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GLYCOLYTIC ENZYME BINDING AND METABOLIC CONTROL

by

JOHN ANDREW DUNCAN

B.Sc. (Hons.), Carleton University, 1986

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

Master of Science

in the department

of

BIOLOGY/BIOCHEMISTRY

CARLETON UNIVERSITY

July, 1988

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Master of Science

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Chairman, Department of Biology

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Supervisor

CARLETON UNIVERSITY

July, 1988

ABSTRACT

Several animals are capable of altering their metabolic rate considerably in order to endure severe environmental stresses. Changes in glycolytic enzyme binding in response to physiologically relevant stresses including anoxia and exercise were examined in three animal models: goldfish (Carassius auratus); crayfish (Orconectes virilis); and freshwater turtles (Pseudemys scripta elegans). To determine the validity of observed binding changes different fractionation procedures were compared including: a) dilution, b) quick press, c) airfuge, and d) triton solubilization methods. All three models exhibited binding changes in response to induced metabolic stresses. Goldfish heart enzymes demonstrated increased binding correlated with increased glycolytic rate in response to anoxia but no significant changes following exercise. Crayfish tail muscle exhibited a general decrease in glycolytic enzyme binding in response to anoxia with drops in percent bound averaging 6-10%. Exercise only yielded significant changes for phosphofructokinase, 20% to 28% bound, and phosphoglycerate kinase, 27% to 23% bound. In turtles, results obtained with the dilution and triton fractionation methods demonstrated that anoxia induced a general increase in glycolytic enzyme binding in all tissues examined (heart, brain, red and white skeletal muscle), the increase being most prominent in red and white skeletal muscle. The

different methodologies used are critically compared and the results discussed with respect to the role of enzyme binding in metabolic rate control.

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LIST OF ABBREVIATIONS

PFK - phosphofructokinase

PK - pyruvate kinase

CK - creatine kinase

LDH - lactate dehydrogenase

HK - hexokinase

GAPDH - glyceraldehyde-3-phosphate dehydrogenase

PGK - phosphoglycerokinase

ADH - alcohol dehydrogenase

AK - arginine kinase

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

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

creatine-P, CrP - creatine phosphate

DNA - deoxyribonucleic acid

m-RNA, r-RNA - messenger and ribosomal ribonucleic
acid, respectively

F-2,6-P₂ - fructose-2,6-bisphosphate

h - hour(s)

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GENERAL INTRODUCTION

An organisms' extraction of maximal chemical bond energy from complete combustion of reduced organic molecules to CO_2 and H_2O typically requires molecular oxygen. However, many organisms experience periods of hypoxia or complete anoxia lasting from minutes to months. While all animals are capable of withstanding short periods of oxygen lack only some species are so tolerant that they can be considered facultative anaerobes. Invertebrate groups demonstrate the most outstanding anaerobic abilities in terms of duration of anoxia tolerance yet some vertebrates are also impressive in their ability to withstand lengthy periods of little or no oxygen. Among these latter animals are the goldfish, Carassius auratus, and some freshwater turtles which are both able to withstand up to months of complete anoxia.

For an animal to survive anoxia certain provisions must be made. Of obvious importance, sufficient substrate stores need to be maintained and mechanisms in place for the regulation of substrate utilization such that internal stores are not depleted fully. Thus the energy requirements of all tissues and organs can be matched to substrate availability. This provision can be achieved by the storage of endogenous substrate in non-central depots, by utilizing energetically more efficient fermentation pathways and/or by depressing metabolic rate and thus the demand for substrate (Hochachka and Somero, 1984).

Typically the consumption of carbohydrate is about seven fold lower under aerobic conditions than under anaerobic ones (Stryer, 1981). This inhibition of glycolysis by aerobic respiration is now termed the Pasteur effect after its discovery in yeast by Louis Pasteur. Much of this effect is accounted for by the inhibition of PFK by citrate and ATP, the levels of which decrease in anoxia leading to the apparent activation of glycolysis (Stryer, 1981). This activation is used to maintain ATP levels by fermentation, which is much less efficient than respiration, to enable survival of anoxic periods. However this mechanism is only effective for very short periods rapidly depleting carbohydrate reserves and building up toxic end products (Krebs, 1972). Yeasts and some bacteria employ the Pasteur effect utilizing potentially unlimited substrate reserves in their growth medium and excreting end products back into the medium permitting longer term anoxic survival (Storey, 1985a).

It was a long held belief that the Pasteur effect was the normal response of cells to anoxia due to its conspicuous appearance in the major model systems used. Animals which did not exhibit this effect were considered anomalies. However most multicellular organisms with a true facility for long-term anaerobiosis do not exhibit a Pasteur effect in response to anoxia and instead display a metabolic depression (Storey, 1985). This effect has the advantage of

reducing the requirement for ATP at the same time as reducing the depletion of carbohydrate reserves and the accumulation of toxic end products and has been well documented in several invertebrates and vertebrates (Storey, 1985a). While these animals may have different triggers initiating entry into the hypometabolic state and unique mechanisms to extend survival in this state, they nevertheless share the basic mechanisms of metabolic depression. Potential mechanisms mediating metabolic rate depression include 1) the covalent modification of regulatory enzymes, 2) control of key regulatory enzymes by changes in the levels of effectors and, 3) changes in the proportion of enzymes associated with the particulate fraction of the cell.

Since anaerobic metabolism results in the production of toxic waste products which can have deleterious effects on the organism and will at the very least limit anoxia tolerance, provision must be made to minimize, store, cycle, tolerate or excrete such products. Again several mechanisms can be employed to effect a solution including: minimizing product accumulation by depressing metabolism; maintaining high buffering capacities of blood or tissues to tolerate high accumulation; and detoxifying end products by further metabolism to less noxious or excretable compounds (Hochachka and Somero, 1984). One of the greatest problems to be dealt with by facultative anaerobes undergoing anoxia

is the accompanying acidification and while the above mentioned mechanisms can reduce the threat, the utilization of H^+ -consuming reaction pathways can be used as a supplementary mechanism.

Adaptation to enable survival of hypoxic or anoxic conditions can be expected to occur in different parts of the energy metabolism encompassing both the way in which ATP is produced as well as the way it is used. Basically metabolism must be altered such that non-essential processes are reduced or inactivated while the balance between essential processes is maintained or re-established (Storey, 1985a). Since glycolysis is the only energy producing pathway capable of operating in the absence of molecular oxygen it constitutes an essential pathway and has been the focus of the majority of research into hypometabolism. Glycolysis is the primary energy producing pathway operating in facultative anaerobes undergoing anoxic stress, including the goldfish, crayfish and turtle examined here, and hence the focus of this research.

6
Conventional wisdom once dictated that the agents of metabolism existed in dispersed array in the aqueous cytoplasm of cells with all reactions aided solely by the physical forces of diffusion. Experimental evidence has since fostered re-examination of this tenet to the extent that a new understanding has emerged.

Recently, a variety of investigative approaches have lent support to the new credo whereby enzymes function in some kind of structured regime. The earliest hints of this philosophy came with the discovery that certain metabolic processes were partitioned off into physical compartments within the cell. DNA replication and m-RNA/r-RNA synthesis occur in the nucleus; glycolysis, the pentose phosphate pathway and fatty acid synthesis occur in the cytosol; fatty acid oxidation, the citric acid cycle and oxidative phosphorylation occur in mitochondria. Higher levels of organization require interactions between metabolic machinery and elements of the cytomatrix or between enzymes of a pathway. Such interactions have been most fastidiously explored for enzymes of the glycolytic sequence although the inherent metabolic advantages which these interactions could confer are not limited to the proverbial 'glycolytic' complex but can be applied to most, if not all, metabolic processes (Srere, 1987). To date, considerable evidence has accumulated demonstrating the metabolic advantages of enzyme binding (Clarke and Masters, 1974, 1975, 1976; Knull, 1971,

Bronstein and Knull, 1981; Clarke and Morton, 1982; Clarke et al., 1984; Plaxton and Storey, 1986).

Possibly the greatest advantages of a structured, organized enzyme system are mechanistic in origin. If enzymes in a pathway are sequentially ordered and in such a way that intermediates are passed directly from one enzyme to the next then the transit times for these intermediates would be reduced by eliminating the random diffusion in three dimensions which is an inherent component of any soluble enzyme system (Hubscher et al., 1971; Ottaway and Mowbray, 1977). This 'channeling' of intermediates in one or two dimensions results in a more rapid overall catalytic rate. It is possible that the development of such a mechanism to escape reliance on random processes such as diffusion may have been an important event in the evolution of eukaryotic cells (Clegg, 1984).

More rapid catalytic rates enable faster equilibration to steady state following perturbations in a pathway such as might be encountered in anoxia. Catalytic rates can also be altered by changes in the kinetic parameters of certain key enzymes. Such allosteric, regulatory effects have been demonstrated to be induced by the binding of an enzyme to a structural component of a cell or to other enzymes (Luther and Lee, 1986; Arnold and Pette, 1970; Walsh et al., 1977; Liou and Anderson, 1980; Kuo et al., 1986). Demonstrated kinetic effects of binding constitutes convincing evidence

supporting enzyme organization within the structural framework of the cell particularly in light of other lines of evidence.

Channeling allows for the segregation of potentially competing pathways preserving an intermediate for the most appropriate pathway at a given time and avoiding the energy costs of futile cycling. Intermediates which are unstable in aqueous environments are also protected and a high effective concentration of intermediate can be maintained with relatively few substrate molecules permitting a low intracellular concentration of intermediates. This latter hypothetical advantage has some experimental support from data indicating that only substrate and product of certain pathways are accumulated in the cell to an appreciable degree with intervening intermediates presumably bound up in the enzyme complex rather than released to the aqueous cytoplasm (Srivastava and Bernhard, 1986; Westerhoff, 1985). Interactions of sequential enzymes with the cytoskeletal matrix of a cell may also help modulate the properties of that cytoskeleton (Morton et al., 1982). All of these potential advantages confer improved efficiency on a system or pathway and have been demonstrated experimentally.

A wide variety of techniques have been applied to determine the presence, extent, and functional significance of enzyme binding. These include isotopic tracer studies,

histochemical techniques,, immunofluorescence, nuclear magnetic resonance, binding studies with purified components, physiochemical and permeabilization experiments, and developmental studies (for examples see Srere, 1987).

The most convincing evidence comes from an abundance of studies whereby enzyme activity was measured directly in separated soluble and particulate fractions of cells. This work has demonstrated changes in binding in response to physiological and non-physiological stresses and has revealed appropriate kinetic effects on specific glycolytic enzymes in response to their reversible association with structural elements or other enzymes of glycolysis (for a review see Srere, 1987; Srivastava and Bernhard, 1986).

In 1965 Green reported the disruption of erythrocytes and yeast cells and the derivation of fractions which were capable of catalyzing the complete sequence of glycolytic reactions with greatly increased specific activity over that of the whole homogenates (Green et al., 1965). Many other animal systems have been studied since by a variety of methods used to separate bound and soluble fractions to the common conclusion that interactions of glycolytic enzymes with cytoskeletal components can and do occur within the cellular environment. The majority of these binding experiments involve the shearing of plasma membranes by homogenization and subsequent centrifugation to sediment out

the particulate fraction. Both supernatant and resuspended pellet are then assayed for soluble and bound enzyme activity respectively.

Arnold and Pette demonstrated the dependence of enzyme association with the particulate phase of the cell on ionic strength when they reported complete desorption of aldolase at the assumed intracellular ionic strength of 150 mM KCl (Arnold and Pette, 1968). Desorption by physiological levels of various metabolites and adenylates as well as inorganic phosphate has also been demonstrated making it theoretically possible that by diluting the levels of these in the microenvironment of an enzyme or enzymes one may induce degrees of binding which are unrelated to those 'in vivo' (Arnold and Pette, 1968). However, studies employing conditions of physiological ionic strength and high protein concentration have demonstrated significant amounts of glycolytic enzyme association (Clarke and Masters, 1975; Westrin and Backman, 1983).

It is commonly understood now that the aqueous cellular environment is a highly concentrated solution and although limited knowledge of the exact nature of this cellular soup renders mimicry of it in buffers impossible recent binding studies have attempted to employ more physiologically relevant solutions. In addition to the highly concentrated thixotropic protein solution that is the cell cytoplasm

there is also the pervasive cytoskeletal network composed of actin, microtubule and intermediate filament systems, which imparts a high degree of structure to the cell and cytoplasm.

Besides using physiologically relevant buffers in fractionation alternate methods of separating soluble and particulate phases have been developed which eliminate some of the aforementioned problems. Employing a detergent to solubilize the cell membrane without disturbing the structural scaffolding of the cytoskeleton may offer an advantage over disruption by homogenization (Lowery et al., 1987). Squeezing of tissue samples under high pressure yields a fractionation without the attendant problems introduced by non-physiological media (Ross and Hultin, 1980; Amberson et al., 1964; Amberson et al., 1965). These extraction methods have produced results which constitute some of the evidence for the existence and functional significance of enzyme binding.

