Division of Comparative Physiology and Biochemistry, Society for Integrative and Comparative Biology

Regulation of Enzymes of Carbohydrate Metabolism during Anoxia in the Salt Marsh Bivalve Geukensia demissus

Author(s): Elizabeth L. Russell and Kenneth B. Storey

Source: *Physiological Zoology*, Vol. 68, No. 4 (Jul. - Aug., 1995), pp. 567-582
Published by: The University of Chicago Press . Sponsored by the Division of Comparative Physiology and Biochemistry, Society for Integrative and Comparative Biology
Stable URL: http://www.jstor.org/stable/30166345
Accessed: 22-03-2016 13:14 UTC

Regulation of Enzymes of Carbohydrate Metabolism during Anoxia in the Salt Marsh Bivalve *Geukensia demissus*

Elizabeth L. Russell
Kenneth B. Storey*
Institute of Biochemistry and Department of Chemistry, Carleton University, Ottawa, Ontario K1S 5B6, Canada

Accepted 11/29/94

Abstract

The effects of anoxia exposure (2 or 12 h at 5°C) on the tissue-specific responses by enzymes of carbohydrate metabolism were analyzed in the mantle, gill, hepatopancreas, and adductor muscle of the intertidal bivalve Geukensia demissus demissus. *Reversible enzyme phosphorylation has been identified in other species of marine molluscs as a mechanism for coordinating the suppression of metabolic rate and the redirection of carbon into fermentative pathways under anoxic conditions. The present study shows patterns of response to anoxia by five enzymes of carbohydrate metabolism, including glycogen synthetase (GS) and pyruvate dehydrogenase (PDH), whose responses to anoxia have not before been analyzed in marine molluscs. Anoxia-induced changes in properties, consistent with reversible phosphorylation modification of the enzymes, were found for pyruvate kinase (PK) and PDH in all tissues and in selected tissues for glycogen phosphorylase (GP) and GS. However, phosphofructokinase did not appear to be modified in any tissue during anoxia. Within 2 h of anoxia exposure, PK showed a sharp drop in the activity ratio (at subsaturating vs. saturating PEP concentrations) that indicated a stable modification of enzyme* K_m *for PEP; for example, in the gill, the ratio (determined at 0.75 and 7.5 mM PEP) fell from 0.39 ± 0.08 for aerobic controls to 0.08 ± 0.01 after 2 h anoxia. The percentage of PDH in the active* a *form also dropped significantly in anoxia from 80%–84% PDH*a *in controls to 65%–75% PDH*a *in anoxic tissues. Changes in both of these enzymes are consistent with anoxia-induced metabolic rate suppression. By contrast, anoxia exposure increased GP activity in the gill and adductor muscle, indicating a need for increased glycogenolysis during anoxia in these tissues. Total phosphorylase (*a + b*) activity increased in both tissues, as did the percentage of* a *in the adductor; for example, in the adductor, active GP*a *content rose from 0.09 ± 0.02 U/g wet wt in controls to 0.24 ± 0.01 U/g wet wt after 2 h and to 0.16 ± 0.02 U/g wet wt after 12 h of anoxia exposure. Glycogen synthetase behaved oppositely in*

* To whom correspondence should be sent: Institute of Biochemistry and Department of Chemistry, Carleton University, 1125 Colonel By Drive, Ottawa, Ontario K1S 5B6, Canada.

Physiological Zoology 68(4):567–582. 1995. 0031-935X/95/6804-9466$02.00

hepatopancreas, showing a significant decrease in total activity in 12-h anoxic tissue, but GS was unaffected by anoxia in the gill or the adductor muscle. In the mantle, oppositely directed changes in total GS activity and the percentage active resulted in no net change in the activity of the active I *form during anoxia.*

Introduction

Many marine invertebrates, particularly intertidal species, exhibit well-developed tolerances for hypoxia and anoxia (de Zwaan 1983; Kreutzer, Siegmund, and Grieshaber 1985; Storey 1993). For intertidal species, this capacity ensures survival during the daily interruptions of oxygen availability dictated by the tidal cycle. The biochemical adaptations supporting anoxia tolerance have been analyzed in various bivalve and gastropod molluscs, with the most extensive studies centering on the sea mussel *Mytilus edulis* and the whelk *Busycon canaliculatum* as the model animals (de Zwaan 1983; Storey 1993). The general features of anoxia tolerance among marine molluscs include (1) fermentation of glycogen and aspartate and accumulation of end products, including alanine, succinate, propionate, and acetate, (2) supplementation of the ATP output of anaerobic glycolysis by alternative fermentative pathways linking ATP production with the synthesis of organic acids, (3) minimizing metabolic acidosis with enhanced buffering capacity and alternative end products, and (4) metabolic rate depression. This last mechanism is quantitatively the most important for anoxia survival and characterizes anoxia tolerance by many other animals as well (Storey and Storey 1990). By sharply suppressing anoxic metabolic rate, generally to less than 10% of the aerobic rate at the same temperature (Kluytmans et al. 1983; Shick, de Zwaan, and de Bont 1983; Brooks et al. 1991; de Zwaan et al. 1991), animals can greatly extend the time that a fixed reserve of fermentable fuel can support the ATP needs of metabolism.

