cellulose column (Figure 4.2). The peaks were identified as the phosphorylated (first) and dephosphorylated (last) forms of FBPase. Each peak was collected as described in Materials and Methods and used to determine kinetic properties of the enzyme.

The data indicated that the phosphorylated form was the more active of the two as it displays a significantly lower K_m for F1,6P₂, K_a for Mg²⁺, and a higher I_{50} for AMP than does the dephosphorylated form. Table 4.1 summarizes the kinetic properties of the two forms of FBPase. Figure 4.5 shows the velocity vs [F1,6P₂] and the activation of FBPase by Mg²⁺ for the two enzyme forms. The K_a for Mg²⁺ and the K_m for F1,6P₂ were calculated to be three fold higher for the dephosphorylated form than the phosphorylated one. Figure 4.6 shows the inhibition of FBPase by AMP. The enzyme was inhibited to 50% of activity at a concentration 10 fold less for the dephosphorylated form than the phosphorylated form by AMP and

TABLE 4.1 PROPERTIES OF PHOSPHORYLATED AND DEPHOSPHORYLATED FORMS OF FBPase FROM -4°C ACCLIMATED PARTIALLY PURIFIED *E. scudderiana*

	DEPHOSPHORYLATED	PHOSPHORYLATED
Km F1,6P ₂ (mM)	0.076 $\pm$ 0.016	0.025 $\pm$ 0.006 **
Ka Mg ²⁺ (mM)	17.1 $\pm$ 1.28	5.6 $\pm$ 0.4 **
I ₅₀ F2,6P ₂ (uM)	0.60 $\pm$ 0.10	0.95 $\pm$ 0.10 *
I ₅₀ AMP (uM)	6.0 $\pm$ 0.15	63.0 $\pm$ 0.75 *

DATA ARE MEANS $\pm$ S.E.M. FOR ALL WITH AT LEAST $n = 3$

DIFFERENT PREPARATIONS OF THE PURIFIED ENZYME. *

SIGNIFICANTLY DIFFERENT FROM THE CORRESPONDING VALUES FOR DEPHOSPHORYLATED FORM OF THE ENZYME BY THE STUDENT'S t-TEST (TWO-TAILED), $P < 0.025$; ** $P < 0.005$.

Figure 4.5 PLOTS OF VELOCITY VS SUBSTRATE CONCENTRATION FOR FBPase

The representative plots illustrate the change in enzyme activity when the substrate concentrations (F1,6P₂ and Mg²⁺, respectively) were varied. Hollow circles give FBPase activity of the dephosphorylated form and filled circles give activity for the phosphorylated form. The velocity was relative and determined with partially purified enzyme.

Figure 4.6 AMP INHIBITION OF FBPase

The representative plot illustrates the effect of varying AMP on FBPase activity at 0.09 mM F1,6P₂. Hollow circles give FBPase activity of the dephosphorylated form and filled circles give activity of the phosphorylated form. The velocity was relative and determined with partially purified enzyme.

F2,6P₂ was found to inhibit both the two forms of the enzyme equivalently.

DISCUSSION OF FRUCTOSE 1,6-BISPHOSPHATASE

Fructose 1,6-bisphosphatase (FBPase) catalyzes the reversible conversion of fructose-1,6-bisphosphate to fructose-6-phosphate. The enzyme is known to be an important regulator of gluconeogenesis. The enzyme is allosterically regulated by AMP (Vidal et al, 1986; Behrisch & Elsner, 1979) and F2,6P₂ (Pilkis et al, 1987; Hue & Rider, 1987), and through reversible protein phosphorylation (Vidal et al, 1986; Meek & Nimmo, 1984; EKdahl, 1992). Churchill and Storey (1989a) have shown that F2,6P₂ levels increase 10-fold during the first few hours of cold exposure. This increase would activate the reciprocal reaction catalyzed by phosphofructokinase (PFK) but inhibit FBPase. Holden and Storey (in press b) have shown that PFK is regulated through a conformational change that allows for activation of the enzyme at lower temperatures; but, the enzyme does not undergo reversible phosphorylation. The same authors (in press a) have shown that FBPase is regulated through different affinities of substrates, and inhibitors at lower temperature. Regulation of the two enzymes may coordinate the production of glycerol in *E. scudderiana*.

In vitro Incubations & Seasonal Changes in the Phosphorylation State of FBPase

The ion exchange column, CM-cellulose, is able to separate the two forms of FBPase from *E. scudderiana*. *In vitro* incubations show that the first peak corresponds to the phosphorylated form, with the second corresponding to the dephosphorylated form of FBPase.

