J Comp Physiol B (1995) 165: 132–142

ORIGINAL PAPER

E. L. Russell · K. B. Storey

Anoxia and freezing exposures stimulate covalent modification of enzymes of carbohydrate metabolism in *Littorina littorea*

Accepted: 20 January 1995

Abstract The effects of anoxia (N_2 atmosphere at 5 °C) or freezing (at −8 °C) exposure in vivo on the activities of five enzymes of carbohydrate metabolism were assessed in foot muscle and hepatopancreas of the marine periwinkle *Littorina littorea*. Changes in glycogen phosphorylase, glycogen synthetase, pyruvate kinase and pyruvate dehydrogenase under either stress were generally consistent with covalent modification of the enzymes to decrease enzyme activity and/or convert the enzyme to a less active form. However, no evidence for a similar covalent modification of phosphofructokinase was found. The metabolic effects of freezing and anoxia were generally similar, suggesting that a primary contributor to freezing survival is the implementation of anaerobic metabolism and metabolic arrest mechanisms that also promote anoxia survival in marine molluscs. However, in hepatopancreas phosphorylase was activated and pyruvate kinase remained in two enzyme forms in freezing-exposed snails, contrary to the results for anoxic animals. Ion exchange chromatography on DE-52 Sephadex revealed the presence of two forms of pyruvate kinase in both tissues of control *L. littorea*, eluting at 30–50 mmol·l^{-1} KCl (peak I) or 90–110 mmol·l^{-1} KCl (peak II). Anoxia exposure converted pyruvate kinase in both tissues to the peak I form, as did freezing for foot muscle pyruvate kinase. Kinetic analysis showed that peak I pyruvate kinase had lower affinities for substrates, phosphoenolpyruvate and ADP, and was very strongly inhibited by L-alanine compared with the peak II enzyme. Peak I pyruvate kinase had an I_{50} value for L-alanine of 0.38 mmol·l^{-1}, whereas peak II pyruvate kinase was unaffected by L-alanine evenat 40 mmol·l^{-1}. In vitro incubation of extracts from control foot muscle under conditions promoting phosphorylation or dephosphorylation identified the peak I and II forms as the low and high phosphate forms, respectively. This result for *L. littorea* pyruvate kinase was highly unusual and contrary to the typical effect of anoxia on pyruvate kinase in marine molluscs which is to stimulate the phosphorylation of pyruvate kinase and, thereby, convert the enzyme to a less active form.

Key words Freeze tolerance · Anoxia tolerance · Marine gastropods · Carbohydrate metabolism · Reversible protein phosphorylation

Abbreviations
AABS *p*-(*p*-aminophenylazo)benzene sulphonic acid · *F2, 6P* fructose-2,6-bisphosphate · *F6P* fructose-6-phosphate · *G6P* glucose-6-phosphate · *GP* glycogen phosphorylase · *GS* glycogen synthase · I_{50} inhibitor concentration reducing enzyme velocity by 50% · *MR* metabolic rate · *PDH* pyruvate dehydrogenase · *PEP* phosphoenopyruvate · *PFK* phosphofructokinase · *PK* pyruvate kinase · *SW* sea water · T_a air temperature · *TCA* trichloroacetic acid · *UDPG* uridine-diphosphate glucose · *WW* wet weight.

Introduction

Marine molluscs inhabiting the intertidal zone are exposed to a harsh and changeable environment and experience wide daily and seasonal fluctuations in O_2 and water availability, salinity and temperature. Most show a well-developed anoxia tolerance that ensures survival when O_2 availability is restricted during aerial exposures at low tide (Livingstone and de Zwaan 1983). Intertidal species also tolerate wide variation in body

E.L. Russell · K.B. Storey (✉)
Institute of Biochemistry and Department of Chemistry,Carleton University, Ottawa, K1S 5B6, Canada

fluid osmolality whether caused by aerial desiccation or by the variable salinity of water in tide pools (Gilles 1979). Various species living at high latitudes have also developed the ability to tolerate the freezing of extracellular body fluids to endure exposures to subzero T_a at low tide (Aarset 1982; Murphy 1983; Loomis 1987). The periwinkle *Littorina littorea* L. (Mollusca, Gastropoda) is one of the most widely distributed intertidal inhabitants and its abilities to endure O_2 deprivation and freezing, as well as other stresses of intertidal life, have been extensively described (Wieser 1980; Kanwisher 1955; Murphy and Johnson 1980; Murphy 1983). For example, these snails readily endured freezing at −8 °C for at least 8 days but mortality increased sharply with lower temperatures, half-time values for survival at −9, −11, and −13 °C being 7 days, 28 h, and 4 h, respectively (Murphy and Johnson 1980; Murphy 1983).

One of the critical biochemical adaptations supporting long-term anaerobic survival among marine molluscs is metabolic rate depression; anoxic MR for different species is only 2–20% of the corresponding aerobic resting rate at the same temperature (Famme et al. 1981; Shick et al. 1983; Kluytmans et al. 1983; de Zwaan et al. 1991). Low temperature and/or freezing also appears to induce metabolic arrest, although for bivalves at least this may be the consequence of low-temperature-induced shell valve closure which rapidly leads to anoxia. Kanwisher (1955) reported that Q_{10} for O_2 consumption by *L. littorea* rose sharply to about 50 at temperatures below 0 °C. By suppressing MR animals can greatly extend the time that fixed endogenous fuel reserves can support anaerobic survival by fermentative metabolism. Carbohydrate fermentation (producing one or more end products including lactate, alanine, succinate, propionate, and acetate) is the primary means of ATP production during anoxia and strict control over the enzymes of glycolysis and associated reactions is key to reducing the rate of ATP generation during anoxia-induced metabolic suppression. Several mechanisms of glycolytic rate depression have been identified and these also appear to apply to the regulation of other cellular functions such that a coordinated suppression of the rates of ATP-producing and ATP-utilizing processes is achieved (Storey and Storey 1990; Storey 1993). The most powerful and widespread of these mechanisms is reversible protein phosphorylation, the actions of protein kinases and protein phosphatases allowing for large, rapid and coordinated changes in the activity states of numerous enzymes and functional proteins in the cell. Reversible phosphorylation regulation of glycolytic enzymes has been shown to play an important role in MR depression, as well as in the control of aerobic versus anaerobic routes of carbohydrate catabolism in numerous species of anoxia-tolerant marine molluscs [review: Storey (1993)].

