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Full Name of Author — Nom complet de l'auteur

William Charles PLAXTON

Date of Birth — Date de naissance

December 16, 1954

Country of Birth — Lieu de naissance

Canada

Permanent Address — Résidence fixe

149 HENDERSON AVE
APT. 2
OTTAWA, ONT.
K1N 7P5

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Dr. K.B. Storey

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A STUDY OF THE METABOLIC ADAPTATIONS
OF MARINE GASTROPOD MOLLUSCS
TO ENVIRONMENTAL ANOXIC STRESS

by

WILLIAM CHARLES PLAXTON, B.Sc.

A thesis submitted to the Faculty of
Graduate studies and Research in partial fulfilment
of the requirements for the degree of
Doctor of Philosophy

Department of Biology

Carleton University

Ottawa, Ontario

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The undersigned hereby recommend to
The Faculty of Graduate Studies and Research
acceptance of the thesis, submitted by
William C. Plaxton, B.Sc.
in partial fulfilment of the requirements
for the degree of Doctor of Philosophy

.....
Chairman, Department of Biology

.....
Thesis Supervisor

.....
External Examiner

Carleton University

ABSTRACT

Catalytic and regulatory properties of alanopine dehydrogenase (ADH) and pyruvate kinase (PK) from tissues of anoxia tolerant marine gastropods were studied. Particular attention was given to those properties of the enzymes which could help explain their potential role(s) in anaerobic energy metabolism so as to provide insights into the adaptive forces involved in the evolutionary development of an extreme tolerance to anoxia.

The physical and kinetic properties of the terminal glycolytic dehydrogenase, ADH, purified to homogeneity from foot muscle of the common periwinkle, Littorina littorea, were examined. The kinetic properties of ADH, in particular the effects of rising co-substrate levels and decreased pH in lowering the apparent K_m 's for substrates, favor enzyme function in cytoplasmic redox balance during the recovery period following long-term environmental anoxia. At this time anaerobic glycolysis is activated and must supplement aerobic respiration in order to fulfill elevated tissue energy demands.

Tissue specific isozymes of ADH were found in another marine gastropod, the channelled whelk, Busycotypus canaliculatum. Three isozymic forms, specific for muscle, gill/kidney and hepatopancreas were separable by polyacrylamide gel electrophoresis and isoelectrofocusing. The three tissue specific isozymes of ADH were purified to homogeneity from foot muscle, gill and hepatopancreas and

their kinetic and physical properties were studied. Muscle ADH showed properties like those of L. littorea foot ADH which appear to gear this isozyme for alanopine synthesis as an end product of glycolysis. The hepatopancreas isozyme, with a high affinity for meso-alanopine and NAD^+ , appears suited for a role in alanopine oxidation in vivo. The properties of gill ADH are intermediate between those of the other two forms.

Tissue specific forms of PK were also found in B. canaliculatum. Three isozymic forms, specific for red muscle, white muscle and soft tissues, were separable by cellulose acetate electrophoresis and isoelectrofocusing and showed differential sensitivities to the allosteric activator fructose-1,6-bisphosphate. Furthermore, each PK isozyme was modified in animals subjected to 21 h of anoxic stress such that electrophoretic mobilities were increased, pI 's were decreased, and activities at low PEP concentrations were greatly reduced.

Aerobic and anoxic forms of red muscle PK (RPK-AER and RPK-ANX) were purified to homogeneity from radular retractor tissue of B. canaliculatum and the physical and kinetic properties of the enzyme were extensively studied. Compared to RPK-AER, RPK-ANX showed an unchanged native molecular weight, but decreased affinity for PEP, increased cooperativity in binding of PEP, increased susceptibility to inhibition by ATP and L-alanine, and decreased sensitivity to activation by fructose-1,6-bisphosphate. The differences

in kinetic properties between RPK-AER and RPK-ANX indicates that red muscle PK activity is probably greatly depressed in vivo during long-term anoxic stress. The anoxia-dependent, in vivo, covalent incorporation of injected [^{32}P] orthophosphate into RPK-ANX demonstrated that the enzyme is a phosphoprotein. Specificity in labelling was strongly suggested by: (i) coincidental elution of PK activity and radioactivity following chromatography of purified RPK-ANX on Sepharose CL-6B, and (ii) comigration of the area containing [^{32}P] phosphate and Coomassie blue staining protein following SDS-polyacrylamide gel electrophoresis of homogenous RPK-ANX. The [^{32}P] phosphate content of the enzyme was calculated to be 7.3 mol phosphate/mol of enzyme (233,000 daltons, 180 units/mg protein). Evidence for the reversibility of this phosphorylation was provided by the kinetic similarities between purified RPK-AER and homogenous alkaline phosphatase treated RPK-ANX. Comparison of the electrophoretic mobilities of products derived from acid hydrolysis of purified ^{32}P -labelled RPK-ANX with authentic substances indicated the presence of an O-phospho-L-threonine residue in the protein.

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List of Abbreviations and Symbols

ADP	- Adenosine diphosphate
ADH	- Alanopine dehydrogenase
AMP	- Adenosine monophosphate
ATP	- Adenosine triphosphate
cpm	- Counts per minute
dpm	- Disintegrations per minute
CoA	- Coenzyme A
EDTA	- Ethylenediaminetetraacetic acid
EGTA	- [Ethylenebis(oxyethylenitrilo)]-tetraacetic acid
FR	- Fumarate reductase
Fru-1,6-P ₂	- Fructose-1,6-bisphosphate
Fru-2,6-P ₂	- Fructose-2,6-bisphosphate
GAP.DH	- Glyceraldehyde-3-phosphate dehydrogenase
Hanes plot	- Substrate conc./velocity versus substrate conc.
HCl	- Hydrochloric acid
Hill plot	- $\log \text{velocity} / (V_{\text{max}} - \text{velocity})$ versus $\log \text{substrate concentration}$
I ₅₀	- Inhibitor concentration producing 50% decrease in enzyme velocity
IDP	- Inosine diphosphate
K _a	- Activator concentration producing 50% of maximal enzyme activation
K _{av}	- Represents the fraction of the stationary gel volume which is available for diffusion of a given solute species
KCl	- Potassium chloride
KHCO ₃	- Potassium bicarbonate
K _i	- Inhibitor-enzyme dissociation constant

LDH	- Lactate dehydrogenase
M	- Molar
mA	- Milliamperes
mCi	- Millicurries
MDH	- Malate dehydrogenase
MES	- [2-(N-Morpholino)ethane sulfonic acid]
MgCl ₂	- Magnesium chloride
MgSO ₄	- Magnesium sulphate
MnCl ₂	- Manganese chloride
mM	- Millimolar
mOsm	- Millosmolar
M _r	- Molecular weight
N ₂	- Dinitrogen
n _H	- Hill coefficient or slope of Hill plot
NaCl	- Sodium chloride
NAD ⁺	- Nicotinamide adenine dinucleotide
NADP ⁺	- Nicotinamide adenine dinucleotide phosphate
NADPH	- Nicotinamide adenine dinucleotide phosphate reduced
NaF	- Sodium fluoride
NH _x DH	- The hypoxanthine derivative of NADH
NH ₄ Cl	- Ammonium chloride
(NH ₄) ₂ SO ₄	- Ammonium Sulphate
OD ₂₈₀	- Optical density @ 280 nm
ODH	- Octopine dehydrogenase
P	- Phosphorus
PEP	- Phosphoenolpyruvate

PEP.CK - Phosphoenolpyruvate carboxykinase
 PK - Pyruvate kinase
 pH_i - Intracellular pH
 pI - Isoelectric point
 P_i - Orthophosphate
 PMSF - Phenylmethylsulfonyl fluoride
 R_f - Relative mobility = distance migrated by protein ÷ distance migrated by tracker dye
 RPK-AER - Red muscle PK from aerobic B. canaliculatum
 RPK-ANX - Red muscle PK from anoxic B. canaliculatum
 $S_{0.5}$ or K_m - Substrate concentration producing 50% of enzyme maximal velocity
 SDH - Strombine dehydrogenase
 SDS - Sodium dodecyl sulphate
 s.e.m. - Standard error of mean
 TAPS - N-tris(hydroxymethyl)methyl-3-aminopropane sulfonic acid
 $t_{1/2}$ - Half life
 Tris - tris(hydroxymethyl)aminomethane
 U. - Unit of enzyme activity; amount of enzyme catalyzing conversion of 1 μmol of substrate/min at specified temperature
 μCi - Microcurries
 μl - Microliters
 μmol - Micromoles
 μM - Micromolar
 V_{max} - Maximum enzyme velocity
 V_0 - Initial enzyme velocity in absence of effectors

CHAPTER I: General Introduction

The concept that animals are anatomically and behaviorally adapted to their environment has been with us since Darwin's time. Only during the past several decades has attention turned to the study of the physiological and biochemical adaptations which have permitted exploitation of all but the most inhospitable terrestrial and aquatic habitats available for life on earth. All such adaptations represent responses to the environment on an evolutionary time scale.

Although there are many exceptions, most animals of the phylum Mollusca are slow moving and confined to wide ranging, but quite specialized habitats. These include: the abysses of the sea, coral reefs, intertidal areas, mudflats, deserts, forests, rivers, lakes, underground and as parasites inside of other animals (Morton, 1979). Molluscan species which inhabit marine coastal areas show adaptations at all levels of biological organization: anatomically (a hard calcareous shell protects most species from predation as well as physical damage brought about by a pounding surf), behaviorally (many bivalve and gastropod species prevent dessication during air exposure at low tide by quickly closing their valves or withdrawing into their shell), physiologically (during anoxic stress the release of bicarbonate from the shell helps buffer body fluid pH), and biochemically. Biochemical adaptations, being less readily apparent, are the least well understood, but are none-the-less a key requirement for adaptation to any

environment. The aim of the present work is to examine several biochemical adaptations which underlie the remarkable tolerance to environmental anoxia commonly displayed by many marine molluscan species. 'Environmental' anoxia has been defined as "exposure of the whole organism to hypoxic or anoxic conditions in the natural environment" (Gade, 1983). This is contrasted with 'functional' anaerobiosis in which temporary anoxic conditions are generated in special tissues (mostly muscle) when initiated by a burst consumption of energy (Zebe et al., 1981).

Animal species adapted for survival of environmental anoxia are found in practically all major phyla, but respective tolerances to anoxic stress can vary tremendously (Table 1). Natural historians have found that many fish species possess impressive capacities for tolerating anoxic or hypoxic conditions. European carp often survive in small bodies of water which freeze over in the winter and through the winter months become totally anoxic (Blazka, 1958). Diving mammals, birds and reptiles also rely heavily upon anaerobiosis (Andersen, 1966; Clark and Miller, 1973; Simon et al., 1974). Other animal species with well developed anaerobic abilities are found amongst many invertebrate groups including the Mollusca, Echinoderma and Arthropoda (Mangum and van Winkle, 1973).

Hochachka and Somero (1973) have postulated that there are two mechanisms of biochemical adaptation in use during 'environmental' or 'functional' anaerobiosis.

Table 1. Approximate time limits of various animal species for survival of environmental anoxia.

<u>Species</u>	<u>Length of Anoxic Stress</u>
bird/duck	3-5 minutes
man	5-10 minutes
sea lion	5-10 minutes
porpoise	10-15 minutes
harbor seal	15-20 minutes
sperm whale	60-90 minutes
marine iguana	2 hours
turtle	days - months in winter
European carp	days - months in winter
bivalve/sea mussel	several days
bivalve/oyster	3 weeks
gastropod/channelled whelk	2 days

1. Exploitative: this strategy is employed by those organisms which undergo long term environmental anaerobiosis and do not depend upon an immediate return to oxygen. Metabolism is characterized by having a relatively high efficiency, but an overall low rate of energy production.

2. Compensatory: Organisms which undergo functional anaerobiosis compensate for temporary depletion of oxygen by increasing the anaerobic metabolic rate. Metabolism based on this strategy characteristically shows a high rate of energy production, but a low overall efficiency. This biochemical strategy depends upon a relatively quick return to aerobic conditions.

Many marine molluscs often experience large environmental fluctuations in oxygen availability. During low tide gill breathing intertidal species must cope with oxygen deprivation brought about by aerial exposure. Both intertidal and subtidal species must also frequently cope with a large reduction in oxygen availability in the sand or mud in which they burrow. During environmental anoxia the tissue demand for energy frequently increases owing to a combination of factors such as reduced cardiac output and muscular activity, and a cessation of activities such as filter feeding (de Zwaan and Wijsman, 1976; Zurburg, 1981). This reduced energy demand is reflected by a drastic reduction in overall metabolic rate; i.e. the 'exploitative strategy' (de Zwaan and Wijsman, 1976; Famme et al., 1981). The remarkable tolerance to oxygen deprivation displayed by

marine molluscs arises from their ability to meet the reduced, but very necessary, tissue energy requirements through a modification of intermediary metabolism.

Fundamental requirements for anaerobic energy production

At the biochemical level there are two fundamental requirements for the survival of anoxia (Hochachka, 1980). First, an adequate source of fermentable substrate must be provided for generation of ATP. The two normal metabolic fuels are carbohydrate and fat. However, it should be emphasized that carbohydrate stores (i.e. glycogen), can be utilized for either oxidative or for fermentative purposes, whereas fat, although a far more efficient storage fuel, can only be utilized aerobically (Weiss-Fogh, 1968). Hence, molluscan tissues are generally rich in glycogen. Amounts of glycogen range up to 35% of tissue dry weight in adductor muscle of the bivalve Mytilus edulis, whereas fat stores are very low (de Zwaan and van Marrewijk, 1973). The second basic requirement for anaerobic energy production is the presence of a standard metabolic 'equipment' (anaerobic glycolysis) for which the fermentable energy source is glycogen. Under the basic metabolic scheme shown in Fig. 1 ATP is generated in substrate-level phosphorylations catalyzed by phosphoglycerate kinase and pyruvate kinase (PK).

An essential requirement for continued energy

Fig. 1. Standard metabolic map of the glycolytic pathway as it occurs in anoxic vertebrate tissues.

production based upon anaerobic glycolysis is the maintenance of cytoplasmic redox balance. NAD^+ which is utilized at the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) reaction must be regenerated. Energy production in anoxic vertebrate tissues depends upon the action of lactate dehydrogenase (LDH) to maintain cytoplasmic redox balance, lactic acid accumulating as the product of carbohydrate fermentation (Fig. 1). The tissues of many marine invertebrates, however, contain activities of one or more imino acid dehydrogenases which functionally replace LDH as the terminal enzyme of anaerobic glycolysis (Fig. 2) (Dando et al., 1981; Livingstone et al., 1983).

Anaerobic energy metabolism in anoxia-tolerant marine molluscs

Many studies have shown that during molluscan environmental anaerobiosis multiple end products accumulate. Aside from species specificity in end product formation, considerable variation can occur between tissues of an individual species. Moreover, end products produced are often dependent on the duration of anaerobiosis and season (Gade, 1983). In general, the major end products are alanine and succinate and to a lesser extent the volatile fatty acids propionate and acetate and the imino acids alanopine and/or strombine (de Zwaan, 1983, Fields, 1983). The metabolic maps shown in Fig. 3 integrate most of the known information on marine molluscan energy metabolism.

Fig. 2. Terminal dehydrogenases of glycolysis in marine molluscs. Redox balance during anaerobic glycolysis in molluscan tissues is maintained through the action of lactate dehydrogenase and/or one of a number of imino acid dehydrogenases such as octopine dehydrogenase, alanopine dehydrogenase, or strombine dehydrogenase.

before, during and following exposure to long term environmental anoxic stress. Under aerobic conditions the catabolism of glycogen leads to pyruvate, which is then fully oxidized to carbon dioxide and water by the Krebs cycle reactions (Hammen, 1969), a metabolic organization not unlike that which occurs in vertebrate tissues (Fig. 3-A).

The initial phase of environmental anaerobiosis has been associated with the immediate mobilization of aspartate reserves for succinate production and glycogen for alanine production (Fig. 3-B) (Collicutt and Hochachka, 1977; Baginski and Pierce, 1977; Zurburg and Ebberink, 1981; de Zwaan et al., 1982; Eberlee et al., 1983; Korycan and Storey, 1983). Alanine is derived from pyruvate via transamination; amino groups for alanine synthesis are partially provided via the mobilization of aspartate reserves. Utilization of aspartate as a substrate for anaerobic metabolism is restricted to the first several hours of anoxic stress because of its relatively small pool size when compared to glycogen (de Zwaan, 1983; Gade and Ellington, 1983). Oxaloacetate formed via transamination of aspartate is reduced to malate by a cytoplasmic malate dehydrogenase (MDH) and is followed by the transport of malate into mitochondria. The MDH reaction serves to maintain cytoplasmic redox balance, regenerating NAD^+ used at the GAPDH reaction. Anaerobic malate metabolism has been studied in detail with in vitro preparations of mantle mitochondria from the sea mussel, M. edulis (de Zwaan et

Fig. 3. A - D: A metabolic map to account for the degradation of carbohydrates in marine molluscs. From left to right the different pathways represent gradual transitions in the carbon flow that may occur in the course of a tidal cycle, except for Fig. 3C (emersed; prolonged), which only occurs with much longer periods of anoxic stress. The width of the bars is an indication of the relative carbon flux through that part of the pathway.

Abbreviations: CH₂O, glycogen +/- glucose; ET, electron-transfer chain; OCT, octopine; PEP, phosphoenolpyruvate; PROP, propionate; PYR, pyruvate; S, malate shuttle; STR; strombine/alanopine; TF₁, transfer of NH₂ by glutamate-oxaloacetate transferase; TF₂, transfer of CoA by acyl-CoA transferase (from: de Zwaan, 1983).

al., 1981). Apparently, mitochondrial malate is converted into succinate by two different routes (Fig. 3-B and 3-C). The major portion is converted into fumarate, which is subsequently reduced to form succinate via a reversal of two reactions of the Krebs cycle. Fumarate reductase (FR) catalyzes the final reaction of this sequence and is the key reaction in the anaerobic mitochondrial production of ATP, during the conversion of malate to succinate (de Zwaan, 1983). Studies on both intact mitochondria and sub-mitochondrial particles of sea mussel tissues have shown that the FR reaction proceeds through a portion of the mitochondrial respiratory chain, namely the complexes NADH-ubiquinone oxidoreductase and ubiquinone-fumarate oxidoreductase, with fumarate replacing oxygen as the terminal electron acceptor (Holwerda and de Zwaan, 1979, 1980). As phosphorylation site I is linked to the electron transfer of NADH to ubiquinone it was expected, and proven, that the FR reaction is coupled to the phosphorylation of one equivalent of ADP (Holwerda and de Zwaan, 1979, 1980). A minor portion of the mitochondrial malate pool follows the Krebs cycle in a forward manner involving several oxidative steps (Fig. 3-C). Mitochondrial redox balance is thereby ensured; the provision of NADH for the reduction of fumarate to succinate being provided by the combined reactions of malic enzyme, MDH, pyruvate dehydrogenase, isocitrate dehydrogenase and α -ketoglutarate dehydrogenase. Partial operation of the Krebs cycle also allows for the

continuation of its role in anabolism as well as for the use of other substances (i.e. amino acids) as temporary energy reserves.

During prolonged anoxia the catabolism of glycogen to the level of phosphoenolpyruvate (PEP) is analogous to the process in vertebrates (Fig. 3-C). However, current evidence strongly suggests that during this period a metabolic rearrangement occurs at the level of PEP. PEP is carboxylated by a cytosolic phosphoenolpyruvate carboxykinase (PEP.CK) to form oxaloacetate thereby replacing aspartate as the precursor for mitochondrial succinate production (de Zwaan, 1977, 1983; Gade, 1983). As in the scheme depicted in Fig. 3-B the cytoplasmic reduction of oxaloacetate to malate reoxidizes NADH produced by the GAPDH reaction. This is again followed by transport of malate into the mitochondria and its subsequent conversion into succinate. As the reduction of fumarate is coupled to ATP production, this pathway of glycogen catabolism is energetically advantageous over the production of the various glycolytic end products derived from pyruvate (5 ATP/glucosyl unit vs. 3 ATP/glucosyl unit) (Zurburg, 1981). In addition, further catabolism of succinate to produce propionate provides 2 extra ATP per glucosyl unit utilized in those species which can produce propionate. However, propionate, being volatile, can be irretrievably lost, whereas succinate is usually not excreted.

That anaerobic aspartate and glycogen catabolism can occur via the pathways outlined above has been demonstrated through the use of radiotracer studies (Stokes and Awapara, 1968; de Zwaan and van Marrewijk, 1973; Collicutt and Hochachka, 1977; Gade and Ellington, 1983). The bulk of the label from [^{14}C -U] aspartate degradation is found in succinate, whereas the bulk of the label from [^{14}C -U] glucose is found in alanine during the early phase of anoxic stress and succinate during the latter stage.

As a Krebs cycle intermediate accumulated succinate should serve to 'prime' the cycle for a quick start upon return to aerobiosis. Not surprisingly, during the initial hours of recovery from long term anoxia succinate levels immediately decline in various bivalve and gastropod marine species (de Zwaan and Zurburg, 1981; Zurburg et al., 1982; Korycan and Storey, 1983; Eberlee et al., 1983; Eberlee and Storey, 1984). During this recovery period a significant accumulation of the glycolytic end products alanine and/or strombine parallels a large increase in the overall rate of glycolytic flux which is concomitant with an enhanced aerobic respiration for the whole animal (Ebberink et al., 1979; Ebberink and de Zwaan, 1980; de Zwaan and Zurburg, 1981; Zurburg et al., 1982; de Zwaan et al., 1983; Eberlee et al., 1983; Eberlee and Storey, 1984). The elevated metabolic demand during recovery appears to necessitate both aerobic and glycolytic energy production to meet the requirements for restoration of ATP, arginine phosphate and

aspartate pools, the removal of accumulated end products, as well as the resumption of normal metabolic activities such as feeding. Although tissue reoxygenation occurs rapidly after anoxia aerobic metabolism is unable to fulfill the entire energy requirements of the recovery period (de Zwaan et al., 1983). Glycolytic fermentation provides additional energy and leads to the accumulation of imino acids. Thus, based on the criteria of Hochachka and Somero (1973) the exploitative strategy used for survival of long term environmental anaerobiosis is replaced by a compensatory strategy (i.e. 'functional' anaerobiosis) during the recovery period when aerobic respiration cannot fulfill tissue energy demands.

Regulation of glycolytic flux and direction of PEP disposal during molluscan environmental anaerobiosis

The exploitative strategy employed by marine molluscs during environmental anaerobiosis generally involves a drastic reduction in energy demand (i.e. metabolic rate) and the use of the succinate pathway of glycogen fermentation with its increased ATP yield per glucosyl unit used. Thus, the flux regulating steps of glycolysis must be tightly regulated in response to the availability of oxygen.

An initial approach to identifying the flux regulating or control sites of glycolysis is to examine the maximal catalytic activities of the component enzymes under optimal

assay conditions. Reactions which have relatively low catalytic activities represent the more probable sites for regulation of the overall flux of the pathway (Krebs, 1963). Another method for locating control sites involves the application of the crossover theorem (Chance et al., 1958) to studies in which the levels of intermediates of a pathway are measured during transition between two steady states. A third approach to the location of rate limiting steps is provided by a determination of those reactions which are far from equilibrium under in vivo conditions (Newsholme and Start, 1973). Such reactions can be identified by calculation of an equilibrium constant* from measurements of the in vivo levels of products and substrates and comparison with the apparent equilibrium constant determined in vitro under similar conditions.

Potential control sites of glycolysis have been examined in the adductor muscle of M. edulis (Ebberink et al., 1979; Ebberink and de Zwaan, 1980) by using metabolite measurements to identify non-equilibrium reactions of this pathway (Newsholme and Start, 1973). As is the case for most animal tissues glycogen phosphorylase, phosphofructokinase (PFK) and PK were shown to catalyze the non-equilibrium reactions. In order to determine what role these non-equilibrium reactions play in the determination of the rate and direction of glycolytic flux during anaerobiosis the concentrations of all intermediates were followed as a function of time during 24h of anoxic stress

(Ebberink et al., 1979). It was concluded that only during the first few hours of anoxia, when glycogen is converted to alanine and aspartate to succinate, is the flow of carbon dependent on the activity of PFK. As the anaerobic period progresses the PK reaction was found to be more and more displaced from equilibrium (i.e. rate limiting), which leads to the accumulation of PEP, and its channelling towards succinate (Ebberink and de Zwaan, 1980).

Only recently has attention turned to examination of the kinetic properties of these rate limiting enzymes. During anoxia the activity of glycogen phosphorylase b from M. edulis adductor muscle is kept in check (Ebberink, 1983). Neither the inactive phosphorylase b kinase, nor the inactive phosphorylase b are phosphorylated to their active forms. However, partly purified phosphorylase b from anoxic M. edulis could be activated by AMP and this allosteric effect was believed to maintain a constant concentration of glucose-1-phosphate for use in anaerobic energy production. Properties of glycogen phosphorylase from aerobic tissues have not yet been examined, but it is possible that during the 'burst' of glycolytic flux associated with recovery from anoxia it is covalently modified to yield a fully active enzyme.

PFK is generally believed to be the key control site of glycolysis and invariably displays complex control properties that allow enzyme activity to be finely tuned to a variety of metabolic signals (Mansour, 1972).

M. edulis adductor PFK shows classical allosteric kinetic behavior with respect to its substrate fructose-6-phosphate, is inhibited by PEP, ATP and H^+ and activated by AMP (Ebberink, 1982). These properties were felt to fit in well with the metabolic events occurring in M. edulis muscle, such that during anaerobiosis PFK activity was decreased (owing mainly to a drop in pH combined with inhibition by PEP) and during recovery its activity was greatly stimulated. No attention was given to possible control of PFK via covalent modification (e.g. phospho-/dephosphorylation) during anaerobiosis or to the role of the potent activator fructose-2,6-bisphosphate.

The control of the carbon flow through the PEP branchpoint during molluscan environmental anaerobiosis has been the subject of many papers and reviews over the past decade. Early studies of PK from muscle tissues anoxia-tolerant molluscs indicated that unlike its mammalian muscle counterpart it is an allosteric enzyme (Mustafa and Hochachka, 1971; de Zwaan 1972; de Zwaan and Holwerda, 1972). Hochachka and Mustafa (1972) proposed that the combined effects of a drop in pH and the increase in alanine concentrations which accompany anaerobiosis serve to inactivate PK. To produce succinate via the pathways shown in Fig. 3-C carboxylation of PEP by PEP.CK must occur. In mammalian tissues, PEP.CK functions in gluconeogenesis (Scrutton and Utter, 1968), but in facultative anaerobes such as intertidal bivalves it is kinetically poised in the

direction of oxaloacetate production (de Zwaan and van Marrewijk, 1973; Mustafa and Hochachka, 1973a,b). PEP.CK is believed to be activated during anaerobiosis owing to increasing alanine and decreasing pH. Recent studies suggest that PK from muscle tissue of M. edulis is also subject to probable control via covalent modification (Sibenaller, 1979; Holwerda et al., 1981; Holwerda et al., 1983; Holwerda et al., 1984). This fact is based primarily on the finding that during the course of anoxia, in the absence of other effectors, the activity of the enzyme becomes greatly depressed at low concentrations of PEP. The reversible covalent modification of a molluscan PK in response to oxygen availability forms an important facet of the present study and as shall be seen is probably the single most important factor for controlling the direction of carbon flow at the PEP branchpoint during long term environmental anaerobiosis of marine molluscs.

All work reported in this thesis was confined to gastropod species for two main reasons. First, despite the fact that about three-quarters of molluscs are gastropods (Morton, 1979), very few studies have been published concerning the metabolic adaptations of marine gastropods during environmental anaerobiosis. The ability of gastropods to withstand experimental anoxia varies between species, but, in general anaerobic functions are thought to be less well developed than in bivalves (von Brand, 1946).