To ascertain any functional purpose for enzyme associations it is best to examine them in tissues or animals which undergo large changes in metabolic state. Thus, if corresponding and reversible changes are noted in enzyme associations one can infer a functional relationship and look for further evidence of its precise nature.

Clarke and coworkers subjected perfused rat hearts and 'in situ' sheep hearts to anoxia or ischaemia measuring the activity of various glycolytic enzymes in the soluble and particulate fractions separated by centrifugation of homogenized tissue. In both animal hearts ischaemia induced significant increases in the association of PFK and aldolase with the particulate fraction (Clarke et al., 1984). The resultant drop in pH in response to ischaemia was thought to be a factor since simple anoxia in the perfused rat heart yielded less drastic effects. Subsequent binding with isolated myofibrils and aldolase confirmed that binding increases considerably as pH is lowered over a range which is physiologically relevant to the ischaemic heart (Clarke et al., 1984). These results implicate changes in glycolytic enzyme organization with the regulation of metabolic rate in response to physiologically relevant stimuli. As with Clarke's earlier work with electrically stimulated skeletal muscle, increased binding was correlated with increased glycolytic activity necessitated by the requirement for glycolysis to meet all energy demands alone (Clarke and Masters, 1973, 1974).

The same techniques have been applied in more natural systems with similar results correlating metabolic rate changes with enzyme binding changes. The channelled whelk, an anoxia tolerant marine gastropod, demonstrated anoxia induced decreases in glycolytic enzyme association with the

particulate cellular fraction in conjunction with a general metabolic rate depression (Plaxton and Storey, 1986). Glycolysis appears to be part of this depression as indicated by other anoxia induced changes including the phosphorylation of the key enzymes PFK and PK which renders them less active and the drop in levels of the key allosteric activator of PFK, F-2,6-P₂ (Storey, 1984; Plaxton and Storey, 1984; Storey, 1985). The effect was reversed upon a return to aerobic conditions thus the general relationship between enzyme associations and metabolic demand fits well with that established by Clarke and co-workers with the degree of glycolytic enzyme binding being directly related to the metabolic rate of a given tissue.

With the abundance of recent evidence it now appears evident that enzyme partitioning between soluble and particulate phases of a cell is one of several components acting in a concerted manner to control cellular metabolism in response to environmental stresses. However, elucidation of the relative importance of enzyme binding to metabolic control remains to be quantitated for any of the numerous organisms the phenomenon has been demonstrated in. The objective of this research is to examine enzyme binding changes in different tissues occurring in a whole animal in response to a physiologically relevant metabolic stress. These changes may operate to effect tissue and organismal specific differences in metabolic rate.

MATERIALS AND METHODS

MATERIALS AND METHODS

Chemicals and animals

All biochemicals and coupling enzymes were purchased from Sigma Chemical Co. or Boehringer Mannheim. All buffers used were adjusted to their respective pH at 20 °C. Note that the quick immersion of animals into anoxic environments represents a shock anaerobiosis and may not induce the same changes as the slow acclimation to anaerobiosis encountered in nature.

Goldfish, Carassius auratus, were obtained locally and maintained in circulating, aerated water at 7 °C. Animals were fed ad lib. on goldfish pellets and fasted overnight prior to use. Control fish were gently netted from the holding tank and killed by a blow to the head. Tissues were dissected out (within seconds) and processed immediately. For anoxia experiments, fish were transferred to sealed aquaria (max. 3 fish per tank) containing water that had been bubbled with nitrogen gas for 9 hours previously. Bubbling with nitrogen gas continued alongside bubbling with carbon monoxide for the next 21 hours. The tanks were bathed in circulating 7 °C water throughout. Fish were sampled at the end of the 21 h anoxic period as for controls. Exercised fish were isolated in a separate tank containing stock tank water at 7°C and induced to perform burst work by tail pinching to exhaustion. Exhaustion was

obtained at a mean time of 30 minutes. Control and experimental fish exhibited little or no struggling upon removal from the tanks thus minimizing effects of stress or muscle activity.

Crayfish, Orconectes virilis were obtained from a local supplier and maintained in circulating water at 7 °C. Animals were fasted for the duration of the experimental period. Control crayfish were picked out of the holding tank with a minimum of disturbance (no tail flips) and sacrificed by severing the head just behind the eyes. Tissues were dissected out and processed immediately (within seconds). Animals to be exercised were placed one at a time in a shallow tray filled with water at 7 °C and chased by hand to induce tail flips which were counted as an indicator of the degree of exercise. The animals were then sampled as for controls. For anoxia experiments, crayfish were placed in containers of water at 7 °C which had been bubbled with N₂ for 9 h and were kept submerged by a wire mesh ceiling suspended 10 cm below the water surface. The 4 h and 12 h anoxic animals were kept in separate containers and sampled as for controls at the end of the anoxic period.

Turtles, Pseudemys scripta elegans, were obtained from Boreal Canada and maintained in circulating water at 7 °C in a large holding tank with a heat lamp and dry area available. Turtles were fed ad lib. on turtle pellets,

lettuce, and egg shells and fasted overnight prior to use. Control animals were taken quickly from the tank and sacrificed by decapitation. Tissues were dissected out and processed immediately. For anoxia experiments, turtles were transferred to sealed containers with water that had been bubbled previously with nitrogen for 9 h. Care was taken not to disturb the nitrogen gas layer on top of the water. The turtles were kept submerged by a wire mesh ceiling suspended 10-15 cm below the water surface and the entire container was placed in an incubator at 7 °C. At the end of the experimental period (5 h or 20 h) the animals were removed and sampled as for controls.

Enzyme assays

All enzyme assays were optimized for each animal and tissue. Enzymes were assayed using the following conditions: Hexokinase (HK): 50 mM Tris-HCl buffer (pH 8.0), 1mM D-glucose, 1 mM ATP, 1.5 mM MgCl₂, 1 mM NADP⁺ and excess NADP⁺-dependent glucose-6-phosphate dehydrogenase; Phosphofructokinase (PFK): 50 mM Tris-HCl (pH 8.0), 10 mM fructose-6-phosphate, 0.5 mM ATP, 5 mM MgCl₂, 50 mM KCl, 0.15 mM NADH and excess aldolase, triose phosphate isomerase and glycerol-3-phosphate dehydrogenase; Aldolase (ALD): 50 mM imidazole-HCl buffer (pH 7.0), 1mM fructose-1,6-bisphosphate, 2mM MgCl₂, 0.15 mM NADH and excess triose phosphate isomerase and glycerol-3-phosphate dehydrogenase;

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH): 100 mM Tris-HCl (pH 8.0), 0.9 mM EDTA, 2 mM $MgCl_2$, 1 mM ATP, 0.15 mM NADH, 20 mM 3-phosphoglycerate and excess phosphoglycerokinase; Phosphoglycerokinase (PGK): 100 mM Tris-HCl (pH 8.0), 0.9 mM EDTA, 2 mM $MgCl_2$, 1 mM ATP, 0.15 mM NADH, 20 mM 2-phosphoglycerate and excess glyceraldehyde-3-phosphate dehydrogenase; Pyruvate kinase (PK): 50 mM imidazole-HCl (pH 7.0), 50 mM KCl, 5 mM $MgCl_2$, 2 mM ADP, 5 mM phosphoenolpyruvate, 0.15 mM NADH and excess lactate dehydrogenase; Lactate dehydrogenase (LDH): 50 mM imidazole-HCl (pH 7.0), 2 mM $MgCl_2$, 0.15 mM NADH, 1 mM pyruvate; Arginine kinase (AK): 0.23 M Glycine-NaOH (pH 8.6), 5 mM $MgSO_4$, 50 mM KCl, 100mM Arginine, 1 mM ATP, 3 mM PEP, 0.15 mM NADH and, excess lactate dehydrogenase and pyruvate kinase; Creatine kinase (CK): 50 mM Tris-KOH (pH 8.2), 3 mM $MgSO_4$, 25 mM K_2SO_4 , 0.1 mM EDTA, 100 mM creatine, 10 mM 2-mercaptoethanol, 1 mM ATP, 3 mM PEP, 0.15 mM NADH and, excess lactate dehydrogenase and pyruvate kinase; Alcohol dehydrogenase (ADH): 50 mM Tris-HCl (pH 8.5), 100mM ethanol and 0.2 mM NAD^+ (Lowry and Passonneau, 1972). All enzyme assays were performed at 23°C and activities determined by following the reduction or oxidation of pyridine nucleotides at 340 nm using a Gilford recording spectrophotometer. Enzyme activities were corrected for non-specific NADP or NADH utilization (by subtraction of blanks run minus substrate) when present. One unit of enzyme activity is defined as the amount of enzyme resulting

in the utilization of 1 μ mol of NADP or NADH per min at 23°C.

Measurement of enzyme binding to the particulate fraction

All separations were performed at 4 °C.

(1) Dilution method based on that of Clarke et al., 1984.

Tissue samples were weighed and homogenized (1:4, w/v) in ice cold homogenization solution (HS): 250 mM sucrose with 10 mM 2-mercaptoethanol and 0.1 mM phenylmethylsulfonyl fluoride (PMSF) for 2 x 15 s using an Ultraturrax homogenizer at 80% of full speed. An aliquot (0.3 ml) of this homogenate was diluted (1:4, v/v) with a stabilization buffer (SB) containing 100 mM potassium phosphate (pH 7.5), 1 mM EGTA, 1 mM EDTA, 25 mM NaF, 0.1 mM fructose-1,6-bisphosphate, 0.1 mM ATP and 10 mM 2-mercaptoethanol. This fraction was used as a measure of total activity of enzymes to check recovery in the soluble and particulate fractions. A second aliquot (0.3 ml) of the crude homogenate was centrifuged at 12,000 xg for 5 minutes in an IEC micro-capillary centrifuge (model MB) with an 837 rotor at room temperature. The resulting supernatant was diluted (1:4, v/v) in stabilization buffer and henceforth is referred to as the free or soluble fraction. The pellet was twice extracted to release bound enzyme with each extraction consisting of resuspension in 0.75 ml of stabilization buffer and centrifugation as above. The combined extracts represent the bound fraction of enzymes. All fractions were

stored on ice until and during activity assay.

Stabilization buffer is used to prevent loss of PFK activity over the course of the experiment. No attempt was made to correct for a maximum of 2 % of activity which pellets because of non-specific trapping. Assuming that the supernatant is a representative fraction of the soluble cytosolic components, then the percentage of free enzyme can be calculated from the weight ratio of supernatant to pellet when the total water content of muscle and the activities of each fraction are known. The equation follows;

$$\% \text{ Free} = \frac{\text{IU/ml}_{\text{free}} * 0.80 * \text{total wgt. sample}}{(\text{IU/ml}_{\text{free}} + \text{IU/ml}_{\text{bound}}) * \text{wgt. snt.}}$$

(2) Pressed method

Tissue samples were dissected out in 0.5-1.0 g pieces and placed on ice. The samples were then transported (3-5 min. total time) on ice to a hydraulic press and pressed under 264 tonnes per square inch for 15 seconds in a French pressure cell. Two layers of 200 mesh nylon screening was placed between the tissue sample and the hole to prevent extrusion of tissue. The fluid passing through the bottom hole was collected and centrifuged at 12,000xg for 5 minutes to remove any cell debris. The pressed juice was diluted (1:24, v/v) in stabilization buffer to maintain the

identical concentrations obtained with the dilution method. Less than 2 % of the total activity was associated with the 12,000xg pellet. The diluted extract was kept on ice until activity assays were performed. Since the remaining pressed tissue was not free of cellular juice due to the imperfect fit of the press it was not homogenized and assayed as a measure of bound enzyme activity. Instead, pressed samples were taken from animals which were also treated with the diluted method. Thus, the pressed extract was compared to the total fraction of the dilution method to obtain a measure of % enzyme activity in the pressed (free) extract. The % free values were determined from the ratio of enzyme activities in the juice versus whole tissue with correction for the percentage of tissue which is water.

$$\% \text{ Free} = (\text{IU/ml}_{\text{free}} * 0.80) / (\text{IU/ml}_{\text{total}})$$

(3) Airfuged method

Tissues were homogenized (1:4, w/v) in homogenization buffer as for the dilution method. Aliquots (150 μ l) of the homogenates were then centrifuged at 100,000 xg for 1 hour in a Beckman air driven microcentrifuge at room temperature. The supernatant was diluted (1:4, v/v) in stabilization buffer (600 μ l) and stored on ice. The pellet was resuspended and homogenized with a ground glass tissue

homogenizer in 750 μ l of stabilization buffer giving final dilutions of 1:25 for both fractions. The airfuged samples were taken from animals that were also treated by the dilution method making the results of the two methods directly comparable with respect to enzyme binding and total recovered activity.