Metabolic rate depression requires the coordinated suppression of the rates of most or all processes in the cell, including both ATP-producing and ATP-utilizing reactions. Many studies to date have focused on the reversible control of the ATP-producing reactions operating in anoxia, for these are few and well understood. The primary pathway involved is glycolysis, and three mechanisms for the reversible suppression of glycolytic rate in anoxia have been identified and characterized: (1) allosteric control by key metabolites, (2) reversible enzyme binding associations with subcellular structural elements, and (3) reversible protein phosphorylation (Storey and Storey 1990; Storey 1993). The third mechanism appears to be the most powerful and extensive for coordinating numerous cellular reactions during transitions

to and from the hypometabolic state. For example, anoxia-induced phosphorylation is known to suppress the activities of glycogen phosphorylase (GP), 6-phosphofructo-1-kinase (PFK), 6-phosphofructo-2-kinase, and pyruvate kinase (PK) in marine molluscs, leading to the reduction of glycolytic rate as well as, due to PK inactivation, a redirection of glycolytic carbon via phosphoenolpyruvate carboxykinase into the reactions of succinate synthesis (Siebenaller 1979; Holwerda et al. 1983; Plaxton and Storey 1984; Storey 1988; Bosca and Storey 1991; Brooks et al. 1991; Whitwam and Storey 1991).

The present study focuses on the enzymatic regulation involved in the control of carbohydrate metabolism during anoxia in tissues of the ribbed mussel *Geukensia demissus demissus* (Dillwyn). The metabolic responses to anoxia by this species have received relatively little attention, but in a recent study we showed that succinate is the anaerobic product of the mantle and hepatopancreas, whereas the adductor muscle accumulated lactate during 12-h anoxia exposure at 5°C (Storey and Churchill 1995). As a sedentary species that lives high in the intertidal zone in temperate climates, this species is also one of a limited number of marine molluscs that are freeze tolerant (Murphy and Pierce 1975; Murphy 1977*a,* 1977*b*), and a good anoxia tolerance would also be of great value for enduring the ischemia that is imposed by the freezing of extracellular body fluids. Indeed, our initial study comparing metabolite changes during freezing and anoxia showed a number of similarities, but also distinct differences, between the two stresses (Storey and Churchill 1995). To further compare the metabolic controls and adaptations that support anoxia tolerance and freeze tolerance by *G. demissus,* we decided to first characterize the responses of enzymes of carbohydrate metabolism to anoxia exposure in this species. The present study assesses the effects of anoxia exposure on five enzymes in the adductor muscle, mantle, gill, and hepatopancreas of *G. demissus.* The study not only analyzes the responses of the regulatory enzymes of glycogen fermentation (GP, PFK, PK) but further examines whether coordinated controls are also in place to suppress other aspects of carbohydrate metabolism during anoxia, such as glycogen synthesis (via control over glycogen synthetase [GS]) and carbohydrate entry into the tricarboxylic acid cycle (via control over pyruvate dehydrogenase [PDH]).

Material and Methods

Chemicals and Animals

All biochemicals and coupling enzymes were obtained from Sigma (St. Louis) and Boehringer-Mannheim (Montreal). Distilled, deionized water

was used throughout for the preparation of biochemical solutions. All ATP and ADP solutions contained added $MgCl_2$ in 1:1 molar amounts. Marine mussels, *Geukensia demissus demissus,* were obtained from Woods Hole Marine Biological Laboratory in Massachusetts in early October. Animals were held in 40-L tanks of aerated artificial seawater (1,000 mosm/L) at 5°C without feeding for 3–4 wk prior to experimentation.

Preparation of Experimental Animals

Control animals were taken directly from the 5°C seawater. Anoxia experiments were conducted in 4-L jars that could be tightly sealed; a small amount of seawater (approx. 50 mL) was placed in the bottom of the jar to maintain humidity during the experiment (but water did not cover the mussels), and nitrogen gas (100%) was then bubbled through the seawater for 15 min. Mussels were then placed in the anoxic jar, which was sealed except for two syringe needle ports, one to continue flushing with nitrogen gas and one to vent the gas. Gassing was continued for a further 20 min, after which these ports were sealed, and the jar was returned to the 5°C incubator. Exposure to the anoxic atmosphere was continued for 2 or 12 h; initial experiments showed that mussels survived both exposures. For tissue sampling, the shell was broken open, and tissues were rapidly removed. The tissues were immediately frozen in liquid nitrogen and stored at −70°C until further use.