Like many marine bivalve species, a number of anaerobic end products including lactate, octopine, alanopine, strombine, alanine, succinate and propionate have been detected in marine gastropod species (Wieser, 1980; Ellington, 1981; Livingstone and de Zwaan, 1983; Eberlee and Storey, 1984). End products produced are dependent upon animal life-style and in the case of alanopine or strombine also vary between phylogenetic groups (Livingstone et al., 1982). Available evidence suggests that the general pattern of carbon flux and time course of end product accumulation occurring in tissues of anoxia-tolerant marine gastropods before, during, and following long term environmental anoxic stress is similar to that depicted in Fig. 3 (Eberlee and Storey, 1984). A second reason for confining the present work to gastropod species is that with their closed circulation system and discrete organs, which appear to function similarly to those of higher animals, the Gastropoda are good candidates for the possible occurrence of tissue specific differences in anaerobic energy metabolism. Such differences have been well documented for cephalopod molluscs (Storey and Storey, 1983) and other higher life forms.

This thesis concerns the study of two enzymes involved in marine molluscan anaerobic energy metabolism, alanopine dehydrogenase (ADH) and pyruvate kinase, in order to determine how their properties have been adapted for the metabolic role they play in vivo. Initially, an examination

of the newly discovered and little studied terminal glycolytic dehydrogenase, ADH, from two gastropods, the common periwinkle, Littorina littorea (Chapter 2), and the channelled whelk, Busycotypus canaliculatum (Chapters 3 and 4), was undertaken. Amongst the data reported are the first-ever: (1) kinetic studies on the reverse, or alanopine-oxidizing, reaction of ADH (Chapters 2 and 4), and (2) demonstration of tissue specific isozymes of ADH (Chapter 3 and 4). The latter part of this thesis deals with the key glycolytic enzyme PK from tissues of B. canaliculatum and the regulatory mechanisms whereby it controls the fate of glycolytic carbon at the PEP branchpoint during exposure to, and recovery from, long term environmental anoxic stress. Chapter 5 provides the first kinetic and physical evidence for: (1) gastropod tissue specific PK isozymes, and (2) the modification of gastropod PK in response to environmental anoxia. Subsequent sections of this thesis report the first-ever: (1) purification to homogeneity and extensive kinetic characterization of aerobic and anoxic forms of molluscan PK (Chapter 6), and (2) conclusive evidence that the anoxia-induced inactivation of a molluscan PK is due to the reversible covalent incorporation of phosphate into the enzyme molecule (Chapter 7).

CHAPTER II: Alanopine dehydrogenase: purification and
characterization of the enzyme from Littorina littorea foot
muscle.

Introduction

Anaerobic energy production in vertebrate tissues depends upon the action of LDH to maintain cytoplasmic redox balance, lactic acid accumulating as the product of carbohydrate fermentation. The tissues of many marine invertebrates, however, contain activities of one or more imino acid dehydrogenases:

pyruvate + amino acid + NADH + H⁺ $\longleftrightarrow$ imino acid + NAD⁺ + H₂O, which functionally replace LDH as the terminal enzyme of anaerobic glycolysis (Regnoui and Thoai, 1970; Fields, 1976, de Zwaan and Zurburg, 1981, Dando et al., 1981). Octopine dehydrogenase (ODH), catalyzing the reductive condensation of pyruvate and L-arginine, has been well characterized (Thoai et al., 1969; Fields et al., 1976; Storey and Storey, 1979a); amongst cephalopod molluscs, high speed swimming, supported by glycogenolysis and arginine phosphate breakdown, results in the accumulation of octopine as the end product of mantle muscle metabolism (Grieshaber and Gade, 1976; Storey and Storey, 1979b; Baldwin and England, 1980).

Alanopine dehydrogenase (ADH), catalyzing the reaction: pyruvate + L-alanine + NADH + H⁺ $\longleftrightarrow$ meso-alanopine + NAD⁺ + H₂O, was first identified in 1976 in the muscle tissues of the oyster, Crassostrea gigas (Fields, 1976) while alanopine (2,2'-iminodipropionic acid) and/or strombine (2-methyliminodiacetic acid) accumulate as end products of

^{14}C -glucose fermentation during anoxia in oyster ventricle (Collicutt and Hochachka, 1977). Subsequently ADH and strombine dehydrogenase (SDH) (utilizing glycine as the amino acid substrate) activities have been identified in tissues of all major phyla except Athropoda, Echinodermata and Chordata (Livingstone et al., 1983). Some kinetic properties of purified oyster adductor muscle ADH have recently been reported (Fields and Hochachka, 1981); the enzyme has broad amino acid specificity, utilizing L-alanine, glycine and several other amino acids at nearly equal rates and probably produces both alanopine and strombine in vivo. Similar results were found for the partially purified adductor muscle enzyme from the bivalves M. edulis (Dando, 1981), Mercenaria mercenaria (Storey et al., 1982) and Modiolus squamosus (Nicchitta and Ellington, 1983). Kinetic properties of SDH from the tissues of the sponge, Halichondria panicea (Barret and Butterworth, 1981) and ADH and SDH from tissues of the sea mouse, Aphrodite aculeata (Storey, 1983), also been reported.

In the present study an examination of the kinetic properties of ADH purified to homogeneity from foot muscle of the common periwinkle, Littorina littorea has been undertaken. L. littorea are small herbivorous gastropod molluscs belonging to the order Meogastropoda and are well adapted to the high-tidal zone (Morton, 1979). Periwinkle foot muscle ADH, unlike the enzyme from bivalves, sponges and polychaetes, was found to be highly specific for both

L-alanine and meso-alanopine as substrates. This specificity may link enzyme function in vivo to the recovery period following anaerobiosis, the production of alanopine coupling two products, pyruvate and alanine, of anaerobic glycolysis. Other kinetic characteristics of the enzyme included strong pH effects on enzyme affinity for pyruvate and alanine which also appear to promote alanopine synthesis during the recovery period.

Materials and Methods

Animals and Chemicals.

Specimens of the common periwinkle, Littorina littorea, were obtained from local fish markets. Foot muscle was dissected out and stored at -80°C until use. No loss of enzyme activity was seen during storage for periods of at least one month.

D-strombine and meso-alanopine were provided by Dr. J. Tempe and Dr. P.R. Dando. ATP, ADP, and D- and L-lactate and NADH were purchased from Boehringer Mannheim Corp. while all other biochemicals were from Sigma Chemical Co. Sephadex G-100, PBE 94 chromatofocusing exchanger and Polybuffer 74 were obtained from Pharmacia Fine Chemicals, Ampholines (pH 4-6) were from LKB products, and molecular weight standard proteins were from Sigma and Pharmacia. Unless otherwise indicated all amino acids used were pure L-isomers. All buffers used in the study were adjusted to pH at 23°C .

Enzyme Assay and Kinetic Studies.

ADH activity was assayed by monitoring NAD(H) utilization at 340 nm using a Gilford recording spectrophotometer attached to a circulating water bath for temperature control of cuvettes. Standard assay conditions for ADH were: 50 mM imidazole-HCl buffer, pH 7.5, 1.3 mM pyruvate, 130 mM alanine and 0.1 mM NADH in the forward direction, and 50 mM Tris-HCl buffer, pH 9.2, 50 mM meso-alanopine and 2 mM NAD⁺ in the reverse direction in a final volume of 1 ml. Assays were started by the addition of enzyme preparation. Standard assay conditions for LDH were: 50 mM imidazole-HCl buffer, pH 7.5, 1.3 mM pyruvate and 0.1 mM NADH. One unit of enzyme activity is defined as the amount of enzyme utilizing 1 μ mol of NADH per minute at 23 °C.

Apparent K_m values were determined from Hanes plots under conditions of constant, saturating concentrations of co-substrate(s) while absolute K_m values for alanine and pyruvate were derived from secondary plots using data from apparent K_m determinations at 3 subsaturating co-substrate concentrations (Cornish-Bowden, 1979). Inhibitor constants (K_i) were determined from Dixon plots and the effect of inhibitors was evaluated using double reciprocal plots. I_{50} 's (inhibitor concentration producing 50% inhibition of enzyme activity) were determined by the method of Job et al. (1978). All kinetic parameters are the means of duplicate determinations on three separate preparations of the

purified enzyme and are reproducible within at least $\pm 10\%$. In most cases, results are expressed as means $\pm$ s.e.m.

Purification of Alanopine Dehydrogenase.

Foot muscle was minced with scissors and homogenized (1:5 w/v) for 2 X 1 min in ice-cold 50 mM imidazole-HCl buffer, pH 7.5, containing 15 mM 2-mercaptoethanol, using a Polytron PT 10 homogenizer. The homogenate was centrifuged at 27,000 X g for 20 min at 4 °C and the supernatant used as the source of crude enzyme.

The crude supernatant was brought to 2.95 M (60% saturation) with ammonium sulphate, stirred at room temperature for 30 min, and then centrifuged as above. The protein pellet was discarded and the resulting supernatant was then adjusted to 4.03 M (80% saturation) with ammonium sulphate and again stirred and centrifuged. The pellet was resuspended in 2-3 ml of 20 mM imidazole-HCl buffer, pH 7.4, containing 10 mM 2-mercaptoethanol and centrifuged to remove insoluble material. The partially purified enzyme solution was then layered onto a column (60 X 0.9 cm) of Sephadex G-100 equilibrated in 20 mM imidazole-HCl buffer, pH 7.4, containing 10 mM 2-mercaptoethanol. The column was eluted at 20 ml/h using the same buffer and 1 ml fractions were collected. Peak fractions, containing ADH activity were pooled and layered onto a column (30 X 0.9 cm) of PBE 94 chromatofocusing exchanger pre-equilibrated in the same buffer. The column was eluted at 20 ml/h with 200 ml

Polybuffer 74 (stock diluted 1:8 with distilled water) adjusted to pH 4.0 and 1 ml fractions were collected. ADH activity eluted in a single sharp peak at pH 5.7 (± 0.05 , $n=3$) on the linear pH gradient. Peak fractions from chromatofocusing were pooled and applied to a second, larger G-100 column (90 X 1.5 cm) equilibrated in 50 mM imidazole-HCl buffer, pH 7.5, containing 10 mM 2-mercaptoethanol and 0.04% sodium azide. The column was eluted with this same buffer and peak fractions, containing purified ADH, were combined and stored at 4°C for use in kinetic studies. The purified enzyme was stable in this form, with no loss of activity or change in kinetic parameters, for at least 2 weeks.

Molecular Weight Determination.

Molecular weight determinations were made on a column (90 X 1.5 cm) of Sephadex G-100 using 50 mM imidazole-HCl buffer, pH 7.5, containing 10 mM 2-mercaptoethanol and 0.04% sodium azide as the equilibration/elution buffer. One ml fractions were collected and assayed for absorbance at 280 nm and ADH activity. The molecular weight of ADH was determined from a plot of K_{av} (partition coefficient) versus $\log M_r$ for the standard proteins: ribonuclease ($M_r = 13,700$), chymotrypsinogen ($M_r = 25,000$), ovalbumin ($M_r = 45,000$) and bovine serum albumin ($M_r = 67,000$).

Electrophoresis.

Polyacrylamide gel electrophoresis using 10% slab gels

was carried out at 4 °C with 40 mA constant current for about 4 h. Tris-HCl/glycine (46 mM/35 mM), pH 8.0 ($\pm$ 1% SDS) was used as the electrode buffer with bromophenol blue as the tracker dye. Duplicate non-SDS gels were stained for protein using the silver stain of Switzer et al. (1979) or for ADH activity. To detect enzyme activity, gels were incubated in the dark at room temperature for 30 min in a mixture of 50 mM Tris-HCl buffer, pH 8.5 (8.0 ml), 50 mM meso-alanopine (2 ml), 10 mg/ml NAD (2 ml) and 1 mg/ml nitroblue tetrazolium (5 ml) followed by visualization of the enzyme band by the addition of 0.5 ml of 1 mg/ml phenazine methosulfate. For SDS gels, protein samples were preincubated in 1% SDS for at least 2 h at 37 °C, then run and stained for protein as described above. For the determination of subunit molecular weight using SDS electrophoresis, a plot of R_f versus log molecular weight was constructed using the following standard proteins: trypsinogen (24,000), ovalbumin (45,000) and bovine serum albumin (66,000).

Isoelectrofocusing.

Isoelectrofocusing was performed by the method of Vesterberg (1971) using an LKB 8101 column (110 ml) and a pH gradient of ampholines of 4.0 to 6.0. Crude enzyme supernatant was run at 400 V for 14 h at 4 °C. The column was then drained and 1 ml fractions collected and assayed for ADH activity.

Protein Assay.

Protein concentration was measured by the method of Bradford (1976) using the Bio-Rad prepared reagent and bovine gamma globulin as the standard.

Results

Foot Muscle Enzyme Activities

L. littorea foot muscle contains two cytoplasmic dehydrogenases acting at the pyruvate branchpoint: ADH (11.1 units/g wet weight) and D-lactate specific LDH (12.2 units/g wet weight). ODH is not present.

Purification and Physical properties.

The purification of ADH is summarized in Table 2. The enzyme was purified about 1,300-fold to a final specific activity of 240 units/mg protein and was judged to be homogenous by SDS polyacrylamide gel electrophoresis (Plate 1).

The molecular weight of L. littorea ADH, determined by gel filtration, was found to be 42,200 $\pm$ 500 (n=4) similar to that reported for other alanopine/strombine dehydrogenases: 47,000 for the oyster (C. gigas) enzyme (Fields and Hochachka, 1981) 40,000 for the sea mussel (M. edulis) enzyme (de Zwaan and Zurburg, 1981) and 44,000 for the sea mouse (A. aculeata) enzyme (Storey, 1983). Like other imino acid dehydrogenases, L. littorea ADH was a

Table 2. Summary of the purification of alanopine dehydrogenase from the foot muscle of the gastropod mollusc, Littorina littorea.

Step	Total Activity (units)	Total Protein (mg)	Specific Activity (units/mg)	Purification (fold)	Yield (%)
Crude supernatant	340	1728	0.19	-	-
Ammonium Sulphate	238	216	1.10	6	70
Sephadex G-100	200	6.9	29.0	153	59
Chromatofocusing	167	1.9	88.0	463	49
Sephadex G-100	116	0.48	240.0	1263	34

Plate 1. SDS polyacrylamide gel electrophoresis of purified
alanopine dehydrogenase from L. littorea foot muscle.

monomer; SDS gel electrophoresis showed a subunit size of 42,400.

Isoelectrofocusing showed an isoelectric point of 5.6 ± 0.02 ($n=3$).

In all preparations only a single band of ADH activity was seen on either SDS or non-SDS gels stained for protein or for enzyme activity. Isoelectrofocusing of the crude enzyme preparation showed only a single peak of ADH activity. There was no evidence, therefore, for multiple molecular forms of ADH in foot muscle.

Substrate Specificity.

The substrate specificity of purified ADH was assessed in terms of apparent K_m values and relative velocities (Table 3). Alanine and pyruvate were the preferred substrates of the enzyme. Low activity was found with four alternative amino acid substrates while three alternative keto acid substrates were shown. Relative activity with oxaloacetate as an alternative substrate was high, but the apparent K_m for this keto acid was too great to suggest a physiological role for this substrate. No enzyme activity was observed with other amino acids including arginine, aspartate, glutamate, histidine, isoleucine, leucine, methionine, phenylalanine, proline, taurine, tyrosine, asparagine, glutamine, lysine, threonine, tryptophan or valine, with D-alanine, with NH_4^+ , or with hydroxypyruvate, α -ketoglutarate, or α -ketovalerate. In the reverse

Table 3. Substrate specificity of *L. littorea* alanopine dehydrogenase.

Substrate	Apparent K_m (mM)	Relative velocity
Alanine	47	100
Glycine	1267	37
L- α -aminobutyrate	67	33
Serine	220	13
Cysteine	35	4
Pyruvate	0.55	100
Oxaloacetate	5.7	94
α -Ketobutyrate	9.4	37
Glyoxylate	10.0	10

Assay conditions were 50 mM imidazole-HCl buffer, pH 7.5, with constant co-substrate concentrations, 1.3 mM pyruvate, 130 mM alanine and 0.1 mM NADH. Relative velocities were measured at saturating levels of each of the amino or keto acids and are expressed relative to the apparent V_{max} with alanine and pyruvate as substrates.

direction, activity was observed with meso-alanopine only. No activity was apparent with up to 30 mM D-strombine or 200 mM iminodiacetic acid. Similarly, gels stained for ADH activity showed no band of enzyme activity when D-strombine was substituted for meso-alanopine. The enzyme was specific for NAD(H) showing no activity in the presence of NADP(H).

pH Effects.

pH profiles of periwinkle muscle ADH are shown in Fig.

1. In the forward, alanopine-producing direction, the enzyme has a pH optimum of 7.5 at high substrate concentrations (130 mM alanine, 1.3 mM pyruvate, 0.1 mM NADH: saturating substrate conditions at pH 7.5), but this is shifted to about 6.5 at lower substrate concentrations (100 mM alanine, 1 mM pyruvate, 0.1 mM NADH: saturating substrate concentrations at pH 6.5). This effect appears to be related to the pyruvate and alanine substrate inhibition characteristics of the enzyme. In the reverse direction, the pH optimum was 9.2 at 50 mM meso-alanopine but dropped to 8.5 at lower (10 mM) levels of meso-alanopine. The ratio of the maximal activities in the forward (at pH 7.5) versus reverse (at pH 9.2) directions was about 5:1.

Kinetic Constants.

L. littorea ADH followed Michaelis-Menten saturation kinetics for all substrates. Kinetic constants for ADH are given in Table 4. Due to the marked effect of pH on the K_m 's for pyruvate and alanine and the possible importance of

Fig. 4. pH profiles for L. littorea alanopine dehydrogenase in the forward and reverse directions. Conditions are:

- , 1 mM pyruvate, 100 mM alanine, 0.1 mM NADH, 50 mM imidazole-HCl buffer (saturating conditions at pH 6.5);
 - , 1.3 mM pyruvate, 130 mM alanine, 0.1 mM NADH, 50 mM imidazole-HCl buffer (saturating conditions at pH 7.5);
 - , 10 mM meso-alanopine, 2 mM NAD^+ , 50 mM Tris-HCl buffer;
 - , 50 mM meso-alanopine, 2 mM NAD^+ , 50 mM TAPS-HCl buffer.
- Results are expressed relative to V_{max} for the forward direction which is set at 100.

Table 4. Michaelis constants for *L. littorea* alanopine dehydrogenase at two pH's.

Substrate	pH 6.5	pH 7.5
Absolute K_m (mM)		
Alanine	14.9 $\pm$ 0.85	23.8 $\pm$ 0.52
Pyruvate	0.17 $\pm$ 0.02	0.26 $\pm$ 0.01
Apparent K_m (mM)		
NADH	0.009 $\pm$ 0.0001	0.009 $\pm$ 0.0001
meso-Alanopine	6.5 $\pm$ 0.59	16.9 $\pm$ 1.73
NAD ⁺	0.15 $\pm$ 0.03	0.18 $\pm$ 0.003

Kinetic constants were derived as outlined in Materials and Methods from analyses of three separate preparations of the purified enzyme. Values are means $\pm$ s.e.m. Assays were performed at 23°C in 50 mM imidazole-HCl buffer with constant co-substrate concentrations, where appropriate, of 1.3 mM pyruvate, 130 mM alanine, 0.1 mM NADH, 30 mM meso-alanopine, and 2 mM NAD⁺ at pH 7.5 and 1.0 mM pyruvate, 100 mM alanine, 0.1 mM NADH, 15 mM meso-alanopine, and 2 mM NAD⁺ at pH 6.5.

intracellular pH in regulating anaerobic metabolism in marine invertebrates, the kinetics of the enzyme in the forward direction were examined at two pH's, 6.5 and 7.5. The apparent K_m 's for pyruvate and alanine were strongly influenced by co-substrate concentration (alanine or pyruvate), the apparent K_m for either substrate being reduced with increasing co-substrate concentrations. This is apparent from Fig. 5 and Fig. 6. This effect is well known for ODH, ADH and SDH from a variety of sources (Thoai et al., 1969; Fields et al., 1976; Storey and Storey, 1979b; Storey, 1983; Nicchitta and Ellington, 1984). Table 4 shows the absolute K_m 's for pyruvate and alanine. K_m 's for both substrates were influenced by pH, decreasing by 35-37% when pH was lowered from 7.5 to 6.5. The apparent K_m for NADH was not affected by pH nor was the apparent K_m for NAD^+ in the reverse direction. However, the apparent K_m for meso-alanopine, like those for pyruvate and L-alanine, was strongly dependent on pH. While enzyme activity in the reverse direction was highest at pH 8.5, the K_m at this pH was also very high (50 mM), but K_m fell with decreasing pH, reaching 6.5 mM at pH 6.5.

Pyruvate and Alanine Substrate Inhibition.

The effect of pyruvate concentration on the activity of ADH is shown in Fig. 5. At saturating concentrations of alanine, pyruvate substrate inhibition was apparent at pyruvate concentrations above 1.0 mM at pH 6.5 and above 1.3

Fig. 5. A + B: Effect of pyruvate substrate concentration on reaction velocity for L. littorea alanopine dehydrogenase at saturating and subsaturating alanine concentrations and two pH's. Assays were performed at 23°C with 0.1 mM NADH and 50 mM imidazole-HCl buffer.

Fig. 5-A: pH 6.5, alanine concentrations:

□, 15 mM; △, 30 mM; ○, 100 mM.

Fig. 5-B: pH 7.5, alanine concentrations:

□, 30 mM; △, 50 mM; ○, 130 mM.

Fig. 6. A + B.: Effect of alanine substrate concentration on reaction velocity for L. littorea alanopine dehydrogenase at saturating and subsaturating pyruvate concentrations and two pH's. Assays were performed at 23°C with 0.1 mM NADH and 50 mM imidazole-HCl buffer.

Fig. 6-A: pH 6.5, pyruvate concentrations:

□, 0.15 mM; △, 0.35 mM; ○, 1.0 mM.

Fig. 6-B: pH 7.5, pyruvate concentrations:

□, 0.20 mM; △, 0.50 mM; ○, 1.3 mM.

mM at pH 7.5. An I_{50} of about 8 mM pyruvate was observed at both pH's, similar to I_{50} 's of about 10 mM reported for pyruvate substrate inhibition of H type LDH and the brain specific isozyme of ODH (Long and Kaplan, 1973; Eichner and Kaplan; 1977; Storey and Storey, 1979a). Muscle forms of LDH and ODH typically do not show pyruvate substrate inhibition. However, muscle ADHs differ in this property, both L. littorea, C. gigas (Fields and Hochachka, 1981) and A. aculeata (Storey, 1983) muscle ADH show substrate inhibition by pyruvate. Substrate inhibition at high pyruvate levels decreased, however, as alanine concentrations were lowered to subsaturating levels (Fig. 5); a similar phenomenon was noted for ADH from muscle tissue of A. aculeata (Storey, 1983).

Comparable effects were found when alanine substrate inhibition was examined (Fig. 6). At saturating concentrations of pyruvate, substrate inhibition by alanine was apparent above 100 mM at pH 6.5 and above 130 mM at pH 7.5. The apparent I_{50} 's were 450 mM at pH 6.5 and 550 mM at pH 7.5. At subsaturating pyruvate concentration, however, alanine substrate inhibition was lost.

Metabolite and Salt Effects.

A variety of metabolites was tested for effects on ADH activity in the forward direction at subsaturating substrate concentrations (50 mM imidazole-HCl buffer, pH 7.5, 0.5 mM pyruvate, 40 mM alanine, and 0.02 mM NADH).. The following

compounds (at 10 mM) had no effects on ADH activity: glucose-6-phosphate, fructose-6-phosphate, phosphoenolpyruvate (PEP), D-lactate, malate, fumarate, succinate, arginine phosphate, carnitine, acetyl-CoA and the amino acids, proline, histidine, methionine and tyrosine. The pyruvate analogue, oxamate (10 mM), the glycolytic intermediates, fructose-1,6-bisphosphate, 3-phosphoglycerate and dihydroxyacetone-phosphate (all 10 mM) and the Krebs cycle intermediates, citrate, and α -ketoglutarate (both 10 mM), all produced 10-25% inhibition of ADH activity. Other amino acids, arginine, glutamate, lysine (all 100 mM), leucine, isoleucine (both 30 mM) and tryptophan (3 mM) inhibited enzyme activity by 25-40% compared to controls. However, the effects of these inhibitors were not further investigated due to the non-physiological concentrations needed to produce the observed inhibitory effects.

ATP, ADP, NAD^+ , meso-alanopine and L-lactate were inhibitors of ADH (Table 5). Product inhibition by NAD^+ was competitive with respect to NADH while meso-alanopine was a competitive inhibitor with respect to both pyruvate and alanine, the K_i for alanopine being the same (35 mM) with respect to either substrate. L-lactate inhibition is probably a result of the structural similarity between L-lactate and the substrates, pyruvate and alanine. Inhibition by L-lactate has no physiological relevance, however, as L. littorea tissues contain a D-LDH. The adenylates, ATP and ADP, as well as their Mg^{2+} complexes

Table 5. Inhibitor effects on *L. littorea* alanopine dehydrogenase.

<u>Inhibitor</u>	<u>Apparent K_i (mM)</u>	<u>Inhibition</u>	
		<u>type</u>	<u>with respect to</u>
NAD ⁺	0.16 $\pm$ 0.012	competitive	NADH
ATP	5.6 $\pm$ 0.25	competitive	NADH
Mg.ATP	3.0 $\pm$ 0.32	competitive	NADH
ADP	1.7 $\pm$ 0.25	competitive	NADH
Mg.ADP	4.2 $\pm$ 0.32	competitive	NADH
meso-Alanopine	35.0 $\pm$ 0.35	competitive	Alanine & pyruvate
L-lactate	3.2 $\pm$ 0.12	competitive	Alanine
	5.0 $\pm$ 0.21	mixed competitive	Pyruvate

Inhibitor constants were determined from Dixon plots with type of inhibition evaluated by double reciprocal plots. Assay conditions were 50 mM imidazole-HCl buffer, pH 7.5, with constant concentrations of co-substrates, 1.3 mM pyruvate, 130 mM alanine and 0.1 mM NADH. Values are means $\pm$ s.e.m. for n = 3 determinations on separate preparations of purified enzyme. Mg²⁺:adenylate ratio was 1:1.

(ratio 1:1 Mg:adenylate) were competitive inhibitors with respect to NADH. ATP effects probably occur within the physiological range of ATP concentrations in vivo although ATP inhibition of dehydrogenases is probably due to structural rather than regulatory effects, reflecting the presence of an adenine nucleotide domain in the coenzyme binding site (McPherson, 1970).

To examine interactions between inhibitors and pH, inhibitor effects (I_{50} 's) were quantitated at pH 6.5 and 7.5 using subsaturating substrate levels (Table 6). The inhibitory effects of ATP, ADP and NAD^+ on ADH activity decreased (I_{50} decreased) at pH 6.5 compared to pH 7.5 while inhibition by meso-alanopine, D-strombine and L-lactate showed the opposite response. Succinate, which had no effect on enzyme activity at pH 7.5, was inhibitory, at high concentrations, at pH 6.5.

Monovalent and divalent ions had inhibitory effects on ADH activity (Fig. 7). Inhibition by 200 mM salt was 16, 23, 33 and 38% for $(\text{NH}_4)_2\text{SO}_4$, NH_4Cl , KCl and NaCl , respectively, at pH 7.5 and subsaturating substrate concentrations (0.4 mM pyruvate, 40 mM alanine, 0.03 mM NADH). Mg^{2+} ions were also inhibitory; at 50 mM salt, inhibition was 30 and 45% in the presence of MgSO_4 and MgCl_2 , respectively.

Table 6. Inhibitor effects on L. littorea alanopine dehydrogenase at two pH's.