(4) Triton method

Tissues were minced finely and uniformly by hand with a sharp single edge razor and stirred on ice (5 min) in 3 volumes of triton extraction buffer (TEB), 100 mM potassium phosphate (pH 7.5), 1 mM EGTA, 2 M glycerol, 1 mM $MgCl_2$, 0.05% triton X-100, 15 mM 2-mercaptoethanol, 20 μ M PMSF. The tissue in TEB was then centrifuged 10 min. at 1000xg and the resulting supernatant was diluted 1:1 in dilution buffer (DB) which is TEB without triton or glycerol. This extract is designated extract #1. The pellet remaining is resuspended in the original volume of TEB and centrifuged again at 1000xg for 10 min. as before. Reextracting the pellet twice in this manner yields diluted supernatant extracts #s 2 and 3. The final pellet remaining is then homogenized (15 s with an ultraturrax at 80 % of full speed) in 8 volumes of myofibril buffer (MB), 10 mM potassium phosphate, 30 mM NaF, 2 mM EDTA, 1 M glycerol, 15 mM 2-mercaptoethanol, 20 μ M PMSF, yielding extract #4. Combining the activities of extracts 1, 2 and 3 gives a measure of the

soluble enzyme activity while that in extract 4 represents the bound activity.

Data Calculations and Statistics:

Data is reported as mean $\pm$ standard error of the mean (s.e.m.). All data points that have no indication of the size of the s.e.m. had a s.e.m. that was within the limits of the symbols of the plot on the scale used. Statistical significance was assessed by the student's t-test (Mendenhall, 1983).

CHAPTER ONE
GLYCOLYTIC ENZYME BINDING AND METABOLIC CONTROL
IN THE GOLDFISH, Carassius auratus

INTRODUCTION

The goldfish is an ideal vertebrate model in which to study anaerobic metabolism as it is able to survive long-term exposure to complete anoxia by employing adaptations in behaviour, oxygen transport and aerobic and anaerobic metabolism. The impressive anaerobic capacity of goldfish is demonstrated by reported survival periods of several hours of anoxia at 20 °C, several weeks at 5 °C and several months at 0 °C (Walker and Johansen, 1977; Blazka, 1958). Obviously survival times are highly temperature dependent.

While few fish are as tolerant to anoxia as the goldfish many other species of teleost fish are capable of surviving periods of relative hypoxia during which time energy demands can be met by a balance of aerobic and anaerobic metabolism. During long term exposure to non-lethal hypoxia four general metabolic features have been distinguished in teleosts: 1) a transient lactic acidosis, 2) improved O₂ extraction efficiency, 3) higher anaerobic capacity and 4) changed enzyme pattern (van den Thillart and van Waarde, 1986). Basically then in hypoxia teleosts deplete glycogen and creatine phosphate stores and accumulate lactate as a result of a greater dependence on anaerobic methods of ATP production. Hypoxia translates to 'low oxygen' and is thus a relative and vague expression. Since most teleosts immediate reaction to hypoxic conditions

is reduced activity and/or improved O_2 extraction, whether or not the hypoxia represents a metabolic stress great enough to activate anaerobic metabolism depends on the capacity of the oxygen transport system and the energy consumption rate (van den Thillart and van Waarde, 1986). It is thus possible to maintain aerobic metabolism in an hypoxic environment. Anoxia is a distinctly different stress which renders the organism completely reliant on anaerobic ATP producing pathways.

While several freshwater and saltwater teleosts are reported to be anoxia-resistant the most impressive by far are those of the genus Carassius particularly the Crucian carp Carassius carassius and the goldfish Carassius auratus.

Early studies demonstrated that under anoxia goldfish depress their glycolytic activity and in the absence of another fuel source couple this to a reduced ATP consumption. Increases in concentrations of lactate, alanine, creatine, total CO_2 and ADP were demonstrated as were decreases in ATP and CrP however, calculations based on these data indicated that ATP produced could account for only 30% of that required for aerobic maintenance metabolism (van den Thillart, 1976). The value could be lower still considering goldfish do remain slightly active, swimming slowly even under anoxic conditions, thus possibly raising their energy requirements above that of maintenance level

(van den Thillart et al., 1980). Calorimetry studies have also shown overall metabolic rate in the anoxic goldfish to be 20%-30% of normoxic rates (Anderson, 1975). This metabolic rate depression is the key adaptive strategy enabling anoxic survival in the goldfish.

Energy charge of goldfish white muscle was shown to drop little during 10h of anoxia stabilizing at a level high enough to permit work by the tissue (van den Thillart et al., 1976; Atkinson, 1968). Since white muscle is little used under anoxia the high energy charge measured could indicate low energy consumption. Other explanations for maintenance of energy charge include 1) the rephosphorylation of ADP via CrP resulting in depletion of CrP stores, 2) by anaerobic glycolysis leading to glycogen breakdown and accumulation of lactate and, 3) the breakdown of AMP resulting in a decline in the size of the adenylate pool (van Waarde et al., 1983).

A later study by the same authors confirmed the importance of liver glycogen as the major fuel reserve supporting anaerobiosis. Adenylate energy charge fell during anoxia in liver but was maintained in red and white skeletal muscle; two mechanisms were operative: a) hydrolysis of CrP reserves and, b) depletion of AMP by AMP deaminase (van den Thillart et al., 1980).

The lactate increase measured in the whole animal was found to predominate in the liver and blood of anoxic goldfish in conjunction with an increase in glucose concentrations and the depletion of liver glycogen levels (van den Thillart et al., 1980; Walker and Johansen, 1977).

The same authors showed a correlation between anaerobic survival time and the size of liver glycogen stores.

However, when measured in whole animals the lactate increase accounted for only 50% of glycogen depletion whereas a significant amount of CO_2 was excreted (van den Thillart and Kesbeke, 1978). The CO_2 excreted was quantitatively too great to be the result of metabolic acidosis acting on the bicarbonate buffer system. In this study the amount of CO_2 excreted was originally found to balance the difference between glycogen and lactate and thus the possibility of an unknown electron acceptor operating in anaerobiosis was introduced.

The discovery of metabolic CO_2 production in anoxia inspired several studies to elucidate its origin. Metabolic CO_2 appeared to result, at least in part, from glucose catabolism although other sources were hypothesized. Since lactate does not accumulate to an extent predicted by glycogen depletion and could not be found in the water of hypoxic fish an alternate pathway for the catabolism of

lactate in anoxia was suggested (Prosser et al., 1957; Shoubridge and Hochachka, 1980). Prosser et al. did find an excreted product in the water of hypoxic fish but were unable to identify it. Shoubridge and Hochachka described this end product as ethanol. They looked at changes in metabolite concentration in goldfish and surrounding water after an extended period of anoxia and found lactate and ethanol were the only significant end products (Shoubridge and Hochachka, 1980). In radiolabel studies the authors showed at least 90% of the ethanol excreted to be the product of lactate catabolism (Shoubridge and Hochachka, 1980).

Further studies with radiolabelled intermediates demonstrated that both the pentose phosphate pathway and the Krebs cycle are operating although CO_2 production from the latter is greatly reduced (Shoubridge and Hochachka, 1981). Glucose carbons were found to be oxidized unequally and only a small percentage of glucose was oxidized fully. The major part of anaerobic CO_2 arises from C_2 to C_5 carbons of glucose which become C_1 and C_2 at the triose phosphate level. Since triose 2 and 3 are oxidized in the Krebs cycle essentially equivalently most CO_2 likely arises from the C_1 triose carbon which is released by decarboxylation in the pyruvate dehydrogenase reaction (Shoubridge and Hochachka, 1981).

An important conclusion from this study was that the production of ethanol and CO_2 are functionally coupled since inhibition of alcohol dehydrogenase inhibits the production of both end products. Additionally the results demonstrate the existence of two different pathways for glucose metabolism which are physically separate but functionally linked through a common intermediate, lactate (Shoubridge and Hochachka, 1981). Thus, while all tissues possess the metabolic machinery for glycolysis producing lactate, only red and white muscle are capable of oxidizing lactate to ethanol and CO_2 . The entire process is in redox balance and functions to prevent the accumulation of lactate (van den Thillart and van Waarde, 1986).

The alcohol dehydrogenase is cytosolic and has as its substrate acetaldehyde which was shown to be the mitochondrial product of decarboxylation by pyruvate dehydrogenase (Mourik et al., 1982). Red muscle cytoplasm was found to have an alcohol dehydrogenase with a low affinity for ethanol and a high affinity for acetaldehyde indicating acetaldehyde is an intermediate in the production of ethanol in goldfish (Mourik et al., 1982). Apparently acetaldehyde acts as the anaerobic electron acceptor allowing the system to maintain redox balance while helping to prevent the accumulation of lactate by permitting its conversion to ethanol and CO_2 . These final products are rapidly diffused to the environment.

The assumption is made that the ~~alcohol~~ alcohol dehydrogenase reaction is coupled with either lactate dehydrogenase or glyceraldehyde-3-phosphate dehydrogenase. The link has been demonstrated in metabolite studies in which a crossover was found at the GAPDH reaction in red muscle indicating competition between GAPDH and LDH reactions for NAD^+ (Shoubridge and Hochachka, 1983). This supports the theory that lactate from other tissues is converted to ethanol in muscle.

Quantitative measurement of ethanol and CO_2 production show a significant imbalance between glycogen depletion and the observed accumulation of end products (van den Thillart et al., 1983). Specifically, ethanol fermentation accounts for only 80% of the CO_2 production measured indicating the operation of another metabolic pathway. Experiments with radiolabelled glucose, lactate, acetate and glutamate demonstrated that at least 20% of the CO_2 released is produced by Krebs cycle activity (van den Thillart and Verbeek, 1982). Further CO_2 release was shown to be much higher over the first 6 h of anoxia than ethanol release can account for indicating that there is a metabolic transition towards ethanol production which is slowly activated (van den Thillart and Verbeek, 1982). Additional evidence for Krebs cycle activity comes from in vitro studies showing anaerobic CO_2 production from glutamate in minced lateral

red muscle and by isolated mitochondria incubated under anaerobic conditions (Mourik, 1984; van Waarde and De Wilde-Van Berge Henegouwen, 1982).

In teleosts, ammonia is the major component of nitrogen excretion and its production rate is directly related to the rate of protein oxidation (van Waarde, 1983). Studies with anoxic goldfish have shown adenylate-IMP conversion to account for less than 50% of ammonia production with the remainder resulting from spinning of the purine nucleotide cycle coupled to the degradation of amino acids from protein, the oxidation of glutamate via a slowly spinning Krebs-cycle and the breakdown of alanine to ethanol (van Waarde et al., 1982; van Waarde, 1983).

Given the disproportionate production of ethanol and CO_2 in comparison with glycogen depletion during anoxia, and the independence of ammonia production on oxygen availability amino acids become the most likely second carbon source during anaerobiosis. Considerable evidence exists implicating the above pathways in ammonia, CO_2 and ethanol production (van Waarde et al., 1982; van den Thillart et al., 1983; van Waarde, 1983, Mourik, 1984). Additionally, goldfish muscle has been shown to possess significant pyruvate carboxylase activity making it unique among vertebrates and enabling aspartate-ethanol and malate-

ethanol conversions (van den Thillart and Smit, 1984). The enzyme may also stimulate pyruvate oxidation by converting it to oxaloacetate thus preventing early lactate accumulation (van den Thillart and Smit, 1984). In this latter conversion the enzyme also catalyses a substrate-level phosphorylation of ADP possibly improving the efficiency of anaerobic metabolism.

The production of ethanol by goldfish during prolonged anoxia is likely not coupled to energy conservation and is actually wasteful of carbon. The diffusion of ethanol and CO_2 at the gills keeps the process running downhill and maintains the lactate at relatively low levels. This is necessary in the goldfish since it has a rather poor bicarbonate buffer system resulting from its existence in an environment where CO_2 is very soluble ($30\times \text{O}_2$). These fish cannot maintain high bicarbonate levels since large quantities of CO_2 rich water must be passed over the respiratory surface to extract O_2 . Thus a reliance on anaerobic glycolysis alone would lead to acidosis in short order significantly reducing the animals anoxia tolerance. Ethanol producing pathways then function mainly to prevent metabolic acidosis and are an important component of the goldfish's exemplary anaerobic capacity.

However, even more important to anoxic survival is the demonstrated general metabolic rate depression. This is the

primary adaptive strategy adopted by facultative anaerobes the effect of which is to lower ATP requirements to a level which can be met by less efficient fermentation pathways (Storey 1985, 1987). In goldfish, the general metabolic rate is depressed to about 30% of the normoxic rate as indicated by calorimetry and metabolite studies (Anderson, 1975; van den Thillart et al., 1976). However, further investigation has shown the depression to be tissue specific. Liver and kidney apparently sustain a metabolic depression while heart and brain increase their glycolytic rate (Shoubridge and Hochachka, 1983). This creates a lactate gradient from the brain and heart to blood and all other tissues (Shoubridge and Hochachka, 1983).