Much less is known, however, about the effects of freezing on metabolism and metabolic regulation in marine intertidal molluscs. One of the consequences of freezing must be anoxia because extracellular freezing halts the circulation of O_2 by the hemolymph; indeed, freezing effectively results in the much more stressful condition of ischemia. Predictably, therefore, biochemical adaptations that support anoxia tolerance should also be expressed during freezing. Furthermore, additional freezing-specific metabolic responses may also occur to deal with other specific demands of this condition, such as the large cell volume reduction initiated by extracellular ice formation. A recent study compared the changes in cellular metabolite levels during anoxia and freezing exposures in foot muscle of *L. littorea* and found significant differences between the two stresses (T.A. Churchill and K.B. Storey, unpublished observations). Both stresses appeared to induce a rapid metabolic arrest. Cellular energy charge was unaffected under either stress but whereas 72 h anoxia exposure at 5 °C led to the accumulation of only small amounts of D-lactate (a net increase of 0.5 $\mu mol \cdot g\,ww^{-1}$), freezing exposure for 72 h at −8 °C resulted in the accumulation of glucose, D-lactate and succinate (net amounts of 1.2, 1.1, and 3.9 $\mu mol \cdot g\,ww^{-1}$, respectively) coupled with a decrease in the fermentative substrate, L-aspartate.

The present study further analyzes the effects of anoxia and freezing on the metabolism of *L. littorea* tissues but with a focus on enzymatic regulation to assess the control of carbohydrate metabolism at five key loci. We hypothesized that both stresses would induce stable modifications to regulatory enzymes of carbohydrate metabolism in order to promote MR depression and that at least some of these should be common to the two stresses, reflecting the important metabolic adjustments needed to deal with O_2 limitation. The results showed coordinated patterns of response by enzymes to both stresses, the occurrence of specific differences in the metabolic responses to anoxia versus freezing exposure, and the importance of reversible protein phosphorylation to coordinated enzyme regulation under both conditions.

Materials and methods

Chemicals and animals

All biochemicals and coupling enzymes were obtained from Sigma (St. Louis, Mo., USA) or Boehringer-Mannheim Montreal, PQ, Canada). Distilled, deionized water was used throughout for the preparation of biochemical solutions. All ATP, ADP and citrate stock solutions contained added $MgCl_2$ in 1:1 molar amounts. Marine periwinkles, *L. littorea*, were obtained from a local seafood supplier. Animals were acclimated in aerated artificial SW (1000 $mosmol \cdot l^{-1}$) at 5 °C for at least 3 weeks prior to experimentation.

Preparation of experimental animals

Control snails were sampled directly from SW at 5 °C. The shell was broken open and tissues were quickly excised, immediately frozen in

liquid N_2 and then transferred to –70 °C for storage. For freezing studies, initial tests were conducted to determine the length of exposure at –8 °C before freezing occurred. To do this a thermistor was placed in contact with the mantle and secured in place with tape around the shell. The thermistor was attached to a YSI telethermometer with output to a linear recorder and cooling in air at –8 °C was monitored over time until the initiation of freezing (nucleation) was observed as an abrupt jump in temperature (exotherm) due to release of the latent heat of fusion. These tests revealed that snails nucleated within 45-min at this temperature and in subsequent studies a 45-min cooling period was allowed before beginning to time the 12- or 24-h experimental freezing exposure times. Initial tests also showed 100% recovery of snails when transferred back to SW at 5 °C after these freezing exposures. For experimental freezing of large numbers of snails, animals were placed in a plastic box with damp paper toweling on the bottom and then put in an incubator set at – 8.0 °C. After freezing exposure, snails were rapidly dissected as described above. For experimental anoxia snails were placed in a jar with about 1 cm of SW in the bottom that had been previously bubbled with N_2 gas (100%) for 15 min. The jar was then flushed with N_2 gas for a further 20 min, after which the lid was sealed and the jar was returned to the 5 °C incubator. Anoxia exposure was continued for 12 or 24 h, followed by rapid dissection as described above. Initial tests showed that 100% of animals survived anoxia at both time points.

Tissue extraction and enzyme assay

Tissue extracts for enzyme assay were prepared by homogenizing frozen tissue samples with an Ultra-Turrax or a Polytron PT10 homogenizer using a 1:2 w/v ratio for hepatopancreas and a 1:10 w/v ratio for muscle. Homogenizing buffer for all enzymes except PDH contained ($mmol \cdot l^{-1}$): 20 imidazole-HCl (pH 7.2), 100 NaF, 10 EDTA, 10 EGTA, 0.1 phenylmethylsulphonyl fluoride (PMSF), and 15 2-mercaptoethanol.

Glycogen phosphorylase

Homogenates were centrifuged for 2 min at 5 °C in a Brinkman 5412 refrigerated microcentrifuge and the supernatant was removed for enzyme analysis. Optimal assay conditions for GP*a* were determined to be 50 $mmol \cdot l^{-1}$ potassium phosphate buffer (pH 7.0), 2 $mg \cdot ml^{-1}$ glycogen (previously dialyzed), 0.25 $mmol \cdot l^{-1}$ EDTA, 10 $mmol \cdot l^{-1}$ $MgCl_2$, 0.4 $mmol \cdot l^{-1}$ NADP, 10μ $mol \cdot l^{-1}$ glucose-1,6-bisphosphate, 0.7 $U \cdot ml^{-1}$ phosphoglucomutase and 0.2 $U \cdot ml^{-1}$G6P dehydrogenase (coupling enzymes previously dialyzed). For total phosphorylase ($a + b$), assays were conducted in the presence of 2 $mmol \cdot l^{-1}$ AMP. Preliminay experiments conducted in the presence versus absence of 5 $mmol \cdot l^{-1}$ caffeine to inhibit phosphorylase *b* showed that this did not change the measured percent *a*. One unit is defined as the amount of enzyme that produces 1 μmol glucose-1-phosphate·min^{-1} at 21 °C.

Glycogen synthetase

Homogenates were allowed to settle on ice for 30 min and then aliquots of the supernatant were removed for assay. Optimal assay conditions for the active *I* form were determined to be 20 $mmol \cdot l^{-1}$ imidazole-HCl buffer (pH 7.0), 5 $mmol \cdot l^{-1}$ UDPG, 2 $mg \cdot ml^{-1}$ oyster glycogen (previously dialyzed), 1 $mmol \cdot l^{-1}$ PEP, 5 $mmol \cdot l^{-1}$ $MgCl_2$, 20 $mmol \cdot l^{-1}$ KCl, 0.15 $mmol \cdot l^{-1}$ NADH, 1 $U \cdot ml^{-1}$ PK, and 1 $U \cdot ml^{-1}$ lactate dehydrogenase. For total synthetase activity ($I + D$), 5 $mmol \cdot l^{-1}$ G6P was included in the assay mixture. One unit is defined as the amount of enzyme that utilizes 1 μmol UDPG at 21 °C.