Inhibitor	<u>I₅₀ (mM)</u>	
	<u>pH 6.5</u>	<u>pH 7.5</u>
NAD ⁺	0.50	0.38
ATP	2.50	1.50
ADP	3.65	2.50
meso-Alanopine	9.50	25.0
D-Strombine	14.50	61.0
L-Lactate	0.90	3.15
Succinate	19.0	no inhibition

Inhibition constants (I_{50}) were determined as outlined in Materials and Methods. Assays were performed in 50 mM imidazole-HCl buffer at constant subsaturating substrate conditions: 0.2 mM pyruvate, 20 mM alanine, 0.025 mM NADH at pH 6.5 and 0.25 mM pyruvate, 25 mM alanine, 0.025 mM NADH at pH 7.5. Values are means of $n = 2$ determinations with variation of $\pm 10\%$.

Fig. 7. Salt effects on L. littorea alanopine dehydrogenase.

Enzyme activity was assayed in 50 mM imidazole-HCl buffer, pH 7.5, with subsaturating substrate concentrations: 0.4 mM pyruvate, 40 mM alanine, 0.03 mM NADH. Additions are: $\square$, $(\text{NH}_4)_2\text{SO}_4$; $\blacksquare$, NH_4Cl ; $\circ$, NaCl ; $\bullet$, KCl ; $\triangle$, MgSO_4 ; $\blacktriangle$, MgCl_2 .

Discussion.

ADH, purified from L. littorea, differs most strongly from purified oyster muscle ADH in the substrate specificities of the enzyme. L. littorea ADH is very specific for alanine and pyruvate as substrates; maximal activities with other amino acids were no more than 1/3 of that found with alanine while alternative keto acids showed high (and unphysiological) apparent K_m values and/or low maximal velocities. By contrast, oyster ADH showed virtually equal maximal activities with alanine, glycine, serine or L- α -aminobutyrate as well as equal apparent K_m values for alanine and glycine (Fields and Hochachka, 1981). The amino acid substrate specificities of imino acid dehydrogenases may be, in part, related to the predominant free amino acids available in vivo in each species; in L. littorea alanine is one of the most abundant free amino acids in foot muscle (at 18 $\mu\text{mol/g}$ wet weight in animals acclimated to 100% seawater; Hoyaux et al., 1976). However, it is now becoming apparent, that of imino acid dehydrogenases using alanine and/or glycine as substrates, two distinct enzymes can be identified. One enzyme has a high specificity for alanine as its substrate and shows very low activity with glycine. This enzyme is exemplified by ADH from L. littorea and also occurs in tissues of another gastropod, the whelk, Busycotypus canaliculatum (see Chapters 2 and 3). The other enzyme, which is now called

SDH by most authors (de Zwaan and Zurburg, 1981; Dando et al., 1981; Dando, 1981; Storey, 1983, Nicchitta and Ellington, 1984) shows glycine $\geq$ alanine activity. The enzyme from oyster adductor muscle, called ADH by Fields and Hochachka (1981), is actually a good example of this enzyme type. Both of these enzymes occur in tissues of M. edulis where they are tissue specific and are electrophoretically separable (Dando et. al., 1981; Dando, 1981). ADH occurs in many of the soft tissues of M. edulis while SDH is restricted to muscle tissues which have high intracellular levels of glycine as the major free amino acid.

Substrate specificities and inhibitor effects provide information about the substrate site of ADH. Carbon chain length is important in determining substrate utilization. C_2 (glycine, glyoxylate) and C_4 (L- α -aminobutyrate, α -ketobutyrate) substrates were utilized but only at reduced rates and with high apparent K_m values compared to pyruvate and alanine. Alterations to a C_3 substrate (the hydroxyl groups of serine and hydroxypyruvate, the sulfhydryl group of cysteine) also reduced enzyme activity indicating the importance of the methyl groups of alanine and pyruvate in substrate binding. Compounds of chain length greater than C_4 or with substitutions on the C_4 chain (threonine, methionine, valine, leucine, isoleucine, α -ketovalerate, α -ketoglutarate) were inactive. The enzyme was also inactive with D-alanine; D-lactate, the product of the D-LDH of L. littorea, had no effect on ADH but L-lactate was

inhibitory with respect to both alanine and pyruvate. This suggests the importance of the L configuration at the substrate site.

As a terminal glycolytic dehydrogenase ADH could play a metabolic role in maintaining cytosolic redox balance during anaerobic glycolysis associated with (1) 'burst' muscle work, (2) environmental anoxia, or (3) the recovery period following environmental anaerobiosis. However, the kinetic properties of L. littorea ADH can suggest a major physiological role for the enzyme in redox regulation during the recovery period which follows long term environmental anaerobiosis. Two properties are particularly persuasive. Firstly, ADH shows a dependence of the apparent K_m for pyruvate and alanine on co-substrate (alanine or pyruvate) concentration, rising levels of either the amino or keto acid co-substrate decreasing the apparent K_m for keto or amino acid, respectively. This effect is well known for ODH and it has been suggested that this property is a key factor influencing enzyme activity in vivo and promoting the synthesis of octopine as the product of glycolytic muscle work (Fields et al., 1976). Alanine and succinate are major products of long term environmental anaerobiosis in L. littorea (Wieser, 1980). The activation of glycolysis concomitant with the redirection of glycolytic carbon away from succinate and towards pyruvate during the recovery period could lead to significant accumulation of alanopine as an end product of glycolysis. Wieser (1980) did not

assay for alanopine in tissues of L. littorea during anoxia and subsequent recovery. However, tissues of another gastropod, Busycotypus canaliculatum, have been shown to accumulate small amounts of alanopine/strombine but significant amounts of alanine during the early stages of anoxic stress, succinate throughout 24 h of anaerobiosis and substantial amounts of alanopine or strombine during the recovery period (Eberlee and Storey, 1984). This also agrees with the pattern of end product accumulation reported for bivalve tissues during exposure to and recovery from long term environmental anoxic stress (de Zwaan and Zurburg, 1981; Korycan and Storey, 1983; Eberlee et al., 1983). Similarly, Ellington and Gade (1983) report that after 3 h of anoxic stress (short term) in vitro preparations of the ventricle of the gastropod, Busycon contrarium, incorporated only 15% of the label from [^{14}C -U] glucose into alanopine, but 76% into alanine. At the same time 64% of the label from [^{14}C -U] aspartate was incorporated into succinate. The above data suggest that during the early phase of oxygen deprivation NADH produced at the glyceraldehyde-3-phosphate dehydrogenase reaction is tightly coupled with the cytosolic malate dehydrogenase reaction used during mobilization of aspartate reserves. It is thus tempting to speculate that during this initial period of anoxic stress ADH activity is probably limited by the availability of NADH.

A second important kinetic property of L. littorea ADH is that the K_m 's for both pyruvate and alanine decrease with

decreasing pH; an effect which could also promote alanopine synthesis during the early stages of recovery when intracellular pH is low owing to the anoxia-induced accumulation of acidic end products, CO_2 and succinate (de Zwaan, 1977). For oyster muscle ADH, this effect of pH on K_m 's for alanine and pyruvate was not found although pH had a slight effect on K_m for NADH (Fields and Hochachka, 1981).

Imino acid dehydrogenases appear to function like LDH in maintaining glycolytic redox balance during functional anaerobiosis. Thus, L. littorina ADH and bivalve SDHs probably play an important metabolic role during the functional anaerobiosis which is associated with the recovery period following long term environmental anaerobiosis. It is during this transition period that aerobic respiration must be supplemented by anaerobic glycolysis in order to fulfill tissue energy demands. Functional anaerobiosis also occurs during 'burst' muscle work when oxygen supplies are insufficient to meet energy demand by contracting muscle. This function appears to be the primary function of ODH in the species in which the enzyme occurs (Grieshaber and Gade, 1976; Storey and Storey, 1979b) and may also apply to ADH or SDH in certain species. For example, production of strombine/alanopine has been reported to occur in muscle tissues of several species of gastropod Strombidae during escape from predatory starfish (Baldwin and England, 1983). An additional accumulation of strombine/alanopine occurred during the

recovery period after exercise. In L. littorea foot muscle, however, both ADH and LDH occur in approximately equal activities suggesting distinct roles for the two enzymes. LDH may function during functional anaerobiosis brought about by muscle work, whereas ADH probably functions during functional anaerobiosis associated with the recovery period immediately following long term environmental anaerobiosis.

CHAPTER III: Tissue specific isozymes of alanopine
dehydrogenase in the channelled whelk, Busycotypus
canaliculatum.

Introduction

A member of the order Neogastropoda, the channelled whelk, Busycotypus (formerly Busycon) canaliculatum, is the largest marine gastropod found on the Atlantic coast and inhabits muddy sediments in the lower intertidal and upper subtidal zone from Cape Cod to the Gulf of Mexico (Pierce, 1950). It can be exposed to seasonal changes of salinity from 500 to 1000 mOsm, temperature extremes varying over 20 °C and cyclic periods of anoxia (Polites and Mangum, 1980). Like all other members of the order Neogastropoda, whelks are carnivores or carrion scavengers with fusiform shells, a strong anterior canal and an inhalent siphon forming a roving tentaculate nostril (Morton, 1979). B. canaliculatum feeds mainly on other molluscs and echinoderms. Attaching itself to the shell of a bivalve by its muscular foot, it pulls or wedges the valves of its prey apart, using its own shell as a lever, then inserts its proboscis into the gap between the valves (Barnes, 1974). The proboscis is a highly extensible organ that allows the whelk to reach and penetrate vulnerable areas of its prey.

The respiratory pigment of Gastropoda and Cephalopoda is haemocyanin, a copper-containing compound carried in the blood, which is colored faintly blue (Morton, 1979). Although similar in O₂ carrying properties to hemoglobin, the various haemocyanins can differ widely in their affinity for oxygen. In comparison with other molluscan species B.

canaliculatum has a relatively high rate of total oxidative metabolism which apparently stems from the fact that the whelk is able to utilize an unusually large fraction of oxygen present in its blood.

The channelled whelk was chosen for the present study because its large and well-defined organs facilitate the study of tissue specific enzyme distribution. Amongst cephalopod molluscs, ODH occurs in tissue specific isozymic forms. The muscle and brain isozymes display kinetic properties analogous to those of M and H forms of LDH, respectively (Storey and Storey, 1979a), and appear to be involved in tissue specific aspects of octopine metabolism in vivo (Storey and Storey, 1979b). Tissue specific isozymes of LDH occur in the horseshoe crab, Limulus polyhemus (Long and Kaplan, 1973). Furthermore, LDH isozymes have been found in the terrestrial gastropod Cepaea nemoralis (Gill, 1978).

In the present study attention was turned to the possible occurrence of tissue specific isozymes of ADH in the channelled whelk B. canaliculatum. Three cytosolic dehydrogenases, ADH, ODH, and LDH, occur with distinct tissue specific activity profiles. Kinetic data, as well as isoelectrofocusing, chromatofocusing, and electrophoretic analyses, show the presence of tissue specific isozymes of ADH. Three forms, specific for muscle tissue, hepatopancreas and kidney-gill, occur.

Materials and Methods

Chemicals and Animals.

Oxamate-Sepharose affinity gel was supplied by Dr. T. Moon (University of Ottawa); meso-alanopine was synthesized by the method of Abderhalden and Haase (1931); all other biochemicals were obtained as outlined in Chapter 1. Specimens of the whelk B. canaliculatum were obtained from Woods Hole Marine Biological Laboratory, MA, and kept in recirculating, aerated, seawater at 12°C without feeding until use.

Tissue Preparation and Enzyme Assay.

Tissues were excised from freshly killed whelks, blotted, weighed, and homogenized (1:5 w/v) in ice-cold 50 mM imidazole-HCl buffer, pH 7.5, containing 15 mM 2-mercaptoethanol using a Polytron homogenizer. For kidney and hepatopancreas, 3 mM PMSF (phenylmethylsulfonyl fluoride), a protease inhibitor, was included in the homogenizing buffer. Homogenates were centrifuged at 27,000 X g for 30 min at 4°C, and the supernatant was removed and dialyzed overnight at 4°C, against 20 mM imidazole-HCl buffer, pH 7.5, containing 15 mM 2-mercaptoethanol to free the preparations of endogenous amino acids. Enzyme activities were stable to dialysis.

Activities of enzymes in the dialyzed preparations were measured by following NADH utilization at 340 nm using a Gilford recording spectrophotometer. Standard assay.

conditions were 50 mM imidazole-HCl buffer (pH 7.0), 2.0 mM pyruvate, and 0.15 mM NADH for LDH, with additions of 4.0 mM arginine for ODH and 50 mM alanine or 300 mM glycine for the alanine versus glycine dependent ADH activities. For tissues containing LDH, an LDH blank was subtracted from the ADH or ODH activity.

Michaelis constants (apparent K_m 's) for alanine and glycine were determined as outlined in Chapter 1. For kinetic studies of ADH in tissues containing LDH activity, the dialyzed supernatant was first passed through an oxamate-Sepharose column to remove the LDH. A column (0.7 X 10 cm) was packed with 2 ml oxamate-Sepharose equilibrated in 10 mM imidazole-HCl buffer, pH 7.5, containing 10 mM 2-mercaptoethanol and 0.4 mM NADH. Under these conditions, LDH was bound to the column while ADH was eluted, with the same buffer, in a single peak with a yield of 70-80%.

Isoelectrofocusing.

Isofocusing was performed by the method of Vesterberg (1971) using a LKB column (110 ml) and LKB ampholines, pH 4 to 6 for hepatopancreas, kidney, and gill preparations and pH 5 to 8 for all other tissues. Preliminary runs using a pH 3.5 to 10 gradient were used for all tissues to establish the best narrow range gradient for use. Enzyme preparations were run at 450 V for 16 h at 4°C. The column was then drained and 1 ml fractions were collected and assayed for ADH, ODH, and LDH activities.

Chromatofocusing.

Enzyme preparations were layered onto a column (0.7 X 50 cm) of PBE 94 chromatofocusing exchanger pre-equilibrated in 25 mM imidazole-HCl buffer, pH 7.3, containing 10 mM 2-mercaptoethanol. The column was eluted at 17 ml/h with 200 ml of Polybuffer 74 (a 1:8 dilution of stock), adjusted to pH 4.0.

Polyacrylamide Gel Electrophoresis.

Electrophoresis of dialyzed enzyme preparations using 7.5% slab gels was carried out at 4°C with 50 mA constant current for 4 h with Tris-HCl/glycine (46:35 mM), pH 7.3, as the electrode buffer and bromophenol blue as the tracker dye. To detect ADH activity, gels were incubated in the dark at room temperature for 30 min with a mixture of 50 mM Tris-HCl buffer (pH 8.4; 24 ml), 50 mM meso-alanopine (6 ml), 10 mg/ml NAD (6 ml), and 10 mg/ml nitro blue tetrazolium (2 ml), followed by visualization of the bands by the addition of 2 ml of 5 mg/ml phenazine methosulfate. Control gels, omitting meso-alanopine, showed no stained bands.

Results

Enzyme Distribution and Activities.

Table 7 shows the activities of LDH, ODH, and ADH in seven tissues of B. canaliculatum. ADH and ODH were present

Table 7. Distribution and activities of lactate dehydrogenase, octopine dehydrogenase and alanopine dehydrogenase in the tissues of the whelk, *B. canaliculatum*.

Tissue	LDH	ODH	ADH		
			V_{ala}	V_{gly}	$V_{\text{ala}}/V_{\text{gly}}$
Hepatopancreas	-	1.9 $\pm$ 0.52	26.4 $\pm$ 2.23	11.9 $\pm$ 0.70	2.2
Gill	-	1.3 $\pm$ 0.65	9.6 $\pm$ 0.47	2.2 $\pm$ 0.25	4.3
Kidney	-	1.8 $\pm$ 0.48	6.5 $\pm$ 0.70	1.4 $\pm$ 0.15	4.6
Mantle	-	7.2 $\pm$ 0.33	17.8 $\pm$ 0.93	4.8 $\pm$ 0.31	3.7
Ventricle	28.9 $\pm$ 2.58	53.1 $\pm$ 5.47	83.7 $\pm$ 7.39	23.3 $\pm$ 2.41	3.6
Foot muscle	0.4 $\pm$ 0.13	40.6 $\pm$ 2.85	47.2 $\pm$ 2.01	12.7 $\pm$ 0.53	3.7
Proboscis muscle	22.5 $\pm$ 0.82	150.6 $\pm$ 12.4	74.5 $\pm$ 1.35	21.4 $\pm$ 0.98	3.5

Enzyme activities were measured under standard assay conditions as described in Materials and Methods. Values are the means $\pm$ s.e.m. of determinations on at least three individual preparations of each tissue.

in all tissues tested; highest activities of both enzymes were found in muscle tissues although ADH occurred in substantial activities in all of the soft tissues (hepatopancreas, gill, kidney) as well. In all tissues except proboscis muscle (radular retractor), ADH exceeded that of ODH. D-LDH activity was detected in three muscle tissues only with substantial activities of the enzyme found in ventricle and proboscis muscle, two "aerobic" (i.e. myoglobin containing red muscle tissue) muscle types.

B. canaliculatum ADH, like ADH from other sources, utilizes glycine as an alternate amino acid substrate. Table 7 shows the V_{ala} versus V_{gly} for ADH from the seven tissues. The activity ratios (V_{ala}/V_{gly}) fall into three groups. The ratio is highest (4.3 to 4.7) in gill and kidney, lower in muscle tissue (3.5 to 3.7) and lowest (2.2) in hepatopancreas.

Enzyme Kinetics.

Table 8 shows the apparent K_m 's for alanine and glycine for ADH from the seven tissues. Again, the enzymes can be grouped into three kinetically distinct forms, a muscle-type enzyme, a gill-kidney form, and a hepatopancreas form. Apparent K_m 's for both amino acids were lowest for the hepatopancreas enzyme. The apparent K_m for alanine was significantly higher in muscle tissues than in hepatopancreas and was higher still in gill and kidney. Kinetic differences between the tissue ADH forms were even.

Table 8. Apparent K_m 's for alanine and glycine of alanine dehydrogenase from tissues of the whelk, B. canaliculatum.

Tissue	Apparent K_m		Ratio $K_{m(gly)}/K_{m(ala)}$
	Alanine	Glycine	
Hepatopancreas	8.84 $\pm$ 0.03	619 $\pm$ 55	70
Gill	13.12 $\pm$ 0.65	2542 $\pm$ 230	194
Kidney	13.35 $\pm$ 0.26	2518 $\pm$ 209	189
Mantle	10.87 $\pm$ 0.58	1367 $\pm$ 87	126
Ventricle	10.64 $\pm$ 0.40	1412 $\pm$ 115	133
Foot muscle	10.31 $\pm$ 0.10	1395 $\pm$ 122	135
Proboscis muscle	10.91 $\pm$ 0.23	1443 $\pm$ 91	132

Assays were performed at 24 °C with 50 mM imidazole-HCl buffer, (pH 7.5), 2.0 mM pyruvate, and 0.15 mM NADH. Values are the means $\pm$ s.e.m. of determinations on $n = 3$ preparations of each tissue.

Based on kinetic properties, three groups of ADH enzymes are apparent: (1) hepatopancreas; (2) gill and kidney; and (3) mantle, ventricle, foot, and proboscis muscle. Apparent K_m 's for both alanine and glycine are significantly different (using Students t-test), $P < 0.05$, between the three groups but are not statistically different within each group.

more strongly evident when comparing the apparent K_m 's for glycine, the K_m (gly) of the muscle and gill-kidney forms being two- and four-fold higher, respectively, than the K_m (gly) of hepatopancreas ADH. The very high apparent K_m 's for glycine found for B. canaliculatum ADH's suggest that the enzyme from this species, like that of another gastropod, L. littorea (Chapter 1), but unlike ADH from bivalve molluscs (Fields and Hochachka, 1981), is highly specific for alanine as the physiological substrate in vivo.

Isoelectrofocusing, Chromatofocusing and Electrophoresis.

In addition to their kinetic differences, the tissue specific forms of ADH were distinctly separable by three techniques: isoelectrofocusing, chromatofocusing, and polyacrylamide gel electrophoresis. Each technique showed three distinct forms of ADH present in the tissues of B. canaliculatum, specific for muscle tissue, gill-kidney, and hepatopancreas.

Table 9 shows the isoelectric points of ADH from the seven whelk tissues and indicates three isozymic forms, differing in pI , corresponding to hepatopancreas, gill and kidney, and the four muscular tissues.

Polyacrylamide gel electrophoresis showed similar results. ADH migrated anodally under the conditions used. Gill and kidney each showed a single, fast migrating band of ADH while muscle tissues had two bands of activity, a major slower moving band and a very minor band corresponding to

Table 9. Isoelectric points of alanopine dehydrogenase from seven tissues of the whelk, B. canaliculatum.

<u>Tissue</u>	<u>pI</u>
Hepatopancreas	5.69 $\pm$ 0.05
Gill	5.58 $\pm$ 0.04
Kidney	5.57 $\pm$ 0.03
Mantle	5.93 $\pm$ 0.01
Ventricle	5.95 $\pm$ 0.02
Foot muscle	5.91 $\pm$ 0.01
Proboscis muscle	5.94 $\pm$ 0.04

Values are means $\pm$ s.e.m. of $n = 3$ preparations of each tissue. pI's of hepatopancreas and gill-kidney are significantly different, $P < 0.1$, and are each statistically different from the pI's of the four muscular tissues, $P < 0.05$.

the gill-kidney enzyme. This minor band was evident in the isoelectrofocusing as a small shoulder on the acidic side of the muscle ADH peak. A well-defined band of hepatopancreas ADH was not achieved, probably as a result of proteolysis.

Chromatofocusing resolved the major and minor ADH peaks in muscle with a ratio of activity in the two peaks of 8:1. The major peak, eluting at pH 6.3, had a K_m for glycine of 1250 mM while the minor peak had a K_m for glycine of 2600 mM. The minor form of ADH in muscle tissue appears, therefore, to be identical with the gill-kidney isozyme by both electrophoretic and kinetic criteria. Chromatofocusing of gill and hepatopancreas ADH showed only a single peak of enzyme activity in each case, gill ADH eluting at pH 5.95 (elution pH in chromatofocusing is dependent upon enzyme pI but is not a quantitative measure of pI) and hepatopancreas ADH eluting at pH 6.15, with apparent K_m 's for glycine of 2480 and 500 mM, respectively.

Discussion.

Three tissue specific isozymes of ADH occur in the whelk identified by differences in their kinetic and electrophoretic properties. A muscle specific form occurred in ventricle, foot, and proboscis muscle and also in mantle. A second form was specific for kidney and gill, two very aerobic tissues. A third form was found in hepatopancreas. A similar distribution of ODH isozymes was found in the

cephalopod mollusc Sepia officinalis; muscle, brain, and hepatopancreas specific isozymes were identified (Storey, 1977). Soft tissues, such as gill and kidney, contained both brain and muscle specific isozymes in varying ratios (Storey, 1977; Dando et al., 1981).

The occurrence of tissue specific isozymes of ADH implies a tissue specific metabolism of alanine in the whelk, similar perhaps to the tissue specific metabolism of lactate in vertebrates or of octopine in cephalopods. The M and H forms of LDH in vertebrates appear to gear tissues for roles in lactate production versus lactate oxidation, respectively. H-type LDH (occurring in heart and brain) displays kinetic properties including substrate inhibition at elevated levels of pyruvate and a low K_m for lactate, which appear to limit the enzyme's function in anaerobic glycolysis but enhance the oxidation of lactate as an aerobic substrate (Everse and Kaplan, 1973). M-type LDH, on the other hand, is kinetically well suited for catalyzing the rapid production of lactate during glycolytic muscle work. Similar kinetic differences characterize the muscle versus brain forms of ODH in cephalopods (Storey and Storey, 1979a). Studies on the tissue metabolism of lactate and octopine back up the roles suggested for LDH or ODH by enzyme studies. Both products are accumulated rapidly during muscle work but are not effectively oxidized in muscle. Instead the end products are released into the bloodstream to be cycled to tissues capable of utilizing

them as substrates for aerobic oxidation of gluconeogenesis (Everse and Kaplan, 1973; Storey and Storey, 1979b). A parallel situation may exist for the ADH-alanine system in B. canaliculatum.

The tissue distributions of ADH, ODH, and LDH in B. canaliculatum suggest differential functions for the three cytosolic dehydrogenases. ADH was present in quite high activities in all tissues of the whelk suggesting a function for ADH in all tissues. A major function could be a role in redox balance during recovery from environmental anaerobiosis. ODH, however, was much more strongly associated with muscle tissues suggesting that octopine, which couples the products of phosphagen (arginine phosphate) breakdown and glycogenolysis, may be the principal product of glycolytic muscle work. LDH was present in substantial activities in the two myoglobin containing tissues. Both of these tissues perform sustained work, the ventricle in pumping blood and the proboscis muscle in fueling the long term grinding action of the radular teeth needed to penetrate the shell or exoskeleton of prey. The presence of high activities of all three dehydrogenases in these two tissues shows that sustained work by these tissues may not always be aerobic. A sustained demand for glycolytic energy production could result in a depletion of tissue arginine-P reserves and alanine pools, thereby halting glycolytic redox regulation by ODH or ADH and necessitating the action of LDH.

CHAPTER IV: Purification and properties of alanine
dehydrogenase isozymes in the channelled whelk, Busycotypus
canaliculatum.

Introduction

Chapter 3 examined the tissue distribution of terminal glycolytic dehydrogenases in the channelled whelk, Busycotypus canaliculatum, and demonstrated the presence of tissue specific isozymic forms of ADH. Three forms, specific for muscle, gill/kidney and hepatopancreas were identified. The present chapter reports the purification and characterization of the three ADH isozymes from foot muscle, gill and hepatopancreas of B. canaliculatum in order to investigate the tissue specific functions of the enzymes.

Materials and Methods

Chemicals and Animals.

All chemicals and animals were obtained as outlined in Chapters 1 and 2.

Enzyme Assay and Kinetic Studies.

ADH was assayed at 24°C by monitoring NAD(H) utilization at 340 nm using a Gilford recording spectrophotometer. Standard assay conditions for ADH were: 50 mM imidazole-HCl buffer, pH 7.5, 2.0 mM pyruvate, 50 mM alanine and 0.15 mM NADH in the forward direction and 50 mM Tris-HCl buffer, pH 8.5, 40 mM meso-alanopine and 2.0 mM NAD⁺ in the reverse direction in a final volume of 1 ml. Assays were started by the addition of enzyme preparation. One unit of enzyme is defined as the amount of enzyme utilizing 1 μ mol of NADH per

min at 24 °C.

Michaelis constants (apparent K_m 's) of substrates were determined as described in Chapter 1, using constant co-substrate concentrations: 2.0 mM pyruvate, 50 mM alanine, 0.15 mM NADH, 40 mM meso-alanopine or 2.0 mM NAD^+ . Product inhibition characteristics were evaluated at subsaturating substrate conditions: 0.2 mM pyruvate, 7.5 mM alanine, 0.035 mM NADH in 50 mM imidazole-HCl buffer, pH 7.0, for the forward direction and 5.0 mM meso-alanopine, 0.12 mM NAD^+ in 50 mM imidazole-HCl buffer, pH 8.0, for the reverse direction.

Purification of Alanopine Dehydrogenase.