Tissue Preparation and Enzyme Assays

Frozen tissue samples were homogenized 1:2 or 1:10 wt/vol with an Ultra-Turrax or a Polytron PT10 homogenizer. Homogenizing buffer for all enzymes except PDH contained 20 mM imidazole-HCl (pH 7.2), 100 mM NaF, 10 mM ethylenediaminetetraacetic acid (EDTA), 10 mM N,N,N′,N′-tetraacetic acid (EGTA), 0.1 mM phenylmethylsulfonyl fluoride (PMSF), and 15 mM 2-mercaptoethanol. For PDH, tissues were homogenized in ice-cold 50 mM potassium phosphate buffer (pH 7.8) containing 2 mM 2-mercaptoethanol, 1 mM EDTA, 0.1 mM PMSF, and 0.1% (vol) Triton X-100.

Glycogen Phosphorylase (E.C. 2.4.1.1). Homogenates were centrifuged for 2 min at 5°C in a Brinkman 5412 refrigerated microcentrifuge. The supernatant was removed and used for enzyme analysis. Assay conditions for GP*a* were 50 mM potassium phosphate buffer (pH 7.0) containing 0.25 mM EDTA, 10 mM $MgCl_2$, 0.4 mM $NADP^+$, 10 μM glucose-1,6-bisphosphate, 2 mg/mL glycogen (previously dialyzed), 0.7 U/mL phosphoglucomutase, and 0.2

U/mL glucose-6-phosphate dehydrogenase. For measurements of total phosphorylase, assays were conducted in the presence of 2 mM AMP.

Glycogen Synthetase (E.C. 2.4.1.11). Homogenates of three tissues were allowed to settle on ice for 30 min, and then aliquots of the supernatant were sampled; adductor muscle homogenates were first centrifuged in a Brinkman 5412 refrigerated microcentrifuge for 2 min at 5°C before sampling. Optimal assay conditions for GS were 20 mM imidazole-HCl buffer, 5 mM uridine 5′-diphosphoglucose (UDPG), 1 mM PEP, 5 mM $MgCl_2$, 20 mM KCl, 0.15 mM NADH, 2 mg/mL glycogen (previously dialyzed), 1 U/mL PK, and 1 U/mL lactate dehydrogenase. For measurements of total synthetase activity, 5 mM G-6-P was included in the assay mixture.

Phosphofructokinase (E.C. 2.7.1.11) and Pyruvate Kinase (E.C. 2.7.1.40). Homogenates for PFK and PK assays were centrifuged for 20 min at 25,000 *g* in a Sorvall RC-5B refrigerated centrifuge at 5°C. Supernatants were collected, and low molecular weight metabolites were removed via passage through 5-mL columns of G-25 Sephadex equilibrated in homogenization buffer (Helmerhorst and Stokes 1980). Sephadex columns were centrifuged in a bench-top centrifuge at top speed for 1 min, and the filtrate was collected, stored on ice, and used for enzyme assay. Optimal assay conditions for PFK were 20 mM imidazole-HCl (pH 7.2) containing 1 mM fructose-6-phosphate (F-6-P), 0.5 mM Mg.ATP, 5 mM $MgCl_2$, 20 mM KCl, 0.15 NADH, 0.2 U/mL triosephosphate isomerase, 2.4 U/mL glycerol-3-phosphate dehydrogenase, and 0.4 U/mL aldolase. Optimal assay conditions for PK were 20 mM imidazole-HCl (pH 7.2), 2 mM ADP, 5 mM $MgCl_2$, 20 mM KCl, 0.15 NADH, and 1 U/mL lactate dehydrogenase, with the following PEP concentrations: 5 mM (adductor), 7.5 mM (gill, hepatopancreas), and 2 mM (mantle).

Pyruvate Dehydrogenase (E.C. 1.2.4.1). Tissue homogenates were frozen at −20°C for 2 h to aid the breakup of mitochondrial membranes. Samples were then placed on ice, and PDH activity was assayed as soon as homogenates had thawed. Assay conditions were 50 mM Tris-HCl (pH 7.8), 1 mM $MgCl_2$, 0.5 mM EDTA, 0.2% wt/vol Triton X-100, 2.5 mM NAD^+, 0.2 mM thiamine pyrophosphate, 0.2 mM CoA, 0.1 mM *p*-[*p*-aminophyeny-azo]benzene sulfonic acid (AABS), and 0.05 U/mL arylamine acetyltransferase. The reaction was initiated by the addition of homogenate and allowed to run for about 5 min to establish a background rate. Activity of PDH*a* was then measured after the addition of 10 mM pyruvate. To measure total PDH activity, samples were incubated in the presence of different concentrations of $MgCl_2$ and $CaCl_2$ at 21°C or 4°C to stimulate conversion of inactive PDH

to active PDH. Initial tests determined that enzyme conversion was optimal after incubation at 21°C ± 1°C with 10 mM $MgCl_2$ and 1 mM $CaCl_2$ for 40 min for adductor muscle extracts or with 20 mM $MgCl_2$ and 1 mM $CaCl_2$ for 20 min for extracts of other tissues. After incubation, total PDH activity was then measured under the same conditions as described above for the *a* form.