Phosphofructokinase and pyruvate kinase

Homogenates were centrifuged for 20 min at 25 000 g in a Sorvall RC-5B refrigerated centrifuge at 5 °C. The supernatant was removed and desalted by centrifugation (benchtop centrifuge at top speed for 1 min) through a 5-ml column of fine Sephadex G-25 equilibrated in homogenization buffer. Optimal assay conditions for maximal PFK activity were determined to be 20 $mmol \cdot l^{-1}$ imidazole-HCl (pH 7.2) containing ($mmol \cdot l^{-1}$): 1 F6P, 1 Mg.ATP, 5 $MgCl_2$, 50 KCl, 0.15 NADH, 2 $U \cdot ml^{-1}$ triosephosphate isomerase, 2.4 $U.ml^{-1}$ glycerol-3-phosphate dehydrogenase, and 0.4 $U \cdot ml^{-1}$ aldolase. Optimal assay conditions for maximal PK activity were 20 $mmol \cdot l^{-1}$ imidazole-HCl (pH 7.2) containing ($mmol \cdot l^{-1}$): 1 PEP, 2 ADP, 5 $MgCl_2$, 20 KCl, 0.15 NADH and 1 $U \cdot ml^{-1}$ lactate dehydrogenase. One unit is defined as the amount of enzyme that produces 1 μmol product (fructose-1,6-bisphosphate or pyruvate)·min^{-1} at 21 °C.

Pyruvate dehydrogenase

For PDH assay, tissues were homogenized in ice-cold 50 $mmol \cdot l^{-1}$ potassium phosphate buffer (pH 7.8) containing 2 $mmol \cdot l^{-1}$ 2-mercaptoethanol, 1 $mmol \cdot l^{-1}$ EDTA, 0.1 $mmol \cdot l^{-1}$ PMSF and 0.1% w/v Triton X-100. Homogenates were then placed at –20 °C for 2 h to break mitochondrial membranes. Samples were then removed, stored on ice and PDH activity was determined immediately after thawing. Optimal assay conditions were determined to be ($mmol \cdot l^{-1}$): 50 TRIS-HCl (pH 7.8), 1 $MgCl_2$, 0.5 EDTA, 0.2% (w/v) Triton X-100, 2.5 NAD^+, 0.2 thiamine pyrophosphate, 0.2 coenzyme A, 0.1 AABS and 0.05 $U \cdot ml^{-1}$ arylamine acetyltransferase. The reaction was initiated by the addition of an appropriate volume of homogenate and allowed to run for approximately 5 min to establish a background rate. Pyruvate (10 $mmol \cdot l^{-1}$) was then added and PDH*a* activity was measured. To measure total PDH activity, samples were incubated in the presence of $MgCl_2$ and $CaCl_2$ to stimulate the action of endogenous phosphatases to convert inactive PDH into active PDH. Initial trials varied salt concentrations, and incubation time and temperature to determine optimal conversion conditions. For foot PDH these were determined to be incubation with 10 $mmol \cdot l^{-1}$ $MgCl_2$ and 1 $mmol \cdot l^{-1}$ $CaCl_2$ on ice for 40 min, whereas optimal conditions for conversion for hepatopancreas PDH were incubation with 20 $mmol \cdot l^{-1}$ $MgCl_2$ and 1 $mmol \cdot l^{-1}$ $CaCl_2$ at 21 °C for 40 min. Total PDH activity was defined as the activity in the sample after the incubation period and was determined under the same conditions as the *a* form of the enzyme. One unit is defined as the amount of enzyme that produces 1 μmol acetyl-CoA·min^{-1} at 21 °C.

All enzyme assays, except PDH, were conducted by monitoring changes in concentration of NADH or NADPH at 340 nm using a Gilford 240 recording spectrophotometer. PDH activity was measured by following the decolourization of AABS at 460 nm. All assays were initiated by addition of the enzyme preparation and performed at 21 ± 1 °C, with a final cuvette volume of 1 ml.

DE-52 chromatography

To determine whether multiple forms of PK and PFK occurred in periwinkle tissues, extracts of foot and hepatopancreas were subjected to DE-52 Sephadex ion-exchange chromatography. For PK analysis, foot and hepatopancreas samples were homogenized 1:5 (w/v) in 20 $mmol \cdot l^{-1}$ imidazole-HCl buffer (pH 7.2) containing ($mmol \cdot l^{-1}$): 3 EDTA, 3 EGTA, 0.1 PMSF, 5 2-mercaptoethanol, 30 NaF and 10% w/v glycerol and then centrifuged and desalted as described previously. The filtrate was placed on a DE-52 Sephadex column (6 cm × 1.5 cm) equilibrated in the homogenization buffer,

and washed twice with several column volumes of this buffer. The column was then developed with a linear salt gradient of 0–250 mmol·l^{-1} KCl in 40 ml homogenization buffer and 1-ml fractions were collected and assayed for PK activity. The same procedure was followed for PFK analysis except that the buffers used also contained 5 mmol·l^{-1} F6P (initial tests showed that this substrate was needed to maintain enzyme stability during chromatography). Enzyme was eluted with a linear KCl gradient in 40 ml, 0–150 mmol·l^{-1} for foot and 0–400 mmol·l^{-1} for hepatopancreas extracts; 1-ml fractions were collected and assayed for PFK activity.

In vitro phosphorylation and dephosphorylation

To prepare enzyme extracts for studies of in vitro phosphorylation or dephosphorylation of PFK and PK, tissue samples were homogenized 1:5 w/v in 20 mmol·l^{-1} imidazole-HCl (pH 7.2) containing 15 mmol·l^{-1} 2-mercaptoethanol, 10% w/v glycerol and 0.1 mmol·l^{-1} PMSF, followed by centrifugation and Sephadex G25 desalting as described previously. Extracts were then divided into three aliquots and incubated under the following conditions for PK(mmol·l^{-1}): (1) 20 imidazole-HCl buffer (pH 7.0), 30 NaF, 3 EDTA, 3 EGTA, and 5 2-mercaptoethanol (control); (2) 20 imidazole-HCl buffer (pH 7.0), 30 NaF, 1 cyclic 3′5′AMP, 10 $MgCl_2$ and 10 ATP; or (3) 20 imidazole-HCl buffer (pH 7.0), and 10 $MgCl_2$. For PFK studies all three incubation conditions also contained 5 mmol·l^{-1} F6P. All samples were incubated overnight at 4 °C and then PFK and PK elution patterns on DE-52 Sephadex columns as analyzed as described above.