Coordination of such a tissue specific reorganization of metabolism requires precise controls. The elucidation of the control points and mechanisms has been the focus of recent research. Work with marine molluscs has demonstrated three key molecular mechanisms acting to control glycolytic rate during anoxia and the accompanying depression. These are: 1) covalent modification via enzyme phosphorylation or dephosphorylation reactions (Storey, 1984 Plaxton and Storey, 1984a,b, 1985a,b); 2) F-2,6-P₂ regulation of PFK for the control of carbohydrate use in biosynthesis (Storey, 1985); and 3) enzyme aggregation/association to form complexes bound to subcellular particles (Plaxton and Storey, 1986). Research to date has dealt only with the

first two potential mechanisms for controlling glycolytic rate in the anoxic goldfish.

The results have shown a significantly increased percentage of glycogen phosphorylase a in brain and a decreased percentage in liver, kidney, spleen and heart with total phosphorylase also reduced in liver and gill (Storey, 1987). This is concurrent with a rapid depletion of glycogen stores in heart and brain and their conservation in skeletal muscle (Merrick, 1954; McDougal et al., 1968; Shoubridge and Hochachka, 1983). Concurrently, there is a drop in F-2,6-P₂ in liver, gill and spleen and a rise in heart and brain (Storey 1987). All this points to an anoxia induced relative activation of glycolysis in heart and brain accompanied by a relative decrease of glycolysis in liver, spleen, gill and kidney.

The aim of the present research is to elucidate the contribution of enzyme partitioning between particulate and soluble fractions of the cell to the control of metabolic rate in the anoxic goldfish.

RESULTS

In response to a physiologically relevant metabolic stress, goldfish demonstrate tissue specific glycolytic enzyme associations. Two fractionation methods were used to compare enzyme distribution between soluble and particulate fractions however both yielded statistically identical measurements of the degree of enzyme binding.

In assaying enzyme binding in goldfish nine enzymes were examined: hexokinase; phosphofructokinase; aldolase; glyceraldehyde-3-phosphate dehydrogenase; phosphoglycerate kinase; pyruvate kinase; lactate dehydrogenase; alcohol dehydrogenase and creatine kinase. Maximal activities are given in Table 1. There were no significant differences between control, anoxic or exercised animals with respect to maximal activities therefore the numbers were pooled.

The percent of activity associated with the particulate fraction is given in Figures 1 and 2 for heart and white muscle respectively. These tissues were examined in three metabolic states: aerobic controls, anoxic (animals exposed to 21 h of anoxia in N_2 and CO bubbled water), and exercised (animals forced to swim for 30 min). Figure 2 shows that there were no significant changes in white muscle following either treatment. In contrast, heart muscle demonstrates significant increases in the binding of aldolase and the regulatory enzymes phosphofructokinase and pyruvate kinase

in response to anoxia as shown in figure 1. PFK showed the greatest redistribution from 35 % to 48 % bound while pyruvate kinase binding increased from 13 % to 18 % and aldolase increased from 24 % to 32 %. As heart glycolytic rate has been shown to increase in response to anoxia, glycolytic enzyme binding does vary directly with metabolic rate in the goldfish heart (Shoubridge and Hochachka, 1983). This relationship is supported by the reported activation of PFK and aldolase upon binding with the components of muscle thin filaments, F-actin, troponin and tropomyosin (Luther and Lee, 1986; Walsh et al., 1977; Liou and Anderson, 1980; Kuo et al., 1986). These findings also indicate a Pasteur effect in the goldfish heart in response to whole animal anoxia which has been demonstrated in different studies (Shoubridge and Hochachka, 1983).

Figure 3 shows that pressing white muscle tissue under 264 tonnes per square inch yielded statistically identical measurements of glycolytic enzyme binding when compared to the results of the dilution method. This method was employed to assess the contribution, if any, of dilution effects and/or homogenization on enzyme binding. The results show that neither the dilution of small metabolites nor the physical forces encountered in homogenization contribute to the propensity of the glycolytic enzymes examined to associate with the particulate fraction of goldfish white muscle cells. Maximal activity obtained with

the dilution method varied from 1.3x to 2.8x that found with the pressed method.

Figures 4 and 5 show heart and white muscle PFK binding to have different pH sensitivities. Heart PFK is released at lower pH values than white muscle PFK and the entire titration curve being shifted towards acid pH by approximately 1 pH unit. Heart and white muscle PFK also differ in their response to KCl. White muscle PFK is insensitive to KCl even at 100 mM while heart muscle PFK is released from the bound state by 35 mM KCl. This indicates different isozymic forms of PFK exist in the goldfish or tissue specific modifications of PFK occur to yield distinguishably different forms.

Table 1. Maximal activities of nine enzymes in goldfish heart and white muscle as determined by the dilution and pressed juice methods.

Enzyme	Dilution method		Pressed method
	Heart	White muscle	White muscle
HK	6.6 $\pm$ 0.5	n.f.	n.f.
PFK	19 $\pm$ 3	16 $\pm$ 1	6.6 $\pm$ 0.9*
Aldolase	41 $\pm$ 6	56 $\pm$ 6	36 $\pm$ 4
GAPDH	374 $\pm$ 52	327 $\pm$ 31	158 $\pm$ 24
PGK	234 $\pm$ 26	118 $\pm$ 15	81 $\pm$ 1*
PK	172 $\pm$ 17	169 $\pm$ 14	83 $\pm$ 9
LDH	415 $\pm$ 45	312 $\pm$ 26	221 $\pm$ 26
ADH	n.f.	7.4 $\pm$ 1.3	4.0 $\pm$ 0.6
CK	94 $\pm$ 9	353 $\pm$ 40	206 $\pm$ 55*

Notes: Dilution values are means of 14-15 determinations

Pressed values are means of 3 determinations

* - significantly different ($p < 0.05$) dilution versus pressed

n.f. - not found

Fig. 1. Percentage of recovered enzyme activity associated with the particulate fraction of the cell in goldfish heart muscle under three metabolic states: aerobic ($\square$), anoxic ($\blacksquare$), and exercised ($\boxtimes$).

● - indicates value significantly different compared to control aerobic value, $p < 0.05$, using the Student's t-test. Data are the means of 5-6 determinations for each treatment with error bars showing the sem.

Fig. 2. Percentage of recovered enzyme activity associated with the particulate fraction of the cell in goldfish white muscle under three metabolic states: aerobic ($\square$), anoxic ($\blacksquare$), and exercised ($\boxtimes$).
● - indicates value significantly different compared to control aerobic value, $p < 0.05$, using the Student's t-test. Data are the means of 5-6 determinations for each treatment with error bars showing the sem.

Fig. 3. Percentage of recovered enzyme activity associated with the particulate fraction of the cell in goldfish white muscle. Results obtained with the dilution and pressed methods are compared. Data are the means $\pm$ sem of $n=5-6$, for dilution, and $n=3$, for pressed, determinations. No significant differences were measured between methods based on Student's t-test.

Fig. 4. pH titration profile of goldfish heart muscle phosphofructokinase binding. No KCl ($\square$), 35 mM KCl ($\blacksquare$). Each data point represents a single determination. The lines represent the best fit of the data points as determined by linear regression.

Fig. 5. pH titration profile of goldfish white muscle phosphofructokinase binding. No KCl (○), 100 mM KCl (●). Each data point represents a single determination. The lines represent the best fit of the data points as determined by linear regression.

DISCUSSION

These investigations into the potential role of glycolytic enzyme binding in metabolic control within the goldfish, Carassius auratus, revealed changes in association occurring in a tissue specific manner in response to a physiologically relevant metabolic stress. The data correlate well with earlier research on metabolic control in the goldfish as well as research on enzyme binding and metabolic control in other animal models.

There were no changes observed in maximal enzyme activity of any enzyme tested in heart or white muscle following either 30 minutes of exercise or 21 hours of complete anoxia (Duncan, unpublished data). Thus all maximal activities measured were pooled to yield the data presented in Table 1. This data shows tissue specific differences for activities of HK (not measureable in white muscle), ADH (not found in heart muscle), and CK (4x lower in heart muscle).

ADH has been well documented to exist only in skeletal muscle and so its absence in heart muscle is expected (Shoubridge and Hochachka, 1981, 1983; Mourik et al., 1982). HK activity was impossible to measure accurately in white muscle extracts due to excessive background activity in the homogenates obtained although its presence there is reported (van den Thillart and Smit, 1984). Heart homogenates were

clearer and accordingly had less background activity allowing measurement of HK activity. Thus any apparent discrepancy between white muscle and heart HK activity may be due only to differences in background activity. The tissue specific differences measured for CK as shown in table 1 are best explained by the specialized role white muscle plays in burst work. Due to limitations on oxygen supply and aerobic ATP production white muscle is periodically reliant on non-aerobic forms of energy production even in aerobic environments. A high level of CK activity in this muscle permits more rapid utilization of CrP stores enabling the animal to engage in burst exercise to escape predators. This capacity is not required in the heart.

The binding studies demonstrated significant increases in particulate fraction association for PFK, PK and aldolase in heart tissue following 21h of complete anoxia. Given that PFK and PK are the regulatory enzymes of the glycolytic pathway and that heart glycolytic rate has been shown to increase in response to anoxia the results indicate that enzyme binding varies directly with metabolic rate in the goldfish heart (Shoubbridge and Hochachka, 1983; Storey, 1987). Additionally, increases in enzyme binding have been correlated with increased enzyme activity lending further support to the involvement of PFK, PK and aldolase binding in the control of glycolytic rate in the goldfish (Clarke

and Masters, 1973, 1974; Clarke et al., 1984; Plaxton and Storey, 1986). Specifically the key glycolytic regulatory enzyme PFK, and aldolase are activated by binding to subcellular components of cells which alone may account for increases in glycolytic rate with or without the benefits of channeling and direct transfer (Luther and Lee, 1986; Liou and Anderson, 1980; Kuo et al., 1986; Arnold and Pette, 1970). Thus, one of the ways that glycolytic rate may be controlled during anoxia in goldfish could be via tissue specific changes in enzyme partitioning between soluble and particulate phases of cells. Other potential regulatory mechanisms include covalent modification of regulatory enzymes and F-2,6-P₂ regulation of PFK.

F-2,6-P₂ levels increase approximately 3.5 fold in goldfish heart tissue during anoxia indicating and in correlation with an activation of glycolysis, the typical Pasteur effect (Storey, 1987). This activation occurs as a result of a switch from aerobic lipid metabolism to anaerobic carbohydrate metabolism which is facilitated in part by the F-2,6-P₂ activation of PFK (Neely and Morgan, 1974; Shoubridge and Hochachka, 1983; Storey, 1987). Investigation into the role of covalent modification, via phosphorylation-dephosphorylation reactions, in regulating glycolytic rate in goldfish heart revealed no apparent effects of PK and inhibitory effects on PFK following anoxic exposure (Rahman and Storey, 1988). Luther and Lee (1986)

reported changes in binding of PFK induced by phosphorylation. Thus, such covalent modification although not directly involved in regulating glycolytic rate in goldfish heart may mediate binding changes which could effect rate alterations.

Glycogen phosphorylase a activity, a good indicator of the relative state of glycogenolysis as a contributor to carbohydrate catabolism, was shown to drop six fold in heart indicating decreased glycogen utilization in that tissue yet skeletal muscle showed no change in total phosphorylase or phosphorylase a content (Storey, 1987). This change in heart may help to preserve glycogen reserves in that tissue.

No significant changes in enzyme binding were measured for white muscle glycolytic enzymes or creatine kinase following 21 h of anoxia. This tissue is known to undergo a decrease in glycolytic rate in response to anoxia although no mediators of this metabolic depression have yet been discovered (Shoubridge and Hochachka, 1983; Storey, 1987). Neither glycogen phosphorylase-a content nor F-2,6-P₂ concentrations changed significantly in response to anoxia (Storey, 1987). Additionally, no kinetic evidence for phosphorylation could be found (Rahman and Storey, 1988). Glycolytic rate in red and white muscle may be regulated by the rate of glucose uptake from blood in the resting state or by adenylate activation of glycolysis during muscle work

(Storey, 1987). However, the mediator(s) of glycolytic depression in skeletal muscle of anaerobic goldfish remain an enigma.

The only significant change in white muscle was a decrease in percent ADH bound following 21 hours of anoxia. Since ethanol production is inoperative in aerobic conditions and is operating at or near its in vivo maximum velocity after 24 hours of anoxia the change in particulate association is assumed to be functional (Shoubridge and Hochachka, 1983). Glycolytic rate is depressed in skeletal muscle during anoxia yet ADH activity increases as it oxidizes lactate, mostly imported by the blood from other tissues (Shoubridge and Hochachka, 1983). This argues against the formation of a glycolytic complex with ADH as a component and thereby argues against an increase in ADH binding in response to anoxia. The decrease noted may liberate enzyme to the cytosol where imported lactate is delivered thus enabling more rapid oxidation to ethanol and CO_2 . The alcohol dehydrogenase has been shown to be cytosolic having as its substrate acetaldehyde, a product of decarboxylation by the mitochondrial pyruvate decarboxylase (Mourik et al., 1982). Acetaldehyde apparently acts as the anaerobic electron acceptor allowing the system to maintain redox balance while serving as an intermediate in the conversion of lactate to ethanol and CO_2 . These final products are rapidly diffused to the environment.