DE-52 Chromatography of PFK

The presence of tissue-specific forms of PFK was assessed by DE-52 Sephadex ion exchange chromatography. Tissues were homogenized 1:5 wt/vol in 10 mM phosphate buffer (pH 8.0) containing 2 mM EDTA, 2 mM ethylene glycol-bis(β-amino-ethyl ether) EGTA, 50 mM NaF, 15 mM 2-mercaptoethanol, 1 mM F-6-P, and 10% wt/vol glycerol. Homogenates were then centrifuged and desalted through G-25 Sephadex, as described above. The filtrate was layered onto a DE-52 Sephadex column (6 cm × 1.5 cm) equilibrated in the homogenization buffer and washed twice with several column volumes of homogenization buffer. Enzymes were then eluted with a linear salt gradient of 0–200 mM KCl in 40-mL homogenization buffer, and 1-mL fractions were collected and assayed for PFK activity.

Data and Statistics

Enzyme activities were assayed at 21°C ± 1°C in 1 mL with a Gilford 240 recording spectrophotometer by monitoring NADH or NADP use at 340 nm or AABS use at 460 nm. Results are presented as means ± SEM. Data were analyzed with ANOVA (model 1) followed by testing of differences between groups with Dunnett's test (two-tailed).

Results

Glycogen Phosphorylase

In the adductor muscle, anoxia exposure led to a nearly twofold increase in total GP activity after 2 h as well as a significant increase in the percentage of the enzyme in the active *a* form (table 1). In combination, therefore, the activity of GP*a* rose from 0.09 ± 0.02 U/g wet wt in the control adductor to 0.24 ± 0.01 U/g wet wt in the 2-h anoxic muscle and remained elevated at 0.16 ± 0.02 U/g wet wt after 12 h (both significantly different from control values, $P < 0.01$). Activity of GP in the gill was very low, but total activity increased by fivefold during anoxia. Activity of GP*a* also rose from 0.003

TABLE 1

Effect of anoxia exposure on total GP (a + b) activities and percentage of the active a form in tissues of Geukensia demissus

	Adductor	Gill	Hepatopancreas	Mantle
Total GP (U/g wet wt):				
Control	.28 ± .02	.007 ± .0003	.39 ± .07	.32 ± .04
2-h Anoxic	.50 ± .02*	.036 ± .003*	.54 ± .05	N.D.
12-h Anoxic	.42 ± .04*	.038 ± .002*	.56 ± .03	.29 ± .02
Percentage of GP*a*:				
Control	32.4 ± .4	40.3 ± 2.3	35.9 ± 1.0	38.0 ± .7
2-h Anoxic	48.9 ± 1.2*	27.8 ± 2.0*	32.1 ± 3.4	N.D.
12-h Anoxic	38.3 ± 1.2*	50.1 ± 1.1	35.4 ± 5.8	35.8 ± 2.7

Note. Data are means ± SEM, $n = 4$–6. N.D. = not determined.

* Significantly different from corresponding control values, $P < 0.05$.

± 0.0001 U/g wet wt in controls to 0.01 ± 0.001 U/g wet wt after 2-h anoxia exposure and to 0.019 ± 0.001 U/g wet wt after 12-h anoxia exposure, but the percentage of GP*a* was actually reduced somewhat after 2-h anoxia exposure (table 1). Activities of GP and percentages of *a* were not altered by anoxia exposure in either the hepatopancreas or mantle.

Glycogen Synthetase

Neither total activities of GS, the sum of the G-6-P-independent active form (*I*) and the G-6-P-dependent inactive form (*D*), nor the percentage of *I* were altered by 12-h anoxia exposure in the adductor muscle or gill (table 2). In the hepatopancreas, total GS activity decreased by 20% after 12-h anoxia exposure, whereas in the mantle, total activity increased by 38%. The percentage of enzyme in the active *I* form also changed in the mantle, decreasing from 27.8% in controls to 19.5% in the anoxic mantle. As the result of oppositely directed changes in total GS and the percentage of *I* in the mantle, there was no significant difference between the amount of the active *I* form in the control and anoxic mantles.

Pyruvate Kinase

Pyruvate kinase from numerous molluscan species is subject to anoxia-induced protein phosphorylation that changes enzyme kinetic properties, no-

TABLE 2
*Effect of 12-h anoxia exposure on total GS (*I + D*) activities and percentage of the active* I *form in tissues of* Geukensia demissus

	Total GS (U/g wet wt)		Percentage of GS*I*	
	Control	Anoxic	Control	Anoxic
Adductor	.22 ± .02	.26 ± .04	37.5 ± 1.8	40.8 ± 4.1
Gill	.18 ± .03	.15 ± .02	33.0 ± 3.9	40.6 ± 2.5
Hepatopancreas	.44 ± .01	.35 ± .02*	18.0 ± .5	23.7 ± 2.3
Mantle	.34 ± .04	.47 ± .06*	27.8 ± 2.6	19.5 ± 1.9*

Note. Activities are means ± SEM, $n = 4$–6.
* Significantly different from corresponding control values, $P < 0.05$.

tably the affinity for the substrate PEP. A change in PEP affinity, implicating anoxia-induced enzyme modification, can be readily detected by comparing the ratio of enzyme activity at subsaturating and saturating PEP concentrations. Table 3 shows such activity ratios for PK from *Geukensia demissus* tissues. In all cases the ratio decreased significantly in extracts from anoxic tissues, indicating a decrease in enzyme affinity for PEP (increase in K_m) during anoxia. The most pronounced change was in the gill, which showed a fivefold change in the ratio between control and 2-h anoxic states.