Kinetics and statistics

K_m was determined by fitting data to the Hill equation using a non-linear least squares regression program (Brooks 1992). A modified Hill equation that introduced a V_0 term (rate at zero activator concentration) was used for determining K_a. I_{50} (inhibitor concentration that reduces enzyme velocity by 50%) was obtained from plots of rate against inhibitor concentration. Data are presented as means ± SEM with statistical testing using analysis of variance (Model I) followed by the Dunnett's test (2-tailed).

Results

Glycogen phosphorylase

Table 1 shows the effects of anoxia and freezing on GP activities in foot muscle and hepatopancreas of *L. littorea* including the total activity (active *a* + inactive *b* forms), the activity of phosphorylase *a*, and the percent *a*. In foot muscle, both stresses resulted in significant decreases (25–50%) in the total activity of GP measurable. Phosphorylase *a* activity also decreased significantly in all but the snails frozen for 12 h. However, the net changes in total and active phosphorylase resulted in significant increases in the percentage of the enzyme in the *a* form after 12 h of anoxia or freezing, but after 24 h of stress the%*a* in foot was reduced again and not significantly different from control values. GP in hepatopancreas showed a different pattern of response to anoxia or freezing. Both total phosphorylase and phosphorylase *a* activities were unaffected by anoxia exposure and also by 12 h freezing. However, both activities were significantly elevated (60–80%) in hepatopancreas of snails frozen for 24 h. A small increase in the%*a* was seen in 12 h anoxic hepatopancreas but was unaltered under other conditions.

Glycogen synthetase

Table 2 shows the total activity of GS in *L. littorea* tissues, the sum of the active G6P-independent *I* form and the inactive G6P-dependent *D* form, as well as the percentage of activity in the *I* form after 24 h of anoxia or freezing exposure. In foot muscle total GS activity was sharply reduced under either stress to 38 or 53% of control values in anoxic and frozen animals, respectively. However, the percentage of the enzyme in the active *I* form increased significantly. Hepatopancreas also showed an increase in the%*I* under both stresses but only a small decrease in total GS activity during freezing.

Phosphofructokinase

Ion exchange chromatography on DE-52 Sephadex was used to determine whether PFK from *L. littorea* tissues existed in multiple molecular forms. Extracts from control, 24 h frozen and 24 h anoxic tissues were

Table 1 Total glycogen phosphorylase (*a* + *b*) and phosphorylase *a* activities and the percentage of the active *a* form in tissues of *L. littorea* during freezing and anoxia. Values are mean ± SEM; $n = 4 - 6$

	Foot			Hepatopancreas		
	Total *a* + *b* U·g ww^{-1}	Phos. *a* U·g ww^{-1}	%*a*	Total *a* + *b* U·g ww^{-1}	Phos. *a* U·g ww^{-1}	%*a*
Control	2.09 ± 0.15	0.73 ± 0.09	34.6 ± 2.3	0.26 ± 0.015	0.10 ± 0.01	36.8 ± 2.7
Anoxic 12 h	1.06 ± 0.07[a]	0.45 ± 0.05[b]	42.1 ± 2.3[b]	0.24 ± 0.036	0.12 ± 0.02	48.4 ± 3.6[b]
Anoxic 24 h	1.57 ± 0.13[b]	0.44 ± 0.02[b]	28.1 ± 1.4	0.22 ± 0.008	0.07 ± 0.01	32.2 ± 4.0
Frozen 12 h	1.16 ± 0.13[a]	0.65 ± 0.07	55.7 ± 1.5[a]	0.29 ± 0.039	0.12 ± 0.01	41.6 ± 2.8
Frozen 24 h	1.17 ± 0.18[a]	0.45 ± 0.04[a]	39.5 ± 2.1	0.41 ± 0.025[a]	0.17 ± 0.02[b]	41.7 ± 1.1

[a] Values are significantly different from corresponding control values, $P < 0.01$; [b]$P < 0.05$

Table 2 Total glycogen synthetase (I + D) activities and the percentage of the active *I* form in tissues of *L. littorea* during freezing and anoxia. Values are mean ± SEM; $n = 4 - 6$

	Foot		Hepatopancreas	
	U·g ww^{-1}	%I	U·g ww^{-1}	%I
Control	1.01 ± 0.11	10.5 ± 1.3	0.19 ± 0.01	26.7 ± 1.7
Anoxic 24 h	0.38 ± 0.03[a]	19.4 ± 2.2[b]	0.17 ± 0.02	45.4 ± 7.5[b]
Frozen 24 h	0.53 ± 0.08[a]	28.7 ± 3.1[a]	0.13 ± 0.01[b]	47.2 ± 2.8[b]

[a] Values are significantly different from corresponding control values, $P < 0.01$; [b] $P < 0.05$

analyzed using elution with a 0–150 mmol·l^{-1} KCl gradient for foot muscle and a 0–400 mmol·l^{-1} gradient for hepatopancreas. In all cases, PFK from foot muscle eluted as a single peak at about 50 mmol·l^{-1} KCl, whereas hepatopancreas PFK eluted as a single peak at about 90 mmol·l^{-1} KCl. Thus, while tissue-specific forms of PFK probably exist, there was no evidence of multiple forms within a single tissue.

PFK is frequently a target of reversible protein phosphorylation but the consequence of covalent modification (such as changes in enzyme kinetic properties or enzyme binding to myofibrils) are very different for different tissue-specific isozymes; hence, kinetic analysis is not a consistent indicator of PFK covalent modification. However, because the addition or removal of phosphate from the enzyme must alter its net charge, a change in the elution profile of PFK on DE-52 Sephadex ion exchange should be a consistent indicator of enzyme covalent modification. To determine whether *L. littorea* PFK could be modified by reversible phosphorylation, extracts of control foot and hepatopancreas were incubated in vitro under conditions that would promote the phosphorylation or dephosphorylation of the enzyme by endogenous protein kinases or phosphatases. Three incubation conditions were compared (each in 20 mmol·l^{-1} imidazole-HCl, pH 7.0, with 5 mmol·l^{-1} F6P) (mmol·l^{-1}): (1) control: 30 NaF, 3 EDTA, 3 EGTA, 5 2-mercaptoethanol; (2) phosphorylation-promoting: 1 cyclic 3′5′AMP, 10 ATP, 10 $MgCl_2$; and (3) dephosphorylation-promoting: 10 $MgCl_2$. After incubation, extracts were analyzed by DE-52 chromatography to determine whether a shift in the elution peak had occurred. However, both foot and hepatopancreas extracts again showed only a single peak, similar to the unincubated extracts, and with no difference in the elution profile and peak for any of the three incubation conditions.