During glycerol production and throughout the seasonal time course, there are changes in the percentage of phosphorylated and dephosphorylated forms of the enzyme. These changes may effectively shut down gluconeogenesis at the onset of cryoprotectant synthesis, and facilitate the resumption of gluconeogenesis when the cryoprotectant is no longer required.

Figure 4.4 shows the changes in the percentage of the phosphorylated-dephosphorylated form of FBPase in the season time course, and between 15 and -4°C acclimated larvae. Study of the 15°C acclimated animals shows that more of the active phosphorylated form of FBPase is present when compared to the dephosphorylated form. With a drop in temperature to -4°C, the percentage of the two forms becomes almost equivalent. FBPase is able to assist in the production of glycerol by slowing the rate of gluconeogenesis thereby preventing the futile recycling of carbon.

The mean weekly temperatures in September fluctuate between a high of 23°C and a low of 13°C (Table 2.1). Due to these warm temperatures, the insect may still be preparing for overwintering and therefore need an active FBPase to assist in normal warm temperature metabolism and gluconeogenesis. As Figure 4.4 indicates, the more active phosphorylated form is predominant in early autumn. As temperatures fall further, to a low of 4°C in November and a low of -20°C in January, the percentage of the two forms became equivalent. These equivalent levels of the phosphorylated and dephosphorylated form of the enzyme ensure gluconeogenesis will be inhibited throughout the winter months by the lower levels of the active phosphorylated form of FBPase. With the resumption of warm temperatures in late March (high of 10°C), there is an increase in the more active phosphorylated form of the enzyme. The increase in the active form of FBPase may facilitate the catabolism of glycerol as high levels of cryoprotectants may no longer be needed.

Kinetic Analysis

Kinetic studies show that the phosphorylated form of FBPase is the active form, while the dephosphorylated form is less active. The K_m of F1,6P₂ for the phosphorylated form is three-fold lower than the dephosphorylated form. The K_a of

Mg^{2+} for the phosphorylated form is three-fold lower than the phosphorylated form. F2,6P_2 and AMP are stronger inhibitors of the dephosphorylated form. F2,6P_2 not only inhibits FBPase but strongly activates PFK. Churchill and Storey (1989a) have shown that F2,6P_2 rises from 0.1 nM at 22°C to 8 nM at -4°C . Therefore, this potent inhibitor would have only minimal effects on FBPase at higher temperatures; but would certainly inhibit the enzyme at lower temperatures regardless of its phosphorylation state. FBPase in either the phosphorylated or dephosphorylated form would be inhibited by the high levels of F2,6P_2 at low temperatures. Reciprocally, PFK is activated by the high levels of F2,6P_2 at lower temperatures (Holden & Storey, in press b). The concerted action of FBPase and PFK allows the insect to effectively shut down gluconeogenesis while glycerol is produced thus facilitating cryoprotectant synthesis. The greater affinities for substrates and activators, and the decreased inhibition of the phosphorylated form give means for strong regulation of the enzyme by reversible phosphorylation.

Conclusion

The ability of the larvae of *Epiblema scudderiana* to maintain FBPase in a less active dephosphorylated form until needed allows for tight control of gluconeogenesis at this locus. FBPase is found in both the more active phosphorylated and less active dephosphorylated form throughout the time course. Kinetic studies show that the phosphorylated form has stronger affinities for substrates and activators, and is less inhibited by AMP and F2,6P_2 . The levels of F2,6P_2 during the initiation of glycerol production are high enough to inhibit both forms of FBPase and activate PFK. Thus, gluconeogenesis can be virtually shut-off at this locus during cryoprotectant synthesis. This effect is furthered by the decrease in the percentage of the active form of FBPase

present at lower temperatures. More importantly, the turning-off of gluconeogenesis by FBPase may prevent the intermediates of glycerol synthesis (eg GAP, F1,6P₂, DHAP) from being sucked back into glycogen synthesis. Metabolic depression of the glycerol pathway throughout the winter months is assisted by the maintenance of close to 50% of the enzyme in the less active dephosphorylated form, thereby reducing the possibility that carbon will be cycled back into gluconeogenesis. During the warmer spring months, glycerol must be catabolized in order alleviate energy needs and resume a normal metabolism. The increased percentage of the more active phosphorylated form assists in activation of gluconeogenesis, thus the catabolism of glycerol.