Foot muscle (1:6 w/v), gill (1:4 w/v) or hepatopancreas (1:3 w/v) was minced, blotted, weighed and homogenized in ice-cold 50 mM imidazole-HCl buffer, pH 7.5, containing 15 mM 2-mercaptoethanol using a Polytron PT 10 homogenizer. For hepatopancreas, 3 mM PMSF was included in the homogenizing buffer. Homogenates were centrifuged at 27,000 $\times$ g for 30 min at 4 °C and pellets were discarded. Each supernatant was adjusted to 2.05 M (50% saturation) with ammonium sulphate, stirred at room temperature for 30 min and then centrifuged as above. Pellets were discarded and supernatants were then adjusted to 3.28 M (80% saturation) with ammonium sulphate and stirred and centrifuged as above. The pellets, containing ADH, were resuspended in 25 mM imidazole-HCl buffer, pH 7.5, containing 10 mM 2-mercaptoethanol and then

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dialyzed against this buffer for 2.5 h at 4 °C. Enzyme preparation was then layered onto a column (50 X 0.7 cm) of PBE 94 chromatofocusing exchanger equilibrated in the dialysis buffer. The column was eluted at 20 ml/h with 160 ml Polybuffer 74 (diluted 1:8) adjusted to pH 4 and 1.5 ml fractions were collected. Peak fractions were pooled and applied to a column (90 X 1.5 cm) of Sephadex G-100 equilibrated in 25 mM imidazole buffer, pH 7.3, containing 10 mM 2-mercaptoethanol and 0.04% sodium azide and eluted with this same buffer. Peak fraction were combined and stored at 4 °C for use in kinetic studies. The purified enzymes were stable, with no significant loss of activity or change in kinetic parameters, for at least 2 weeks.

Electrophoresis.

SDS-polyacrylamide gel electrophoresis of the purified enzyme preparations were carried out using 7.5% tube gels containing 1% SDS with 1.5 mA constant current applied per tube for 6 h at 24 °C. Tris-HCl/glycine (46 mM/35 mM), pH 8.3, containing 1% SDS was used as the electrode buffer with bromophenol blue as the tracker dye. Prior to application enzyme samples were pre-incubated in 1% SDS for 10 min at 80 °C. Following electrophoresis gels were fixed overnight in a solution of 40% isopropanol and 10% glacial acetic acid followed by staining for protein using Coomassie Brilliant Blue G by the method of Blakesley and Boezi. (1977).

Molecular Weight Determination and Protein Assay.

Molecular weight determinations and protein assays were performed as described in Chapter 1.

Results

Enzyme Purification.

Final specific activities of the purified enzymes were 340, 387 and 318 U/mg protein for foot muscle, gill and hepatopancreas representing purifications of about 300-, 2700- and 700-fold, respectively. All three enzyme preparations were judged to be homogenous by SDS-polyacrylamide gel electrophoresis⁴ (Plate 2).

Molecular Weight Determination.

Sephadex G-100 gel filtration yielded molecular weights of $43,000 \pm 2,000$, $41,900 \pm 1,600$ and $41,000 \pm 2,500$ ($n = 2$ for each) for foot muscle, hepatopancreas and gill ADH, respectively, similar to molecular weights of 38,000 to 47,000 reported for ODH (Olomucki et al., 1972; Fields et al., 1976), ADH (Fields and Hochachka, 1981; Storey, 1983; Chapter 1) and SDH (Dando, 1981, de Zwaan and Zurburg, 1981; Nicchitta and Ellington, 1984) monomers from other sources.

pH Effects.

In the forward direction pH optima of the foot and hepatopancreas enzymes varied with changing substrate concentrations. The pH optimum of the foot muscle enzyme

Plate 2. SDS polyacrylamide gel electrophoresis of purified
alanopine dehydrogenase isozymes from B. canaliculatum.
From left to right: foot muscle, hepatopancreas, gill.

decreased from 7.05 at high (saturating) substrate concentrations (50 mM alanine, 2 mM pyruvate) to 6.45 at low substrate levels (35 mM alanine, 1.4 mM pyruvate). A similar effect was noted for L. littorea foot muscle ADH (Chapter 1). The pH optima of the hepatopancreas enzyme responded oppositely, however, increasing from 6.5 at high substrate to 7.1 at low substrate concentrations. The pH optimum of the gill isozyme (6.5-6.6) was not affected by substrate concentration.

In the reverse direction differential interactions between substrate concentrations and pH optima were not apparent. All three isozymes showed pH optima of about 9 at high substrate levels (40 mM meso-alanopine, 2 mM NAD⁺). At low substrate levels (15 mM meso-alanopine, 1.2 mM NAD⁺), all three pH optima decreased, the foot and gill optima to pH 8.5 and 8.6, respectively, and the hepatopancreas to 8.1.

Michaelis Constants.

Apparent K_m 's for pyruvate and alanine were strongly dependent upon co-substrate (alanine or pyruvate) concentration; for all three isozymes apparent K_m decreased with increasing co-substrate concentration. This effect also occurs for ADH and ODH from other sources (Storey and Storey, 1979a; Fields and Hochachka, 1981; Storey, 1983; Chapter 1).

Apparent K_m 's for substrates for the three isozymes are shown in Table 10. As intracellular pH drops during

Table 10. Michaelis constants (apparent K_m) for substrates of alanine dehydrogenase isozymes from foot muscle, gill and hepatopancreas of *B. canaliculatum* at high and low pH.

	Foot muscle			Gill			Hepatopancreas		
	pH 6.5	pH 7.5	pH 8.5	pH 6.5	pH 7.5	pH 8.5	pH 6.5	pH 7.5	pH 8.5
Alanine (mM)	16.1 $\pm$ 0.28	23.3 $\pm$ 0.60 ^a	19.8 $\pm$ 1.03	22.9 $\pm$ 0.75 ^b	20.5 $\pm$ 0.61	10.6 $\pm$ 0.44 ^a			
Pyruvate (mM)	0.29 $\pm$ 0.006	0.66 $\pm$ 0.026 ^a	0.36 $\pm$ 0.019	0.71 $\pm$ 0.077 ^a	0.30 $\pm$ 0.013	0.34 $\pm$ 0.014 ^b			
NADH (μ M)	7.6 $\pm$ 0.4	7.1 $\pm$ 0.4	8.3 $\pm$ 0.3	6.8 $\pm$ 0.5	7.7 $\pm$ 0.4	6.8 $\pm$ 0.3			
meso-Alanopine (mM)	8.3 $\pm$ 0.18	12.1 $\pm$ 0.35 ^a	5.9 $\pm$ 0.30	15.0 $\pm$ 0.42 ^a	3.7 $\pm$ 0.13	7.9 $\pm$ 0.55 ^a			
NAD ⁺ (μ M)	175 $\pm$ 6.0	146 $\pm$ 6.0 ^a	128 $\pm$ 5.0	83 $\pm$ 3.0 ^a	61 $\pm$ 2.0	60 $\pm$ 2.0			

Apparent K_m 's were determined at constant co-substrate concentrations given in Materials and Methods.

Data are means $\pm$ s.e.m. for $n = 3$ determinations on each of 2 preparations of each enzyme.

^a - significantly different from value at the lower pH, $P < 0.01$; ^b - $P < 0.10$.

anaerobiosis in marine invertebrates (de Zwaan; 1977; Ellington, 1983a), apparent K_m 's were determined at two pH's, 6.5 and 7.5. Apparent K_m 's for alanine and pyruvate of foot muscle ADH were strongly dependent upon pH, decreasing by 31 and 55% at pH 6.5 compared to pH 7.5. Similarly, the apparent K_m 's for alanine and pyruvate of the gill enzyme also decreased at the lower pH, by 14 and 49% respectively. This pH effect on the apparent K_m 's for the substrates of the forward reaction could function to promote alanopine synthesis as a glycolytic end product during the recovery period following long term environmental anaerobiosis. By contrast, the apparent K_m 's for substrates of hepatopancreas ADH responded differently. Apparent K_m for pyruvate was affected only slightly by pH while the response of the apparent K_m for alanine was opposite to that seen for the gill or muscle isozymes; K_m for alanine increased by 93% with the pH drop from 7.5 to 6.5. Apparent K_m 's for NADH were similar for all three isozymes and were not affected by pH.

In the reverse direction, hepatopancreas ADH showed the lowest apparent K_m 's for both meso-alanopine and NAD^+ . Muscle showed the highest apparent K_m 's, 2.2- and 2.9-fold greater than hepatopancreas apparent K_m 's for meso-alanopine and NAD^+ , respectively, at pH 7.5 while apparent K_m 's for the gill enzyme were intermediate between those of the other two isozymes. Apparent K_m for meso-alanopine decreased with decreasing assay pH for all three isozymes; the same result

was seen for L. littorea foot muscle ADH (Chapter 1). Apparent K_m for NAD^+ of the hepatopancreas isozyme was not affected by pH but K_m increased with decreasing pH for the other two isozymes.

Substrate Inhibition.

All three isozymes of ADH showed substrate inhibition by high levels of either pyruvate or alanine when assayed at saturating co-substrate concentrations. I_{50} for pyruvate was 10, 9, and 11 mM for foot, gill and hepatopancreas ADH, respectively, at pH 6.5, values similar to I_{50} 's of about 10 mM seen for pyruvate substrate inhibition of H-type LDH (Everse and Kaplan, 1973) or the brain specific isozyme of squid ODH (Storey and Storey, 1979a). At pH 7.5, I_{50} 's for pyruvate substrate inhibition were 12-15 mM. Alanine substrate inhibition was also similar amongst the three isozymes, I_{50} for alanine being 250 mM at pH 7.5 and 300-350 mM at pH 6.5. When assayed at subsaturating co-substrate concentrations (0.35 mM pyruvate or 10 mM alanine), however, little or no substrate inhibition was found at elevated pyruvate or alanine concentrations, a situation which was also seen for foot muscle ADH from L. littorea (Chapter 1).

Product Inhibition.

Table 11 summarizes the product inhibition characteristics of the three ADH isozymes. In the forward direction, the hepatopancreas enzyme showed the strongest product inhibition. Inhibition by meso-alanopine was

Table 11. A comparison of the product inhibition characteristics of alanopine dehydrogenase isozymes from B. canaliculatum.

	Percentage Inhibition by			
	meso-Alanopine	NAD ⁺	Alanine	Pyruvate
	7.5 mM	0.25 mM	50 mM	1 mM
Foot muscle	47.4 $\pm$ 3.2	12.6 $\pm$ 0.1	37.2 $\pm$ 2.0	29.4 $\pm$ 2.9
Gill	48.2 $\pm$ 0.9	9.4 $\pm$ 0.1	11.8 $\pm$ 0.2	12.5 $\pm$ 0.1
Hepatopancreas	64.8 $\pm$ 4.6	36.2 $\pm$ 1.03	3.9 $\pm$ 0.1	3.5 $\pm$ 0.1

Product inhibitions were measured using subsaturating substrate conditions given in Materials and Methods. Data are means $\pm$ s.e.m. for duplicate determinations on two preparations of each enzyme.

significantly greater than that seen for the foot muscle or gill enzymes (which show equivalent levels of inhibition), while inhibition by NAD^+ was 3- and 4-fold greater, respectively, than NAD^+ inhibition of the foot muscle or gill enzymes. In the reverse, alanine oxidizing, direction, however, hepatopaneas ADH showed very little product inhibition by pyruvate and alanine. The greatest product inhibition of the reverse direction was seen for the foot muscle enzyme, where inhibition was 10-fold greater than that found for the hepatopaneas enzyme. Gill ADH showed product inhibition characteristics intermediate between those of the foot muscle and hepatopaneas enzymes.

NHxDH Substrate Analogue Studies.

M and H isozymes of LDH, as well as the brain and muscle isozymes of ODH, show differential responses to the hypoxanthine derivative of NADH. Dawson et al. (1964) found activity ratios (NHxDH/NADH) of 0.5 for M and 3 for H LDH while ODH showed activity ratios of 0.63 and 2.43 for the muscle and brain isozymes (at high pyruvate concentrations), respectively (Storey and Storey, 1979a). Activity ratios, NHxDH/NADH for the three ADH isozymes of B. canaliculatum, measured at two pyruvate concentrations, are shown in Table 12. With increasing pyruvate concentration, the activity ratio decreased for the foot muscle enzyme similar to the findings for muscle ODH (Storey and Storey, 1979a). Both gill and hepatopaneas ADH showed opposite reactions, the

Table 12. Utilization of NHxDH , the hypoxanthine derivative of NADH , by muscle, gill and hepatopancreas isozymes of alanine dehydrogenase from B. canaliculatum.

	Activity Ratio NHxDH/NADH	
	At 2 mM pyruvate ^a	At 15 mM pyruvate
Foot muscle	0.98 $\pm$ 0.006 ^a	0.92 $\pm$ 0.012 ^b
Gill	0.94 $\pm$ 0.007	1.11 $\pm$ 0.009 ^b
Hepatopancreas	0.94 $\pm$ 0.008	1.05 $\pm$ 0.006 ^b

Assay conditions were 50 mM imidazole-HCl buffer, pH 7.5, 50 mM alanine, 0.15 mM coenzyme and pyruvate as shown. Data are means $\pm$ s.e.m. for n = 4 determinations on 2 separate preparations of each purified enzyme.

^a - significantly different from values for gill and hepatopancreas enzymes at 2 mM pyruvate, $P < 0.01$.

^b - significantly different from corresponding value at 2 mM pyruvate, $P < 0.01$.

activity ratios increasing significantly with increased pyruvate levels.

Discussion

ADH occurs in three tissue specific isozymic forms in the tissues of the whelk. Muscular tissue, including ventricle, proboscis (radular retractor), mantle and foot contain one form, gill and kidney exhibit a second form and hepatopancreas shows the third form of the enzyme (Chapter 2).

Data in the present study indicate that ADH from hepatopancreas shares a number of kinetic features in common with the H-type LDH. These include: (1) low apparent K_m 's for the substrates of the reverse reaction, a property which would favor alanopine oxidation in vivo, (2) low product inhibition of the reverse reaction by alanine and pyruvate, again favoring alanopine oxidation by the enzyme, and (3) a high product inhibition of the forward reaction by alanopine and NAD^+ , a feature which could limit alanopine accumulation in vivo as the product of anaerobic glycolysis. Muscle type ADH, however, like M-type LDH (Everse et al., 1971; Everse and Kaplan, 1973) or muscle type ODH (Storey and Storey, 1979a), shows kinetic properties which would gear the enzyme for alanopine production in vivo as the product of anaerobic glycolysis. Compared to hepatopancreas ADH, then, the muscle isozyme shows: (1) a much lower affinity (higher apparent

K_m 's) for the substrates of the reverse reaction, alanopine and NAD^+ , (2) lower product inhibition of the forward reaction by alanopine and NAD^+ , thus allowing continued alanopine synthesis in muscle despite an accumulation of tissue alanopine and (3) much higher product inhibition of the reverse reaction by alanine and pyruvate. These features, as first postulated for LDH (Everse et al., 1971; Everse and Kaplan, 1973) would enhance the formation of alanopine under conditions of high glycolytic flux but are believed to limit the reversibility of the enzyme in vivo and the potential for reoxidation of accumulated end product in muscle.

The gill isozyme of ADH has properties which are intermediate between those of the muscle and hepatopancreas forms. Product inhibition of the forward reaction is low, similar to that seen for the muscle enzyme, suggesting a good capacity for alanopine accumulation during anaerobic glycolysis. However, apparent K_m 's for the reverse reaction and product inhibition characteristics of this direction are both intermediate between the hepatopancreas and foot muscle kinetics suggesting that the enzyme also has a good capacity for reversing the reaction in vivo and utilizing alanopine as an aerobic substrate for gill oxidative metabolism.

Apparent K_m 's for both pyruvate and alanine of foot muscle ADH are strongly influenced by pH, both decreasing as pH is lowered from 7.5 to 6.5. A fall in intracellular pH during anaerobiosis is well documented amongst marine

invertebrates (de Zwaan, 1977, Ellington, 1983a). The production of alanopine as the end product of anaerobic glycolysis in muscle during recovery from long term environmental anoxic stress would be favored, then, by two effects, both lowering the apparent K_m 's for substrates: (1) decreased intracellular pH, and (2) increased co-substrate concentrations arising from increased glycolytic flux and the previous accumulation of alanine as an anaerobic end product. This pH effect on the apparent K_m 's for pyruvate and alanine is less pronounced for the gill enzyme while hepatopancreas ADH shows only a slight decrease in K_m with decreasing pH. Apparent K_m for alanine of the hepatopancreas enzyme reacts oppositely to decreasing pH, doubling with the pH drop from 7.5 to 6.5. This property coupled with the high product inhibition of the enzyme by meso-alanopine and NAD^+ suggests that enzyme function during recovery from anoxic stress could be limited.

ADH isozymes also differed in their utilization of the hypoxanthine analogue of NADH. Muscle ADH behaved like the muscle isozyme of ODH, the activity ratio, $NHx\text{DH}/NADH$, decreasing when pyruvate substrate concentration was increased. Gill and hepatopancreas isozymes, which were similar in this characteristic, showed an analogous behavior to that seen for brain type ODH (Storey and Storey, 1979a), the activity ratio increasing at higher pyruvate concentrations.

The three isozymes of ADH in B. canaliculatum appear to be geared by differences in their kinetic properties, for tissue specific functions in the metabolism of alanopine in vivo. Co-substrate and pH effects on the apparent K_m 's for pyruvate and alanine of muscle ADH suggest that the enzyme may have its major function as the cytosolic dehydrogenase responsible for redox regulation during muscle glycolytic function. The kinetic characteristics of the hepatopancreas isozyme, however, particularly the low apparent K_m 's for the substrates of the reverse reaction, suggest that the role of the enzyme is chiefly in the oxidation of alanopine. Hepatopancreas could make effective use of blood alanopine (derived from alanopine release from other tissues or from dietary intake) as an oxidative or gluconeogenic substrate. The kinetics of the gill isozyme suggest that the enzyme may have a bidirectional function. As the major terminal glycolytic dehydrogenase activity in the tissue (see Chapter 2), the enzyme should play a major role in redox regulation during anaerobiosis; the kinetic properties of the forward direction (pH effects on apparent K_m 's, low product, inhibition) would promote this function. However, the low apparent K_m 's for substrates of the reverse reaction also imply a good capacity for the oxidation of alanopine as an aerobic substrate just as vertebrate heart and brain can make effective use of blood lactate as an aerobic fuel.

Recent studies on the time-course of changes in metabolite levels in tissues and blood of B. canaliculatum

before, during, and following exposure to 24 h of anoxic stress (Eberlee and Storey, 1984) are consistent with the kinetic properties of whelk ADH isozymes reported here. Although hepatopancreas tissue showed no significant accumulation of alanopine or strombine at any time during the experimental period, gill and muscle tissues did show significant accumulation of alanopine or strombine. The most convincing data which supports the concept for tissue specific metabolism of alanopine in the whelk is that of blood metabolite levels. Bivalve molluscs do not show tissue specific isozymes of ADH or SDH (Gade, 1983), and do not appear to excrete any nitrogen-containing end products (Ellington, 1983b). However, cephalopod molluscs which maintain tissue specific ODH isozymes do excrete considerable quantities of octopine into their blood during functional anaerobiosis (Storey and Storey, 1983). Likewise, B. canaliculatum blood (sampled from foot sinus) was shown to contain significant amounts of alanopine (concentration = 2 mM) during the recovery period (Eberlee and Storey, 1984). The presence of alanopine in whelk blood demonstrates that the capacity for intertissue transport of this compound is present in these animals. Experiments which could examine potential tissue specific metabolism of injected [^{14}C]-labelled alanopine are needed in order to substantiate the proposed metabolic roles for the tissue specific ADH isozymes of B. canaliculatum.

CHAPTER V: Tissues specific isozymes of pyruvate kinase in the channelled whelk, Busycotypus canaliculatum, and their modification during environmental anaerobiosis.

Introduction

Pyruvate kinase (PK) ($\text{PEP} + \text{ADP} \xrightarrow{\text{K}^+ \text{Mg}^{2+}} \text{pyruvate} + \text{ATP}$) is a key regulatory enzyme of the glycolytic pathway and in mammals, two basic forms are found. L-type PK is present mostly in liver and kidney, both gluconeogenic tissues (Munday et al., 1980). This isozyme is activated by fructose-1,6-bisphosphate (Fru-1,6-P_2), exhibits cooperative kinetics with respect to phosphoenolpyruvate (PEP), and is known to be subject to phosphorylation by a hormonally stimulated protein kinase, which reduces the activity of the enzyme at intracellular concentrations of metabolites (Engstrom, 1978). The other main mammalian PK isozyme is M-type PK. Found in non-gluconeogenic tissues this isozyme is insensitive to Fru-1,6-P_2 , exhibits Michaelis-Menten kinetics with PEP and has never been shown to be subject to phosphorylation in vivo. Electrophoretically distinct tissue specific PK isozymes have also been identified in birds, fish and frogs (Guderley et al., 1978; Guderley and Cardenas, 1979; Schloen et al., 1969), as well as in the bivalve mollusc Crossostrea gigas (Mustafa and Hochachka, 1971) and the cephalopod mollusc, Octopus cyanea (Guderley et al., 1976).

In contrast with the enzyme from most vertebrate species, PK from many invertebrates often functions in anoxic environments and its activity must be coordinated with the organism's metabolic strategy for anaerobic energy

production. As discussed in the introduction to this thesis anaerobic fermentation of glycogen can lead to a variety of metabolic end products (see Fig. 3). PEP occupies a central position in the metabolic maps depicted in Fig. 3 and PK is believed to control not only the direction of PEP disposal (i.e. pyruvate versus succinate), but also in conjunction with phosphofructokinase (PFK), overall glycolytic flux. Moreover, PK of gluconeogenic tissues may be subject to additional regulatory controls. It is important to note that most of the previous work on PK and its control during anoxia in marine invertebrates has been based on kinetic studies of the enzyme prepared from aerobic animals in which no precautions were taken to control the phosphorylation state of the enzyme (Mustafa and Hochachka, 1971; de Zwaan, 1972; de Zwaan and Holwerda, 1972). Only recently has circumstantial evidence been reported which suggests that PK from adductor muscle (Holwerda et al., 1981, 1983) and mantle (Holwerda et al., 1984) of the bivalve mollusc, Mytilus edulis, is inactivated during long term environmental anaerobiosis via a reversible covalent modification.

The work reported in this chapter was performed for several reasons. A survey of PK from various tissues of the channelled whelk, B. canaliculatum was conducted in order to first ascertain if, as is the case for ADH (Chapter 2 and 3), tissue specific forms of PK occur, and secondly, to determine whether or not PK and/or its competitor for the

conversion of PEP, phosphoenolpyruvate carboxykinase (PEP.CK), are subject to regulation via covalent modification in response to long term anoxic stress. The electrophoretic, isoelectrofocusing and kinetic data reported herein demonstrate the presence of tissue specific isozymes of PK, specific for red muscle tissue (ventricle and radular retractor), white muscle tissue (foot and mantle), and soft tissues (gill, kidney and hepatopancreas). This is the first demonstration of tissue specific PK isozymes from a gastropod mollusc. The results of the present study also show that all three tissue specific forms of whelk PK undergo a clear-cut modification of physical and kinetic properties when animals are subjected to 21 h of environmental anoxic stress. This oxygen-dependent modification of PK has never before been demonstrated in any gastropod and may play an important role in controlling the fate of glycolytic carbon at the PEP branchpoint. In contrast to PK, whelk PEP.CK does not appear to be modified in response to long term environmental anoxic stress in any tissue.

Materials and Methods

Chemicals and Animals.

Coupling enzymes were from Boehringer Mannheim and cellulose acetate strips (Seprapore III, 1 X 6") were from Gelman Instrument Co. Other chemicals, biochemicals and

specimens of the channelled, whelk, Busycotypus canaliculatum, were obtained as outlined in Chapters 2 and 3. Whelks were held in recirculating sea water (1000 mOsm) at 12-15 °C without feeding until use. Control, aerobic animals were sampled directly from the sea water tank. To impose anoxia animals were placed in large jars containing sea water and nitrogen gas was continuously bubbled through the sea water for 21 h. Anoxic animals were held at room temperature (20 °C).

Tissue Preparation and Enzyme Assay.

Tissues from aerobic and anoxic whelks were rapidly dissected out, blotted and frozen in liquid nitrogen until use. No loss in enzyme activity was seen for periods of at least one month. Quick frozen tissues were ground to a powder under liquid nitrogen using a mortar and pestle, weighed and homogenized for 2 X 30 s in ice cold 50 mM imidazole-HCl buffer (pH 7.5) containing 0.1 mM PMSF, 10 mM 2-mercaptoethanol, 50 mM NaF, 3 mM EDTA and 3 mM EGTA, using a Polytron PT 10-35 homogenizer. The ratio of tissue weight to volume of homogenization buffer was as follows: hepatopancreas (1/3 w/v), gill and kidney (1/4 w/v), foot and mantle (1/5 w/v), and heart and radular retractor (proboscis muscle) (1/10 w/v). Homogenates were centrifuged at 27,000 X g for 30 min at 4 °C, and the supernatant was removed and dialyzed overnight at 4 °C against 4 l of homogenization buffer minus PMSF.

Tissue extracts used for cellulose acetate electrophoresis were prepared as described above except the homogenization buffer was 15 mM Tris-HCl buffer (pH 7.2) containing 250 mM sucrose, 0.1 mM PMSF, 1.5 mM EDTA, 1.5 mM EGTA, 25 mM NaF and 10 mM 2-mercaptoethanol and ratio of tissue weights to volume homogenization buffer used was: kidney, gill and hepatopancreas (1/1 w/v), mantle and foot (1/2 w/v), and ventricle and radular retractor (1/10 w/v).

Activities of enzymes in the dialyzed preparations were determined by following NADH utilization at 340 nm using a Gilford recording spectrophotometer attached to a circulating water bath for temperature control of cuvettes. Temperature for all assays was maintained at 21°C. Standard assay conditions for PK were: 50 mM imidazole-HCl buffer (pH 7), 5 mM PEP, 5 mM ADP, 100 mM KCl, 10 mM $MgCl_2$, 0.15 mM NADH and excess dialyzed LDH in a final volume of 1 ml. Standard assay conditions for PEPCK were: 50 mM MES-HCl buffer (pH 6; extensively degassed), 3 mM PEP, 1.5 mM IDP, 20 mM $KHCO_3$, 1 mM $MnCl_2$, 1 mM $MgCl_2$, 15 mM NaF, 0.15 mM NADH and excess dialyzed malate dehydrogenase. A blank was conducted without $KHCO_3$, and thus corrects for PK which may show activity under the assay conditions employed. All assays were initiated by addition of enzyme preparation. One unit of enzyme activity is defined as the amount of enzyme resulting in the utilization of 1 μ mol of NADH per min at 21°C.

Isoelectrofocusing.

Isoelectrofocusing was performed as described in Chapter 2 using a pH 3.5 to 10 gradient. Dialyzed enzyme preparations were run at 300 V for 20 h at 4 °C. The column was drained and 1.5 ml fractions were collected and assayed for PK activities.

Electrophoresis.

Electrophoresis was performed on cellulose acetate strips for 1 h at 4 °C with 18 V per strip using 15 mM Tris-HCl buffer (pH 7.2) containing 250 mM sucrose, 10 mM 2-mercaptoethanol and 1.5 mM EDTA as the electrophoresis buffer. Aliquots (kidney, gill and hepatopancreas = 1 µl; mantle and foot = 0.7 µl; ventricle and radular retractor = 0.5 µl) were applied to the center of the strips using a 1 µl Hamilton syringe. Prior to sample application the strips had been soaked for 2 h in electrophoresis buffer containing 0.1% (w/v) bovine serum albumin. PK activity was detected by a modification of the method of Susor and Rutter (1971). Following electrophoresis the strips were laid on a plexiglass ruler and were covered with another strip which had been soaked in a solution of 50 mM imidazole-HCl buffer (pH 7) containing 5 mM PEP, 5 mM ADP, 100 mM KCl, 10 mM MgCl₂, 20 µM Fru-1,6-P₂, 1 mM NADH and 10 I.U./ml LDH. Opposing strips were then placed in a humidified box and PK activity visually observed under UV light owing to the loss of fluorescence associated with NADH oxidation.

Results

Pyruvate Kinase.