Table 3 summarizes the kinetic properties of PFK from crude extracts of foot muscle and hepatopancreas of control *L. littorea*. The two enzymes showed different kinetic properties. PFK from foot muscle had a higher affinity for the substrate F6P (K_m was only half of the hepatopancreas value) and was more sensitive to the allosteric activators AMP and F2,6P_2, as well as to activation by K^+. However, the foot muscle enzyme was also more sensitive to inhibition by Mg.ATP, Mg.citrate and PEP than the hepatopancreas enzyme.

Table 3 Kinetic properties of PFK from foot muscle and hepatopancreas of control *L. littorea*. Values are mean ± SEM; $n = 4$ different animals

	Foot Muscle[a]	Hepatopancreas[b]
K_m F6P (mmol·l^{-1})	0.22 ± 0.03	0.44 ± 0.01[c]
K_a AMP (mmol·l^{-1})	0.016 ± 0.002	0.045 ± 0.001[c]
K_a F2,6P_2 (μmol·l^{-1})	1.7 ± 0.2	8.0 ± 0.6[c]
K_aKCl (mmol·l^{-1})	11 ± 0.7	17 ± 0.5[c]
K_aAspartate (mmol·l^{-1})	14 ± 1.2	16 ± 1.6
I_{50} Mg.ATP (mmol·l^{-1})	0.30 ± 0.03	0.51 ± 0.06[c]
I_{50} Mg.Citrate (mmol·l^{-1})	1.6 ± 0.14	4.9 ± 0.19[c]
I_{50} PEP (mmol·l^{-1})	0.90 ± 0.10	1.34 ± 0.08[c]

[a] Foot muscle K_m (F6P) was determined at 0.1 mmol·l^{-1} Mg.ATP; I_{50} and K_a at 0.2 mmol·l^{-1} F6P and 0.1 mmol·l^{-1} Mg.ATP
[b] Hepatopancreas K_m (F6P) was determined at 0.1 mmol·l^{-1} Mg.ATP; I_{50} and K_a at 0.4 mmol·l^{-1} F6P and 0.1 mmol·l^{-1} Mg.ATP
[c] Significantly different from the corresponding foot muscle value by the Student's *t*-test (2-tailed), $P < 0.025$

Pyruvate kinase

Analysis of PK from *L. littorea* tissues by DE-52 Sephadex ion exchange chromatography showed a different result. Two peaks of enzyme activity were present in extracts of control tissues (Figs. 1, 2). In foot muscle, the two peaks eluted from DE-52 at approximately 30 and 90 mmol·l^{-1} KCl for peak I and II, respectively, whereas in hepatopancreas extracts the peaks eluted at about 50 and 110 mmol·l^{-1} KCl, respectively. The proportions of the two enzyme forms changed when animals were subjected to 24 h anoxia or freezing exposures. Under either stress in foot muscle only a single peak, corresponding to the peak I form of controls, was detected. The same was true for anoxic hepatopancreas, but hepatopancreas from freezing-exposed snails still showed the two forms.

To determine whether the two forms of PK in *L. littorea* tissues were interconvertible by reversible phosphorylation, extracts of control foot and hepatopancreas were incubated in vitro under conditions that would promote protein phosphorylation by endogenous protein kinases (added 1 mmol·l^{-1} cAMP + 10 mmol·l^{-1} Mg.ATP + 30 mmol·l^{-1} NaF) or protein dephosphorylation by endogenous protein phosphatases (added 10 mmol·l^{-1} $MgCl_2$), as described in "Materials and methods". Figure 3 shows that incubation of foot extracts under conditions that promote enzyme phosphorylation resulted in a shift from two peaks in control extracts to a single peak eluting at the higher KCl concentration characteristic of the peak II form of controls. Conditions of in vitro phosphorylation had the same effect on PK in