The evidence thus far indicates that enzyme partitioning between soluble and particulate phases of a cell is one of several components acting in a concerted manner to control cellular metabolism in response to environmental stress in the goldfish. The binding of key regulatory enzymes, possibly mediated by covalent modification, and changes in effector concentrations lead to tissue specific alterations in glycolytic rate that enable long term survival under anoxic conditions.

Exercise consisted of 30 minutes of forced swimming during which time the fish were at best lackadaisical in their approach. This may explain the lack of binding changes in white muscle, the tissue responsible for the type of burst work stimulated by a predator threat such as that posed by a hand grabbing at the tail. Trout white muscle on the other hand, demonstrates a 50% increase in the percentage of PFK, GAPDH, aldolase and 3-PGA associated with the particulate fraction within 30 seconds of the onset of exercise (Brooks and Storey, in press). It should be noted that trout are much more vigorous in their response to forced swimming, so much so that they are completely exhausted after approximately 4 minutes. Either the sluggish swimming of goldfish does not involve white muscle activity to an appreciable degree or enzyme binding is not involved in the regulation of glycolytic rate during exercise in this animal as it appears to be in trout.

While heart tissue typically increases its metabolic output during exercise results for goldfish heart in figure 1 show no binding changes following exercise for any enzymes tested. Ischaemic heart tissue does demonstrate increased binding in several glycolytic enzymes including PFK although the difference may be due to differing substrates utilized by heart tissue under the different metabolic stresses (Clarke et al., 1984). Ischaemic conditions likely lead to a reliance on carbohydrate fermentation necessitating an activation of glycolysis while the increased work demands of burst exercise are probably met by increased aerobic ATP production from fatty acid oxidation without requiring an activation of glycolysis (Brooks and Storey, in press).

Another technique used in this study to investigate enzyme associations with the particulate phase of the cell and the functional significance of it in a facultative anaerobe involved the pressing of tissue under high pressure. This technique avoids diluting the sample while the soluble fraction is being extracted which has been a point of contention with dilution methods such as those employed for the collection of the goldfish data and those of Clarke et al. (1984). However, no statistically significant differences are found between goldfish white muscle assayed by either method. This suggests that neither the dilution of small metabolites nor the physical forces

encountered in homogenization contribute to the degree of enzyme binding.

Tissue anoxia is known to effect drops in cellular pH in several organisms due to the accumulation of acidic end products of anaerobic metabolism. In goldfish, CO_2 and lactate are produced under anoxic conditions and although lactate is converted to ethanol and CO_2 in skeletal muscle and excreted, decreases in pH still occur in goldfish tissue. Such changes in pH may be the trigger for the controlling mechanisms of glycolytic rate including changes in particulate association of regulatory enzymes. In goldfish, pH sensitivities of PFK are as tissue specific as binding changes with heart enzyme binding being less sensitive to acid pH than white muscle enzyme. While the evidence is tenuous at best it may be that the increased resistance of heart PFK to decreased pH is a reflection of that enzymes' propensity for binding under anoxic stress when pH is acidic.

Titration with KCl revealed further tissue specificity with heart PFK released by 35 mM KCl while white muscle PFK remains insensitive to KCl up to 100 mM. The heart data concurs with other studies demonstrating the elution of PFK by high ionic strength buffer indicating polar binding forces are involved (Gerlach and Hofer, 1986). The resistance of white muscle PFK may indicate the presence of

different binding forces although it is more likely that the forces involved in such a pervasive phenomenon as enzyme binding would be ubiquitous. Alternately, heart and white muscle PFK may have different binding sites on the enzyme bestowing differing binding and elution properties by virtue of the nature of the particular amino acid residues surrounding the site. Notwithstanding, the results suggest the existence of different isozymic forms of PFK in heart and white muscle of goldfish having apparent functional significance with respect to association with the cell particulate phase in response to the physiologically relevant stress of anaerobiosis.

CHAPTER TWO
GLYCOLYTIC ENZYME BINDING AND METABOLIC CONTROL
IN THE CRAYFISH, Orconectes virilis

The crayfish, Orconectes virilis, is a decapod crustacean inhabiting freshwater ponds and slow flowing streams and rivers. In these environments O. virilis often encounters and survives periods of hypoxia or anoxia indicating the existence of the requisite metabolic adaptations (Gade, 1984). Additionally tissues of crayfish can become hypoxic as a consequence of burst exercise. The tail muscles in particular are specially adapted for performing maximal work in a few contractions such as may be required for predator avoidance (Webb, 1979). The powerful contractions of burst work occur in rapid succession to propel the animal from danger. However, burst activity occurs infrequently in the life of crayfish leaving the tail muscles inactive for long periods of time (Onnen and Zebe, 1983). The energy producing capacity of aerobic respiration and the oxygen supply capabilities of the circulatory system are insufficient to meet the energy demands of burst activity (Gade and Grieshaber, 1986). In anoxic waters the oxygen supply is non-existent and aerobic glycolysis can contribute nothing to satisfy the energy needs of an animal. In these instances crayfish obtain their energy from the degradation of phosphagen reserves and from anaerobic glycolysis (Gade, 1984).

The crayfish used in this study were obtained from Bishop's Mills Creek. In this environment the crayfish encounter extremely low O_2 from December through to March with oxygen levels dropping to 0 ppm throughout January and February (Fred Schueler, pers. comm.). While little is known about the mechanisms which enable survival of O. virilis over such long term anoxia two important mechanisms appear to be the absence of a Pasteur effect and the depression of metabolic rate.

In response to anoxia crayfish demonstrate some arginine phosphate depletion (13 mM to 5 mM over 20 hours) and lactate accumulation (Weibust, unpubl. data). The arginine phosphate depletion is not great under the circumstances indicating energy may be being obtained from some other source. Anaerobic glycolysis is the most likely source however the minor accumulation of lactate indicates that in anoxia glycolysis is running slowly (Weibust, unpubl. data; Gade and Grieshaber, 1986). Crossover studies substantiate this and give reason to believe that glycolytic flux is decreased in the crayfish in response to anoxia (Weibust, unpubl. data; Churchill pers. comm.).

Crossover analysis can be used to show any changes in glycolytic flux rates in response to some stimulus and can be used to indicate control points in multienzyme sequences (Williamson, 1970). The ratio of experimental to control

levels of a metabolite are plotted against the sequence of metabolites in the pathway and wherever the plot crosses the axis of 100% of control level an activation or inhibition is indicated. If an enzyme is activated then there will be a decrease in substrate levels and an increase in product levels in the experimental sample which will be reflected by a positive slope on the plot. The reverse holds true for enzyme inhibition. The mathematical assumptions underlying this analysis are defined by Williamson (1970).

For crayfish tail muscle such an analysis has demonstrated negative crossovers at both PFK and PK, key regulatory points in the glycolytic pathway (Weibust unpubl. data; Churchill pers. comm.). This indicates an inhibition of the pathway although it gives no clue as to the mechanism(s) acting at these points in glycolysis.

In another study the levels of F-2,6-P₂ in crayfish were examined with no significant differences found in tail muscle between control and anoxic or exercised animals (Brooks, unpubl. data.). Decreases in the level of this potent activator of PFK and hence glycolysis were found in hepatopancreas and gill (Brooks, unpubl. data.). Such decreases have been demonstrated in marine invertebrates (Storey, 1985). The same tissue specific differences have been shown in goldfish with the soft tissues exhibiting decreases in F-2,6-P₂ in conjunction with decreases in

glycolytic rate and the skeletal muscle tissue showing no such significant difference in F-2,6-P₂ levels (Storey, 1987). Thus in crayfish as in goldfish there is reason to believe that a mechanism other than F-2,6-P₂ regulation is controlling the drop in glycolytic rate.

Covalent modification is one of the alternatives that could be regulating glycolytic rate. However, this mechanism could not be demonstrated in crayfish tail muscle. PFK and PK were tested from the tail muscle with both control and anoxic yielding the same kinetic parameters (Duncan unpubl.data). Similar results have been recorded from goldfish where phosphorylation/dephosphorylation was shown to act in all tissues but for skeletal muscle (Rahman, 1988). This indicates that covalent modification of glycolytic regulatory enzymes is not responsible for controlling the depression of glycolytic rate observed during anoxia and constitutes further evidence for a non-F-2,6-P₂, non-covalent modification mechanism controlling the glycolytic rate depression in anoxic crayfish and goldfish.

Enzyme partitioning is the only remaining factor known to affect glycolytic rate. Thus changes in the percentage of enzyme bound to the particulate fraction of the cell maybe the primary factor controlling glycolytic rate in crayfish tail muscle. If this is not the case then there would have to be an as yet unknown regulator of glycolytic

rate operating in the crayfish tail muscle and the direction of future research would be clear.

RESULTS

Crayfish tail muscle examined by the dilution method demonstrated a general decrease in glycolytic enzyme binding in response to long term, whole animal anoxia. Table 2 and Figure 6 show for PFK and HK differential responses to short(4h) and long(12h) term anoxia. PFK binding increases after 4h of anoxia from 20 % to 27 % bound then drops to control values after 12h while HK increases dramatically, 9 % to 23 % bound, following 4h of anoxia then drops to 14 % bound which is still significantly greater than that found in control animals. PGK binding decreases from 27 % to 20 % bound after 4h of anoxia with no further change after 12h. PK binding drops from 16 % to 10 % bound after 4 anoxic hours then rises to 11%, a value statistically indistinguishable from either control or 4h values. Binding of LDH only changes significantly following 12h of anoxia which induces a drop from 21 % to 11 % bound. AK followed the same pattern with a final decrease from 45 % to 33 %. Thus all glycolytic enzymes tested demonstrated a decrease in the percent of recovered enzyme activity associated with the particulate fraction but for aldolase, GAPDH and HK. Aldolase and GAPDH showed no significant changes in response to anoxia or exercise while HK demonstrated increased binding. The results indicate a depressed metabolic rate in crayfish tail muscle following 12h of anoxia. There were no significant differences in maximal activity measured in

control, anoxic or exercised animals. Exercise, defined here as 20 tail flips in an animal in which 30-40 flips is exhausting (maximal), yields significant changes only in PFK, 20 % to 28 % bound, and PGK, 27 % to 23 % bound.

Increased binding of PFK has been correlated with increased glycolytic rate although activation of glycolysis typically involves changes at both the PFK and PK loci which are not found in the binding results.

Table 2. Percentage of recovered enzyme activity associated with the particulate fraction of crayfish, (*Orconectes virilis*) tail muscle cells as determined by the dilution method.

Enzyme	units/g	Percent bound activity			
		Control	Exercised	Anoxic	
				4 hours	12 hours
PFK	5 ± 1	20 ± 2	28 ± 2 ^a	27 ± 3 ^a	19 ± 2
Aldolase	25 ± 3	18 ± 1	16 ± 2	16 ± 1	15 ± 1
GAPDH	68 ± 6	18 ± 1	17 ± 1	15 ± 1	16 ± 2
PGK	18 ± 2	27 ± 2	23 ± 1 ^a	20 ± 2 ^a	20 ± 1 ^a
PK	22 ± 2	16 ± 2	16 ± 2	10 ± 2 ^a	12 ± 1
LDH	38 ± 3	21 ± 2	21 ± 2	24 ± 2	11 ± 2 ^{a, b}
HK	0.6 ± 0.1	9 ± 2	17 ± 5	23 ± 2 ^a	14 ± 2 ^{a, b}
AK	6 ± 1	45 ± 4	44 ± 2	46 ± 2	33 ± 4 ^{a, b}

Notes: Values are means of 6 determinations

^a - significantly different (p<0.05) as compared to controls

^b - significantly different (p<0.05) 4 h versus 12 h

Fig. 6. Percentage of recovered enzyme activity associated with the particulate fraction of the cell in crayfish tail muscle under four metabolic states: aerobic ($\square$), exercised ($\boxtimes$), four hour anoxic ($\blacksquare$), and twelve hour anoxic ($\boxplus$). Data are the means of 5-6 determinations for each treatment with error bars showing the sem.

● - indicates value significantly different, $p < 0.05$, compared to control aerobic value. Δ - indicates value significantly different, $p < 0.05$, comparing four hour and twelve hour anoxic data. All data analyzed by the Student's t-test.

DISCUSSION

Work with crayfish, Orconectes virilis, demonstrates that environmental anoxia stimulates a general decrease in the percentage of glycolytic enzymes associated with the particulate fraction of tail muscle cells. This corresponds with a general metabolic rate depression during anoxia while the increases in PFK binding following exercise may reflect the involvement of glycolysis in ATP generation in this state. Nevertheless, the results indicate significantly different responses to anoxia and exercise in tail muscle.