CHAPTER FIVE
PYRUVATE KINASE
FROM THE LARVAE OF FREEZE AVOIDING GALL MOTH
EPIBLEMA SCUDDERIANA

INTRODUCTION TO PYRUVATE KINASE

Pyruvate kinase (PK) (ATP: pyruvate O²-phosphotransferase, EC 2.7.1.40) plays an important role in metabolism as its activity can influence the rates of both gluconeogenesis and glycolysis (Chang et al, 1987). PK catalyses the phosphoryl-group transfer from PEP to ADP forming ATP and pyruvate. Four isozymes of PK have been isolated from mammalian tissues, L, M¹, M², and R, with M¹ being the most stable (Zamora et al, 1992). PK from various sources displays reversible phosphorylation-dephosphorylation (Siebenaller, 1979; Hakim et al, 1984). Generally, the phosphorylated form of the enzyme has been found to be the less active than the dephosphorylated form (Zomara et al 1992; Siebenaller, 1985; Brooks & Storey, 1990; Papadopolus et al, 1989) For example, the phosphorylated form of PK from anoxia tolerant whelk (*B. canaliculatum*) displays a higher K_m value for PEP and a lower I⁵⁰ for alanine (Brooks & Storey, 1992).

Two studies have shown how reversible covalent modification of PK allows for tight regulation of the enzyme. A study of the earthworm *Allolobophora calliginosa* reveals that PK is regulated when the worm experiences anaerobic conditions such as natural habitat flooding. The results indicate that anoxia may cause a covalent modification of PK. The modification results in a less active form of PK and thus the reduction in the production of L-lactate. After a few hours of exposure to anoxia, the PK from the earthworm becomes phosphorylated to its inactive form. This regulation of PK (conversion to the less active form) allows the earthworm to slow down metabolism, by controlling the amount of carbon that enters the citric acid cycle, thereby metabolically depressing in order to survive anaerobic conditions (Papadopoulos et al, 1989).

Studies of the land snail *Otala lactea* have shown that reversible phosphorylation of PK is one mechanism contributing to the overall metabolic rate

depression of the estivating state. PK from three tissues of the land snail underwent stable modification (phosphorylation) upon entry into estivation as a response to anoxia. Protein kinases were able to phosphorylate PK while phosphatases were able to dephosphorylate PK from foot muscle in estivating snails. Overall, the changes in the kinetic properties of PK, brought about by estivation or anoxia in the three tissues of the snail, can be attributed to the less active phosphorylated form of the enzyme (Whitwam & Storey, 1990).

Overall, the snail, whelk, and worm studies show that anoxia and/or estivation leads to a less active enzyme form that has characteristics that include lower affinities of substrates and activators, as well as a decrease in sensitivity to inhibitors. Thus there is evidence of phosphorylation-dephosphorylation regulation with respect to environmental stresses, as well as glycolytic/gluconeogenic demands.

Pyruvate kinase from the larvae of *Epiblema scudderiana* may play an important role in the overwintering of the insect. Covalent modification through reversible phosphorylation may inhibit the enzyme during glycerol production. The less active enzyme would slow down the flow of carbon into the citric acid cycle, thus carbon may be diverted from glycolytic flow into glycerol production. The less active form of pyruvate kinase, by regulating the flow of carbon into the citric acid cycle, may assist in depression of carbon flow as it occurs in other animals.

MATERIALS AND METHODS

Animals

The animals used for the study of pyruvate kinase were the seasonal time course larvae and the 15 and -4°C acclimated laboratory larvae.

Enzyme Preparation & Purification

Samples of the frozen larvae were homogenized, with a few crystals of PMSF added, 1:10 w/v in buffer A. Buffer A contained 20 mM imidazole-HCl, 100 mM NaF, 5 mM EDTA, 5 mM EGTA, and 15 mM 2-mercaptoethanol, pH 6. The homogenate was stored on ice until it was centrifuged at 13,000 r.p.m. for 20 min in a refrigerated Sorvall RC-5B centrifuge set at 5°C. The supernate was separated from the pellet and fat layer and desalted by centrifugation (1 min at top speed on a desk top centrifuge) through a G-25 Sephadex column equilibrated in buffer A. The eluant was collected and placed on a CM-cellulose column (7 cm x 1.5 cm) equilibrated in buffer A. The column was washed with 20 ml of buffer A and then pyruvate kinase was eluted with a 20 ml linear gradient of 0-300 mM KCl in buffer A. One ml fractions were collected and assayed for PK activity.

This procedure was performed for samples of the larvae from the entire time course, as well as the 15 and -4°C laboratory acclimated larvae, in order to determine the percentage of phosphorylated and dephosphorylated enzyme forms present. The CM-cellulose column was able to separate the two forms and incubations were performed to determine the order in which they were eluted.