Pyruvate Dehydrogenase

Table 4 shows the effects of anoxia exposure on the percentage of PDH in the active *a* form in *G. demissus* tissues. All four tissues showed PDH*a* values of 80%–84% for controls, and 2-h anoxia exposure reduced PDH*a* to 65%–70% in three tissues (2-h mantle not determined). The percentage of PDH*a* remained reduced in all tissues of 12-h anoxic animals. Total PDH activity in *G. demissus* tissues remained constant during anoxia at 0.082 ± 0.003, 0.057 ± 0.003, 0.168 ± 0.011, and 0.091 ± 0.005 U/g wet wt ($n = 4$) in the adductor, gill, hepatopancreas, and mantle, respectively.

PFK Analysis by DE-52 Chromatography

Multiple forms of PFK can occur, including tissue-specific isozymic forms as well as phosphorylated and dephosphorylated variants within each tissue. Phosphorylation is also known to have tissue-specific effects on enzyme

TABLE 3
Effect of anoxia exposure on the ratio of enzyme activities at low and at saturating PEP concentrations for PK from the tissues of Geukensia demissus

	Adductor	Gill	Hepatopancreas	Mantle
Control	.12 ± .006	.39 ± .080	.18 ± .020	.23 ± .020
2-h Anoxic	.04 ± .006*	.08 ± .010*	.08 ± .010*	N.D.
12-h Anoxic	.05 ± .008*	.11 ± .040*	.05 ± .004*	.16 ± .010*

Note. Data are means ± SEM, $n = 3$–4. N.D. = not determined. Enzyme activity ratios were determined at 0.50 and 5.0 mM PEP for adductor, 0.75 and 7.5 mM PEP for gill and hepatopancreas, and 0.40 and 2.0 mM PEP for mantle.
* Significantly different from corresponding controls, $P < 0.05$.

TABLE 4
Effect of anoxia exposure on the percentage of PDH in the active a *form in tissues of* Geukensia demissus

	Control	2-h Anoxic	12-h Anoxic
Adductor	83.5 ± 1.5	69.9 ± 1.1*	75.2 ± 1.5
Gill	80.2 ± .6	65.1 ± 5.1*	57.6 ± 4.5*
Hepatopancreas	81.3 ± 2.1	70.6 ± 3.0*	65.8 ± 4.4*
Mantle	84.2 ± 1.9	N.D.	70.8 ± 2.4*

Note. Data are means ± SEM, $n = 4$. N.D. = not determined.
* Significantly different from corresponding control values, $P < 0.05$.

properties, in some cases altering kinetic properties and in others altering enzyme-binding affinity for F-actin. Because the possible effects of phosphorylation on *G. demissus* PFK were unknown, it was decided to use DE-52 Sephadex anion exchange chromatography (with elution by a KCl gradient) to determine whether multiple forms of PFK were present in mussel tissues. Because phosphorylation changes the charge characteristics of an enzyme, an anoxia-induced change in the elution profile of PFK off DE-52 Sephadex should be a good indicator of a covalent modification of the enzyme, whereas tissue-specific differences in the PFK elution profile would suggest the occurrence of isozymic forms. In all cases, however, PFK activity from each of the four *G. demissus* tissues eluted in a single peak, indicating that multiple enzyme forms were not present in any tissue. Anoxia exposure also had no effect on the elution profile of the enzyme in any tissue, suggesting that the enzyme was probably not subject to anoxia-induced covalent modification (see fig. 1 for data for the gill). Furthermore, the enzyme in all four tissues eluted at about 90–100 mM KCl (fig. 1), which suggests a lack of tissue-specific isozymic forms.

Discussion

Posttranslational modification via the addition or removal of covalently attached phosphate from proteins is one of the most powerful ways of controlling the activity of individual enzymes and of coordinating the responses of multiple enzymes in response to an extracellular signal (Cohen 1980; Woodford, Taylor, and Corbin 1992). Anoxia-induced phosphorylation of