Although the percent bound of PFK is increased after exercise it is apparently not correlated with an activation of glycolytic rate. First an activation of glycolysis is typically accompanied or initiated by activation of both PFK and PK. Also from earlier studies of arginine phosphate and metabolites, covalent modification and examinations of F-2,6-P₂ levels it appears that crayfish tail muscle is reliant on phosphagen reserves as the primary source of high-potential phosphoryl groups for the production of the required ATP (Weibust unpubl. data; Gade, 1984; Churchill pers. comm.). Glycogen is only a secondary source of ATP in exercise and though carbon flux is increased by exercise, these studies indicate no activation of glycolysis. The results mirror those found for the shrimp, Crangon crangon thus the importance of phosphagen reserves and the relative unimportance of anaerobic glycolysis to anoxic survival are

ubiquitous themes in crustaceans (Onnen and Zebe, 1983), and in Mytilus edulis (Ebberink and De Zwaan, 1980). With the tail flap not routinely used for locomotion it is entirely possible that tail muscle is thoroughly dependent on phosphagen reserves for this activity while the glycolytic machinery exists to preserve phosphagen levels and for maintenance metabolism. The absence of binding changes in arginine kinase suggest this enzyme is not regulated by particulate association and is not part of a glycolytic complex. Given the similar results found for CK in goldfish it appears that this is a general condition of phosphagen kinases.

HK binding increases during anoxia are an anomaly given the reported binding characteristics and role in providing fuel for glycolysis which is not activated but depressed in this state. This enzyme is primarily associated with the outer mitochondrial membrane where it catalyzes the conversion of glucose to glucose-6-phosphate the rate of which is regulated by control of an equilibrium between free and bound forms of HK (Wilson, 1980). By improving the catalytic properties of HK upon binding, substrate could be transformed more efficiently. Substrate availability would then act as the limiting factor regulating glycolytic rate. This may explain the lack of any other evident mediators of glycolytic rate changes occurring in this tissue.

In other animal models, intense muscular exercise increases energy demand in muscle necessitating the activation of glycolysis which hastens an accumulation of lactate as oxygen becomes limiting. The increase in glycolytic flux is mediated by PFK and PK and accompanied by an activation of glycogen phosphorylase and increased glycogen breakdown. Phosphagen reserves are depleted but energy charge falls nonetheless (Newsholme and Stewart, 1973). Dramatic binding increases have been demonstrated in response to exercise in direct correlation with increases in glycolytic rate (Brooks and Storey, in press; Helga Gudderly pers. comm.). Thus sharp contrasts exist in energy metabolism between crayfish tissue and that of the other animals which are reflected by the different enzyme binding responses. This indirectly demonstrates the relationship between changes in flux through a pathway and the degree of enzyme association.

In response to anoxia crayfish exhibited a general decrease in glycolytic enzyme binding with drops in percent bound for PGK, PK and LDH as well as arginine kinase. PFK binding was increased after 4 h although it dropped to control values by 12 h. These results coupled with present knowledge of crayfish tail muscle metabolism argues against an activation of glycolysis as the primary mechanism maintaining energy charge in anaerobiosis. In fact crossover studies have demonstrated negative crossovers at

PFK and PK suggesting decreased glycolytic flux (Churchill pers. comm.). Regulation by enzyme partitioning may be the controlling factor since no effect on F-2,6-P₂ levels or phosphorylation state of enzymes could be detected in tail muscle (Brooks pers. comm.; Duncan unpubl. data). Changes in these parameters were found in gill and hepatopancreas indicating decreased glycolytic flux in these tissues much the same as in marine invertebrates (Brooks pers. comm.; Duncan unpubl. data; Storey, 1985). If crayfish share a metabolic strategy with marine invertebrates then enzyme binding is likely involved in mediating glycolytic rate in muscle tissues although the results are not as dramatic for crayfish as they are for the channelled whelk (Plaxton and Storey, 1986). Additionally, earlier studies with the crayfish have demonstrated a reliance on phosphagen reserves in anoxia thus it may be that metabolic rate is depressed in general as mediated by enzyme association changes thereby reducing the demand for ATP and conserving arginine phosphate supplies to extend survival (Gade, 1984).

Enzyme particulate associations are generally decreased during the course of anoxia however, rearrangements also occur as anoxia progresses. Between 4 and 12 hours of anaerobiosis significant binding decreases occur for PFK, LDH, HK and AK. Although supporting data is scarce it may be that the glycolytic depression becomes stronger as anoxia progresses. A mutable metabolic rate varying with the

length of anoxic stress would be adaptive for a facultative anaerobe particularly one that encounters both short and long term anoxia. The crayfish may encounter short anoxic periods in the warm summer lakes and rivers and certainly contends with months of complete anoxia overwinter (Schueler, pers. comm.). Perhaps anaerobiosis greater than several hours such as may be faced in a hot afternoon is functionally different from that of greater duration. More information is required on the metabolic state of overwintering crayfish with respect to metabolic rate and potential controllers of it including substrate availability.

CHAPTER THREE
GLYCOLYTIC ENZYME BINDING AND METABOLIC CONTROL
IN THE TURTLE, Pseudemys scripta

INTRODUCTION

The freshwater turtles, Pseudemys scripta and Chrysemys picta, are lower vertebrates that inhabit lakes and rivers and possess extraordinary tolerance to prolonged anoxia. In completely anoxic conditions which would lead to rapid degenerative events and death in other animals, P. scripta and C. picta can survive for up to 177 days (Ultsh and Jackson, 1982). The metabolic adaptations that permit this are utilized by the animals over winter when they are often kept anoxic beneath the ice for up to six months each year. Additionally this anaerobic capacity makes the freshwater turtles P. scripta and C. picta ideal models in which to examine metabolic strategies employed in maintaining life without oxygen.

P. scripta have demonstrated anoxic survival times of 24 hours at 22°C and up to 2 weeks at 16-18°C (Penney, 1974; Robin et al., 1964). C. picta demonstrate similar survival times with the greatest reported times exceeding 25 weeks for those animals acclimated to 3°C (Ultsh and Jackson, 1982). Clearly diving survival is temperature dependant in both species although C. picta is able to extend its diving survival time at all temperatures in oxygenated water (Ultsh and Jackson, 1982a,b). On the other hand P. scripta is unable to take up significant quantities of O₂ by non-pulmonary respiration even in oxygenated water and is thus

reliant on metabolic adaptations to support such facultative anaerobiosis (Belkin, 1968). . In the turtles these adaptations include 1) sizable reserves of fermentative substrate (glycogen), particularly in liver, and substantial activities of glycolytic enzymes in all organs, 2) a considerable buffering capacity within tissues and in extracellular fluids and 3) the ability to profoundly depress metabolic rate.

For freshwater turtles the beginning of an anoxic dive is accompanied by the onset of the 'diving syndrome' whereby blood oxygen stores are preserved for essential organs by extensive peripheral vasoconstriction (Bentley et al., 1983). Even so turtles experience a drop in lung and plasma O_2 to essentially zero after about 45 minutes and a progressive rise in CO_2 levels that continues for at least 6 hours (Jackson, 1968; Robin et al., 1981). Thus available internal oxygen stores are depleted within the first hour and survival beyond this is wholly dependent on anaerobic energy production (with its attendant consequences). There is also a significant bradycardia although blood pressure is unaffected (except at $3^{\circ}C$) (Penney, 1974; Herbert and Jackson, 1985b). Within the first hour a 39% reduction in the O_2 utilization rate occurs indicating a metabolic rate depression that averages 78% over a 6 hour dive (Caliguir et al., 1981). Metabolic rate depressions of greater than 90% have been reported based on calorimetry studies and measurements of lactate accumulation (Jackson, 1968; Herbert

and Jackson, 1985b). Such a considerable depression of metabolic rate is the key to the long term survival of anoxic turtles, as it is for goldfish and crayfish.

Similarly as for goldfish and crayfish turtles rely on fermentative glycolysis as the mechanism of ATP generation in anaerobiosis since alternate pathways are of minor quantitative importance at best (Penney, 1974). While creatine phosphate provides a readily available reservoir of high potential phosphoryl groups to supplement ATP stores these reserves are rapidly depleted within the early hours of anoxia (Clark and Miller, 1973; Lutz et al., 1984). ATP stores drop early in anoxia rising again as anoxia continues although energy charge is maintained in all tissues throughout this time (Lutz et al., 1984; Kelly and Storey, submitted). Corresponding changes in total adenylates maintain the energy charge in the face of changing ATP levels (Kelly and Storey, submitted). The adenylate data suggests that ATP is not inhibitory to key regulatory enzymes of glycolysis and hence that some other factor must be controlling glycolytic rate in anoxia.

Glycogen is the primary source of carbon used to fuel anaerobic glycolysis with the quantitatively most important reserve in the turtle being that of the liver (Gatten, 1987). Cardiac glycogen is utilized early in anoxia and is essentially exhausted after 3 hours (Clark and Miller,

1973). This reflects a rapid initiation of glycolysis in anoxia and is facilitated by a level of glycogen phosphorylase a which is 8x that found in mammalian heart, although turtles have less total phosphorylase (McNeill et al., 1971). Brain glycogen stores are low and are apparently not utilized as a substrate for glycolysis (McDougal et al., 1968; Clark and Miller, 1973). However, liver glycogen falls progressively over at least 15 hours of anoxia and is accompanied by a rise in blood glucose suggesting that this is the primary substrate for anaerobic glycolysis and ATP production (Clark and Miller, 1973; Penney, 1974). Blood glucose levels begin to rise shortly after the onset of anoxia and continue progressively attaining 5-6x normoxic levels in a matter of hours (Penney, 1974; Clark and Miller, 1973). The measured rapid glycolytic inhibition in liver in the first hour also argues for glucose export from glycogenolysis in the liver (Kelly and Storey, submitted).

Concomitant with a fall in liver glycogen and rise in blood glucose is an increase in the levels of lactate measured in the blood and tissues of anoxic turtles. Lactate accumulation is the primary factor responsible for the metabolic acidosis measured in anoxic turtles and can result in blood and tissue lactate levels up to 8x (100x in plasma, Herbert and Jackson, 1985b Table 4). greater than normoxic (Penney, 1974; Clark and Miller, 1973; Kelly and

Storey, submitted). The levels and rate of lactate accumulation demonstrate tissue specificity thus indicating organ specific metabolic responses to anaerobiosis involving both the magnitude and timing of metabolic depression (Clark and Miller, 1974; Kelly and Storey, submitted). These organ specific differences are expected to be reflected in the regulatory mechanisms employed by the turtle.

The metabolic acidosis resulting from lactate accumulation can contribute as much as 75% of the pH drop in anoxia although the contribution by respiratory acidosis increases with temperature (Herbert and Jackson, 1985a). While metabolic depression slows the pH drop turtles are only able to survive the combined respiratory and metabolic acidosis due to the existence of substantial buffering capacities in the blood and tissues. Changes in lactate concentrations are directly related to changes in HCO_3^- levels and strong ion concentrations including Ca^{++} , Mg^{++} , Na^+ , K^+ and Cl^- which markedly retard the development of acidosis (Jackson and Ultsh, 1982; Jackson and Heisler, 1983; Herbert and Jackson, 1985a). By far the greatest changes occur in lactate, HCO_3^- and Ca^{++} with the latter two falling and rising respectively in response to rising lactate levels suggesting these are the most important ionic species interacting in turtle anaerobiosis (Jackson and Ultsh, 1982; Jackson and Heisler, 1983; Herbert and Jackson, 1985a). These changes are observed intracellularly in all

tissues to varying degrees as well as in plasma, and pericardial and peritoneal fluids indicating extracellular fluid participation in buffering lactate (Jackson and Ultsh, 1982). Coelomic fluid is apparently not part of the buffer system as no changes in lactate, buffer or ions could be found there, however significant amounts of lactate and Ca^{++} are found in bladder urine suggesting excretion of some acid may occur (Penney, 1974; Jackson and Ultsh, 1982).

While a drop in pH or changes in metabolite levels may be the cue initiating compensatory mechanisms in anaerobiosis little is known regarding the components of the regulatory system in turtles. Potentially glycolytic rate could be controlled by covalent modification of regulatory enzymes, by changes in the levels of glycolytic activators such as F-2,6- P_2 , or by a redistribution of glycolytic enzymes between the soluble and particulate phases of the cell. The absence of significant decreases in F-2,6- P_2 levels in anoxic turtle tissues suggests an alternate controlling factor must be responsible for the measured depression of glycolytic rate (Brooks and Storey, in press; Brooks unpubl. data).