In vitro Phosphorylation and Dephosphorylation Incubations

Incubations with the following compounds were used in order to convert the enzyme into either its phosphorylated or dephosphorylated form. Homogenates were prepared as described through to the centrifugation step. At this point, the supernatant was separated into two portions. The first was centrifuged through a G-25 column equilibrated in buffer B (Buffer A minus NaF, EGTA, and EDTA) at pH 6. A 0.5 ml portion of the eluant was mixed with 0.5 ml of buffer B plus 10 mM MgCl_2 ; this incubation promoted the dephosphorylation of the enzyme. The second portion was centrifuged through a G-25 Sephadex column equilibrated in buffer C (buffer A minus EDTA and EGTA). A 0.5 ml fraction was mixed with 0.5 ml buffer C plus 1 mM cAMP, 10 mM MgCl_2 , and 10 mM ATP; this procedure promoted phosphorylation of the enzyme. Both portions were left to incubate 24 h in a 4°C refrigerator. The next day the samples were desalted by centrifuging through a G-25 column equilibrated in buffer A. The eluants were then chromatographed on CM-Cellulose columns as described above.

Enzyme Assay & Kinetics

Pyruvate kinase activity was determined via a coupled enzyme assay that monitored the conversion of NADH to NAD^+ at 340 nm using a Gilford 260 spectrophotometer. The optimal assay conditions were found to be: 2 mM PEP, 2 mM ADP, 5 mM MgCl_2 , 10 mM KCl, 0.15 mM NADH, and 1 unit of the coupling enzyme lactate dehydrogenase.

SCHEME 5.1 PYRUVATE KINASE ANALYSIS COUPLED TO LACTATE DEHYDROGENASE

Kinetic studies measuring the K_m of PEP, K_a of aspartate, K_a of phosphate, I_{50} of L-alanine, and I_{50} of citrate-Mg were carried out on the partially purified phosphorylated and dephosphorylated forms of pyruvate kinase from the 15°C laboratory acclimated larvae. K_a values were determined at suboptimal K_m of PEP concentrations, and I_{50} values at above optimal K_m of PEP concentrations. All values were reported as means $\pm$ S.E.M. with at least $n=3$. Statistical differences were determined via the Students-t test (two-tailed) with $P < 0.005$.

RESULTS

Enzyme Preparation and Incubations

CM-cellulose column chromatography showed that there were two forms of pyruvate kinase in *E. scudderiana*. Figure 5.1 shows the elution profile of a typical separation of the two forms from the 15°C acclimated animals. To determine whether these forms were isozymic forms or phosphorylated vs dephosphorylated forms of a single enzyme (PK from many sources is subject to reversible phosphorylation), *in vitro* incubations of the enzyme preparation were performed with conditions that promoted either the phosphorylation or the dephosphorylation of the enzyme by endogenous protein phosphatases or protein kinases. The results shown in Figure 5.2 indicate that the two peaks represented phosphorylation vs dephosphorylation enzyme forms. Incubations under conditions that promoted phosphorylation shifted virtually all the enzyme activity into a single peak eluting at 88 ± 5 mM KCl; whereas, incubations under conditions that promoted dephosphorylation shifted activity into a peak that eluted later at 144 ± 7 mM KCl. These two peaks each corresponded with one of the peaks identified in Figure 5.1 and allowed for identification of these two peaks as phosphorylated (eluting first) and dephosphorylated (eluting second) forms of the enzyme.

Figure 5.1 ELUTION PROFILE OF PYRUVATE KINASE ON A CM-CELLULOSE COLUMN

The enzyme, from 15⁰C acclimated larvae, was eluted with 0-300 mM KCl gradient. One ml fractions were collected and 100 ul of each fraction was assayed. Activities are given relative to the velocity in the peak tube.

Figure 5.2 EFFECTS OF in vitro INCUBATIONS UNDER CONDITIONS PROMOTING PHOSPHORYLATION OR DEPHOSPHORYLATION ON THE SUBSEQUENT ELUTION PROFILE OF PYRUVATE KINASE ON A CM-CELLULOSE COLUMN.

The representative plot shows the results of two separate incubations of 15°C acclimated larvae. The filled circles represent the forced phosphorylation activity by promoting endogenous cAMP-dependent protein kinase activity. The hollow triangles represent the forced dephosphorylation by promoting endogenous phosphatase activity. The enzyme forms were eluted with a linear gradient of 0-300 mM KCl. One ml fractions were collected and 100 ul of each fraction was assayed. Activities are given relative to the velocity in the peak tube.