PK was present in all tissues tested; highest activities occurred in muscle tissues, whereas much lower activities were found in the soft tissues (Table 13). In all cases, when examined from animals subjected to 21 h of anoxic stress, maximal activity was decreased significantly, whereas the ratio of enzyme activity with high (5 mM) versus low (0.5 and 0.25 mM) PEP concentrations was dramatically increased (Table 13). These results suggest that, like PK from adductor muscle and mantle of *M. edulis* (Holwerda et al., 1981, 1983, 1984), a modification of whelk PK's occurs in response to long term anoxic stress. A similar kinetic response to 21 h of anoxic stress was noted for whelk PKs when assayed at pH 8 (data not shown).

Table 13 also lists the activity ratios for PKs prepared from tissues of aerobic and anoxic animals when assayed with and without 1 mM Fru-1,6-P₂ at saturating PEP (5 mM) and ADP (5 mM). Under these assay conditions PK from the red muscle tissues (ventricle and radular retractor) of both aerobic and anoxic animals is Fru-1,6-P₂ insensitive. By contrast, PK from white muscle tissues (foot and mantle) and soft tissues (gill, kidney and hepatopancreas) of anoxic animals was more sensitive to activation by Fru-1,6-P₂ than was the enzyme from the respective aerobic tissues, further indication that an anoxia-dependent modification of a

Table 13. *B. Canaliculatum* pyruvate kinase: maximal enzyme activities and enzyme activity ratios at high (5 mM) versus low (0.5 and 0.25 mM) PEP concentrations and plus or minus 1 mM Fru-1,6-P₂.

Tissue	Status	Maximal Enzyme Activity units g wet weight	Enzyme Activity Ratios			
			$\frac{V_5 \text{ mM PEP}}{V_{0.5 \text{ mM PEP}}}$	$\frac{V_5 \text{ mM PEP}}{V_{0.25 \text{ mM PEP}}}$	$\frac{V_1 \text{ mM Fru-1,6-P}_2}{V_{\text{control}}}$	$\frac{V_1 \text{ mM Fru-1,6-P}_2}{V_{\text{control}}}$
Radular Retractor	Aerobic Anoxic	116 $\pm$ 33 39 $\pm$ 7	1.4 $\pm$ 0.1 36 $\pm$ 13	1.9 $\pm$ 0.1 88 $\pm$ 20	1.03 $\pm$ 0.01 1.00 $\pm$ 0.03	
Ventricle	Aerobic Anoxic	75 $\pm$ 12 28 $\pm$ 5	1.5 $\pm$ 0.1 27 $\pm$ 8	1.9 $\pm$ 0.2 67 $\pm$ 7	1.01 $\pm$ 0.01 1.05 $\pm$ 0.04	
Foot	Aerobic Anoxic	45 $\pm$ 4 17 $\pm$ 7	1.5 $\pm$ 0.2 30 $\pm$ 5	1.7 $\pm$ 0.2 79 $\pm$ 10	1.19 $\pm$ 0.04 1.34 $\pm$ 0.05	
Mantle	Aerobic Anoxic	4.4 $\pm$ 0.9 1.6 $\pm$ 0.6	1.6 $\pm$ 0.2 20 $\pm$ 4	2.3 $\pm$ 0.4 53 $\pm$ 8	1.22 $\pm$ 0.06 1.43 $\pm$ 0.08	
Gill	Aerobic Anoxic	1.7 $\pm$ 0.2 0.1 $\pm$ 0.03	3.2 $\pm$ 0.5 ∞^a	5.5 $\pm$ 1.5 ∞^a	2.71 $\pm$ 0.60 4.36 $\pm$ 0.60	
Kidney	Aerobic Anoxic	1.1 $\pm$ 0.2 0.05 $\pm$ 0.01	3.5 $\pm$ 0.6 ∞^a	4.4 $\pm$ 0.3 ∞^a	2.64 $\pm$ 0.12 4.78 $\pm$ 0.36	
Hepatopancreas	Aerobic Anoxic	1.6 $\pm$ 0.1 0.18 $\pm$ 0.03	2.9 $\pm$ 0.4 5.5 $\pm$ 2	6.9 $\pm$ 0.8 24 $\pm$ 9	2.37 $\pm$ 0.20 3.94 $\pm$ 0.46	

All assays were performed at 21°C and pH 7.0 using otherwise standard assay conditions as described in Materials and Methods. Values are means $\pm$ s.e.m. of $n = 3$ preparations of each tissue obtained from aerobic animals and whelks subjected to 21 h of anoxic stress.

^a - PK activity ratios for extracts prepared from gill and kidney tissue of anoxic animals = ∞ because PK at 0.5 and 0.25 mM PEP was undetectable.

gastropod PK can occur. Furthermore, in the presence of saturating PEP and ADP a tissue specific sensitivity to activation by 1 mM Fru-1,6-P₂ occurs such that PKs from red muscle tissues are Fru-1,6-P₂ insensitive, white muscle tissue PKs are slightly activated whereas soft tissue PKs are uniformly susceptible to strong activation by Fru-1,6-P₂.

Phosphoenolpyruvate Carboxykinase.

Like PK, PEP.CK activity was also found in all tissues tested (Table 14). However, although muscle tissues contained the highest maximal activities, differences in maximal activity with respect to the soft tissues were not nearly so dramatic as was the case for PK. In contrast to PK, in no case was maximal PEP.CK activity or the ratio of PEP.CK activity at high (3 mM) versus low (0.3 and 0.15 mM) PEP levels significantly altered when analyzed at pH 6 (Table 14) or pH 7 (data not shown) from dialyzed extracts prepared from animals subjected to 21 h of anoxic stress.

Isoelectrofocusing and Electrophoresis of Whelk Pyruvate Kinase.

Table 15 shows the isoelectric points of PK from seven tissues in aerobic and 21 h anoxic whelks. As is the case for Fru-1,6-P₂ effects (Table 13) three isozymic forms, differing in pI, corresponding to red muscle tissue (ventricle and radular retractor), white muscle tissue (foot and mantle) and soft tissues (gill, kidney and

Table 14. *B. canaliculatum* PEP.CK: maximal enzyme activities and enzyme activity ratios at high (3 mM) versus low (0.3 and 0.15 mM) PEP concentrations.

Tissue	Status	Maximal Enzyme Activity	Enzyme Activity Ratios	
			$\frac{v_{3 \text{ mM PEP}}}{v_{0.3 \text{ mM PEP}}}$	$\frac{v_{3 \text{ mM PEP}}}{v_{0.15 \text{ mM PEP}}}$
Radular Retractor	Aerobic	3.0 $\pm$ 0.8	2.4 $\pm$ 0.2	4.2 $\pm$ 1.4
	Anoxic	2.5 $\pm$ 0.2	2.6 $\pm$ 0.1	3.5 $\pm$ 0.4
Ventricle	Aerobic	5.3 $\pm$ 3.0	2.5 $\pm$ 0.6	3.6 $\pm$ 0.9
	Anoxic	3.6 $\pm$ 2.3	2.7 $\pm$ 0.4	3.4 $\pm$ 0.3
Foot	Aerobic	4.5 $\pm$ 0.4	2.2 $\pm$ 0.1	4.8 $\pm$ 1.6
	Anoxic	3.1 $\pm$ 1.0	2.7 $\pm$ 0.4	4.4 $\pm$ 1.2
Mantle	Aerobic	1.5 $\pm$ 0.1	2.5 $\pm$ 0.2	3.6 $\pm$ 0.4
	Anoxic	1.3 $\pm$ 0.2	2.6 $\pm$ 0.5	3.8 $\pm$ 0.6
Gill	Aerobic	1.5 $\pm$ 2.0	2.2 $\pm$ 0.1	3.1 $\pm$ 1.1
	Anoxic	1.3 $\pm$ 0.5	1.8 $\pm$ 0.4	2.5 $\pm$ 0.1
Kidney	Aerobic	1.2 $\pm$ 0.6	2.0 $\pm$ 0.5	3.0 $\pm$ 0.6
	Anoxic	1.1 $\pm$ 0.5	2.0 $\pm$ 0.4	2.9 $\pm$ 0.5
Hepatopancreas	Aerobic	0.55 $\pm$ 0.25	1.1 $\pm$ 0.1	1.5 $\pm$ 0.1
	Anoxic	0.40 $\pm$ 0.14	1.2 $\pm$ 0.1	1.9 $\pm$ 0.4

All assays were performed at 21°C and pH 6.0 using otherwise standard assay conditions as described in Materials and Methods. Values are means $\pm$ s.e.m. of $n = 2$ preparations of each tissue obtained from aerobic animals and whelks subjected to 21 h of anoxic stress.

Table 15. Effect of 21 h of environmental anaerobiosis on isoelectric points of pyruvate kinase from seven tissues of the channelled whelk, *B. canaliculatum*.

<u>Tissue</u>	<u>pi</u>	
	AEROBIC	ANOXIC
Radular retractor	5.81 $\pm$ 0.06	5.42 $\pm$ 0.03
Ventricle	5.84 $\pm$ 0.06	5.43 $\pm$ 0.04
Foot muscle	4.21 $\pm$ 0.05	3.81 $\pm$ 0.06
Mantle	4.28 $\pm$ 0.07	3.72 $\pm$ 0.09
Gill	4.91 $\pm$ 0.03	4.52 $\pm$ 0.02
Kidney	4.92 $\pm$ 0.05	4.56 $\pm$ 0.05
Hepatopancreas	4.98 $\pm$ 0.12	4.53 $\pm$ 0.06

Values are means $\pm$ s.e.m. of $n = 2$ preparations of each tissue. pi's of foot-mantle and gill-kidney-hepatopancreas are significantly different, $P < 0.05$, and each are statistically different from pi's of the two red muscle tissues (radular retractor and ventricle), $P < 0.05$.

hepatopancreas) were identified. In each case when analyzed from anoxic animals, respective pI 's were decreased uniformly by about 0.4 pH units.

Cellulose acetate electrophoresis showed similar results (Fig. 8). All PK's migrated towards the anode; PK from white muscle tissues showed a fast migrating band of activity, red muscle PK showed a slow migrating band of activity, and soft tissue PK showed an intermediate migrating band of activity. Moreover, the extent of anodal migration of all whelk PKs was increased to the same degree when analyzed from extracts prepared from animals subjected to 21 h of anoxic stress.

Discussion

Three tissue specific isozymic forms of PK occur in B. canaliculatum identified by differences in their: (1) sensitivities to activation by Fru-1,6- P_2 , (2) isoelectric points, and (3) electrophoretic mobilities on cellulose acetate strips. A red muscle form occurred in the two myoglobin containing tissues, ventricle and radular retractor. A second form was specific for the two white muscle tissues, foot and mantle, whereas a third form occurred in the soft tissues, gill, kidney and hepatopancreas. This is the first evidence for the occurrence of tissue specific isozymes of PK in a gastropod mollusc. A similar distribution of PK isozymes was found in the cephalopod mollusc, Octopus cyanea (Guderley et al.,

Fig. 8. Electrophoretic resolution of pyruvate kinase isozymes from red muscle (radular retractor and ventricle), white muscle (foot and mantle), and soft tissues (gill, kidney and hepatopancreas) of aerobic and 21 h anoxic specimens of B. canaliculatum. Cellulose acetate electrophoresis was carried out for 1 h at 18 V per strip as described in Materials and Methods.

1976); mantle muscle and hepatopancreas specific isozymes were identified, and the kinetic differences between these two PK isozymes were found to be reminiscent of the distinctions between mammalian M and L-type PK.

The kinetic data of the present study clearly shows that, as is the case for adductor muscle and mantle PK from the bivalve mollusc M. edulis (Holwerda et al., 1981; 1983; de Zwaan and Dando, 1984), an in vivo modification of B. canaliculatum PKs occurs in response to long term environmental anaerobiosis (Table 13). The tissue specific isozymes from anoxic whelks showed a decrease in activity at saturating (5 mM) PEP and a much larger decrease in activity at subsaturating (0.5 and 0.25 mM) PEP levels compared to the enzyme from aerobic animals. This is the first evidence for an oxygen dependent modification of gastropod PK and is analogous to the significant reduction in activity at low PEP concentrations shown by mammalian L-type PK when phosphorylated by a cyclic AMP dependent protein kinase (Engstrom, 1978). Whelk white muscle and soft tissue PKs also showed increased sensitivity to Fru-1,6-P₂ activation at saturating levels of PEP and ADP in response to long term anoxic stress (Table 13).

Apart from the kinetic changes which were induced during long term anaerobiosis, each tissue specific B. canaliculatum PK isozyme showed decrease in pI of about 0.4 pH units which was reflected by a uniform increase in anodal mobility during electrophoresis on cellulose acetate strips

at pH 7.2. This suggests that the molecular nature of the anoxia induced PK modification is identical for all three isozymes, and, in the absence of other factors, is consistent with the covalent incorporation of phosphate(s) into the isozyme molecules. Hence, whether PK activity, as is believed to be the case for PKs from tissues of the bivalve mollusc M. edulis (Holwerda et al., 1981, 1983, 1984), may be quickly and effectively controlled during exposure to, and recovery from, long term environmental anoxic stress via reversible phospho-/dephosphorylation reactions in vivo.

Until recently it was generally believed that a gradual reduction in tissue pH during anaerobiosis was the major factor controlling glycolytic flux and the pathway of PEP conversion via H^+ inhibition of PK activity (Hochachka and Mustafa, 1972; Holwerda et al., 1973; Wijsman, 1975; de Zwaan, 1977). However, current evidence indicates that intracellular pH reductions which occur during molluscan long term environmental anaerobiosis are not as drastic as was first believed (Ellington, 1983a; Walsh et al., 1983). On the other hand, it is becoming increasingly apparent that the anoxia dependent modulation of PK activity via reversible covalent modification is probably the major regulatory mechanism whereby PEP.CK can successfully compete with PK for glycolytic carbon during environmental anaerobiosis. In contrast to the results for PK, in no instance was the activity

of PEP.CK at either high (3 mM) or low (0.3 or 0.15 mM) PEP

levels influenced by 21 h. of anoxic stress in any tissue (Table 14). Similarly, PEP.CK activity from M. edulis adductor muscle was unaffected by 12 h of anoxia (Holwerda et al., 1981).

The maximal activities of PK greatly exceeded those of PEP.CK in muscle tissues of B. canaliculatum (i.e. radular retractor: PK = 116 U/g wet weight; PEP.CK = 3 U/g wet weight). High activities of PK in muscle tissue of the channelled whelk may be an important contributing factor to the unusually high rate of oxidative metabolism which has been associated with these animals (Polites and Mangum, 1980) and would also provide part of the necessary metabolic equipment which could allow for a 'burst' of glycolytic flux during the functional anaerobiosis associated with either: (1) the recovery period following long term environmental anoxic stress, or (2) the vigorous muscular activity which occurs during predation. The relatively low activities of PEP.CK found in tissues of B. canaliculatum (Table 14), M. edulis (de Zwaan and De Bont, 1975), and other marine molluscs (Zammit and Newsholme, 1978) may be a contributing factor for the very low 'overall metabolic rate which has been associated with molluscan long term environmental anaerobiosis (Pamatmat, 1978; Hammen, 1980; Famme et al., 1981; Hammen, 1983).

The presence of tissue specific forms of PK in the channelled whelk implies the occurrence of tissue specific metabolism. Indeed, tissue specific changes in metabolite

levels have been demonstrated to occur in B. canaliculatum during exposure to, and recovery from, 24 h of environmental anoxia (Eberlee and Storey, 1984). Furthermore, tissue specific isozymes of ADH also exist (Chapter 2); muscle type ADH is kinetically suited for alanopine production as a glycolytic end product, whereas hepatopancreas ADH is kinetically suited for the oxidation of alanopine in vivo (Chapter 3). The susceptibility of mammalian L-type PK to activation by low concentrations of Fru-1,6-P₂ is believed to be related to a capacity by that tissue to perform gluconeogenesis (Munday et al., 1980). It is possible that the significantly elevated sensitivity of whelk soft tissue PK to activation by Fru-1,6-P₂ at saturating concentrations of substrates and co-factors (Table 13) is related to an analogous tissue specific role in gluconeogenesis. Obviously, kinetic studies of the purified aerobic and anoxic forms of tissue specific B. canaliculatum PK isozymes are required in order to further ascertain their tissue specific function(s). Furthermore, conclusive evidence has yet to be obtained which clearly demonstrate that the anoxia induced modification of a molluscan PK is indeed due to the reversible covalent incorporation of phosphate(s) in vivo.

CHAPTER VI: Purification and properties of aerobic and anoxic variants of pyruvate kinase from red muscle tissue of the channelled whelk, Busycotypus canaliculatum.

Introduction

Regulation of the activity of PK is believed to play a key role in the control of anaerobic energy metabolism in animals with well developed anaerobic capacities (de Zwaan, 1977; Hochachka, 1980; Gade, 1983). Unlike anoxic vertebrate tissues, whose sole end product during anaerobic glycolysis is L-lactate, many anoxia tolerant invertebrates employ an alternate route for anaerobic disposal of PEP provided by glycogen breakdown. During anaerobiosis PEP may be converted to oxaloacetate by a cytosolic PEP-CK, thereby initiating the mitochondrial production of succinate and other end products of anaerobic carbon metabolism (see Introduction; Fig. 3). In muscle tissues of marine invertebrates capable of surviving extended periods of environmental anaerobiosis maximal activities of PEP-CK are often well below those of PK (Zammit and Newsholme, 1978; Chapter 5). Thus, following the aerobic to anoxic transition PK activity must be greatly depressed.

Recently it has been shown that aside from allosteric effects, PK from muscle tissue of several anoxia tolerant marine molluscs, like mammalian L-type PK, is also subject to probable control via covalent modification (Sibenaller, 1979; Holwerda et al., 1981; Chapter 5). This fact is based primarily on the observation that during the course of anoxia the activity of the enzyme, in absence of other effects becomes greatly reduced at low PEP concentrations.

Subsequently, Holwerda and co-workers (1983) have partially purified and characterized two interconvertible forms of PK from the adductor muscle of the sea mussel Mytilus edulis. The proportion of these variants changed in response to anoxic stress or adaptation to higher temperatures. The variant predominating after either stress was enzymatically less active owing to: (a) reduced affinity for the substrate PEP and (b) increased sensitivity to the allosteric inhibitors L-alanine and H^+ .

Work reported in the previous chapter of this thesis demonstrated that PK from tissues of the channelled whelk, Busycotypus canaliculatum, is likely subjected to covalent modification in response to long term anoxic stress. The three tissue specific PK isozymes which were identified were distinguished from their respective anoxic counterparts on the basis of affinity for PEP, isoelectrofocusing data and electrophoretic mobilities on cellulose acetate strips at pH 7.2. Subsequent attention has been concentrated on PK from the myoglobin containing radular retractor (proboscis muscle) muscle of B. canaliculatum. The present chapter reports the purification to homogeneity and extensive kinetic characterization of the aerobic and anoxic forms of this enzyme. The following chapter provides conclusive evidence that the anoxia-induced inactivation of whelk red muscle PK is due to the covalent incorporation of phosphate into the enzyme molecule.

Materials and Methods

Chemicals and Animals.

Phosphocellulose was purchased from Sigma Chemical Co.; Affi-Gel Blue was from Bio-Rad; Sepharose CL-6B, and Sephadex G-25 were from Pharmacia Fine Chemicals. All other chemicals, biochemicals and specimens of the channelled whelk, B. canaliculatum, were obtained as described in Chapter 4. All buffers used in the present study were adjusted to their respective pH at 20 °C. Sampling of radular retractor tissue from aerobic and 21 h anoxic whelks was performed as previously described (Chapter 5).

Enzyme Assays and Kinetic Studies.

The PK reaction was coupled with the LDH reaction and assayed by monitoring NADH utilization at 340 nm using a Gilford Recording spectrophotometer attached to a circulating water bath for temperature control of cuvettes. Temperature for all assays was maintained at 20 °C. Standard assay conditions for PK from aerobic animals were: 50 mM imidazole-HCl buffer (pH 7.0), 1 mM PEP, 2 mM ADP, 50 mM KCl, 5 mM MgCl₂, 0.15 mM NADH and 2 I.U./ml dialyzed LDH in a final volume of 1 ml. Standard assay conditions for PK from anoxic animals were as above except that a PEP concentration of 5 mM was used. Assays were initiated by addition of enzyme preparation. One unit of enzyme activity is defined as the amount of enzyme resulting in the utilization of 1 μ mol NADH per min at 20 °C. $K_{0.5}$'s and Hill

coefficients for all substrates and co-factors were calculated from Hill plots. Activation constants (K_a) were determined using double reciprocal plots ($1/(V-V_0)$ vs. $1/[\text{activator}]$). I_{50} 's and inhibitor constants (K_i) were determined as described in Chapter 2. All kinetic parameters are the mean of duplicate determinations on two separate preparations of the purified enzymes and are reproducible to within $\pm 10\%$. In most cases results are expressed as means $\pm$ s.e.m.

Purification of Pyruvate Kinase from Red Muscle of Aerobic and Anoxic Animals.

(i) Aerobic Pyruvate Kinase.

Quick frozen radular retractor tissue from 5 aerobic whelks was ground to a powder under liquid nitrogen using a mortar and pestle, weighed and homogenized (1/5 w/v) for 2 X 30 s in ice-cold 50 mM imidazole-HCl buffer (pH 7.5), containing 0.1 mM PMSF, 10 mM 2-mercaptoethanol, 50 mM NaF, 3 mM EDTA and 3 mM EGTA, using a Polytron PT'10 homogenizer. The homogenate was centrifuged at 28,000 X g for 20 min at 4 °C and the pellets discarded. The supernatant fraction was adjusted to 1.23 M (30% saturation) with ammonium sulphate, stirred at room temperature for 30 min and then centrifuged as above. Pellets were discarded and supernatants were then adjusted to 2.05 M (50% saturation) with ammonium sulphate, stirred and centrifuged as above. The pellets containing PK were resuspended in 10 ml of 25 mM imidazole-HCl buffer (pH

7.3), containing 10 mM 2-mercaptoethanol, 50 mM NaF and 3 mM EDTA and dialyzed against 2 l of the same buffer for 1.5 h at 4 °C. Following dialysis the resuspended ammonium sulphate pellets were centrifuged as above to remove insoluble material. This partially purified enzyme solution was then layered onto a column (1.5 X 4.0 cm) of Affi-Gel Blue equilibrated in the dialysis buffer. The column was washed with buffer until the OD₂₈₀ dropped to a minimum value. PK was eluted from the column using a 0 to 1.5 M gradient of KCl (50 ml total volume), run at 25 ml/h. Peak fractions were pooled and dialyzed for 1.5 h at 4 °C against 2 l of 25 mM imidazole-HCl buffer (pH 7.0) containing 10 mM 2-mercaptoethanol, 25 mM NaF and 1.5 mM EDTA. The dialyzed peak fractions were then layered onto a column (1.5 X 3.0 cm) of phosphocellulose equilibrated in the same buffer and the column was washed with buffer until the OD₂₈₀ dropped to a minimum value. This was followed by a wash with 10 ml of column buffer containing 10 mM ADP and PK activity was then eluted in a sharp peak with 10 ml of 20 mM ADP and 20 mM PEP in column buffer. Peak fractions were pooled and concentrated against solid sucrose in a dialysis sack. The concentrated enzyme preparation was then diluted two to three fold, with 20 mM imidazole-HCl buffer (pH 6.7), containing 10 mM 2-mercaptoethanol and 1 mM EDTA and layered onto a column (0.9 X 50 cm) of PBE 94 chromatofocusing exchanger pre-equilibrated in the same buffer. The column was eluted at 20 ml/h with 200 ml Polybuffer 74 (stock

diluted 1:8 with distilled water) adjusted to pH 4.0. Peak fractions from chromatofocusing, eluting between pH 5.8 and pH 5.0, were pooled and concentrated against solid sucrose in a dialysis sack. This highly purified enzyme preparation was then layered onto a column (1.5 X 50 cm) of Sepharose CL-6B equilibrated in 25 mM imidazole-HCl buffer (pH 7.1), containing 10 mM 2-mercaptoethanol, 1 mM EDTA, 500 mM sucrose and 0.04% sodium azide. The column was eluted with the same buffer and peak fractions, containing purified aerobic PK were combined, concentrated against solid polyethylene glycol 6,000 in a dialysis sack and stored at 4 °C until use. The purified enzyme was stable in this form with no loss in activity or change in kinetic parameters for at least 4 weeks.

(ii) Anoxic Pyruvate Kinase.

PK was purified from radular retractor tissue of anoxic animals using the above purification scheme with the following modifications. Extensive desalting of the resuspended 50% ammonium sulphate pellets was required in order that the enzyme could be completely absorbed by the Affi-Gel Blue affinity column. This was quickly accomplished by centrifuging as above to remove insoluble material followed by layering of the enzyme extract onto a column (2.5 X 20 cm) containing Sephadex G-25 equilibrated in the resuspension buffer. This column was eluted using the same buffer (flow rate 30 ml/h) and peak fractions were pooled

and layered directly onto the Affi-Gel Blue affinity column (1.5 X 4.0 cm). A 0 to 0.8 M KCl gradient (50 ml total volume) was used to elute bound PK from this column. Washing of the phosphocellulose affinity column with column buffer was followed by a 10 ml wash with 1 mM ADP in column buffer followed by elution of bound PK with 10 mM ADP and 10 mM PEP in the column buffer. As chromatofocusing completely inactivated the anoxic enzyme this purification technique was replaced by preparative isoelectrofocusing performed as previously described (Chapter 1) using a pH gradient of 4.0 to 6.0. Pooled peak phosphocellulose fractions were run at 400 V for 16 h at ~~4~~ °C. The column was then drained and 1.5 ml fractions collected and assayed for PK activity. Peak fractions containing the anoxic form of the red muscle isozyme (pI = 5.4) were combined ~~and~~ concentrated by dialysis against solid polyethylene glycol 6,000. This was followed by Sepharose CL-6B gel filtration and concentration as described above.

Electrophoresis.

SDS polyacrylamide gel electrophoresis was performed using a discontinuous system based on the procedure of Laemmli (1970). Stock solution of acrylamide (monomer) and Bis (cross-linker) used for both stacking and separating gels contained 30% acrylamide and 0.8% Bis. Final monomer concentration in the 0.75 mm thick slab gel was 7.5% for the stacking gel (containing 125 mM Tris-HCl (pH 6.8) and 0.1%

SDS) and 15% for the separating gel (containing 375 mM Tris-HCl (pH 8.3) with 0.1% SDS). Tris/glycine (25 mM/197 mM) (pH 8.3) containing 0.1% SDS was used as the electrode buffer with bromophenol blue as the tracking dye. All samples were preincubated in the presence of 2% (w/v) SDS and 5% (v/v) 2-mercaptoethanol for 15 min at 80 °C and 3 h at room temperature. Gels were precooled to 4 °C before use and were maintained at this temperature during electrophoresis. Gels were run with 100 V constant voltage applied for 15 h. Following electrophoresis gels were fixed for at least 4 h in a solution of 40% methanol and 10% acetic acid followed by staining for protein using Coomassie Brilliant Blue G by the method of Blakesley and Boezi (1977). For the determination of subunit molecular weights using SDS polyacrylamide gel electrophoresis a plot of R_f versus log molecular weight was constructed using the following standard proteins: trypsinogen (24,000), ovalbumin (45,000), rabbit muscle PK (57,000 per subunit; Cottam et al., 1969) and bovine serum albumin (66,000).

Non-SDS polyacrylamide gel electrophoresis was performed using tube gels (5 mm I.D.) with the discontinuous system of Davis (1964). Gels were precooled to 4 °C before use and were maintained at this temperature during electrophoresis. Samples containing sucrose and added bromophenol blue were run at 4 mA/gel for 8 h. Following electrophoresis gels were placed directly into the protein staining solution as described above.

Protein Assay.

Protein concentration was measured as described in Chapter 1.