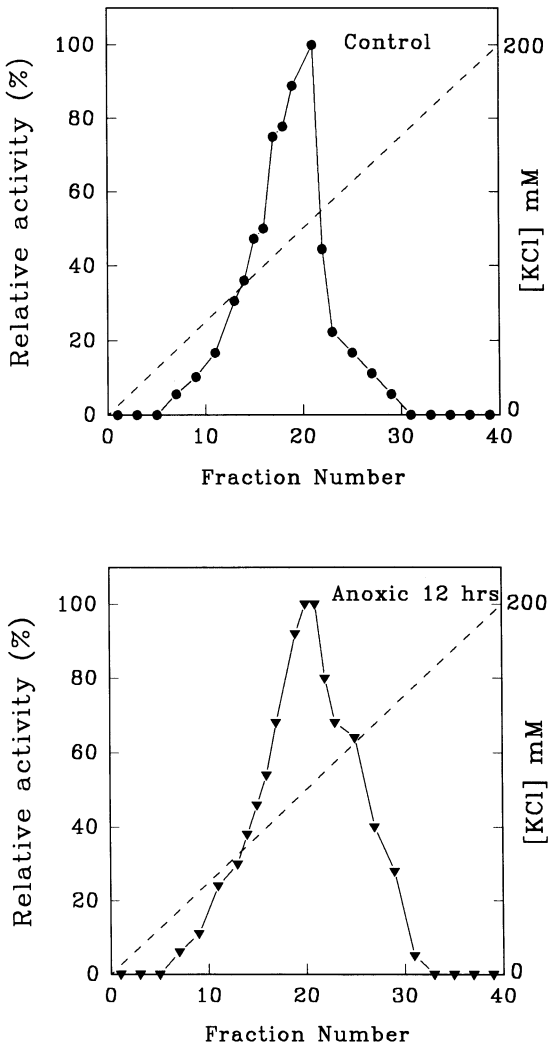

Fig. 1. Chromatography of PFK from the gill of control (circles) and 24-h anoxic (triangles) Geukensia demissus *on DE-52 Sephadex. The PFK was eluted with a linear gradient of 0–200 mM KCl (dashed line) in 10 mM P_i buffer (pH 8.0) containing 50 mM NaF, 2 mM EDTA, 2 mM EGTA, 15 mM 2-mercaptoethanol, 1 mM F-6-P, and 10% vol/vol glycerol.*

enzymes of carbohydrate metabolism is well known in marine molluscs and serves in general as a mechanism of metabolic rate depression (for review, see Storey [1993]). Strict control of PK is also critical to the redirection of carbon at the PEP branch point to allow carbon derived from glycogen to be directed into the ATP-producing reactions of succinate and propionate synthesis. For many enzymes, phosphorylation or dephosphorylation acts as an on/off switch by converting an inactive enzyme into the active forms;

GP, GS, and PDH are typical examples of this (Woodford et al. 1992). Although the activities of the inactive forms of GP and GS can be expressed in vitro in the presence of high concentrations of allosteric activators, it is doubtful whether GP*b* or GS*D* have any activity in vivo under natural cellular concentrations of metabolites. The phosphorylated form of PK in marine mollusc tissues is also likely to be functionally inactive in vivo because of its extreme sensitivity to L-alanine inhibition and the high concentrations of this amino acid in anaerobic tissues (Storey 1993).

Four of the five enzymes assessed clearly showed altered properties in response to anoxia exposure in *Geukensia demissus*. In response to anoxia, PK and PDH changed in all tissues, and anoxia-induced covalent modification of the enzymes was implicated in both instances. Both GP and GS were modified during anoxia in two tissues. The PFK charge state, as assessed by elution off DE-52, did not change in any tissue; while not absolutely conclusive, this implies that the enzyme was probably not modified by phosphorylation during anoxia. A similar assessment of PFK from the foot and hepatopancreas of the intertidal snail *Littorina littorea* showed a lack of change in DE-52 elution profile in response to anoxia, and additional analyses further confirmed that the enzyme was not subject to anoxia-induced reversible phosphorylation (Russell and Storey 1995). Thus, PFK kinetic properties were not altered by anoxia, and in vitro incubation of *L. littorea* PFK under conditions that would promote protein kinase or protein phosphatase action did not alter the DE-52 elution profile of the enzyme. By contrast, the kinetic properties of PK from *L. littorea* were greatly changed by anoxia exposure, and this was correlated with a strong shift in the DE-52 elution profile (by 10–20 fractions) of the enzyme (Russell and Storey 1995).

The activity of PDH in both mammals and molluscs is well known to be regulated by reversible phosphorylation, interconverting the active, dephosphorylated and inactive, phosphorylated forms of the enzyme (Brooks and Storey 1992; Woodford et al. 1992). The percentage of PDH in the active form decreased in all four *G. demissus* tissues during anoxia, an effect consistent with anoxia-induced metabolic arrest. This is, to our knowledge, the first demonstration that the activity of a mitochondrial enzyme is coordinated with those of glycolytic enzymes during anoxia exposure in a marine mollusc. Pyruvate dehydrogenase gates the use of carbohydrate by the oxidative reactions of the tricarboxylic acid cycle. Because the lack of oxygen would itself arrest oxidative metabolism, it might seem redundant to apply regulatory controls to mitochondrial oxidative enzymes. However, natural anoxia exposures for intertidal bivalves generally begin with shell valve closure when animals are exposed to air at low tide; internal reserves of oxygen are

then consumed, and the animal experiences increasingly severe hypoxia, finally reaching anoxia. In this situation of declining oxygen tension, the glycolytic contribution to ATP production gradually increases, while the contribution by oxidative phosphorylation gradually decreases until a critical P_{O_2} is reached at which the combined ATP output can no longer match the ATP demand of normal metabolism (de Zwaan et al. 1991; Storey 1993). At this critical P_{O_2}, metabolic arrest mechanisms are initiated to sharply reduce ATP demand and bring energy use into the range that can be supported over an indefinite term by fermentation pathways. Hence, when a species-specific critical level of hypoxia is reached (de Zwaan et al. 1991), metabolic arrest mechanisms are applied at many key regulatory loci of metabolism (including mitochondrial enzymes such as PDH) to achieve a rapid and coordinated suppression of metabolism.