Seasonal Changes in the Percentage of the Enzyme in the Phosphorylated vs Dephosphorylated form

Analysis of the seasonal changes in the proportion of the phosphorylated and dephosphorylated forms of PK in *E. scudderiana* (Figure 5.3) showed an early predominance of the dephosphorylated form as over 70% of the enzyme was in this form on September 15. The November 1 and January 4 time points showed the majority of PK in the phosphorylated form with the colder fall and winter temperatures. The spring months showed a return to a prominence of the dephosphorylated form. The figure also illustrates that at 15°C there was slightly more of the dephosphorylated form, but with a reduction of temperature to -4°C, PK was close to 60% in the phosphorylated form. To determine if the two enzyme forms differed in kinetic properties, kinetic studies were carried out.

Kinetics Analysis

Kinetic properties of both the phosphorylated and dephosphorylated forms of partially purified PK from the 15°C laboratory acclimated larvae are given in Table 5.1. Figure 5.4 shows the relative activity vs [PEP] for both enzyme forms. The K_m for the phosphorylated form was two fold greater than the dephosphorylated form. Activation of PK by L-aspartate and potassium phosphate are shown in Figure 5.5. The K_a values for L-aspartate and potassium phosphate increased 3 and 10 fold respectively, for the phosphorylated enzyme when compared with the

Figure 5.3 SEASONAL CHANGES IN THE PERCENTAGE OF PK IN THE PHOSPHORYLATED VS DEPHOSPHORYLATED FORMS IN EPIBLEMA SCUDDERIANA LARVAE

The forms of the enzyme were eluted from a CM-cellulose column with a 0-300 mM KCl linear gradient. The percentages were calculated through determination of areas of phosphorylated and dephosphorylated peaks. The filled bars represent the dephosphorylated form while the hollow bars, the phosphorylated enzyme. All values are means $\pm$ S.E.M.

* Significantly different from the corresponding value of the dephosphorylated enzyme form by the Student's t-test (two-tailed) $P < 0.01$; ** $P < 0.001$.

TABLE 5.1 PROPERTIES OF PHOSPHORYLATED AND DEPHOSPHORYLATED FORMS OF PYRUVATE KINASE FROM 15°C ACCLIMATED PARTIALLY PURIFIED *E. scudderiana*

	DEPHOSPHORYLATED	PHOSPHORYLATED	
K _m PEP (mM)	0.06 ± 0.006	0.15 ± 0.009	**
K _a L-ASPARTATE (mM)	0.69 ± 0.06	2.15 ± 0.89	**
K _a PHOSPHATE (mM)	0.49 ± 0.01	4.94 ± 0.49	**
I ₅₀ ALANINE (mM)	23.6 ± 0.64	13.0 ± 0.46	**
I ₅₀ CITRATE-Mg (mM)	2.21 ± 0.09	1.07 ± 0.02	*

DATA ARE MEANS ± S.E.M. FOR ALL WITH AT LEAST n = 3

DIFFERENT PREPARATIONS OF THE PURIFIED ENZYME. *

SIGNIFICANTLY DIFFERENT FROM THE CORRESPONDING VALUES FOR THE DEPHOSPHORYLATED ENZYME FORM BY THE STUDENT'S t-TEST (TWO-TAILED), P < 0.025; ** P < 0.005.

**Figure 5.4 PLOT OF VELOCITY VS SUBSTRATE
CONCENTRATION FOR PYRUVATE KINASE**

The representative plot illustrates the change in enzyme activity when substrate concentrations (PEP) are varied. Hollow circles give PK activity of the dephosphorylated form and filled circles give activity for the phosphorylated form. The velocity was relative and determined with partially purified enzyme.

Figure 5.5 ACTIVATION OF PYRUVATE KINASE

The representative plots illustrates the change in activity of PK when the concentration of aspartate and phosphate respectively, was varied while the optimal assay conditions remain the same (except 0.2 mM PEP). Hollow circles give PK activity of the dephosphorylated form and colored circles give activity of the phosphorylated form. The velocity were relative and determined with partially purified enzyme.

dephosphorylated enzyme. I_{50} values for L-alanine and citrate-Mg decreased 1.7 and 2 fold respectively, when comparing the dephosphorylated and phosphorylated forms of PK. The difference in inhibition is illustrated in Figure 5.6. The kinetics show that the dephosphorylated form of pyruvate kinase was the more active form.