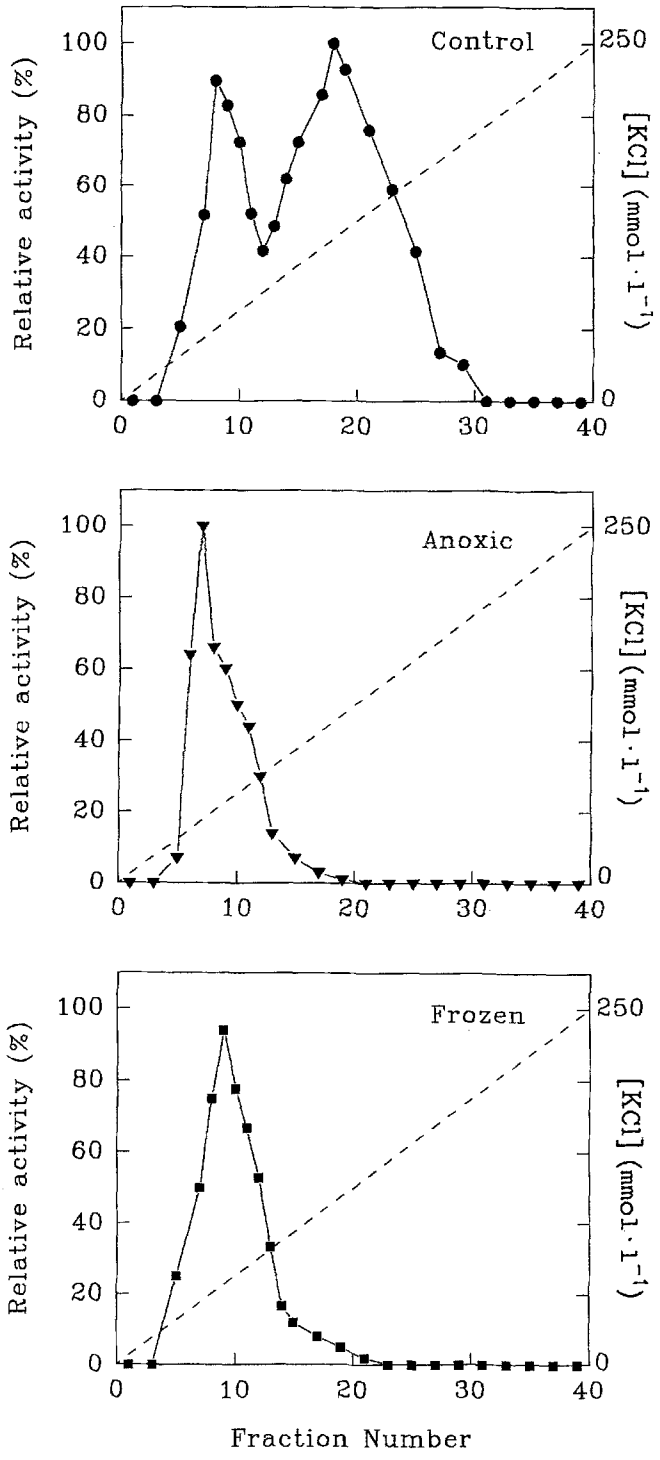

Fig. 1 Chromatography of *L. littorea* foot muscle pyruvate kinase on DE-52 Sephadex. Symbols represent PK from control (*circles*), 24 h frozen (*squares*) and 24 h anoxic (triangles) snails. PK was eluted with a linear gradient of 0–250 mmol·l^{-1} KCl (*dashed line*)

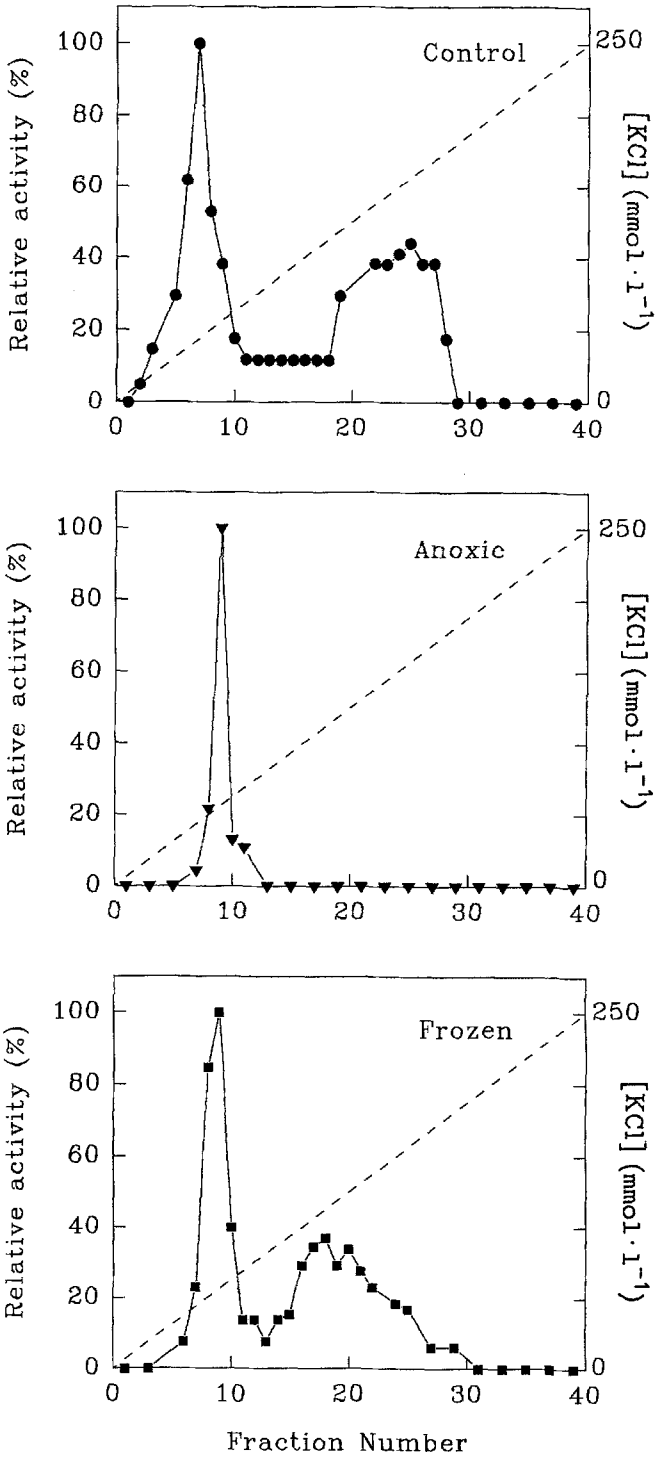

Fig. 2 Chromatography of *L. littorea* hepatopancreas PK on DE-52 Sephadex. Other conditions as in Fig. 1

hepatopancreas extracts (Fig. 4). Conditions that promoted in vitro dephosphorylation of PK had the opposite effect. In foot muscle extracts the proportions of the two peaks changed, with a strong decrease in the proportion of peak II enzyme relative to peak I. The foot extract incubation was repeated at 21 °C (the above incubations were at 4 °C) to try to promote a greater conversion of the enzyme but results were identical with the those of Fig. 3C. In hepatopancreas, dephosphorylation-promoting conditions led to the disappearance of the peak II form with all activity in a single peak corresponding to the peak I form (Fig. 4C).