Results

Purification and Physical Properties.

Tables 16 and 17 present typical purifications for red muscle PK from muscles of aerobic (RPK-AER) and anoxic (RPK-ANX) animals. The purified enzymes had specific activities that varied from 330 to 388 units/mg protein for RPK-AER and 135 to 186 units/mg protein for RPK-ANX. As seen in Plate 3-A following electrophoresis on polyacrylamide gels under non-dissociating conditions both preparations yielded a single protein band, but there was consistently a slight difference in the electrophoretic mobilities between the two purified enzymes, such that RPK-ANX had the faster anodal migration. The subunit molecular weight of purified RPK-AER and RPK-ANX were examined by SDS polyacrylamide gel electrophoresis. As shown in Plate 3-B both RPK-AER and RPK-ANX ran as a single peptide band which migrated with an apparent molecular weight of $64,400 \pm 200$ ($n = 2$). The native molecular weights for RPK-AER and RPK-ANX determined by gel filtration on Sepharose CL-6B were found to be $237,500 \pm 8,500$ ($n = 2$) and $232,600 \pm 12,000$ ($n = 2$), respectively. Thus, as is the case for PK from muscles of a variety of vertebrate and

Table 16. Summary of the purification of red muscle pyruvate kinase from aerobic wheelks.

Step	Total Activity (units)	Total Protein (mg)	Specific Activity (units/mg)	Purification (fold)	Yield (%)
Crude supernatant	720	372	1.9	1.0	100
Ammonium Sulphate	619	103	6.0	3.1	86
Affl-Gel Blue	526	11.2	47.0	34	73
Phosphocellulose	403	2.6	197.0	102	56
Chromatofocusing	180	0.55	325.0	168	25
Sepharose CL-6B	86	0.23	368.0	190	12

Table 17. Summary of the purification of red muscle pyruvate kinase from wheelks subjected to 21 h of anoxic stress.

Step	Total Activity (units)	Total Protein (mg)	Specific Activity (units/mg)	Purification (fold)	Yield (%)
Crude supernatant	434	414	1.0	1.0	100
Ammonium Sulphate	360	171	2.1	2.0	83
Affl-Gel Blue	264	7.9	33.4	33	61
Phosphocellulose	187	1.44	130.0	130	26
Isoelectrofocusing	52		-	-	12
Sepharose CL-6B	26	0.14	186.0	186	6

Plate 3. A: Non-SDS polyacrylamide gel electrophoresis
of purified RPK-AER (left gel) and RPK-ANX
(right gel).

B: SDS-polyacrylamide gel electrophoresis of
purified RPK-AER (left lane) and RPK-ANX
(right lane).

invertebrate species (Munday et al., 1980; Leon et al., 1982), whelk red muscle PK from both aerobic and anoxic tissues appears to be a multimeric protein composed of four identical subunits.

Effect of pH.

The pH activity profiles of purified RPK-AER and RPK-ANX in the presence of saturating concentrations of substrates and co-factors are shown in Fig. 9. Under these conditions both PK forms showed a fairly broad pH optima of about pH 7.0. PK from other molluscan species also shows a typically broad pH optimum when examined under optimal assay conditions (Zammit and Newsholme, 1978; Leon et al., 1982). All kinetic parameters for both purified RPK-AER and RPK-ANX have been determined at pH 7.0.

Cation Requirements.

In common with PK from other species, both RPK-AER and RPK-ANX showed absolute requirements for both a monovalent and divalent cation. In the presence of saturating concentrations of PEP and ADP the activation by K^+ was hyperbolic for RPK-AER ($KCl_{0.5} = 4.1 \pm 0.4$ mM; $n_H = 1.04 \pm 0.10$) but was slightly sigmoidal for RPK-ANX ($KCl_{0.5} = 6.12 \pm 0.16$; $n_H = 1.4 \pm 0.05$). In both cases NH_4^+ could replace K^+ ; however V_{max} in the presence of saturating NH_4^+ was only 50% of that with K^+ . By contrast, the binding characteristics of Mg^{2+} followed a slightly sigmoidal pattern for RPK-AER ($MgCl_{0.5} = 0.46 \pm 0.03$ mM; $n_H = 1.7 \pm$

Fig. 9. Activity of RPK-AER and RPK-ANX as function of pH.

Standard reaction mixtures were used. Enzyme activities are relative to the maximal activity obtained for each respective enzyme preparation. ▲, RPK-AER; ○, RPK-ANX.

0.06) and became strongly cooperative for RPK-ANX ($\text{MgCl}_{0.5} = 0.80 \pm 0.04 \text{ mM}$; $n_H = 2.50 \pm 0.02$). Mn^{2+} could replace Mg^{2+} as the divalent cation; however, when tested at saturating concentrations, in both cases this substitution lead to an approximately 60% decrease in V_{max} .

Dependence of Red Muscle Pyruvate Kinase on Concentration of PEP.

The activity of RPK-AER and RPK-ANX as a function of PEP is shown in Fig. 10. At saturating concentrations of PEP RPK-AER showed an approximately 2 fold greater maximal activity than did RPK-ANX. Furthermore, RPK-AER showed hyperbolic kinetics ($n_H = 0.99 \pm 0.10$) whereas RPK-ANX displayed a strongly sigmoidal pattern of PEP utilization ($n_H = 2.57 \pm 0.01$). $\text{PEP}_{0.5}$ for RPK-AER was $0.07 \pm 0.002 \text{ mM}$ and this constant was increased to $0.85 \pm 0.02 \text{ mM}$ for RPK-ANX. With $50 \text{ } \mu\text{M}$ Fru-1,6- P_2 both curves became hyperbolic and $\text{PEP}_{0.5}$'s decreased to the same value, $0.040 \pm 0.001 \text{ mM}$ for RPK-AER ($n_H = 1.15 \pm 0.01$) and $0.040 \pm 0.004 \text{ mM}$ for RPK-ANX ($n_H = 1.10 \pm 0.06$). Thus the modification of RPK-AER which is apparently induced by anoxic stress causes a decrease in both maximal enzyme velocity and affinity for PEP. Both these effects have been documented for the phosphorylated form of rat liver type PK (Ljungstrom et al., 1974; Titanji et al., 1976). As seen in Fig. 11 both RPK-AER and RPK-ANX showed a similar response of PEP affinity to a pH change from pH 6.75 to pH 7.25 such that in each case

Fig. 10. Dependence of PK activity on concentration of PEP. Standard assay conditions were used. Open and filled symbols represent PK activity in the absence and presence of 50 μ M Fru-1,6-P₂, respectively. Δ , $\blacktriangle$, RPK-AER; $\circ$, $\bullet$, RPK-ANX.

Fig. 11. Effect of pH on affinities of RPK-AER and RPK-ANX for PEP. Standard assay conditions were used. ▲, RPK-AER; ○, RPK-ANX. Results are means ($\pm$ s.e.m.) of duplicate determinations performed on two separate preparations of each purified enzyme.

maximal affinity for PEP occurred at pH 7.0 with respective $PEP_{0.5}$'s increasing from 40 to 75% at the lower and higher pH values.

Pyruvate Kinase Activity as a Function of ADP Concentration.

The effect of ADP on activities of RPK-AER and RPK-ANX is shown in Fig. 12. When saturating concentrations of PEP were used both PK forms reach maximal activity at 2 mM ADP. The substrate saturation curves for ADP followed regular hyperbolas with identical K_m 's for ADP for both RPK-AER ($K_m = 0.27 \pm 0.01$ mM; $n_H = 1.13 \pm 0.01$) and RPK-ANX ($K_m = 0.25 \pm 0.01$ mM; $n_H = 1.10 \pm 0.03$). High concentrations of ADP were inhibitory, this inhibition being more pronounced for RPK-ANX than for RPK-AER. In both cases the addition of $1 \mu M$ Fru-1,6- P_2 had no effect on the K_m for ADP or the pattern of ADP utilization but partially relieved the ADP substrate inhibition.

Metabolite Effects.

A variety of metabolites were tested for effects on RPK-AER and RPK-ANX at subsaturating PEP (0.07 mM and 0.80 mM respectively) and ADP (0.3 mM for both PK forms). The following substances had no effects ($\pm 10\%$ of control velocity) on either PK preparation: acetyl-CoA (0.03 mM); NADP (1 mM); oxaloacetate and 3-phosphoglycerate (both 2 mM); $(Mg)_2$ -citrate, DL-isocitrate, succinate, malate, α -ketoglutarate, glucose-6-phosphate, α -glycerolphosphate, 3-phosphoglycerate, fructose-6-phosphate, D-octopine,

Fig. 12. Effect of ADP concentration on activities of RPK-AER and RPK-ANX. Standard assay conditions were used. Enzyme activities are relative to the maximal activity obtained for each of the two respective enzyme preparations. Open and filled symbols represent PK activity in the absence and presence of $1 \mu\text{M}$ Fru-1,6- P_2 , respectively. Δ , $\blacktriangle$, RPK-AER; $\circ$, $\bullet$, RPK-ANX.

meso-alanopine, NH_4Cl (all 5 mM); arginine, glycine, taurine, creatine phosphate (all 10 mM); and inorganic phosphate (20 mM). L-lactate (10 mM) and MgAMP (5 mM) caused 20-25% inhibition of both RPK-AER and RPK-ANX. Phenylalanine (10 mM) had no effect on RPK-AER but caused 95% inhibition of RPK-ANX. Six other compounds were found to be good effectors: Fru-1,6- P_2 , Fru-2,6- P_2 , MgATP, arginine phosphate, alanine and proline. Under the test conditions described above Fru-1,6- P_2 (0.5 mM) and its analogue Fru-2,6- P_2 (0.25 mM) had no effect on RPK-AER but activated RPK-ANX about 20%. However, when tested at saturating ADP (2 mM) but subsaturating PEP, activation of both RPK forms was dramatically enhanced (Table 19). Table 18 lists apparent K_a 's for these activators. As the K_a for Fru-2,6- P_2 is well beyond probable physiological concentrations of this compound it was not further investigated.

MgATP, alanine, proline and arginine-phosphate were inhibitors of both RPK forms (Table 19). As was the case for phenylalanine RPK-ANX was far more sensitive to inhibition by alanine, proline and to a lesser extent MgATP than was RPK-AER. The K_i of RPK-ANX for alanine (with respect to pyruvate) was about 500 fold lower than the corresponding value for RPK-ANX (Table 18). This greatly differing sensitivity to inhibition by alanine was one of the most dramatic kinetic differences between the two red muscle PK forms. The effect of varying pH from pH 6.75 to pH 7.25 on inhibition of both red muscle PK forms by alanine was also

Table 18. Inhibition and activation constants for metabolic effectors of RPK-AER and RPK-ANX.

Inhibitor	Apparent K_i (mM)		Inhibition	
	RPK-AER	RPK-ANX	Type	With Respect to
Alanine	24.5 $\pm$ 2.5	0.05 $\pm$ 0.004	Competitive	PEP
	84.5 $\pm$ 4.5	1.28 $\pm$ 0.13	Competitive	ADP
MgATP	2.68 $\pm$ 0.13	0.78 $\pm$ 0.08	Competitive	PEP
	5.63 $\pm$ 0.60	8.00 $\pm$ 0.18	Competitive	ADP
Activator	Apparent K_a (μ M)			
	RPK-AER	RPK-ANX		
Fru-2,6-P ₂	12.5 $\pm$ 3.0	154 $\pm$ 21		
Fru-1,6-P ₂	0.054 $\pm$ 0.008	1.3 $\pm$ 0.1		
Fru-1,6-P ₂ (plus 1.5 mM ATP & 1.0 mM alanine)	0.018 $\pm$ 0.004	5.2 $\pm$ 0.4		

Inhibitor constants were determined from Dixon plots and pattern of inhibition evaluated by double reciprocal plots. Activation constants were evaluated at 0.07 mM PEP and were determined from double reciprocal plots ($1/V - V_0$) vs. $1/[FDP]$. Results are means $\pm$ s.e.m. for $n = 2$ determinations on two separate preparations of each purified enzyme.

Table 19. The inhibition of purified RPK-AER and RPK-ANX by arginine phosphate, MgATP, alanine and proline. Standard assay conditions, were used except that the concentration of PEP was 0.07 mM and 0.8 mM for RPK-AER and RPK-ANX, respectively. Enzyme velocity in the presence of inhibitors is expressed relative to control set at 100.

Addition	Concentration (mM)	RPK-AER		RPK-ANX	
		minus	+ 1 μ M	minus	+ 1 μ M
		Fru-1,6-P ₂	Fru-1,6-P ₂	Fru-1,6-P ₂	Fru-1,6-P ₂
Control (none)	-	100	153	100	205
Arginine Phosphate	5	92	143	96	188
	10	73	114	74	156
	15	55	92	46	131
MgATP	1.0	74	140	61	184
	2.5	64	119	31	164
	5.0	44	98	18	116
Alanine	0.025	-	-	77	203
	0.10	-	-	39	160
	0.50	-	-	10	110
	5.0	74	127	-	-
	20.0	57	115	-	-
	50.0	49	107	-	-
Proline	2.5	-	-	27	146
	5.0	-	-	18	132
	10.0	74	150	11	91
	25.0	58	142	-	-
	50.0	50	106	-	-

studied. As seen in Fig. 13 both RPK-AER and RPK-ANX showed a uniform increase in I_{50} with increasing pH. Thus, at lower pH values inhibition by alanine was enhanced.

As an activator Fru-1,6- P_2 functioned in several ways. For both enzymes it facilitated binding of the substrate PEP (Fig. 10) and partially relieved inhibition by the substrate ADP (Fig. 12) and by the allosteric effectors alanine, arginine-phosphate, proline and MgATP (Table 19). In order to investigate whether activation by Fru-1,6- P_2 could be relevant in vivo it was tested in the presence and absence of alanine and ATP (Fig. 14, Fig. 15, Table 18). In the absence of these inhibitors the K_a of RPK-AER for Fru-1,6- P_2 was about 20 fold lower than the corresponding value for RPK-ANX. However, in the presence of 1.0 mM alanine and 1.5 mM ATP: (1) the K_a for Fru-1,6- P_2 of RPK-AER was decreased whereas the K_a for RPK-ANX was increased, resulting in an approximate 300 fold difference between the two, and (2) both red muscle PK forms showed a 15-20% reduction in maximal activity when tested at saturating Fru-1,6- P_2 . Note that, under the assay conditions used, despite the presence or absence of the two inhibitors, in the absence of Fru-1,6- P_2 RPK-ANX activity was totally abolished whereas RPK-AER retained at least 45% of its maximal activity when determined at saturating Fru-1,6- P_2 .

Fig. 13. Effect of pH on inhibition of RPK-AER and RPK-ANX by alanine. Standard assay conditions were used except that PEP was subsaturating. I_{50} 's for RPK-AER ($\blacktriangle$) and RPK-ANX ($\circ$) were determined at 0.1 mM and 1.0 mM PEP, respectively. Results are means ($\pm$ s.e.m.) of duplicate determinations performed on two separate preparations of each purified enzyme.

Fig. 14. The activity of RPK-AER as a function of Fru-1,6-P₂ in the presence (▲) and absence (△) of 1.5 mM ATP and 1.0 mM alanine; determined at 0.07 mM PEP and saturating concentrations of ADP and co-factors.

Fig. 15. The activity of RPK-ANX as a function of Fru-1,6-P₂ in the presence (●) and absence (○) of 1.5 mM ATP and 1.0 mM alanine; determined at 0.07 mM PEP and saturating concentrations of ADP and co-factors.

Discussion

The aim of this investigation was to examine how the kinetic properties of whelk red muscle PK change upon exposure of the animal to anoxic stress in order to obtain information of the physiological significance of the modification which has been shown to occur (Chapter 5).

Like muscle PK from other molluscan sources (Holwerda and de Zwaan, 1973; Leon et al., 1982), RPK-AER and RPK-ANX are both homotetramers with a molecular weight of around 235,000. This indicates that the modification of B. canaliculatum red muscle PK which accompanies anoxic stress does not involve subunit disassociation-reassociation. RPK-AER and RPK-ANX also share several similar kinetic properties which include: (i) affinities for ADP, K^+ and Mg^{2+} , (ii) sensitivities to inhibition by MgAMP, arginine phosphate and L-lactate, and (iii) a pH optima of pH 7.0 under optimal assay conditions. Also both red muscle PK forms showed an identical $PEP_{0.5}$ of 0.04 mM and Hill coefficient of 1.1 when activated by saturating concentrations of Fru-1,6- P_2 . However, in most other respects RPK-AER and RPK-ANX differ markedly.

Preliminary studies have shown that RPK-AER and RPK-ANX differ both in pI as well as electrophoretic mobilities on cellulose acetate strips at pH 7.2 (Chapter 5). During the course of the present study it was also noted that their behaviors on several chromatographic columns used during the

development of their respective purification schemes was quite different. RPK-ANX was not only bound much less tightly to the Affi-Gel Blue and phosphocellulose affinity columns, but was completely inactivated by chromatofocusing. These differences in physical characteristics are accompanied by even larger differences in many of their kinetic properties.

The two B. canaliculatum red muscle PK variants differed greatly in kinetics of PEP utilization; RPK-AER has an approximate two-fold greater maximal velocity, a ten-fold lower $PEP_{0.5}$ and displays Michaelis-Menten kinetics as opposed to the strongly sigmoidal kinetics of RPK-ANX (Fig. 10). Holwerda et al. (1983) noted a similar relationship between $PEP_{0.5}$'s of active and inactive PK variants from adductor muscle of M. edulis but, in contrast to the present study, found consistently larger values of the Hill coefficient for the more active PK variant than for the less active variant. However, like RPK-ANX, the phosphorylated, or less active, form of L-type PK from rat liver has been reported to have a lower maximal velocity, a higher PEP and enhanced cooperative effect with PEP (Ljungstrom et al., 1974; Titanji et al., 1976). Another kinetic difference between the two PK variants was that RPK-AER showed significantly less cooperativity in the pattern of Mg^{2+} binding. Likewise, although their respective affinities for ADP were identical RPK-ANX was more sensitive to substrate inhibition by high concentrations of ADP (Fig. 12).

RPK-AER and its counterpart, RPK-ANX, differed most strongly in their respective responses to a variety of allosteric effectors. RPK-ANX was less susceptible to activation by Fru-1,6-P₂, somewhat more sensitive to inhibition by MgATP and was tremendously more sensitive to inhibition by alanine, phenylalanine and proline (Fig. 14, Fig. 15, Table 18, Table 19). Similarly, the less active form of M. edulis adductor PK showed increased susceptibility to inhibition by alanine but differed from whelk RPK-ANX in having an unchanged response to activation by Fru-1,6-P₂ (Holwerda et al., 1983). Like RPK-ANX the phosphorylated, less active forms of pig and rat liver L-type PK and human erythrocyte PK all proved to be more sensitive to the inhibitors alanine and MgATP but were less readily stimulated by Fru-1,6-P₂ with subsaturating PEP and otherwise standard conditions (Engstrom, 1978; Marie et al., 1980). However, the magnitude of differences in sensitivity to inhibition by phenylalanine and alanine noted in the present study for RPK-AER and RPK-ANX are far greater than that which was observed for the analogous PK variants from M. edulis adductor muscle, pig and rat livers or human erythrocytes.

In the presence of near-physiological concentrations of PEP, ADP, ATP and alanine about 300 times as much Fru-1,6-P₂ was required to reach half maximal stimulation of RPK-ANX as compared to RPK-AER (Table 18). This is paralleled by the considerable alanine and ATP inhibition of RPK-ANX in the

range of 1-2 μM Fru-1,6- P_2 (Fig. 15). The free concentration of this metabolite in whelk red muscle tissue during long term environmental anaerobiosis is not known but is probably very low. Recent studies with B. canaliculatum foot muscle indicate that the key glycolytic enzyme phosphofructokinase, responsible for production of Fru-1,6- P_2 , is partially inactivated by covalent modification (probably phosphorylation) in response to anoxic stress (K.B. Storey, manuscript in preparation). The concomitant reduction in phosphofructokinase activity should be manifested in a negative crossover at the enzyme locus which would result in reduced Fru-1,6- P_2 levels during anoxia. Such an effect has been demonstrated in M. edulis adductor muscle tissue during the first several hours of exposure to anoxic stress (Ebberink and de Zwaan, 1980). Even very high, and likely non-physiological, concentrations of Fru-1,6- P_2 do not completely abolish the inhibitory effects caused by the apparent modification of whelk red muscle PK in response to anoxic stress (Fig. 14, Fig. 15). Thus it can be convincingly argued that the effect of this modification, in the presence of physiological concentrations of substrates and effectors, is to inhibit the enzyme. This suggests that a reversible covalent modification of whelk red muscle PK, in response to availability of oxygen, may not only play a crucial role in regulating enzyme activity in vivo, but consequently, may also control the direction of carbon flow at the PEP branchpoint.

Because several workers have stressed the importance of a decrease in intracellular pH (pH_i) in the control of molluscan PK during long term anoxic stress the effect of H^+ concentration on the respective $\text{PEP}_{0.5}$'s and susceptibility to inhibition by alanine of the two whelk red muscle PK variants was examined. Recent ^{31}P -NMR studies with the isolated perfused ventricle (like radular retractor a red muscle tissue) of the anoxia tolerant whelk, Busycon contrarium, have demonstrated a decrease in pH_i from about pH 7.1 to pH 6.9 during 6 h of experimental anoxia (Ellington, 1983a). Hence, a pH range of 6.75 to 7.25 was chosen for examining pH effects on red muscle PK in the present study. At the lower pH affinities of both PK forms for PEP were decreased and respective sensitivities to inhibition by alanine were increased (Fig. 11, Fig. 13). However, these individual pH induced kinetic changes in no way approach the large, pH-independent, differences in affinity for PEP and sensitivity to inhibition by alanine which RPK-AER and RPK-ANX show. Therefore, the reduction in pH_i which occurs during anoxic stress probably plays an insignificant role in the control of R. canaliculatum red muscle PK activity in vivo. Consequently, possible influences of whelk red muscle PK activity on the direction of glycolytic carbon flux during anaerobiosis are probably pH_i -independent. Similarly, in the absence of other factors, the extent of pH_i decrease in M. edulis adductor muscle during 8 h of anaerobiosis was shown to have a minimal

effect on the direction of carbon flow at the PEP branchpoint (Walsh et al., 1983).

The kinetic properties of RPK-AER and RPK-ANX are ideally suited to their physiological role, in vivo. During the first few hours of anoxia whelk red muscle glycolytic carbon is directed towards pyruvate, which in turn is metabolized mainly into alanine; meanwhile aspartate reserves are mobilized for succinate production (Eberlee and Storey, 1984). After 4-8 h of anoxic stress alanine concentrations stabilize, aspartate utilization rates greatly decline, but glycogen catabolism and succinate production continue unabated. During this apparent shift of glycolytic carbon away from alanine, and towards succinate, phosphofructokinase and PK are probably undergoing covalent modifications. The consequent partial inactivation of phosphofructokinase may play a dual role in the overall metabolic strategy of whelks. First it will lower the total rate of glycolytic flux, thereby allowing conservation of fuels for more efficient use during aerobic periods. Famme and coworkers (1981) have estimated that anaerobic metabolism in M. edulis may represent only 5 to 10% of total metabolism at normoxia. Secondly, decreased phosphofructokinase activity should result in lower free Fru-1,6-P₂ levels, for possible activation of PK. Major kinetic effects resulting from the anoxia-induced modification of red muscle PK include reduced affinity for PEP, decreased sensitivity to activation by Fru-1,6-P₂, as

well as greatly increased susceptibility to inhibition by alanine (which increases in concentration to about 10 mM during the early phase of anoxia) and proline. These kinetic effects will ensure that PK is effectively inhibited, allowing glycolytic carbon to be redirected towards succinate via PEP.CK and the reversal of several Krebs cycle reactions. During the initial stages of recovery from anoxic stress RPK-ANX is likely to be reconverted back to RPK-AER, which with its high affinity for PEP, increased susceptibility to activation by Fru-1,6-P₂ (concentrations which probably rise owing to concomitant phosphofructokinase reactivation; see Ebberink and de Zwaan, 1980), and insensitivity to alanine, would then be fully active. This should allow for both redirection of carbon towards pyruvate as well as for the increased glycolytic rate characteristic of metabolism during the first few hours of recovery from long term environmental anoxia (Famme and Knudsen, 1981). Time-course studies on changes in metabolite levels which occur in whelk red muscle tissue during 24 h of anoxia and subsequent recovery support the conclusions presented above (Ellington, 1982; Eberlee and Storey, 1984). Radiotracer experiments which could demonstrate the channelling of glycolytic carbon into succinate during long term environmental anaerobiosis are required in order to clearly establish the metabolic significance of the anoxia-induced inactivation of B. canaliculatum red muscle PK demonstrated by the present study.

CHAPTER VII: In vivo phosphorylation of red muscle pyruvate kinase from the channelled whelk, Busycotypus canaliculatum, in response to anoxic stress.

Introduction

The previous chapter reported the purification and kinetic characterization of aerobic and anoxic forms of PK from red muscle tissue of the channelled whelk, B. canaliculatum. The anoxic form of B. canaliculatum red muscle PK shared many properties common to mammalian liver-type PK when phosphorylated by a cyclic AMP-dependent protein kinase. These include an unchanged native molecular weight but a lower pI, decreased affinity for PEP, increased cooperativity in binding of PEP, increased susceptibility to inhibition by ATP and alanine, decreased sensitivity to activation by fructose-1,6-bisphosphate and, furthermore, in the presence of saturating concentrations of fructose-1,6-bisphosphate, an affinity and Hill constant for PEP identical to that for the corresponding fructose-1,6-bisphosphate activated unmodified enzyme (Ekman et al., 1976; Engstrom, 1978; Blair et al., 1982). These striking similarities led to the question of whether or not the anoxia-induced inactivation of B. canaliculatum red muscle PK is accompanied by the reversible covalent incorporation of phosphate into the enzyme molecule. The present chapter demonstrates that the aerobic and anoxic forms of whelk red muscle PK are interconvertible dephospho- and phosphoenzymes. Moreover, evidence is presented which suggests that protein phosphorylation may be an important metabolic strategy employed by anoxia-tolerant molluscs for

the control of metabolism following the onset of anaerobiosis.

Materials and Methods

Chemicals and Animals.

Rabbit muscle Peak I cyclic AMP-dependent protein kinase and E. coli alkaline phosphatase were purchased from Sigma Chemical Co. Carrier free [32 P] orthophosphate (9.18 μ Ci/ μ mol) and Aquasol II liquid scintillation cocktail were from New England Nuclear. Other chemicals, biochemicals and specimens of the channelled whelk, B. canaliculatum, were obtained as outlined in the preceeding chapter.

In vitro Incubations of Purified Red Muscle Pyruvate Kinases with Protein Kinase and Alkaline Phosphatase.

Separate aliquots of homogenous red-muscle PK purified from aerobic and anoxic whelks (see Chapter 5) were dialyzed for 3 h at 4°C against 1.5 l of either 50 mM imidazole-HCl (pH 6.5; buffer A) or 50 mM Tris-HCl (pH 8.4; buffer B), each containing 750 mM sucrose, 10 mM 2-mercaptoethanol and 1.0 mM EDTA. Each dialyzed PK preparation (2-4 units/ml) was then incubated in the respective dialysis buffer (minus EDTA) plus 2.5 mg rabbit muscle peak I cyclic AMP-dependent protein kinase (900 units), 2 mM ATP, 50 μ M cyclic AMP, 10 mM $MgCl_2$ with and without 20 mM alanine or 10 μ M fructose-1,6-bisphosphate. Control / incubations were

conducted in the absence of protein kinase, cyclic AMP, MgCl_2 , ATP, alanine or fructose-1,6-bisphosphate. Final volume of incubation mixtures was 0.5 ml and incubation was continued for up to 48 h at 30 °C. PK preparations which had been dialyzed against buffer B were also incubated with or without dialyzed E. coli type III alkaline phosphatase (20 units) for 4 h at 37 °C in dialysis buffer (minus EDTA) containing 25 mM KCl and 10 mM MgCl_2 in a total volume of 0.5 ml.