FIGURE 5.6 INHIBITION OF PYRUVATE KINASE

The representative plots illustrate the change in the activity of PK when the concentrations of alanine and citrate-Mg were varied, respectively, while the optimal assay conditions remain the same (except 0.04 mM PEP). Hollow circles give PK activity for the dephosphorylated form and filled circles give activity for the phosphorylated form. The velocity was relative and determined with partially purified enzyme.

DISCUSSION OF PYRUVATE KINASE

Pyruvate kinase (PK) may play an important role in the production of cryoprotectants by regulating carbon flow into the citric acid cycle. The enzyme is known to be regulated allosterically by a number of activators and inhibitors. Reversible phosphorylation has also been shown to regulate pyruvate kinase in numerous species (Siebenaller, 1979; Hakim et al, 1984; Papadopolus et al, 1989). PK exists in a more active dephosphorylated and a less active phosphorylated form. Inhibition of PK during cryoprotectant synthesis may help divert glycolytic carbon flow into the synthesis of glycerol. As shown with other invertebrates (Whitwam & Storey, 1990), inhibition of pyruvate kinase also facilitates metabolic depression by suppressing the carbon flow into the citric acid cycle during periods of low metabolic needs.

In vitro Incubations & Seasonal Changes in the Percentage of the Phosphorylated and Dephosphorylated Form of PK

The ion exchange column, CM⁻ cellulose column, is able to separate two forms of pyruvate kinase. *In vitro* incubations show that the first peak corresponds to the less active phosphorylated form, whereas the second peak corresponds to the more active dephosphorylated form.

There are changes in the percentage of PK in the phosphorylated and dephosphorylated forms during the seasonal time course and when comparing the 15 and -4°C acclimated larvae. Figure 5.3 illustrates the changes in the percentage in the phosphorylated form of the enzyme. Comparison of the 15 and -4°C acclimated animals show that after prolonged exposures to low temperatures PK exists almost 60% in the less active phosphorylated form. The predominance of the less active form of PK

may allow for carbon to be diverted from this locus and redirected for the production of cryoprotectants. With fluctuating temperatures in September (see Table 2.1), there are corresponding changes to levels of PK as the insect may be maintaining a normal metabolism or preparing for the colder winter temperatures. The Figure shows that through the cold months of November and January there is a predominance of the less active phosphorylated form of PK, up to 80%. The large amount of the less active form of PK may assist the insect in the depression of the flow of carbon into the citric acid cycle. With most of PK maintained in the less active form, there would be less carbon moving into the citric acid cycle due to the reduced energy needs. During the later warmer months, there is a predominance of the active phosphorylated form of PK. This increase in the percentage of the active form of PK may be due to the return of a normal metabolism with the onset of warm temperatures and glycerol catabolism as high levels of the cryoprotectant are no longer needed.

Kinetic Analysis

The kinetic properties show that the dephosphorylated form is the more active form of PK. The phosphorylated form of the enzyme is less susceptible to activation and more susceptible to inhibitors. The K_m for PEP of the dephosphorylated form is two-fold lower than the phosphorylated form. Churchill and Storey (1989a) have shown that an initial increase in the levels of PEP and pyruvate (the end product of the reaction PK catalyzes) occurs in response to a temperatures change from 22 to -4°C in *E. scudderiana*; but, both PEP and pyruvate returned to normal control levels after a few days at the lower temperature. These results may be correlated to the presence of both forms of PK. With the initial activation of glycolysis, there may be an increased flux of carbon to this locus; but, as time goes by, more of the active dephosphorylated

form of PK is converted to the less active phosphorylated form and pyruvate levels should decline. This would assist the animal in depressing the carbon flow through this locus.

The I_{50} values for L-alanine and citrate Mg^{2+} for the dephosphorylated form are 1.7 and 2-fold higher respectively, than the phosphorylated. Churchill and Storey (1989a) have showed that L-alanine increase over time in response to cold temperatures in *Epiblema scudderiana*. The study shows that with the decrease in PEP and pyruvate levels there is a corresponding increase in the levels of L-alanine. As the enzyme was being converted into its less active form in response to prolonged cold temperatures, it is furthered inhibited by the high levels of L-alanine thereby preventing carbon from entering the citric acid cycle.