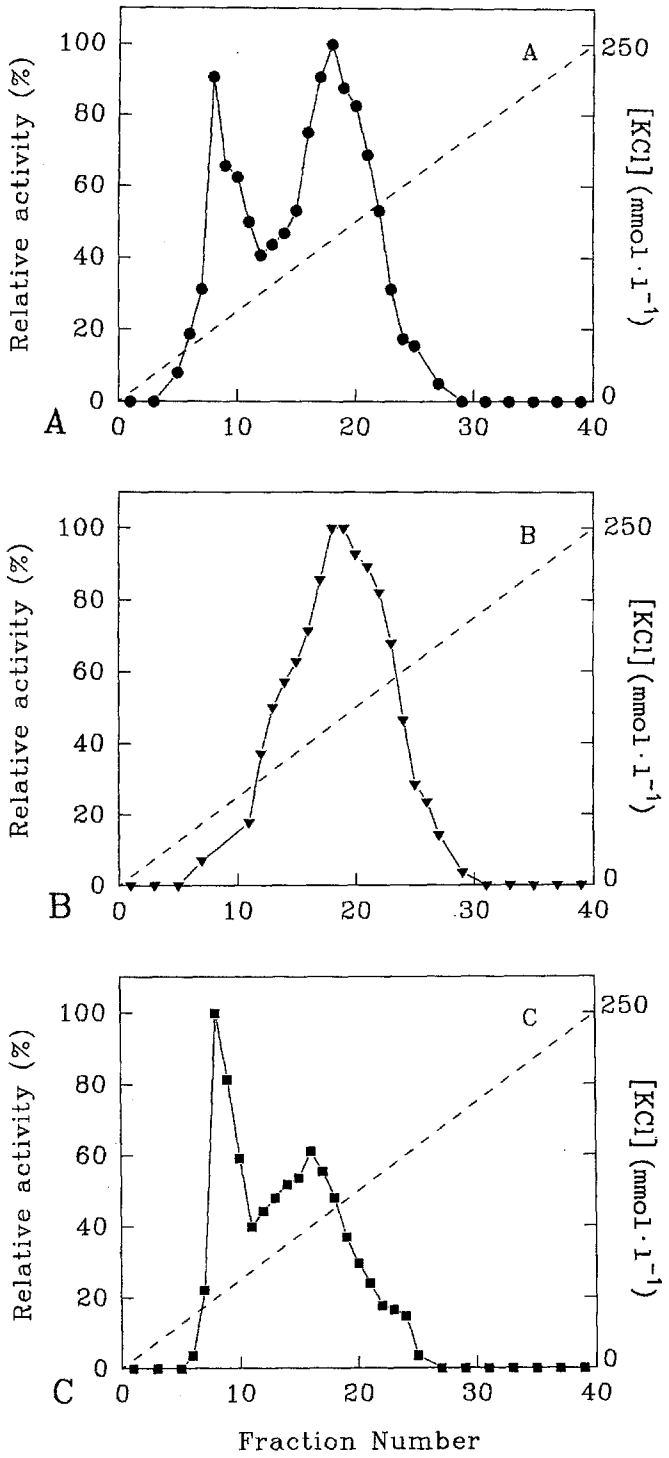

Fig. 3A–C Effect of in vitro incubation under conditions promoting enzyme phosphorylation or dephosphorylation on the elution profile of foot muscle PK from control *L. littorea*. Incubations were conducted overnight at 4 °C followed by DE-52 chromatography with a linear gradient of 0–250 mmol·l^{-1} KCl (*dashed line*): **A** control incubation (*circles*; conditions are 20 mmol·l^{-1} imidazole-HCl, pH 7, 30 mmol·l^{-1} NaF, 3 mmol·l^{-1} EDTA, 3 mmol·l^{-1} EGTA, 5 mmol·l^{-1} 2-mercaptoethanol); **B** in vitro phosphorylation (*triangles*; conditions are 20 m mol·l^{-1} imidazole-HCl, pH 7, 1 mmol·l^{-1} cyclic 3′5′AMP, 10 mmol·l^{-1} ATP, 10 mmol·l^{-1} $MgCl_2$, 30 mmol·l^{-1} NaF); **C** in vitro dephosphorylation (*squares*; conditions are 20 mmol·l^{-1} imidazole-HCl, pH 7, 10 mmol·l^{-1} $MgCl_2$)

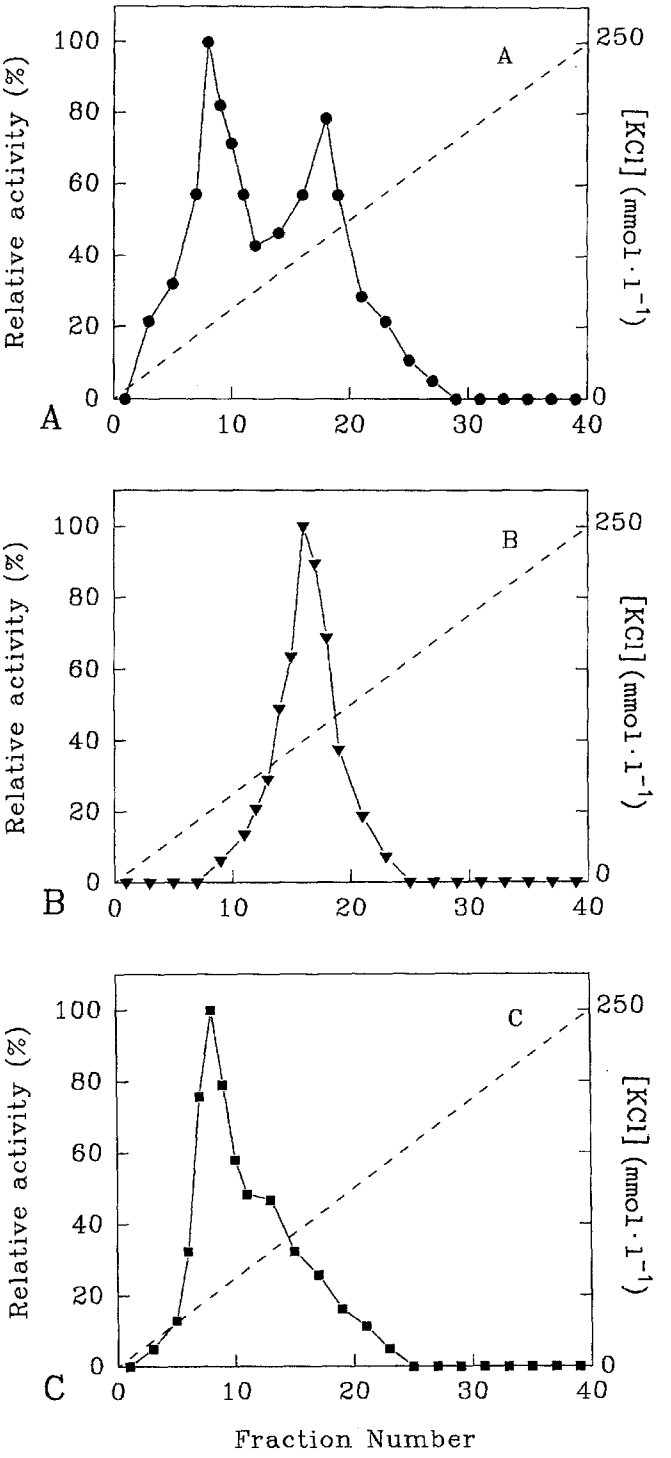

Fig. 4A–C Effect of in vitro incubation under conditions promoting enzyme phosphorylation or dephosphorylation on the elution profile of hepatopancreas PK from control *L. littorea*. Other information as in Fig. 3

Table 4 Kinetic properties of two forms of PK from foot muscle of control *L. littorea*. Values are mean ± SEM, $n = 4$ different animals. K_m PEP was determined at 2 mmol·l^{-1} ADP; K_m ADP at 1 mmol·l^{-1} PEP. I_{50} was determined at 1 mmol·l^{-1} ADP and 0.3 mmol·l^{-1} PEP. (*N.E.* the peak II enzyme showed no inhibition by L-alanine even at the highest alanine concentration tested, i.e. 40 mmol·l^{-1})