Measurements of metabolite changes in turtle tissues demonstrates positive crossovers at the PFK and PK loci indicating these are the control points employed in a glycolytic activation reported in brain, heart and skeletal

muscle in the first hour of anaerobiosis (Kelly and Storey, submitted). Data from the same study suggests that PK, and not PFK, is an important control point for the characteristic metabolic depression which follows this short term activation. Early studies suggest turtle PK may be controlled by changes in metabolite concentrations at least in the heart although no such evidence exists for other tissues and several typical effector concentrations in turtle tissues are essentially maintained in anaerobiosis (Storey and Hochachka, 1974b; Lutz et al., 1984; Kelly and Storey, in press). Turtle brain PK does not appear to be controlled by covalent modification since characteristic differences are not found between aerobic and anaerobic enzyme forms (Brooks and Storey, in press). Evidence does indicate PFK, the key regulatory enzyme of glycolysis, is controlled in part by covalent modification in brain and red and white skeletal muscle (Brooks and Storey, in press; Brooks unpubl. data). Such modification by phosphorylation/dephosphorylation, besides altering kinetic parameters and enzyme activity at physiological levels of substrates and effectors, can alter PFK binding interactions with the subcellular particulate fraction (Luther and Lee, 1986). Covalent modification where it occurs may then be acting in conjunction with or as a trigger for enzyme associations/dissociations to effect the glycolytic rate depression characteristic of facultative anaerobiosis.

RESULTS

The results suggest that changes in glycolytic enzyme binding is one of the turtles responses to complete, whole animal anoxia. In an attempt to validate the bound/free status of the enzymes and the available fractionation methods, three different experimental protocols were examined: 1) the dilution method; 2) the triton method; and 3) the airfuged method.

Maximal activities measured by either of the methods did not change significantly in response to anoxia indicating no new enzyme production was responsible for any increased glycolytic flux (Table 7a,b). Differences between the methods were found. These demonstrated that the lengthy centrifugation of the airfuge method did not result in any loss of absolute activity. The triton method yielded consistently higher activities than the other methods. Results obtained with the dilution and triton methods indicate that anoxia induces a general increase in enzyme binding in all tissues (Tables 3 and 4). PFK exhibited the greatest increases at 40-50% (3-4x) as measured by the dilution method (Table 3). CK demonstrated the fewest changes as the only significant one was a 1.5-2x increase in red muscle as determined by both the dilution and triton methods. The greater majority of changes were increases of approximately 10% and 1.5x. These methods agreed that

increased binding was most prominent in red and white skeletal muscle, particularly so in the former, while heart and brain changes were less extensive. Of note, the dilution method showed increased binding of both PFK and PK in the skeletal muscles following 5 hr of anoxia indicating an activation of glycolysis there. The near absence of binding changes in heart tissue argues for little if any change in heart glycolytic, and hence metabolic, rate. No significant redistribution of enzyme was measured in any tissue by the airfuge method but for an increase brain PGK binding and a decrease in red muscle PK binding (Table 5). This may be a reflection of changes within the tissue over the long centrifugation time. Results of a comparison of the three methods presented in Table 6 show the dilution method to yield the lowest estimate of enzyme binding followed by the triton method and finally the airfuge method which yields the highest estimation. PGK is the notable anomaly in this table as its solubility was not significantly different as measured by any of the three methodologies. This may be related to the same factor which makes PGK solubility one of the least variable in response to anoxia as well.

Table 3. Percentage of recovered enzyme activity associated with the particulate fraction of turtle (*Pseudemys scripta elegans*) cells as determined by the dilution method.

Enzyme	Tissue	Control	Anoxic(5h)	Anoxic(20h)
PFK	Heart muscle	13 ± 3	15 ± 1	19 ± 2
	Red muscle	14 ± 2	52 ± 5 ^a	62 ± 3 ^a
	White muscle	18 ± 3	42 ± 6 ^a	60 ± 7 ^{a,b}
	Brain	18 ± 3	24 ± 2	27 ± 2 ^a
Aldolase	Heart muscle	26 ± 2	42 ± 2	56 ± 3 ^{a,b}
	Red muscle	16 ± 3	34 ± 5 ^a	34 ± 3 ^a
	White muscle	17 ± 3	36 ± 3 ^a	33 ± 6 ^a
	Brain	21 ± 2	31 ± 2 ^a	36 ± 7 ^a
GAPDH	Heart muscle	15 ± 2	15 ± 1	13 ± 1
	Red muscle	19 ± 4	41 ± 6 ^a	31 ± 3 ^a
	White muscle	29 ± 3	36 ± 4	30 ± 4
	Brain	21 ± 1	26 ± 2 ^a	23 ± 2
PGK	Heart muscle	27 ± 4	24 ± 1	28 ± 3
	Red muscle	30 ± 5	28 ± 3	20 ± 3 ^b
	White muscle	33 ± 7	27 ± 2	18 ± 3 ^a
	Brain	31 ± 4	32 ± 2	27 ± 5
PK	Heart muscle	15 ± 1	13 ± 1	12 ± 1 ^a
	Red muscle	17 ± 1	24 ± 3 ^a	14 ± 2 ^b
	White muscle	17 ± 2	24 ± 2 ^a	18 ± 3
	Brain	19 ± 1	19 ± 1	20 ± 2
LDH	Heart muscle	19 ± 4	14 ± 1	10 ± 1 ^a
	Red muscle	16 ± 3	26 ± 3 ^a	17 ± 2 ^b
	White muscle	17 ± 2	29 ± 3 ^a	23 ± 5
	Brain	27 ± 6	22 ± 1	22 ± 2
HK	Heart muscle	65 ± 4	73 ± 3	79 ± 2 ^{a,b}
	Red muscle	50 ± 9	73 ± 3 ^a	64 ± 4
	White muscle	46 ± 3	67 ± 5 ^a	69 ± 6 ^a
	Brain	51 ± 3	60 ± 3 ^a	63 ± 3 ^a
CK	Heart muscle	22 ± 3	19 ± 1	17 ± 2
	Red muscle	17 ± 3	19 ± 1 ^a	18 ± 1
	White muscle	22 ± 2	26 ± 2	20 ± 2
	Brain	27 ± 2	30 ± 1	27 ± 1

Notes: Values are means of 5 determinations

^a - significantly different (p<0.05) compared to controls

^b - significantly different (p<0.05) 5h anoxic versus 20h anoxic

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Table 4. Percentage of recovered enzyme activity associated with the particulate fraction of turtle (*Pseudemys scripta elegans*) cells as determined by the triton method.

Enzyme	Tissue	Control	Anoxic (20h)
PFK	Heart muscle	37 ± 5	33 ± 3
	Red muscle	51 ± 10	52 ± 3
	White muscle	53 ± 6	50 ± 6
	Brain	53 ± 5	59 ± 2
Aldolase	Heart muscle	24 ± 4	21 ± 4
	Red muscle	33 ± 6	38 ± 3
	White muscle	27 ± 5	38 ± 2*
	Brain	46 ± 5	56 ± 2*
GAPDH	Heart muscle	27 ± 4	23 ± 3
	Red muscle	29 ± 5	35 ± 3
	White muscle	32 ± 6	31 ± 3
	Brain	42 ± 4	52 ± 1*
PGK	Heart muscle	23 ± 3	19 ± 2
	Red muscle	23 ± 5	34 ± 3*
	White muscle	26 ± 4	31 ± 2
	Brain	40 ± 5	51 ± 2*
PK	Heart muscle	26 ± 4	28 ± 4
	Red muscle	33 ± 4	43 ± 3*
	White muscle	33 ± 4	42 ± 3*
	Brain	46 ± 4	55 ± 2*
LDH	Heart muscle	32 ± 6	23 ± 4
	Red muscle	33 ± 5	46 ± 2*
	White muscle	38 ± 6	35 ± 3
	Brain	49 ± 9	51 ± 2
CK	Heart muscle	26 ± 6	26 ± 3
	Red muscle	26 ± 6	38 ± 3*
	White muscle	30 ± 3	35 ± 3
	Brain	50 ± 4	53 ± 2

Notes: Values are means of 5 determinations

* - significantly different ($p < 0.05$) as compared to control values

Table 5. Percentage of recovered enzyme activity associated with the particulate fraction of turtle (*Pseudemys scripta elegans*) cells as determined by the airfuge method.

Enzyme	Tissue	Control	Anoxic (5h)
PFK	Heart muscle	64 ± 8	76 ± 3
	Red muscle	72 ± 10	77 ± 3
	White muscle	73 ± 9	76 ± 3
	Brain	71 ± 7	81 ± 2
Aldolase	Heart muscle	52 ± 3	48 ± 1
	Red muscle	51 ± 3	45 ± 4
	White muscle	45 ± 4	47 ± 7
	Brain	48 ± 3	54 ± 3
GAPDH	Heart muscle	41 ± 4	43 ± 5
	Red muscle	55 ± 2	55 ± 3
	White muscle	51 ± 4	56 ± 7
	Brain	48 ± 4	54 ± 2
PGK	Heart muscle	27 ± 2	30 ± 2
	Red muscle	29 ± 9	24 ± 2
	White muscle	19 ± 3	23 ± 3
	Brain	32 ± 2	42 ± 2*
PK	Heart muscle	52 ± 6	48 ± 5
	Red muscle	51 ± 6	36 ± 5*
	White muscle	49 ± 5	48 ± 10
	Brain	67 ± 3	69 ± 6
LDH	Heart muscle	36 ± 5	35 ± 4
	Red muscle	44 ± 3	40 ± 2
	White muscle	46 ± 4	45 ± 8
	Brain	57 ± 4	63 ± 4
HK	Heart muscle	93 ± 1	93 ± 2
	Red muscle	87 ± 3	91 ± 2
	White muscle	82 ± 5	92 ± 1
	Brain	94 ± 2	97 ± 1
CK	Heart muscle	26 ± 2	26 ± 3
	Red muscle	27 ± 3	23 ± 1
	White muscle	27 ± 3	29 ± 4
	Brain	41 ± 2	38 ± 1

Notes: Values are means of 5-6 determinations

* - significantly different ($p < 0.05$) as compared to control values

Table 6. Percentage of recovered enzyme activity associated with the particulate fraction of turtle (*Pseudemys scripta elegans*) cells as determined by three different methods.

Enzyme	Tissue	Fractionation Method		
		Dilution	Triton	Airfuge
PFK	Heart muscle	13 ± 3	37 ± 5	64 ± 8
	Red muscle	14 ± 2	51 ± 10 ^b	72 ± 10
	White muscle	18 ± 3	53 ± 6 ^b	73 ± 9
	Brain	18 ± 3	53 ± 5	71 ± 7
Aldolase	Heart muscle	26 ± 2	24 ± 4	52 ± 3
	Red muscle	16 ± 3	33 ± 6	51 ± 3
	White muscle	17 ± 3 ^a	27 ± 5	46 ± 4
	Brain	21 ± 2	46 ± 5 ^b	48 ± 3
GAPDH	Heart muscle	15 ± 2	27 ± 4	41 ± 4
	Red muscle	19 ± 4 ^a	29 ± 5	55 ± 2
	White muscle	29 ± 3 ^a	32 ± 6	51 ± 4
	Brain	21 ± 1	42 ± 4 ^b	48 ± 4
PGK	Heart muscle	27 ± 4 ^a	23 ± 3 ^b	27 ± 2 ^c
	Red muscle	30 ± 5 ^a	23 ± 5 ^b	29 ± 9 ^c
	White muscle	33 ± 7 ^a	26 ± 4 ^b	19 ± 3
	Brain	31 ± 4 ^a	40 ± 5 ^b	32 ± 2 ^c
PK	Heart muscle	15 ± 1	26 ± 4	52 ± 6
	Red muscle	17 ± 1	33 ± 4	51 ± 6
	White muscle	17 ± 2	33 ± 4	48 ± 5
	Brain	19 ± 1	46 ± 4	67 ± 3
LDH	Heart muscle	19 ± 4	32 ± 6 ^b	36 ± 5
	Red muscle	16 ± 3	33 ± 5	44 ± 3
	White muscle	17 ± 2	36 ± 6 ^b	46 ± 4
	Brain	27 ± 6	49 ± 9 ^b	57 ± 4
HK	Heart muscle	65 ± 4	NA	93 ± 1
	Red muscle	50 ± 9	NA	87 ± 3
	White muscle	46 ± 3	NA	82 ± 5
	Brain	51 ± 3	NA	94 ± 2
CK	Heart muscle	22 ± 3 ^a	26 ± 6 ^b	26 ± 2
	Red muscle	17 ± 3 ^a	26 ± 6 ^b	27 ± 3
	White muscle	22 ± 2	30 ± 3 ^b	27 ± 3 ^c
	Brain	27 ± 2	50 ± 4	41 ± 2

Notes: Values are means of 5-6 determinations on control animals

^a - not significantly different ($p > 0.05$) from triton

^b - not significantly different ($p > 0.05$) from airfuge

^c - not significantly different ($p > 0.05$) from dilution

NA - no measureable activity (high background activity)

Table 7a. Maximal activities (units/g wet weight $\pm$ sem) of nine enzymes in turtle tissues

Tissue	Enzyme	Fractionation Method		
		Dilution	Triton	Airfuge
Heart	PFK	23 $\pm$ 4 ^a	22 $\pm$ 6 ^b	19 $\pm$ 2 ^c
	Aldolase	19 $\pm$ 2	52 $\pm$ 5	15 $\pm$ 1
	GAPDH	168 $\pm$ 29	255 $\pm$ 32	140 $\pm$ 13 ^c
	PGK	93 $\pm$ 18	158 $\pm$ 26	61 $\pm$ 12 ^c
	PK	129 $\pm$ 19	225 $\pm$ 14	110 $\pm$ 10 ^c
	LDH	326 $\pm$ 116 ^a	516 $\pm$ 113	264 $\pm$ 27 ^c
	HK	3.8 $\pm$ 0.6	6.4 $\pm$ 0.6 ^b	5.6 $\pm$ 0.5
	CK	71 $\pm$ 13	187 $\pm$ 34	72 $\pm$ 7 ^c
Brain	PFK	12 $\pm$ 2 ^a	25 $\pm$ 14 ^b	17 $\pm$ 3 ^c
	Aldolase	14 $\pm$ 2	46 $\pm$ 14	19 $\pm$ 3 ^c
	GAPDH	95 $\pm$ 27	178 $\pm$ 30	103 $\pm$ 11 ^c
	PGK	79 $\pm$ 42 ^a	153 $\pm$ 25	62 $\pm$ 9 ^c
	PK	107 $\pm$ 16	301 $\pm$ 45	142 $\pm$ 24 ^c
	LDH	112 $\pm$ 26	286 $\pm$ 87 ^b	149 $\pm$ 23 ^c
	HK	3.8 $\pm$ 1.4 ^a	8.0 $\pm$ 4.9 ^b	7.1 $\pm$ 1.2 ^c
	CK	29 $\pm$ 7 ^a	63 $\pm$ 19 ^b	34 $\pm$ 6 ^c

Notes: Values are means of 5-6 determinations.