Conclusion

The ability of the larvae of *Epiblema scudderiana* to convert PK to a more active dephosphorylated form or less active phosphorylated form allows for tight regulation at this locus. The enzyme in the dephosphorylated form shows a greater affinity for substrates and activators and is less affected by inhibitors. The enzyme, after prolonged exposure to $-4^{\circ}C$, is converted to the less active form (close to 60%) to assist in the depression of carbon flow into the citric acid cycle and help redirect carbon into glycerol production. This effect is furthered by the presence of high levels of the inhibitor L-alanine which inhibits the less active phosphorylated form to a greater extent at lower concentrations. In mid-winter, with temperatures nearing $-20^{\circ}C$, PK is found to be maintained in the less active phosphorylated form (close to 80%). This may assist the insect to further depress carbon flow at this locus. With the return of warmer spring temperatures there is a predominance of the more active phosphorylated form, close to 90%. This may allow for the normal return of metabolism and the catabolism

of glycerol as high levels of glycerol would not longer be needed. The results show that PK is carefully regulated by reversible phosphorylation. This covalent modification allows the enzyme to be converted to a more/less active form depending on the environmental and physiological need.

CHAPTER SIX
GENERAL DISCUSSION

Larvae of the insect *Epiblema scudderiana* are able to survive ambient winter temperatures as low as -40°C . These animals survive prolonged exposure to low temperature by a strategy known as freeze avoidance. The insect maintain a liquid state by depression of freezing and supercooling points, and by inhibiting the formation of ice with the aid of thermal hysteresis proteins (Zachariassen, 1985; Storey & Storey, 1989). The insect use the cryoprotectant glycerol to help maintain a liquid state. Glycerol is a polyhydric alcohol that can be accumulated up to 2 M, or 20% of the insects body mass (Zachariassen, 1985). Glycerol provides excellent protection for the insect, it facilitates the depression of the insects freezing point to -40°C at concentrations of 2 M; is nontoxic and highly soluble in aqueous solutions; and is able to penetrate cellular surfaces easily. These features enable glycerol to move around macromolecules, such as proteins and cellular organelles, thus protecting them from denaturation or damage due to cold temperatures. In addition, glycerol has few effects on metabolic processes even at high concentration (Zachariassen, 1985; Storey & Storey, 1988).

Epiblema scudderiana has been studied extensively in the past. Rickards et al, (1987) (Figure 1.1) have illustrated that with the drop in mean temperature there is a rapid conversion of glycogen to glycerol with a corresponding decrease in the supercooling point of the insect. Churchill and Storey (1989 a, b) have shown the changes in the levels of intermediates and metabolites associated with glycerol synthesis in response to low temperatures. Figure 1.5 illustrates that with prolonged exposure to freezing glycerol and F2,6P₂ levels increase. The levels of G6P, F1,6P₂, and glucose all rose with freezing but fell to control levels after prolonged exposure to cold temperatures. The same authors have shown that PEP and pyruvate levels increase as a response to cold temperature, but quickly fall to below control levels. The levels of L-alanine increase and remain high with exposure to cold temperatures.

Holden and Storey (in press a, b, c, d) have explored a number of enzymes that play important roles in the insects overwintering survival. They have shown that with the onset of cold temperatures glycogen phosphorylase is converted to its more active form, and with lower temperatures the enzyme is temperature modulated to an even more active form. The more active glycogen phosphorylase assisted in the rapid conversion of glycogen to glycerol (in press d). The same authors (in press b) have shown that PFK is activated at lower temperatures. High levels of F2,6P₂ are able to effectively activate PFK to produce F1,6P₂ (the substrate for aldolase) at cold temperatures. The enzyme aldolase, which produces G3P, is temperature modulated to a more active form thus allowing for the production of glycerol (in press c). The authors (in press a) also have shown that FBPase is effectively shut-off during glycerol production but is once again activated during the warmer temperatures to assist in glycerol catabolism.

This thesis studied the regulation of four enzymes that play key roles in the production of glycerol at low temperatures, and are involved in catabolism of glycerol with the return of warm temperatures.

Glycogen synthetase from *Epiblema scudderiana* exists only in the less active D form throughout the winter time course. The presence of the less active form of the enzyme ensures that carbon is not futilely recycled while glycerol is being produced. These effects are further magnified by the temperature modulation to a less active form of glycogen synthetase D. At low temperatures, the enzyme is regulated with lower affinities for the substrates UDPG and glycogen, and the allosteric activator G6P. A combination of inhibition of glycogen synthetase and activation of glycogen phosphorylase, at lower temperatures, allows for the quick conversion of glycogen to G1P. The two enzymes in concert allow for tight control of this locus.

Hexokinase undergoes a conformational change at low temperatures. The enzyme is kinetically regulated at low temperatures showing a stronger affinity for

activators and being less sensitive to inhibitors. Glycerol synthesis results in the production of glucose. Glucose is a strong inhibitor of glycogen phosphorylase and an activator of glycogen synthetase. If glucose is accumulated and levels remain high, glycogenolysis may be inhibited. These effects are nullified by the action of hexokinase. The enzyme converts glucose to G6P. The end product of the reaction, G6P, is a feedback inhibitor of hexokinase. The lower sensitivity of the enzyme for this inhibitor at low temperatures allows hexokinase to assist in the prevention of the accumulation of glucose at the onset of cryoprotectant synthesis.