Changes in PK activity during anoxia were assessed by comparing the ratio of activity at saturating and subsaturating concentrations of the substrate PEP. One of the consistent effects of phosphorylation on PK from many sources is to change the affinity of the enzyme for PEP (Holwerda et al. 1983; Plaxton and Storey 1984; Engstrom et al. 1987; Storey 1993); generally, phosphorylation reduces PEP affinity (increases K_m). The effect of anoxia in significantly reducing the activity ratios in all tissues of *G. demissus* is consistent with an increase in the K_m PEP of the enzyme during anoxia exposure and implicates probable covalent modification of the enzyme as the cause of the change in substrate affinity. The higher-K_m form of the enzyme would be less active under in vivo PEP concentrations, and this enzyme form (phosphorylated) is also typically strongly inhibited by L-alanine in mollusc tissues (Storey 1993). Hence, the data for PK show strong evidence of anoxia-induced covalent modification of the enzyme in all four tissues of *G. demissus* with a change in enzyme properties that would produce a less active form of the enzyme in anoxic tissues. As for the data on PDH, this is consistent with a suppression of the rate of carbohydrate catabolism in anoxic tissue as part of a general metabolic rate depression response to anoxia.

In contrast to the highly consistent effects of anoxia exposure on PK and PDH in *G. demissus* tissues, the effect of anoxia on GP was tissue specific, with two tissues showing no effect of anoxia on the content of active GP*a* and the adductor and gill showing an increase in enzyme activity during anoxia. A variable response by GP to anoxia exposure was also seen in *Busycon canaliculatum* tissues, with muscle tissues showing enzyme activation in anoxia, whereas soft tissues showed no change or decreased activity over a time course of anoxia (Storey 1988). This tissue-specific variation in GP response to anoxia probably arises as the net result of at least

three pressures on GP: (1) stress-induced depression of metabolic rate, (2) the need for glycogenolysis to meet the anoxic ATP requirements of individual tissues, and (3), in some cases, a switch to anoxic glycogen fermentation from the aerobic oxidation of an alternative fuel. The relative importance of each factor in each tissue determines the net amount of active GP*a* during anoxia. Clearly, the present results show that both the gill and the adductor muscle must increase the rate of glycogenolysis under anoxia to meet anoxic ATP requirements, whereas the hepatopancreas and the mantle need not.

The response of GS to anoxia was also tissue specific. The amount of the active, G-6-P-independent form did not change significantly in three tissues, but total GS activity decreased by about 20% in the hepatopancreas. A reduction in GS activity is consistent with metabolic arrest during anoxia since the use of ATP by anabolic pathways such as glycogen synthesis should, predictably, be restricted when ATP availability is limiting. Furthermore, glycogen synthesis is typically restricted under metabolic conditions in which glycogen is also being catabolized as a major fuel, as is the case during anoxia in molluscan tissues. Anoxia-induced changes in GS activity have not previously been assessed in intertidal molluscs, but seasonal changes in GS activity associated with feeding and/or gametogenesis have been observed (Gabbott, Cook, and Whittle 1979; Gabbott and Whittle 1986). As was also seen in *G. demissus,* GS regulation in *Mytilus edulis* involved changes in both total enzyme activity and the percentage of the enzyme in the active *I* form (Gabbott et al. 1979; Gabbott and Whittle 1986).

Acknowledgments

Thanks to J. M. Storey for editorial comments on the manuscript. This study was supported by a National Sciences and Engineering Council of Canada research grant to K.B.S.

Literature Cited

Bosca, L., and K. B. Storey. 1991. Inactivation of 6-phosphofructo-2-kinase during anaerobiosis in the marine whelk *Busycon canaliculatum.* Am. J. Physiol. 260: R1168–R1175.

Brooks, S. P. J., A. de Zwaan, G. van den Thillart, O. Cattani, P. Cortesi, and K. B. Storey. 1991. Differential survival of *Venus gallina* and *Scapharca inaequivalvis* during anoxic stress: covalent modification of phosphofructokinase and glycogen phosphorylase in anoxia. J. Comp. Physiol. 161:207–212.

Brooks, S. P. J., and K. B. Storey. 1992. Properties of pyruvate dehydrogenase from *Otala lactea:* control of enzyme activity during estivation. Physiol. Zool. 65:620–633.