^a - not significantly different ($p > 0.05$) from triton^b - not significantly different ($p > 0.05$) from airfuge^c - not significantly different ($p > 0.05$) from dilution

Table 7b. Maximal activities (units/g wet weight $\pm$ sem) of nine enzymes in turtle tissues

Tissue	Enzyme	Fractionation Method		
		Dilution	Triton	Airfuge
Red muscle	PFK	22 $\pm$ 5 ^a	26 $\pm$ 3 ^b	19 $\pm$ 3 ^c
	Aldolase	46 $\pm$ 2	130 $\pm$ 12	57 $\pm$ 1
	GAPDH	249 $\pm$ 25	518 $\pm$ 96	271 $\pm$ 16 ^c
	PGK	81 $\pm$ 13	166 $\pm$ 17	81 $\pm$ 8 ^c
	PK	188 $\pm$ 36	399 $\pm$ 29	167 $\pm$ 8 ^c
	LDH	241 $\pm$ 62	569 $\pm$ 90	284 $\pm$ 20 ^c
	HK	2.2 $\pm$ 0.5 ^a	2.4 $\pm$ 0.2 ^b	2.0 $\pm$ 0.2 ^c
	CK	77 $\pm$ 14	208 $\pm$ 41	96 $\pm$ 7 ^c
White muscle	PFK	12 $\pm$ 1	33 $\pm$ 4	19 $\pm$ 2
	Aldolase	15 $\pm$ 1	42 $\pm$ 7	19 $\pm$ 3 ^c
	GAPDH	95 $\pm$ 12	173 $\pm$ 14	103 $\pm$ 10 ^c
	PGK	99 $\pm$ 8	138 $\pm$ 18	62 $\pm$ 8
	PK	107 $\pm$ 7	279 $\pm$ 28	142 $\pm$ 21 ^c
	LDH	102 $\pm$ 9	274 $\pm$ 40	149 $\pm$ 42
	HK	3.8 $\pm$ 0.6	10 $\pm$ 1	7 $\pm$ 1
	CK	29 $\pm$ 3	56 $\pm$ 10	34 $\pm$ 5 ^c

Notes: Values are means of 5-6 determinations

- ^a - not significantly different ($p > 0.05$) from triton
^b - not significantly different ($p > 0.05$) from airfuge
^c - not significantly different ($p > 0.05$) from dilution

DISCUSSION

Turtles display changes in enzyme particulate associations in direct response to a physiologically relevant metabolic stress. Increased binding during anaerobiosis affects most glycolytic enzymes. This phenomenon is corroborated by two different methodologies yielding the same general results.

All four turtle tissues examined in this study exhibit a metabolic depression in response to anoxia (Kelly and Storey, in press). The lack of significant changes in $F-2,6-P_2$ levels in all turtle tissues argues against the involvement of this effector (Brooks and Storey in press; Brooks unpubl. data). Evidence suggests covalent modification via phosphorylation/dephosphorylation may play a part in controlling glycolytic rate in turtle brain, heart and red skeletal muscle although its occurrence in white muscle is not observed (Brooks and Storey, in press; Brooks unpubl. data). However, widespread increases in glycolytic enzyme binding reported here suggest their involvement in modulating glycolytic rate in each of the tissues examined. Changes in the phosphorylation state of an enzyme may mediate the binding changes observed.

Typically, glycolytic enzyme binding changes are correlated with metabolic requirements such that increased

glycolytic rate is associated with increased binding.

However, metabolite studies give clear evidence of depressed metabolic rate in all four tissues studied here (Kelly and Storey, in press). In heart, brain and red muscle PK seems a likely control point for this depression as demonstrated by metabolite crossovers although PFK involvement is not indicated (Kelly and Storey, in press). In support of this, enzyme binding changes are more prominent for PK than PFK in the anoxic turtle as measured by the dilution and triton methods. White muscle metabolites exhibit little change during 5 hours of anoxia. These same studies suggest that glycolytic rate may be regulated in red and white muscle by control over the provision of hexose phosphates. Thus increased glycolytic binding may improve the efficiency and rate of the pathway intrinsically while limited substrate regulates the observed rate. This may explain the reverse correlation of enzyme binding and metabolic demands particularly in skeletal muscle.

In this scenario anoxia would induce a transition to anaerobic metabolism which would be manifested by immediate increases in glycolytic rate as mediated by enzyme binding. Glycolytic rate would then be regulated by the availability of hexose phosphates for the pathway. In fact all four tissues demonstrate transient increases in glycolytic rate following the first hour of anoxia followed by a decrease occurring between 1 and 5 hours (Kelly and Storey, in press).

Brain and white muscle normally oxidize carbohydrate aerobically so the transition to anoxia involves only changes in the rate of the always active glycolytic pathway. Heart and red muscle, however, typically oxidize lipid in the aerobic state and thus would have to switch over to carbohydrate as the primary fuel and activate the glycolytic pathway normally repressed during lipid oxidation. Binding increases involved in the early activation in all these tissues would remain throughout anoxia until a signal for return to aerobic metabolism is received.

Support for this proposal is found in the enzyme binding patterns observed, particularly those of skeletal muscle. Increases in binding of the glycolytic regulatory enzymes PFK and PK typically associated with an activated glycolysis are found in red and white muscle. However, these tissues are at the same time undergoing a documented metabolic depression (Kelly and Storey, in press; Caligiuri et al., 1981; Herbert and Jackson, 1985; Jackson, 1968). Levels of glucose-6-P are greatly reduced in both tissues following anoxia suggesting reduced rates of endogenous glycogenolysis and lending support to the notion that regulation of glycolytic rate may result from control over the provision of hexose phosphates to glycolysis (Kelly and Storey in press). Regulation by substrate availability may not act in all tissues. For instance, decreases in binding

of PK and LDH may be related to control of the glycolytic rate decrease observed in the heart.

In turtles only red muscle demonstrated significant changes in CK binding. Measured by the triton method 20h anoxia induced increased CK binding although by the dilution method the increased level of binding measured after 5h had returned to the control value by 20h. Evidence from turtles, crayfish and goldfish demonstrates that CK (AK in crayfish) is an essentially soluble enzyme making no rate related particulate associations.

Three methods were employed to estimate enzyme binding in vivo: dilution, triton and airfuged. Significant differences were found between the proportion of particulate associated enzymes measured by each of the methods. The differences were generalized for virtually all enzymes and tissues with dilution consistently yielding the lowest estimate of enzyme binding. Triton method estimates were nearly always double those of the dilution method while the airfuged method yielded even higher values. The one notable exception to this trend is found in PGK which shows similar values for binding by each method. The higher maximal activities measured by the triton method as compared to those of the dilution and airfuged methods indicates that either homogenization disrupts enzyme structure, denaturing

a portion of the total enzyme complement or that triton acts as a stabilizing agent preserving activity.

By the dilution method enzyme binding alterations have been measured in several animal models in direct correlation with glycolytic rate. This is the most rapid of the methods used thereby minimizing any in vivo changes which may occur post mortem. However, the resulting dilution of the cellular milieu can reduce metabolite and neutral salts to 25% of their values in vivo and may thus alter the degree of binding which involves largely ionic interactions (Brooks and Storey, in press). Dilution has been shown to yield higher estimates of binding than those measured by procedures which do not dilute the cytoplasm (Brooks and Storey in press; Plaxton and Storey, 1986; Ross and Hultin, 1980). Since all techniques used here involve some dilution another explanation for differences must be found. The considerable forces encountered in homogenization certainly disrupt the cytoskeletal lattice which is presumed to provide the support for binding and so may alter the apparent degree of enzyme association. However, the airfuge technique utilized the same homogenate as the dilution procedure and yielded considerably greater estimates of binding giving reason to believe homogenization and dilution effects are not responsible for the differences. The relatively lengthy period of centrifugation encountered with the airfuged method likely results in an alteration of the

original cellular conditions so that enzyme binding is ultimately measured under conditions of reduced ATP and phosphagen levels. Given that decreases in pH have been correlated with increases in enzyme binding (see goldfish data, Brooks and Storey in press, Somero, 1986) drops in homogenate pH due to anaerobic fermentation may also be a factor leading to the relatively high degree of binding measured by the airfuge method.

A desire to minimize the mechanical disruption of the cytoplasmic matrix during the extraction stage led to the use of triton. This detergent solubilizes the plasma membrane permitting the movement of cytosolic components out of the cell. This method is more rapid than the airfuged technique decreasing the potential effects of continued anaerobic metabolism in the tissue or homogenate. It also gives lower estimates of enzyme binding as does the rapid dilution procedure than the airfuged technique which may constitute indirect evidence of fermentation effects operating in samples. The longer the preparation time the greater the degree of enzyme particulate association. However, the differences measured between dilution and triton extracted samples are more likely due to one or both of the following: a) the presence of the plasma membrane may inhibit free movement of enzyme from the cytosol to the buffer; and b) cells situated at the centre of the minced tissue are not likely to be exposed to detergent. Either of

these could account for the higher binding estimates measured relative to those obtained by the dilution method. PGK, being the smallest glycolytic enzyme as well as existing as a monomer would be least affected by the presence of the plasma membrane and least liable to be entrapped during centrifugation. This likely explains the stability of PGK binding estimates obtained with all methods.

From these data it seems clear that increased glycolytic enzyme binding is a physiological response in the turtle to an anoxic stimulus. Two of three methods corroborate this although the third method (airfuge) is so fraught with potential for inaccuracies that it is deemed unreliable. Estimates obtained with the dilution method are likely the most reliable of the three owing to the rapidity of sample preparation and complete homogenization of tissues. Problems apparent with each of the available methods put considerable constraints on the development of a workable ideal procedure. Such a method would not involve dilution of the cellular milieu during extraction, and would reach all cells in a sample rapidly and with minimal disruption to the cytoskeletal matrix.

The results presented here demonstrate changes in glycolytic enzyme binding directly related to documented alterations in glycolytic rate in turtles as well as

goldfish and crayfish. This implicates enzyme binding in metabolic rate control although the relative importance of this component remains unknown. Elucidation of the contribution made by enzyme binding requires a more ideal fractionation procedure than those presently available. However, in light of recent evidence and the results of this study it is evident that enzyme partitioning between soluble and particulate phases of a cell is one of several components acting in a concerted manner to control cellular metabolism in response to environmental stresses.

SUMMARY

Changes in glycolytic enzyme binding in response to physiologically relevant stresses including anoxia and exercise were examined in three animal models: goldfish (Carassius auratus); crayfish (Orconectes virilis); and freshwater turtles (Pseudemys scripta elegans). To determine the validity of observed binding changes different fractionation procedures were compared including: a) dilution, b) quick press, c) airfuge, and d) triton solubilization methods. All three models exhibited binding changes in response to induced metabolic stresses. Goldfish heart enzymes demonstrated increased binding correlated with increased glycolytic rate in response to anoxia but no significant changes following exercise. Crayfish tail muscle exhibited a general decrease in glycolytic enzyme binding in response to anoxia with drops in percent bound averaging 6-10%. Exercise only yielded significant changes for phosphofructokinase, 20% to 28% bound, and phosphoglycerate kinase, 27% to 23% bound. In turtles, results obtained with the dilution and triton fractionation methods demonstrated that anoxia induced a general increase in glycolytic enzyme binding in all tissues examined (heart, brain, red and white skeletal muscle), the increase being most prominent in red and white skeletal muscle. The different methodologies used are critically compared and the

results discussed with respect to the role of enzyme binding,
in metabolic rate control.

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