FBPase is found to be reversibly phosphorylated in *Epiblema scudderiana*. Kinetic studies have shown that the phosphorylated form is the more active form with stronger affinities for substrates and activators, and is less inhibited by AMP and F2,6P₂. During glycerol production, FBPase is predominantly in its less active form, thereby not interfering with glycolysis. This effect is furthered by the high levels of F2,6P₂. The high levels of F2,6P₂ not only inhibits FBPase but activate PK, thus furthering aiding in the production of glycerol. The high levels of F2,6P₂, coupled to high levels of FBPase maintained in the less active form (up to 50%) allow the insect to slow down F1,6P₂ production, thereby aiding in depression of gluconeogenesis. With warmer spring temperatures, glycerol must be catabolized. FBPase is converted to the more active form in order to facilitate gluconeogenesis and glycerol catabolism.

Pyruvate Kinase exists in two reversible phosphorylated forms in *Epiblema scudderiana*. Kinetic studies have shown the dephosphorylated form of the enzyme to be more active with stronger affinities for substrate and activators; and, to be less affected by inhibitors. With prolonged exposure to low temperatures, the animal converts PK to the less active phosphorylated form (close to 60%). This results in a decrease in the flow of carbon into the citric acid cycle, and the possible diversion of glycolytic carbon into glycerol production. These effects are illustrated by the low levels of pyruvate and PEP after prolonged exposure to cold temperatures (Churchill

and Storey, 1989a). High levels of the inhibitor L-alanine also help to regulate the enzyme at lower temperatures by inhibiting PK. With stable mean temperatures reaching -20°C , the larva converts PK to the less active phosphorylated form (close to 80%). This ensures the flow of carbon is stopped at this locus. With the return of warmer temperatures, PK is converted to the more active dephosphorylated form (close to 90%). The presence of the more active form of PK may indicate that catabolism of glycerol has begun, and the insect has resumed normal metabolic rates.

Glycerol synthesis can be regulated by a complex set of controls. This study shows that all four enzymes have a role in the synthesis of glycerol in response to cold temperatures. The two enzymes involved in glycogen synthesis, glycogen synthetase and FBPase, are effectively shut-off by reversible phosphorylation and temperature modulation that allows for a kinetically less active enzyme. Hexokinase is activated to prevent inhibition by glucose, and directs carbon back into glycolysis; pyruvate kinase is shut down thereby diverting carbon to the production of glycerol. Once cold temperatures have been obtained, depression of the pathways allows the animal to survive throughout the winter. With the return of warmer temperatures, the insect must catabolize glycerol through gluconeogenesis and return to normal warm temperature metabolism. PK and FBPase are converted to their active phosphorylation forms in order to compensate for the increased metabolic activity.

The study has shown that the four enzymes from the insect *Epiblema scudderiana* are tightly regulated to control metabolic needs and survive overwintering. The enzymes studied may be regulated by thermal modulation, by covalent modification (reversible phosphorylation-dephosphorylation), by different affinities for substrate and allosteric effectors at low temperatures, or by any combination of the three. These regulations allow the insect to produce high levels of glycerol to supercool, survive the winter, and recover during the warmer temperatures.

FUTURE STUDIES

Although many studies have provided clues to the understanding of freeze avoidance in the insect *Epiblema scudderiana*, there are many questions left to be answered. First, studies of the late summer larvae may show how enzymes can control the production and storage of large quantities of glycogen. These studies may elucidate a further understanding of the regulation of the enzymes already studied, and provide clues needed for a complete understanding of the metabolic control of glycerol synthesis.

Second, synthesis of polyols requires reducing power in the form of NADPH or NADH. Reducing equivalents are produced through the hexose monophosphate shunt (HMS). The most effective method of polyol synthesis is through both glycolytic and HMS routes. If this is the case with *Epiblema scudderiana* then the enzymes glucose 6-phosphatase and polyol dehydrogenase (both involved in HMS) should be present and may be studied.

Finally, in order to produce glycerol the insect must be able to control the levels of glycerol-3-phosphatase (the enzyme that converts G3P to glycerol). Studies on the regulation of this enzyme, as well as its interactions with other key regulatory enzymes may give a better understanding into glycerol production in *Epiblema scudderiana*.

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