In vivo ^{32}P -labelling Studies.

Carrier free [^{32}P] orthophosphate (4 mCi in 0.5 ml full strength sea water) was injected directly into the proboscis (radular retractor muscle) of two aerobic whelks. One animal was held in aerated sea water at 15 °C for 41 h. The other animal was held in aerated sea water for 20 h followed by transfer to water made anoxic by bubbling N_2 (g) through the water for a further 21 h. The radular retractor muscle of each animal was rapidly excised, blotted and frozen in liquid N_2 . Frozen tissues were ground in liquid N_2 using a mortar and pestle and homogenized (1/4 w/v) for 2 X 30 s in 15 mM Tris-HCl buffer (pH 7.2) containing 10 mM 2-mercaptoethanol, 250 mM sucrose, 1.5 mM EDTA, 25 mM NaF and 0.1 mM PMSF using a Polytron PT 10 homogenizer at 0-4 °C. Homogenates were centrifuged at 28,000 X g for 20 min at 4 °C and pellets discarded. Aliquots (0.5 ml) of each extract

were dialyzed together overnight against 4 l of homogenization buffer minus PMSF.

Cellulose Acetate and Polyacrylamide Electrophoresis of ³²P-labelled Extracts.

Electrophoresis was performed on cellulose acetate strips for 1 h at 4 °C with 40 V per strip using 15 mM Tris-HCl buffer (pH 7.2) containing 250 mM sucrose, 10 mM 2-mercaptoethanol and 1.5 mM EDTA as the electrophoresis buffer. Approximately, 5 µl samples containing 20-30 units/ml were applied to the center of the strips using a 10 µl Hamilton syringe. PK activity was detected as previously described (Chapter 4).

Non-SDS polyacrylamide gel electrophoresis was performed using tube gels (5 mm I.D.) with the discontinuous system of Davis (1964). Gels were precooled to 4 °C before use and were maintained at this temperature during electrophoresis. Approximately 10 µl of dialyzed crude ³²P labelled extract containing added sucrose and bromophenol blue was put on each gel and gels were run at 2.5 mA/tube for 1 h and 5 mA/tube for a further 2 h. Following electrophoresis gels were removed from the tubes and placed into test tubes and areas containing PK visually observed by incubation in the dark at room temperature for 30 min in a solution of 50 mM imidazole-HCl buffer (pH 7.0) containing 5 mM PEP, 2 mM ADP, 50 mM KCl, 5 mM MgCl₂, 0.02 mM fructose-1,6-bisphosphate, 2.0 mM NAD⁺, 5 mM D-glucose, 0.6 units/ml hexokinase, 0.6

units/ml glucose-6-phosphate dehydrogenase, and 0.5 mg/ml nitro blue tetrazolium followed by visualization of two enzyme bands by the addition of 0.5 mg/ml phenazine methosulfate. Replicate gels were stained as above, except no D-glucose was added. This allowed, in those gels stained with solutions containing D-glucose, identification of the faster anodally migrating band as adenylate kinase and the slower anodally migrating band as PK. Following staining for PK activity gels were frozen at -20°C and sliced into discs of 2 mm thickness. Each gel slice was placed into a glass scintillation vial and digested with 0.3 ml of 30% hydrogen peroxide at 50°C overnight. The vials were cooled, 6 ml of Aquasol II was added, and radioactivity determined in a Beckman scintillation counter.

Purification and Analysis of ^{32}P -labelled Pyruvate Kinase from Anoxic Whelks.

Carrier free [^{32}P] orthophosphate was injected directly into proboscis of five whelks (5 mCi/animal in 0.4 ml full strength sea water). These animals were held in aerated sea water at 15°C for 20 h and were then made anoxic by bubbling N_2 (g) through the water for a further 21 h at which point radular retractor muscle tissue was rapidly dissected, blotted, and frozen in liquid N_2 . Assay and purification of PK from this tissue and analysis by SDS electrophoresis on polyacrylamide slab gels was performed as in the previous chapter. Determination of ^{32}P distribution in the SDS gel

was performed as described above except that 4 mm sections of the frozen gel were digested with 0.4 ml 30% hydrogen peroxide.

A portion of the purified ^{32}P -labelled enzyme was dialyzed overnight against 1.5 l of 20 mM imidazole-HCl (pH 7.0) containing 10 mM 2-mercaptoethanol and 1.0 mM EDTA. Following dialysis the sample was hydrolyzed under vacuum for 3.5 h at 110 °C in the presence of 6 N HCl and 1 mg each of authentic O-phospho-L-threonine and O-phospho-L-serine. The hydrolysate was concentrated by evaporation and applied to Whatman 3MM paper. Electrophoresis was performed in a single dimension at pH 2.1 for 50 min at 1600 V in 8% acetic acid/2% formic acid. Cold carrier phosphoamino acids were visualized by staining with ninhydrin following autoradiography for 5 days using Kodirex X-ray film.

Results

In vitro Incubations of Purified Pyruvate Kinase from Red Muscle Tissue of Aerobic and Anoxic B. canaliculatum with Protein Kinase and Alkaline Phosphatase.

Purified PK from red muscle tissue of aerobic animals (RPK-AER) was incubated for up to 48 h at 30 °C with rabbit muscle peak I cyclic AMP-dependent protein kinase and for up to 24 h at 30 °C with 30 units of E. coli type III alkaline phosphatase. However, neither of the above treatments affected the enzyme, with no change in maximal reaction

velocity or affinity for PEP with respect to control incubation mixtures.

Similar in vitro incubations were conducted with purified PK from red muscle of anoxic animals (RPK-ANX). Although treatment with rabbit muscle protein kinase resulted in no kinetic difference from the control, incubation with alkaline phosphatase produced an activated enzyme whose PEP saturation curve and susceptibility to inhibition by alanine were very similar to RPK-AER (Fig. 16, Table 20). These results suggested that RPK-AER is a dephosphorylated and RPK-ANX a phosphorylated form of PK.

³²P Studies.

In order to further test our assumption that the inactivation of red muscle PK caused by anoxic stress results from phosphorylation reactions, in vivo labelling studies were performed using [³²P] orthophosphate.

Cellulose acetate and polyacrylamide gel electrophoretic analysis of dialyzed crude enzyme extracts prepared from radular retractor muscle tissue of aerobic and anoxic animals previously injected with 4 mCi of ³²P_i was initially conducted in order to ascertain whether or not, following electrophoresis, areas containing PK were associated with ³²P-activity. As seen in Fig. 17B and Fig. 18B, following analysis of the enzyme extract from anoxic animals both electrophoresis systems showed a major peak of ³²P-activity which was coincident with that segment which stained for PK

Fig. 16. Activation of RPK-ANX following incubations with 20 units of E. coli type III alkaline phosphatase. Incubations were conducted for 4 h at 37 °C in 50 mM Tris-HCl (pH 8.3) containing 10 mM MgCl₂, 25 mM KCl and 750 mM sucrose. Enzyme activity was measured at different PEP concentrations with untreated purified RPK-AER (■) and purified RPK-ANX incubated with (○) and without (●) alkaline phosphatase.

Table 20. Effect of incubation of purified RPK-ANX with and without alkaline phosphatase (E. coli, type III, 20 I.U.).

Sample	Control	Purified RPK-ANX	Alkaline Phosphatase Treated RPK-ANX
V_{max}^a (units/mg protein)	155 $\pm$ 20	368 $\pm$ 29	349 $\pm$ 25
PEP a (mM)	0.88 $\pm$ 0.03	0.067 $\pm$ 0.002	0.27 $\pm$ 0.01
$\eta_{H_2}^b$	2.5 $\pm$ 0.1	0.99 $\pm$ 0.10	1.25 $\pm$ 0.03
I_{50} Alanine b (mM)	0.15 $\pm$ 0.02	72 $\pm$ 7	88 $\pm$ 12

Results are means $\pm$ s.e.m. of duplicate determinations on two separate preparations of purified enzyme.

^a V_{max} and PEP $_{0.5}$'s determined at optimal assay conditions.

^b I_{50} 's for alanine determined at 1.0 mM PEP, 2.0 mM ADP, 50 mM KCl, 5 mM MgCl $_2$, 0.15 mM NADH, 50 mM imidazole-HCl buffer (pH 7.0) and excess dialyzed LDH.

activity. However, extracts analyzed from aerobic animals showed much lower amounts of ^{32}P -activity associated with the area which stained for PK (Fig. 17A and Fig. 18A). Furthermore, Fig. 17 (A and B) and Fig. 18 (A and B) suggest that the amount of macromolecular covalently bound (non-dialyzable) [^{32}P] phosphate in radular retractor muscle tissue of anoxic animals is somewhat higher than is the case for aerobic animals. This is probably a reflection of the fact that 5.7% versus 10.4% of total ^{32}P -activity associated with crude radular retractor muscle extracts from aerobic versus anoxic animals, respectively, remained following overnight dialysis. The radioactivity found in non-dialyzed radular retractor muscle cell free extracts from aerobic versus anoxic animals corresponded to about 1.2% versus 4.4%, respectively, of the total radioactivity injected into the animals. These data suggest that PK, as well as other proteins, may be phosphorylated in response to anoxic stress.

Purification and Analysis of ^{32}P -labelled Pyruvate Kinase.

Chromatography on Sepharose CL-6B.

The final step in the purification of RPK-ANX from anoxic animals previously injected with 5 mCi ^{32}P , was gel filtration chromatography on Sepharose CL-6B (see Chapter 6). As seen in Fig. 19 the elution profile for the enzyme exactly parallels the ^{32}P -activity profile, indicating probable covalent incorporation of phosphate into the

Fig. 17. A + B: Cellulose acetate electrophoretic analysis of PK in dialyzed crude enzyme extracts prepared from $^{32}\text{P}_i$ -injected radular retractor tissue of aerobic (Fig. 17-A) and anoxic (Fig. 17-B) animals. Segment which stained for PK is indicated by arrow; 50 cpm subtracted for background.

Fig. 18. A + B: Polyacrylamide gel electrophoretic analysis of PK in dialyzed crude extracts prepared from $^{32}\text{P}_i$ -injected radular retractor tissue of aerobic (Fig. 18-A) and anoxic (Fig. 18-B) animals. The amount of radioactivity in each 2 mm slice is presented relative to the migration of the tracking dye, bromophenol blue. Segment which stained for PK is indicated by arrow; 60 cpm subtracted for background.

Fig. 19. Chromatography of ^{32}P -labelled RPK-ANX on Sepharose CL-6B. The column (1.5 X 50 cm) was loaded with 17 units of enzyme in 5 ml buffer and run at 25 ml/h. Fractions (2.2 ml) were collected and assayed for PK activity (O) and counted for ^{32}P content ($\blacktriangle$).

enzyme. Based on a final specific activity of 180 units/mg the specific radioactivity of the final preparation was 3.55×10 cpm/mg. From the specific radioactivity of the $^{32}\text{P}_i$ used (at time of chromatography on Sepharose CL-6B S.A. = 6.5 $\mu\text{Ci}/\mu\text{mol}$) the molar amount of [^{32}P] phosphate bound to the enzyme was calculated to be 7.3 mol/mol enzyme. The calculation was based on a ^{32}P counting efficiency of 80% and the assumption that the native enzyme has a molecular weight of 233,000 daltons (see Appendix I).

SDS Electrophoresis.

Electrophoresis of purified ^{32}P -labelled RPK-ANX in the presence of 1% SDS followed by staining with Coomassie blue yielded a single protein band (Plate 4). This position is also identical with the peak of radioactivity found in the sliced gel by liquid scintillation counting (Fig. 20).

Effect of Incubation with Substrates or Alkaline Phosphatase.

Incubation of purified ^{32}P -labelled RPK-ANX in the presence of PEP, ADP, Mg^{2+} and K^+ did not affect the amount of radioactivity in the incubation mixture following overnight dialysis (Table 21). Therefore an exchange of the [^{32}P] phosphate on the protein with unlabelled phosphate did not occur during the catalytic reaction. Incubation of labelled PK in the presence of alkaline phosphatase led to an approximately 60% reduction in the amount of radioactivity following overnight dialysis (Table 21).

Plate 4. SDS-polyacrylamide gel electrophoresis of purified ^{32}P -labelled RPK-ANX. Protein staining was performed with Coomassie Blue.

Fig. 20. Distribution of ^{32}P -radioactivity in a SDS gel. The gel shown in Plate 4 was sliced into 4 mm thick sections which were digested with hydrogen peroxide and counted for ^{32}P content. The amount of radioactivity in each slice is presented relative to the migration of the tracking dye, bromophenol blue. 50 cpm was subtracted for background.

3 3

OF / DE

Table 21. Incubations of purified ^{32}P -labelled pyruvate kinase with substrates or alkaline phosphatase. Following incubation period respective mixtures (1 ml) were divided into equal portions. One portions was dialyzed overnight against 2 l of 50 mM imidazole-HCl buffer (pH 7.0). Dialyzed and undialyzed samples were added to 10 ml Aquasol II and counted for ^{32}P .

Incubation	Substrates ^a	Alkaline Phosphatase ^b
cpm Undialyzed	1143	1011
cpm Dialyzed (Protein Bound)	1090	394
% cpm Protein Bound	95%	39%

^a - Substrates: Incubations were done with 50 mM imidazole-HCl buffer (pH 7.0) containing 5 mM PEP, 2 mM ADP, 50 mM KCl, 5 mM MgCl_2 at 20°C for 40 min (100 cpm subtracted for background).

^b - Alkaline Phosphatase: Incubations were done with 50 mM Tris-HCl buffer (pH 8.4) containing 25 mM KCl, 10 mM MgCl_2 , 750 mM sucrose and 20 units E. coli type II alkaline phosphatase at 37°C for 6 h (60 cpm subtracted for background).

Tentative Identification of the Site of Phosphate on RPK-ANX.

Purified ^{32}P -labelled RPK-ANX was hydrolyzed in the presence of 6 N HCl and the products analyzed by high voltage electrophoresis at pH 2.1. The radioactive products, authentic O-phosphoserine and O-phosphothreonine migrated to the anode under these conditions. The radioactive material had the same electrophoretic velocity as phosphothreonine (Fig. 21). Furthermore, the area stained by ninhydrin, was of the same size and shape as the radioactive spot detected via autoradiography.

Discussion

The results presented in this chapter are consistent with the reversible covalent incorporation of phosphate into B. canaliculatum red muscle PK in response to 21 h of severe environmental anoxic stress. This is the first report which provides definite proof that PK from a muscle can be phosphorylated. Unlike mammalian L-type PK, M-type PK of mammals typically show non-allosteric properties and has never been demonstrated to undergo phospho-/dephosphorylation reactions in vivo (Munday et al., 1980).

Proof that the modification of whelk red muscle PK, brought about by anoxic stress, is due to the covalent incorporation of phosphate was provided by ^{32}P -labelling

Fig. 21. High voltage electrophoresis of an acid hydrolysate of ^{32}P -labelled RPK-ANX. Reference samples of commercial phosphoserine and phosphothreonine were added prior to acid hydrolysis and were also run in parallel. Ninhydrin staining followed autoradiography. The shade spot indicates radioactive area.

work reported in the present study. As the ^{32}P injected into the animals is diluted by the phosphate pool of the body, which is itself in continual exchange with the surrounding sea water, high initial radioactivities are necessary for detecting phosphate bound to the enzyme. This condition was met when about 1-2 mCi per g was injected into the radular retractor muscle of an animal. Maintenance of animals under aerobic conditions for one day following $^{32}\text{P}_i$ injections was required in order that $^{32}\text{P}_i$ could become incorporated into the gamma phosphate of intracellular ATP pools. Specificity of the labelling of radular retractor muscle PK following the subsequent 21 h of anoxic stress was confirmed in two ways: (a) upon elution of PK from the Sepharose CL-6B column used as the final purification step the peak of enzyme activity and radioactivity exactly coincided, and (b) [^{32}P] phosphate comigrated with the Coomassie blue staining protein following SDS-polyacrylamide gel electrophoresis of the final homogenous preparation (Fig. 19; Plate 4 and Fig. 20). Unlike its $^{32}\text{P}_i$ injected anoxic counterpart the aerobic (control) animal showed no peak of radioactivity associated with that segment of the cellulose acetate strip or polyacrylamide gel which stained for PK activity following electrophoresis of dialyzed crude extracts under non-denaturing conditions (Fig. 17 A and B, Fig. 18 A and B). This indicates that the incorporation of [^{32}P] phosphate into PK purified from radular retractor muscle of anoxic animals is specifically due to the

imposition of anoxic stress, and is not due to some artifact generated by experimental procedures. That this anoxia induced phosphorylation of whelk red muscle PK is a reversible process is suggested by the fact that in vitro treatments of unlabelled homogenous RPK-ANX with E. coli alkaline phosphatase consistently results in an enzyme with several kinetic properties which are similar to those seen for purified RPK-AER (Table 20, Fig. 1). Identical treatment of purified ^{32}P -labelled RPK-ANX with alkaline phosphatase led to an approximate 60% reduction in the amount of protein bound [^{32}P] phosphate (Table 21).

Comparison of the electrophoretic mobilities of products derived from acid hydrolysis of purified ^{32}P -labelled RPK-ANX with authentic substances suggests the presence of an O-phosphothreonine residue in the protein (Fig. 21). However, one cannot rule out possible incorporation of phosphate in phosphotyrosine as under the electrophoretic conditions used phosphothreonine may overlap with phosphotyrosine (Manai and Cozzone, 1982). Unfortunately, sufficient quantities of the purified ^{32}P -labelled enzyme were not available for further characterization. That this threonine residue is not an intermediate in the catalytic reaction of the enzyme was shown by the lack of phosphate exchange in the presence of all substrates (Table 21). The role of the phosphate amino acid residues present on RPK-ANX may therefore be in an interconversion mechanism. Presumably, conformational changes which accompany

phosphorylation result in the dramatic kinetic differences between RPK-AER and RPK-ANX reported in the previous chapter. To date, all studies concerning covalent modification of mammalian L-type PK report specific phosphorylation of serine residues (Engstrom, 1978) and, hence, whelk RPK-ANX may be the first PK to be phosphorylated on an amino acid residue other than serine. This would explain the inability of rabbit muscle protein kinase to alter the kinetic properties of purified whelk RPK-AER following in vitro incubations under a variety of conditions. The molar incorporation of phosphate has not been definitely determined, but on the basis of several assumptions it is possible to calculate that there are a total of 7.3 mol ^{32}P /mol RPK-ANX. If one further assumes equal distribution of eight phosphate groups over the four subunits, this result suggests that there are at least two different phosphorylation sites per subunit present in the enzyme. This differs from the phosphorylated form of mammalian L-type PK which has been shown to incorporate a maximum of one phosphate per subunit (Blair et al., 1982).

Preliminary studies on B. canaliculatum PK have established the occurrence of three tissue specific PK isozymes, specific for red muscle (ventricle and radular retractor muscle), white muscle (foot and mantle) and soft tissues (gill, kidney and hepatopancreas) (Chapter 5). The effect of 21 h of anoxic stress on several properties of all three isozymes was shown to be similar; in each case PK

activity at low concentrations of PEP was greatly diminished, isozyme pI's were decreased uniformly by about 0.4 pH unit and the amount of anodal migration during electrophoresis on cellulose acetate strips at pH 7.2 was increased to the same extent. Hence, by analogy, PK's from all tissues of B. canaliculatum are probably reversibly phosphorylated in response to long term anoxic stress.

Results presented in the present study also support the contention that protein phospho-/dephosphorylation may be an important regulatory mechanism not just for the control of PK in response to oxygen availability, but in the quick and efficient control of other aspects of metabolism during the aerobic to anoxic transistion. Famme and co-workers (1981) studied the effect of oxygen on the aerobic-anaerobic metabolism of M. edulis and have demonstrated that anaerobic metabolic rate is only 5 to 10% of metabolic rate in normoxia. A similar metabolic response to anoxia has been reported for the bivalve Modiolus demissus (Pamatmat, 1979). These findings imply that key regulatory enzymes responsible for metabolic control are completely or partially inactivated during anaerobiosis. Support for this concept is provided by recent work in this lab which has demonstrated that B. canaliculatum foot muscle phosphofructokinase is partially inactivated, probably via covalent modification, during long term anoxic stress (K.B. Storey, manuscript in preparation). In the present study it was found that the total amount of macromolecularly bound (non-dialyzable)

[^{32}P] phosphate was significantly greater for a cell free extract prepared from radular retractor muscle tissue of a $^{32}\text{P}_i$ -injected anoxic animal than for the corresponding extract prepared from a $^{32}\text{P}_i$ -injected aerobic animal. This is paralleled by the radioactivity profiles shown in Fig. 17 (A and B) and Fig. 18 (A and B) in which it can be seen that following electrophoresis of the crude extract prepared from a $^{32}\text{P}_i$ -injected anoxic, but not aerobic, animal many areas of the cellulose acetate strip and polyacrylamide gel show large peaks of ^{32}P -activity. It is thus tempting to speculate that anoxia tolerant molluscs contain a whole family of phospho-/dephosphoenzymes whose activities can be tightly regulated in a coordinated, rapid and relatively simple fashion in response to an intracellular messenger which becomes prevalent during the aerobic to anoxic transition. During their work with M. edulis adductor muscle Holwerda and co-workers (1981) demonstrated that following 2 h of anoxic stress there is a significant increase in intracellular concentrations of cyclic AMP which parallels the concomitant reduction in PK activity at low PEP concentrations. Obviously the role(s) and mechanism(s) of potential cyclic AMP-dependent protein phosphorylation in the control of marine invertebrate anaerobic metabolism remains to be elucidated.

CHAPTER VIII: Summary and Conclusions.

During periods of limiting oxygen availability the survival of any organism is dependent upon the operation of a self-sustaining life-support system. At the cellular level the basic design of such life-support systems must include sufficient fermentable substrate (glycogen), as well as the necessary metabolic equipment (anaerobic glycolysis) needed to mobilize this substrate and produce ATP by substrate level phosphorylations. At the same time provisions must be made for redox maintenance in cytosol and mitochondria. These primary requirements for living without oxygen are universal, but for the sake of improving anoxia tolerance they have been readily modified by the most capable animal anaerobes. At the biochemical level such adaptive modifications have occurred in many species of the phylum Mollusca. That a clear-cut metabolic reorganization does occur during molluscan environmental anaerobiosis was initially demonstrated by the finding of multiple anaerobic end products. This metabolic reorganization invariably provides an increased ATP yield and greater diversity of coupling intermediates necessary to sustain anabolic function. The 'exploitative' strategy which is commonly employed by anoxia tolerant molluscs during long term anaerobiosis also involves a drastic reduction of overall metabolic rate so that metabolic fuels can be conserved and survival times extended.

At the heart of all such metabolic reorganizations are the catalytic units which make it all possible - enzymes.

Not only are the kinds of enzymes, and hence available metabolic pathways, regulated, but amounts and kinetic properties are also precisely controlled. It is for this reason that many comparative biochemistry studies, including those of the present work, concern the examination of enzyme catalytic and regulatory properties. The studies of this thesis on gastropod ADH and PK provide good examples of enzyme adaptation to fluctuation in oxygen availability.

A fundamental characteristic of the metabolic restructuring which occurs during molluscan long term environmental anaerobiosis is the shifting of glycolytic carbon away from pyruvate and towards succinate. Regulation of the so-called 'PEP branchpoint' has been examined in some detail over the past decade and until recently a drop in pH and a gradual increase in alanine concentration were felt to control the direction of PEP disposal via inhibition of PK and activation of PEP.CK. However, results of this thesis strongly suggest that the oxygen dependent shift in the direction of carbon flux during molluscan long term anoxic stress is the result of the phosphorylation state of PK. The metabolic maps shown in Fig. 22 integrate most of the current information concerning the control of the PEP branchpoint and the function of ADH in B. canaliculatum red muscle tissue during exposure to and recovery from long term environmental anoxia.

During the early stages of anoxic stress aspartate reserves are mobilized to produce succinate and glycogen is

Fig. 22. A - C: Summary of the control, of the phosphoenolpyruvate branchpoint and role of alanine dehydrogenase, in B. canaliculatum red muscle tissue during exposure to (Fig. 22 A + B) and recovery from (Fig. 22-C) long term environmental anoxic stress.

(A) EARLY ANOXIA

(B) LONG TERM ANOXIA

(C) RECOVERY

converted to alanine with redox balance ensured by the 1:1 coupling of glyceraldehyde-3-phosphate dehydrogenase and cytosolic malate dehydrogenase (Fig. 22-A). During this period PK is in its fully active, unphosphorylated state, and ADH activity is probably limited by the availability of NADH. After 4 to 8 h of anoxic stress alanine concentrations stabilize, aspartate utilization rates greatly decline, but glycogen catabolism and succinate production continue unabated (Fig. 22-B). During this period PK is probably phosphorylated. Consequent kinetic effects on PK include a ten fold lower affinity for PEP, a decreased susceptibility to activation by Fru-1,6-P₂, and greatly increased sensitivity to inhibition by alanine. These kinetic effects are pH independent over the range likely occurring in vivo and should ensure that PK is effectively 'shut off' thereby redirecting glycolytic carbon towards succinate via PEP.CK. Reversible covalent control of PK provides a fast, and relatively simple method of acquiring a completely 'different' enzyme whose properties are well adapted to the needs of the cell at that time.

During the initial stages of recovery from long term anoxic stress PK is reconverted to its dephosphorylated form, which with its high affinity for PEP, increased susceptibility to activation by Fru-1,6-P₂, and insensitivity to alanine, would then be fully active (Fig. 22-C). This should allow for redirection of carbon towards pyruvate and facilitates the increased glycolytic rate

characteristic of metabolism during the first few hours of recovery from anoxic stress.

The kinetic properties of B. canaliculatum and L. littorea muscle ADH suggest a probable function during the recovery period when aerobic respiration must be supplemented by anaerobic glycolysis. ADH from both sources showed a dependence of the apparent K_m for pyruvate and alanine on co-substrate (alanine or pyruvate) concentration, rising levels of either the amino or keto acid co-substrate decreasing the apparent K_m for keto or amino acid, respectively. This property would favor alanopine production during recovery from anoxia owing to the redirection of carbon towards pyruvate and the previous accumulation of alanine as an anaerobic end product. A second important kinetic property of these gastropod muscle ADH's is that the apparent K_m 's for both co-substrates decrease with decreasing pH, an effect which could also promote alanopine synthesis during the early stages of recovery when pH is low owing to the previous accumulation of acidic anaerobic end products.

Results of this thesis also point to another strategy which organisms can utilize during oxygen deprivation. This strategy depends upon the fact that most organs and tissues are not totally closed systems during anoxic stress. Through the use of the blood they remain in close communication with one another. The finding of tissue specific isozymes of ADH and PK in the whelk implies that

tissue specific metabolism may occur in gastropods. Presumably, survival of the whole animals is partially dependent upon the establishment of key metabolic interactions or exchanges between individual, specialized, tissues. Achieving metabolic integration of different organs and tissues in anoxia requires that the synthesis and maintenance of key metabolic enzymes (i.e. ADH and PK) be closely coordinated. The tissue-specific metabolic functions of muscle and non-muscle tissues, as well as the biochemical signals whereby metabolic coordination between tissues can occur, remains a fruitful area for future research.