Cohen, P. 1980. Recently discovered systems of enzyme regulation by reversible phosphorylation. Elsevier/North Holland Biochemical, Amsterdam.

de Zwaan, A. 1983. Carbohydrate metabolism in bivalves. Pages 137–175 in K. M. Wilbur, ed. The Mollusca. Vol. 1. Academic Press, New York.

de Zwaan, A., P. Cortesi, G. van den Thillart, J. Roos, and K. B. Storey. 1991. Differential sensitivities to hypoxia by two anoxia-tolerant marine molluscs: a biochemical analysis. Mar. Biol. 111:343–351.

Engstrom, L., P. Ekman, E. Humble, and O. Zetterqvist. 1987. Pyruvate kinase. Pages 47–75 in P. D. Boyer and E. G. Krebs, eds. The enzymes. Vol. 18. Academic Press, New York.

Gabbott, P. A., P. A. Cook, and M. A. Whittle. 1979. Seasonal changes in glycogen synthetase activity in the mantle tissue of *Mytilus edulis* L.: regulation by tissue glycogen. Biochem. Soc. Trans. 7:895–896.

Gabbott, P. A., and M. A. Whittle. 1986. Glycogen synthetase in the sea mussel *Mytilus edulis* L. II. Seasonal changes in glycogen content and glycogen synthetase activity in the mantle tissue. Comp. Biochem. Physiol. 83B:197–207.

Helmerhorst, E., and G. B. Stokes. 1980. Microcentrifuge desalting: a rapid, quantitative method for desalting small amounts of protein. Anal. Biochem. 104:130–135.

Holwerda, D. A., P. R. Veenhof, H. A. A. van Heugten, and D. I. Zandee. 1983. Modification of mussel pyruvate kinase during anaerobiosis and after temperature acclimation. Mol. Physiol. 3:225–234.

Kluytmans, J. H., A. M. T. de Bont, E. C. J. Kruitwagen, H. J. L. Ravestein, and P. R. Veenhof. 1983. Anaerobic capacities and anaerobic energy production of some Mediterranean bivalves. Comp. Biochem. Physiol. 75B:171–179.

Kreutzer, U., B. Siegmund, and M. K. Grieshaber. 1985. Role of coupled substrates and alternative end products during hypoxia in marine invertebrates. Mol. Physiol. 8:371–392.

Murphy, D. J. 1977*a*. Metabolic and tissue solute changes associated with changes in the freezing tolerance of the bivalve mollusc *Modiolus demissus.* J. Exp. Biol. 69:1–12.

———. 1977*b*. A calcium-dependent mechanism responsible for increasing the freezing tolerance of the bivalve mollusc *Modiolus demissus.* J. Exp. Biol. 69:13–21.

Murphy, D. J., and S. K. Pierce. 1975. The physiological basis for changes in the freezing tolerance of intertidal molluscs. I. Responses to subfreezing temperatures and the influence of salinity and temperature acclimation. J. Exp. Zool. 193:313–322.

Plaxton, W. C., and K. B. Storey. 1984. Purification and properties of aerobic and anoxic forms of pyruvate kinase from red muscle tissue of the channeled whelk, *Busycotypus canaliculatum.* Eur. J. Biochem. 143:257–265.

Russell, E. L., and K. B. Storey. 1995. Anoxia and freezing exposures stimulate covalent modification of enzymes of carbohydrate metabolism in *Littorina littorea.* J. Comp. Physiol. B (in press).

SHICK, J. M., A. DE ZWAAN, and A. M. T. DE BONT. 1983. Anoxic metabolic rate in the mussel *Mytilus edulis* L. estimated by simultaneous direct calorimetry and biochemical analysis. Physiol. Zool. 56:56–63.

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

STOREY, K. B. 1988. Mechanisms of glycolytic control during facultative anaerobiosis in a marine mollusc: tissue-specific analysis of glycogen phosphorylase and fructose-2,6-bisphosphate. Can. J. Zool. 66:1767–1771.

———. 1993. Molecular mechanisms of metabolic arrest in mollusks. Pages 253–269 in P. W. HOCHACHKA, P. L. LUTZ, T. SICK, M. ROSENTHAL, and G. VAN DEN THILLART, eds. Surviving hypoxia: mechanisms of control and adaptation. CRC, Boca Raton, Fla.

STOREY, K. B., and T. A. CHURCHILL. 1995. Metabolic responses to freezing and anoxia by the freeze tolerant marine mussel, *Geukensia demissus*. J. Exp. Mar. Biol. Ecol. (in press).

STOREY, K. B., and J. M. STOREY. 1990. Facultative metabolic rate depression: molecular regulation and biochemical adaptation in anaerobiosis, hibernation, and estivation. Q. Rev. Biol. 65:145–174.

WHITWAM, R. E., and K. B. STOREY. 1991. Organ-specific regulation of phosphofructokinase during facultative anaerobiosis in the marine whelk, *Busycotypus canaliculatum*. Can. J. Zool. 69:70–75.

WOODFORD, T. A., S. J. TAYLOR, and J. D. CORBIN. 1992. The biological functions of protein phosphorylation and dephosphorylation. Pages 453–607 in E. BITTAR, ed. Fundamentals of medical cell biology. Vol. 3B. JAI, Greenwich, Conn.