APPENDIX

Calculation of molar extent of ^{32}P incorporation in RPK-ANX

[$^{32}\text{P}_i$]: Specific activity (S.A.) on 9/12/83

$$= 6.5 \mu\text{Ci}/\mu\text{mol } ^{32}\text{P}_i.$$

$$6.5 \mu\text{Ci}/\mu\text{mol} = (2.2 \times 10^6) \times 6.5 \text{ dpm}/\mu\text{mol } ^{32}\text{P}_i$$

$$= 1.43 \times 10^7 \text{ dpm}/\mu\text{mol } ^{32}\text{P}_i.$$

^{32}P counting efficiency = 80%, therefore

$$1.43 \times 10^7 \text{ dpm}/\mu\text{mol}$$

$$= (1.43 \times 10^7) \times 0.8 \text{ cpm}/\mu\text{mol } ^{32}\text{P}_i$$

$$= 1.14 \times 10^7 \text{ cpm}/\mu\text{mol } ^{32}\text{P}_i.$$

(I) Assume S.A. of purified RPK-ANX = 180 units/mg protein:

On 9/12/83 purified [^{32}P]-labelled RPK-ANX

$$= 3.55 \times 10^5 \text{ cpm/mg},$$

$$\text{and } 3.55 \times 10^5 \text{ cpm/mg}$$

$$= (3.55 \times 10^5 / 1.14 \times 10^7) \mu\text{mol } ^{32}\text{P}/\text{mg}$$

$$= 3.11 \times 10^{-2} \mu\text{mol } ^{32}\text{P}/\text{mg}.$$

(II) Assume Mol. Wt. of RPK-ANX = 2.33×10^5 daltons:

$$= 2.33 \times 10^8 \text{ mg/mol}$$

$$= 2.33 \times 10^2 \text{ mg}/\mu\text{mol},$$

and therefore, 1 mg of RPK-ANX

$$= 4.29 \times 10^{-3} \mu\text{mol enzyme}.$$

Because we have $3.11 \times 10^{-2} \mu\text{mol } ^{32}\text{P}/\text{mg}$ RPK-ANX,

therefore extent of ^{32}P incorporation

$$= (3.11 \times 10^{-2} \mu\text{mol } ^{32}\text{P} / 4.29 \times 10^{-3} \mu\text{mol enzyme}),$$

$$= 7.3 \mu\text{mol } ^{32}\text{P}/\mu\text{mol RPK-ANX}.$$

(III) Assume 4 subunits/enzyme, and

(IV) Equal distribution of ^{32}P 's between all 4 subunits:

therefore, molar extent of ^{32}P incorporation into purified

$$^{32}\text{P-labelled RPK-ANX} = 1.8 \text{ mol } ^{32}\text{P}/\text{mol RPK-ANX subunit}.$$

REFERENCES

References

Abderhalden, E. and Haase, E. 1931. Gewinnung von iminodicarbonsauren aus aminosauern und halogenfettsauren. Z. Physiol. Chem. 202: 49-55.

Andersen, H. 1966. Physiological adaptations in diving vertebrates. Physiol. Rev. 46: 212-243.

Baginski, R. and Pierce, S. 1977. The time course of intracellular free amino acid accumulation during high salinity adaptation with anaerobic metabolism in the ribbed mussel, Modiolus demissus. Comp. Biochem. Physiol. 57A: 407-442.

Baginski, R. and Pierce, S. 1978. A comparison of amino acid accumulation during high salinity adaptation with anaerobic metabolism in the ribbed mussel, Modiolus demissus. J. Exp. Zool. 203: 419-428.

Baldwin, J. and England, W. 1980. A comparison of anaerobic energy metabolism in mantle and tentacle muscle of the blue-ringed octopus, Hapalochlaena maculosa during swimming. Aust. J. Zool. 28: 407-412.

Baldwin, J. and England, W. 1982. The properties and functions of alanopine dehydrogenase and octopine dehydrogenase from the pedal retractor muscle of Strombidae (class Gastropoda). Pac. Sci. 36: 381-344.

Barnes, R. 1974. In Invertebrate Zoology. Third Edition. W.B. Saunders Co., Philadelphia. Chapter 11.

Barrett, J. and Butterworth, P. 1981. A novel amino acid linked dehydrogenase in the sponge Halochondria panicea (Pallas). Comp. Biochem. Physiol. 70B: 141-146.

Blair, J., Cimbala, M. and James, M. 1982. Hepatic pyruvate kinase. Quantitative measurements of phosphorylation in vitro and in the isolated rat hepatocyte. J. Biol. Chem. 257: 7595-7602.

Blakesley, R. and Boezi, J. 1977. A new staining technique for proteins in polyacrylamide gels using Coomassie Brilliant Blue G250. Analyt. Biochem. 82: 580-582.

Blazka, P. 1958. The anaerobic metabolism of fish. Physiol. Zool. 31: 117-128.

Bradford, M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analyt. Biochem. 72: 248-254.

Brand, T. von. 1946. In Anaerobiosis in Invertebrates. B. Luyet, Ed. Biodynamica, Missouri. pp. 87-94.

Chance, B., Holmes, W., Higgins, J. and Connelly, C. 1958. Localization of interaction sites in multicomponent transfer systems: Theorems derived from analogues. Nature 182: 1190-1193.

Chih, C. and Ellington, W. 1983. Energy metabolism during contractile activity and environmental hypoxia in the phasic adductor muscle of the bay scallop Argopecten irradians concentricus. Physiol. Zool. 56: 623-631.

Clark, V. and Miller, A. 1973. Anaerobic metabolism in the fresh-water turtle (Pseudemys scripta elegans). Comp. Biochem. Physiol. 44A: 55-62.

Cornish-Bowden, A. 1979. In Fundamentals of Enzyme Kinetics. Butterworths, London. pp. 109-116.

Cottam, G., Hollenburg, P. and Coon, M. 1969. Subunit structure of rabbit muscle pyruvate kinase. J. Biol. Chem. 244: 1481-1486.

Collicutt, J. and Hochachka, P. 1977. The anaerobic oyster heart: Coupling of glucose and aspartate fermentation. J. Comp. Physiol. 115: 147-157.

Dando, P. 1981. Strombine (N-(carboxymethyl)-D-alanine) dehydrogenase and alanopine (meso-N-(1-carboxyethyl)-alanine) dehydrogenase from the mussel Mytilus edulis L. Biochem. Soc. Trans. 9: 297-298.

Dando, P., Storey, K., Hochachka, P. and Storey, J. 1981. Multiple dehydrogenases in marine molluscs: electrophoretic analysis of alanopine dehydrogenase, strombine dehydrogenase, octopine dehydrogenase and lactate dehydrogenase. Mar. Biol. Lett. 2: 249-259.

Davis, B. 1964. Disc electrophoresis-II. Method and Application to human serum proteins. Ann. N.Y. Acad. Sci. 121: 404-427.

Dawson, D. Goodfriend, T. and Kaplan, N. 1964. Lactic dehydrogenases: Functions of the two types. Science, N.Y. 143: 929-933.

Ebberink, R. 1982. Control of adductor muscle phosphofructokinase activity in the sea mussel Mytilus edulis during anaerobiosis. Mol. Physiol. 2: 345-355.

Ebberink, R. 1983. Regulation of anaerobic carbohydrate degradation in the sea mussel Mytilus edulis L. Ph.D. Thesis. Univ. of Utrecht, Utrecht.

Ebberink, R., Zurburg, W. and Zandee, D. 1979. The energy demand of the posterior adductor muscle of Mytilus edulis in catch during exposure to air. Mar. Biol. Lett. 1: 23-31.

Ebberink, R. and de Zwaan, A. 1980. Control of glycolysis in the posterior adductor muscle of Mytilus edulis in catch during exposure to air. J. Comp. Physiol. 137: 165-172.

Eberlee, J., Storey, J. and Storey, K. (1983) Anaerobiosis, recovery from anoxia, and the role of strombine and alanopine in the oyster Crassostrea virginica, Can. J. Zool. 61: 2682-2687.

Eberlee, J. and Storey, K. 1984. Organ specific changes in metabolism during anaerobiosis in the whelk, Busycotypus canaliculatum. Manuscript in preparation.

Eichner, R. and Kaplan, N. 1977. Catalytic properties of lactate dehydrogenase in Homarus americanus. Arch. Biochem. Biophys. 181: 501-507.

Ekman, P., Dahlqvist, E., Humble, E. and Engstrom, L. 1976. Comparative kinetic studies on the L-type pyruvate kinase from rat liver and the enzyme phosphorylated by cyclic 3',5'-AMP-stimulated protein kinase. Biochim. Biophys. Acta. 429: 374-382.

Ellington, W. 1979. Evidence for a broadly-specific, amino acid requiring dehydrogenase at the pyruvate branchpoint in sea anemones. J. Exp. Zool. 209: 151-159.

Ellington, W. 1981. Energy metabolism during hypoxia in the isolated, perfused ventricle of the whelk, Busycon contrarium Conrad. J. Comp. Physiol. 142: 457-464.

Ellington, W. 1982. Metabolism at the pyruvate branch point in the radula retractor muscle of the whelk, Busycon contrarium. Can. J. Zool. 60: 2973-2977.

Ellington, W. 1983a. Phosphorus nuclear magnetic resonance studies of energy metabolism in molluscan tissues: Effect of anoxia and ischemia on the intracellular pH and high energy phosphates in the ventricle of the whelk, Busycon contrarium. J. Comp. Physiol. 153: 159-166.

Ellington, W. 1983b. The recovery from anaerobic metabolism in invertebrates. J. Exp. Zool. 228: 431-444.

Everse, J., Barnett, R., Thorne, C. and Kaplan, N. 1971. The formation of ternary complexes by diphosphopyridine nucleotide-dependent dehydrogenases. Archs. Biochem. Biophys. 143: 444-460.

Everse, J. and Kaplan, N. 1973. Lactate dehydrogenase: Structure and function. IN Advances in Enzymology. A. Meister, Ed. Interscience, N.Y. Vol. 37: pp. 61-148.

Engström, L. 1978. The regulation of liver pyruvate kinase by phosphorylation-dephosphorylation. IN Current Topics in Cellular Regulation. B. Horecker and E. Stadtman, Eds. Academic Press, N.Y. Vol. 13: pp. 29-51.

Famme, P., Knudsen, J. and Hansen, E. 1981. The effect of oxygen on the aerobic-anaerobic metabolism of the marine bivalve, Mytilus edulis L. Mar. Biol. Lett. 2: 345-351.

Famme, P. and Knudsen, J. 1983. Transitory activation of metabolism, carbon dioxide production and release of dissolved organic carbon by the mussel Mytilus edulis L., following periods of self-induced anaerobiosis. Mar. Biol. Lett. 4: 183-192.

Fields, J. 1976. A dehydrogenase requiring alanine and pyruvate as substrates from oyster adductor muscle. Fed. Proc. 35: 1687.

Fields, J. 1977. Anaerobic metabolism in the cockle Clinocardium nuttali. Am. Zool. 17: 943.

Fields, J. 1983. Alternatives to lactic acid: Possible advantages. J. Exp. Zool. 228: 445-457.

Fields, J., Baldwin, J. and Hochachka, P. 1976. On the role of octopine dehydrogenase in cephalopod mantle muscle metabolism. Can. J. Zool. 54: 871-878.

Fields, J. and Hochachka, P. 1981. Purification and properties of alanopine dehydrogenase from the adductor muscle of the oyster, Crassostrea gigas. Eur. J. Biochem. 114: 615-621.

Gade, G. 1980. The energy metabolism of the foot muscle of the jumping cockle Cardium tuberculatum: sustained anoxia versus muscular activity. Comp. Physiol. 137: 177-182.

Gade, G. 1983. Energy metabolism of arthropods and mollusks during environmental and functional anaerobiosis. J. Exp. Zool. 228: 415-429.

Gade, G. and Ellington, W. 1983. The anaerobic molluscan heart: Adaptation to environmental anoxia. Comparison with energy metabolism in vertebrate hearts. Comp. Biochem. Physiol. 76A: 615-620.

Gill, P. 1978. Non-genetic variation in isoenzymes of lactate dehydrogenase of Cepaea nemoralis. Comp. Biochem. Physiol. 59B: 271-276.

Grieshaber, M. and Gade, G. 1976. The biological role of octopine in the squid, Loligo vulgaris. J. Comp. Physiol. 108: 225-232.

Guderley, H., Storey, K., Fields, J. and Hochachka, P. 1976. Catalytic and regulatory properties of pyruvate kinase isozymes from octopus mantle muscle and liver. Can. J. Zool. 54: 863-870.

Guderley, H., Fields, J., Cardenas, J. and Hochachka, P. 1978. Pyruvate kinase isozymes from liver and kidney of Arapaima gigas. Can. J. Zool. 56: 852-859.

Guderley, H. and Cardenas, J. 1979. Developmental changes in pyruvate kinase isozymes of coho salmon. J. Exp. Zool. 208: 1-12.

Hammen, C. 1969. Metabolism of the oyster, Crassostrea virginica. Am. Zool. 9: 309-318.

Hammen, C. 1980. Total energy metabolism of marine bivalve molluscs in anaerobic and aerobic states. Comp. Biochem. Physiol. 67A: 617-621.

Hammen, C. 1983. Direct calorimetry of marine invertebrates entering anoxic states. J. Exp. Zool. 228: 397-403.

Hochachka, P. 1980. In Living without oxygen. Harvard Univ. Press, Cambridge, Mass. Chapters 1, 3 and 5.

Hochachka, P. and Mustafa, T. 1972. Invertebrate facultative anaerobiosis. Science 178: 1056-1060.

Hochachka, P. and Somero, G. 1973. In Strategies of Biochemical Adaptation. W.B. Saunders, Toronto. p. 32.

Holwerda, D., de Zwaan, A. and van Marrewijk, W. 1973. The regulatory role of pyruvate kinase in the sea mussel Mytilus edulis L., Neth. J. Zool., 23: 225-228.

Holwerda, D. and de Zwaan, A. 1973. Kinetic and molecular characteristics of allosteric pyruvate kinase from muscle tissue of the sea mussel Mytilus edulis L. Biochim. Biophys. Acta. 309: 296-306.

Holwerda, D. and de Zwaan, A. 1979. Fumarate reductase of Mytilus edulis L. Mar. Biol. Lett. 1: 33-40.

Holwerda, D. and de Zwaan, A. 1980. On the role of fumarate reductase in anaerobic carbohydrate catabolism of Mytilus edulis L. Comp. Biochem. Physiol. 67B: 447-453.

Holwerda, D., Kruitwagen, E. and De Bont, A. 1981. Regulation of pyruvate kinase and phosphoenolpyruvate carboxykinase activity during anaerobiosis in Mytilus edulis L. Mol. Biol. 1: 165-171.

Holwerda, D., Veenhof, P., van Heugten, H. and Zandee, D. 1983. Regulation of mussel pyruvate kinase during anaerobiosis and in temperature acclimation by covalent modification. Mol. Physiol. 3: 225-234.

Holwerda, D., Veenhof, P. and de Zwaan, A. 1984. Physiological and biochemical investigations of the ecological relevance of anaerobiosis in bivalves. I. The changes in activity of mussel adductor muscle and mantle pyruvate kinase during aerial exposure and reimmersion. Mar. Biol. Lett. (in press).

Hoyaux, J., Gilles, R. and Jeuniaux, C. 1976. Osmoregulation in molluscs of the intertidal zone. Comp. Biochem. Physiol. 53A: 361-365.

Job, D., Cochet, C., Dhien, A. and Chambaz, E. 1978. A rapid method for screening inhibitor effects: Determination of I_{50} and its standard deviation. Analyt. Biochem. 84: 68-77.

Krebs, H. 1965. Renal gluconeogenesis. Adv. Enz. Regul. 1: 385-398.

Koorman, R. and Grieshaber, M. 1980. Investigations on the energy metabolism and on octopine formation of the common whelk, Buccinum undatum L., during escape and recovery. Comp. Biochem. Physiol. 65B: 543-547.

Korycan, S. and Storey, K. 1983. Organ-specific metabolism during anoxia and recovery from anoxia in the cherrystone clam, Mercenaria mercenaria. Can. J. Zool. 61: 2674-2681.

Laemmli, U. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-685.

Leon, O., Moran, A. and Gonzalez, R. 1982. Purification and characterization of pyruvate kinase from muscle of the sea mollusc Concholepas concholepas. Comp. Biochem. Physiol. 72B: 65-69.

Livingstone, D. and de Zwaan, A. 1983. In The Mollusca. P. Hochachka, Ed. Academic Press, N.Y. Vol. 1: Chapter 5.

Livingstone, D., de Zwaan, A., Leopold, M. and Marteiijn, E. 1983. Studies on the phylogenetic distribution of pyruvate oxidoreductases. Biochem. Syst. Ecol. 11: 415-425.

Ljungstrom, O., Hjelmqvist, G. and Engstrom, L. 1974. Phosphorylation of purified rat liver pyruvate kinase by cyclic 3'-5'-AMP- stimulated protein kinase. *Biochim. Biophys. Acta.* 422: 98-108.

Long, G. and Kaplan, N. 1973. Lactate dehydrogenase from the horseshoe crab, *Limulus polyhemus*, and the seaworm, *Nereis virens*. *Arch. Biochem. Biophys.* 154: 696-710.

Manai, M. and Cozzone, J. 1982. Two-dimensional separation of phosphoamino acids from nucleoside monophosphates. *Analyt. Biochem.* 124: 12-18.

Mangum, C. and van Winkle, W. 1973. Responses of aquatic invertebrates to declining oxygen conditions. *Am. Zool.* 13: 529-541.

Mangum, C. and Polites, G.-1980. Oxygen uptake and transport in the prosobranch mollusc *Busycon canaliculatum*: I. Gas exchange and the response to hypoxia. *Biol. Bull. Woods Hole* 158: 77-90.

Mansour, T. 1972. Phosphofructokinase. *Curr. Top. Cell. Reg.* 5: 1-44.

Marie, J., Buc, H., Simon, M. and Kahn, A. 1980. Phosphorylation of human erythrocyte pyruvate kinase by soluble cyclic-AMP-dependent protein kinases. Comparison with human L-type enzyme. *Eur. J. Biochem.* 108: 251-260.

McPherson, A. 1970. Interaction of lactate dehydrogenase with its coenzyme, nicotinamide-adenine dinucleotide. *J. Mol. Biol.* 51: 39-46.

Morton, J. 1979. *In* Molluscs, Hutchinson and Co. Ltd., London. Chapter 1.

Munday, K. Giles, I. and Poat, P. 1980. Review of the comparative biochemistry of pyruvate kinase. *Comp. Biochem. Physiol.* 67B: 403-411.

Mustafa, T. and Hochachka, P. 1971. Catalytic and regulatory properties of pyruvate kinases in tissues of a marine bivalve. *J. Biol. Chem.* 246: 3196-3203.

Mustafa, T. and Hochachka, P. 1973a. Enzymes in facultative anaerobiosis of molluscs - II. Basic catalytic properties of phosphoenolpyruvate carboxykinase in oyster adductor muscle. *Comp. Biochem. Physiol.* 45B: 639-656.

Mustafa, T. and Hochachka, P. 1973b. Enzymes in facultative anaerobiosis of molluscs - II. Phosphoenolpyruvate carboxykinase and its role in aerobic-anoxic transition. *Comp. Biochem. Physiol.* 45B: 657-667.

Newsholme, E. and Start, C. 1973. In Regulation in Metabolism. Wiley, N.Y. pp. 148-2.

Nicchitta, C. and Ellington, W. 1984. Partial purification and characterization of a strombine dehydrogenase from the adductor muscle of the mussel Modiolus squamosus. Comp. Biochem. Physiol. 77B: 233-236.

Olomucki, A., Huc, C., Lefebure, F. and van Thoai, N. 1972. Octopine dehydrogenase. Evidence for a single chain structure. Eur. J. Biochem. 28: 261-268.

Pamatmat, M. 1979. Anaerobic heat production of bivalves (Polymesoda caroliniana and Modiolus demissus) in relation to temperature, body size, and duration of anoxia. Mar. Biol. 53: 223-229.

Pierce, M. 1950. In Selected Invertebrate Types. F. Brown, Ed. John Wiley and Sons, Inc., N.Y. pp.336-344.

Polites, G. and Mangum, C. 1980. Oxygen uptake and transport in the prosobranch mollusc Busycon canaliculatum: II. Influence of acclimation salinity and temperature. Biol. Bull. Woods Hole 158: 118-128.

Regnouf, F. and van Thoai, N. 1970. Octopine and lactate dehydrogenase in mollusc muscles. Comp. Biochem. Physiol. 32: 411-416.

Schloen, L., Bamburg, J. and Sallach, H. 1969. Isozymes of pyruvate kinase in tissues and eggs of Rana pipiens. Biochem. Biophys. Res. Commun. 36: 823-829.

Scrutton, C. and Utter, M. 1968. The regulation of gluconeogenesis in animal tissues. Ann. Rev. Biochem. 37: 249-302.

Sibenaller, J. 1979. Regulation of pyruvate kinase in Mytilus edulis by phosphorylation-dephosphorylation. Mar. Biol. Lett. 1: 105-110.

Simon, L., Robin, E., Elsner, R., van Kessel, A. and Theodore, J. 1974. A biochemical basis for differences in maximal diving time in aquatic mammals. Comp. Biochem. Physiol. 47B: 209-215.

Stokes, T. and Awapara, J. 1968. Alanine and succinate as end products of glucose degradation in the clam Rangia cuneata. Comp. Biochem. Physiol. 25B: 883-892.

Storey, K. 1977. Tissue specific isozymes of octopine dehydrogenase in the cuttlefish, Sepia officinalis. The roles of octopine dehydrogenase and lactate dehydrogenase in Sepia. J. Comp. Physiol. 115: 159-169.

Storey, K. 1983. Tissue specific alanopine dehydrogenase and strombine dehydrogenase from the sea mouse, Aphrodite aculeata (Polychaeta). J. Exp. Zool. 225: 369-378.

Storey, K. and Storey, J. 1979a. Kinetic characterization of tissue specific isozymes of octopine dehydrogenase from mantle muscle and brain of Sepia officinalis. Eur. J. Biochem. 93: 545-552.

Storey, K. and Storey, J. 1979b. Octopine metabolism in the cuttlefish, Sepia officinalis: Octopine production by muscle and its role as an aerobic substrate for non-muscular tissues. J. Comp. Physiol. 131: 311-319.

Storey, K., Miller, D., Plaxton, W. and Storey, J. 1982. Gas-liquid chromatography and enzymatic determination of alanopine and strombine in tissues of marine invertebrates. Analyt. Biochem. 125: 50-58.

Storey, K. and Storey, J. 1983. In The Mollusca. P. Hochachka, Ed. Academic Press, N.Y. Vol. 1: Chapter 3.

Susor, W. and Rutter, W. 1971. A method for the detection of pyruvate kinase, aldolase, and other pyridine nucleotide linked enzyme activities after electrophoresis. Analyt. Biochem. 43: 147-155.

Switzer, R., Merrill, C. and Shifrin, S. 1979. A highly sensitive stain for detecting proteins and peptides in polyacrylamide gels. Analyt. Biochem. 98: 231-237.

Thoai, N. van, Huc, C., Pho, D. and Olomucki, A. 1969. Octopine dehydrogenase. Purification et propriétés catalytiques. Biochim. Biophys. Acta. 191: 46-57.

Titanji, V., Zetterqvist, O. and Engstrom, L. 1976. Regulation in vitro of rat liver pyruvate kinase by phosphorylation-dephosphorylation reactions, catalyzed by cyclic-AMP dependent protein kinases and a histone phosphatase. Biochim. Biophys. Acta. 422: 98-108.

Vesterberg, O. 1971. Isoelectrofocusing of proteins. In Methods in Enzymology. S. Colowick and N. Kaplan, Eds. Academic Press, N.Y. pp. 389-412.

Walsh, P., Booth, B. and McDonald, D. 1983. The effect of anoxia on intracellular pH in the sea mussel, Mytilus edulis L. Am. Zool. 23: 939.

Weiss-Fogh, T. 1968. In Insects and Physiology. J. Beamont and J. Treherme, Eds. American Elsevier Publ. Co., N.Y. pp. 143-159.

Wieser, W. 1980. Metabolic end products in three species of marine gastropods. J. Mar. Biol. Ass. U.K. 60: 175-180.

Wijsman, T. 1975. pH fluctuations in Mytilus edulis L. in relation to shell movements under aerobic and anoxic conditions. In Proc. 9th Eur. Mar. Biol. Symp., H. Barnes, Ed. Aberdeen Press, Aberdeen. pp. 139-149.

Zammit, V. and Newsholme, E. 1978. Properties of pyruvate kinase and phosphoenolpyruvate carboxykinase in relation to direction and regulation of phosphoenolpyruvate metabolism in muscles of the frog and marine invertebrates. Biochem. J. 174: 979-987.

Zebe, E., Salge, C., Wiemann, C. and Wilps, H. 1981. The energy metabolism of the leech Hirudo medicinalis in anoxia and muscular work. J. Exp. Zool. 218: 157-163.

Zurburg, W. 1981. Environmental influences on the energy metabolism in different organs of Mytilus edulis L. Ph.D. Thesis. Univ. of Utrecht, Utrecht.

Zurburg, W. and Ebberink, R. 1981. The anaerobic energy demand of Mytilus edulis. Organ specific differences in ATP-supplying processes and metabolic routes. Mol. Physiol. 1: 153-164.

Zurburg, W., De Bont, A. and de Zwaan, A. 1982. Recovery from exposure to air and the occurrence of strombine in different organs of the sea mussel Mytilus edulis. Mol. Physiol. 2: 135-147.

Zwaan, A. de 1972. Pyruvate kinase in muscle extracts of the sea mussel Mytilus edulis L. Comp. Biochem. Physiol. 42B: 7-14.

Zwaan, A. de 1977. Anaerobic energy metabolism in bivalve molluscs. Oceanogr. Mar. Biol. Ann. Rev. 15: 103-187.

Zwaan, A. de 1983. In The Mollusca. P. Hochachka, Ed. Academic Press, N.Y. Vol. 1: Chapter 4.

Zwaan, A. de and Holwerda, D. 1972. The effect of phosphoenolpyruvate, fructose-1,6-diphosphate and pH on allosteric pyruvate kinase in muscle tissue of the bivalve Mytilus edulis L. Biochim. Biophys. Acta. 276: 430-433.

Zwaan, A. de and van Marrewijk, W. 1973. Anaerobic glucose degradation in the sea mussel Mytilus edulis L. Comp. Biochem Physiol. 44B: 429-439.

Zwaan, A. de and De Bont, A. 1975. Phosphoenolpyruvate carboxykinase from adductor muscle of the sea mussel Mytilus edulis L. J. Comp. Physiol. 96: 85-94.

Zwaan, A. de and Wijsman, T. 1976. Anaerobic metabolism in bivalva (Mollusca). Characteristics of anaerobic metabolism. Comp. Biochem. Physiol. 54B: 313-324.

Zwaan, A. de, Kluytmans, J. and Zandee, D. 1976. In Biochemical Adaptation to Environmental Change. R. Smellie and J. Pennock, Eds. Biochemical Society Symposium No. 41. pp. 133-169.

Zwaan, A. de and Zurburg, W. 1981. The formation of strombine in the adductor muscle of the sea mussel, Mytilus edulis. Mar. Biol. Lett. 2: 179-192.

Zwaan, A. de, Holwerda, D. and Veenhof, P. 1981. Anaerobic malate metabolism in mitochondria of the sea mussel Mytilus edulis L. Mar. Biol. Lett. 2: 131-140.

Zwaan, A. de, De Bont, A. and Verhoeven, A. 1982. Anaerobic energy metabolism in isolated adductor muscle of the sea mussel Mytilus edulis L. J. Comp. Physiol. 149: 137-143.

Zwaan, A. de, De Bont, A., Zurburg, W., Bayne, B. and Livingstone, D. 1983. On the role of strombine formation in the energy metabolism of a sessile bivalve. J. Comp. Physiol. 149: 557-563.

Zwaan, A. de and Dando, P. 1984. Phosphoenolpyruvate-pyruvate metabolism in bivalve molluscs. Mol. Physiol. (in press).

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