	Peak I	Peak II
K_m PEP (mmol·l^{-1})	0.21 ± 0.03	0.10 ± 0.02[a]
K_m ADP (mmol·l^{-1})	0.20 ± 0.01	0.15 ± 0.02[a]
I_{50} L-alanine (mmol·l^{-1})	0.38 ± 0.02	N.E.
I_{50} KCl (mmol·l^{-1})	208 ± 40	356 ± 25[a]
I_{50}Mg.Citrate (mmol·l^{-1})	2.79 ± 0.40	3.82 ± 0.34
I_{50} Mg.ATP (mmol·l^{-1})	2.78 ± 0.45	4.04 ± 0.55
I_{50} NH_4Cl (mmol·l^{-1})	244 ± 50	231 ± 37
$I_{50}(NH_4)_2SO_4$ (mmol·l^{-1})	138 ± 26	112 ± 22

* Significantly different from the corresponding Peak I values by the Student's *t*-test (2-tailed), $P < 0.05$

Table 5 Effect of freezing and anoxia exposures on the percentage of pyruvate dehydrogenase in the active *a* form in *L. littorea* tissues. Values are mean ± SEM, $n = 4$–6

	Foot %[a]	Hepatopancreas %[a]
Control	72.6 ± 1.3	69.5 ± 2.8
Anoxic 24 h	61.5 ± 3.0[a]	49.3 ± 1.4[b]
Frozen 24 h	59.4 ± 3.6[a]	55.3 ± 3.4[a]

[a] Significantly different from corresponding control values, $P < 0.05$; [b] $P < 0.01$

Kinetic properties of PK peaks I and II from control foot muscle are shown in Table 4. The two forms were separated on DE-52 using a 0–125 mmol·l^{-1} KCl gradient in order to maximize the peak separation. Peak II PK showed a significantly greater affinity for both substrates, PEP and ADP, than did the peak I enzyme; K_m PEP of peak I PK was double the peak II value and K_m ADP was 33% higher for the peak I enzyme. In addition, peak II PK was less sensitive to KCl inhibition and was not inhibited by L-alanine, whereas peak I PK was highly sensitive to L-alanine inhibition. Thus, peak I PK showed an I_{50} for L-alanine of 0.38 mmol·l^{-1}, whereas peak II PK showed no inhibition even at 40 mmol·l^{-1} L-alanine.

Pyruvate dehydrogenase

Table 5 shows the effects of 24 h anoxia or freezing exposure on the percentage of PDH in the active *a* form in foot muscle and hepatopancreas. Both experimental stresses led to significant decreases in the percentage of active PDH. However, total PDH activity remained constant in both tissues under either experimental condition: maximal activity was 0.149 ± 0.008 U·g ww^{-1} in foot muscle and 0.066 ± 0.006 U·g ww^{-1} in hepatopancreas.

Discussion

Covalent modification of enzymes via protein phosphorylation or dephosphorylation is a powerful mechanism for making rapid and large changes in the activity state of individual enzymes as well as for creating coordinated responses by numerous enzymes and pathways to exogenous stimuli (Woodford et al. 1992). The mechanism is widely used in animal systems that undergo metabolic arrest to coordinate reversible transitions to and from the hypometabolic state (Storey and Storey 1990). The role of reversible phosphorylation in anoxia-induced glycolytic rate depression has been studied in various species of marine molluscs (Storey 1993). The present study shows that this mechanism is also an integral part of the response to environmental stress by the periwinkle *L. littorea*, including both anoxia and freezing stress. Both stresses induced changes in GP, GS, PK and PDH in both tissues. In all cases, these changes were consistent with covalent modification of the enzymes; changes in the percentage of active enzyme for GP, GS and PDH are well known to be determined by reversible protein phosphorylation in animal systems (Cohen 1980; Woodford et al. 1992). In most cases, the enzyme modifications induced by anoxia or freezing were those that led to less active enzymes and thus appeared to contribute to MR depression in the stress state. In general, the enzyme response to freezing was similar in pattern to the response to anoxia although there were various quantitative differences in enzyme parameters between the two states. Hence, a number of the enzymatic responses to freezing may be attributed to the effects of the anoxia/ischemia created by the frozen state. However, exceptions to this were seen in hepatopancreas; 24 h freezing led to an increase in GP activity and PK remained in two peaks, contrary to the results for anoxia. These enzymatic responses may serve somewhat different demands on carbohydrate metabolism by the two states. Differences in end product accumulation in anoxic versus frozen foot muscle of *L. littorea* also indicated differences in the response to the two stresses (T.A. Churchill and K.B. Storey, unpublished data).

Glycogen is the primary fuel of anaerobic metabolism in molluscs (de Zwaan 1983). Regulation of substrate supply must be precise and provide a balanced response in each tissue to two pressures: (1) the stress-induced depression of MR that lowers ATP demand, and (2) in some cases, a switch to anoxic glycogen fermentation from aerobic oxidation of alternative fuels. The importance of each factor in each tissue

determines the relative change in glycogenolytic rate and the amount of active GP expressed. Foot muscle GP showed a compromise between these two pressures (Table 1). Thus, the total activity of GP in foot decreased in line with an overall metabolic arrest but the %*a* was elevated after 12 h anoxia or freezing; nonetheless, the result was a net decrease in phosphorylase *a* activity. After 24 h exposure time, however, the %*a* dropped to further lower phosphorylase *a* activity as part of the profound metabolic arrest that develops over time. The change in total GP activity in foot muscle could result from at least two mechanisms: (1) degradation of GP by proteolysis, or (2) dissociation of the subunits of GP to produce an inactive enzyme that is not detectable even in the presence of the allosteric activator AMP. Interestingly, Ebberink and Salimans (1982) reported that GP*b* purified from the posterior adductor muscle of *Mytilus edulis* consisted of two forms, probably a monomer and a dimer. Whereas mammalian muscle GP is primarily regulated by the interconversion of *a* and *b* forms, unusual features of marine mollusc GP have been noted. These include the presence of a third form of GP in *Pecten maximus*, which requires AMP at a concentration 20-fold higher than phosphorylase *b* for activation, and unusual AMP activation kinetics for phosphorylase *b* from *M. edulis* adductor muscle (Vazquez-Baanate and Rosell-Perez 1979; Ebberink and Salimans 1982).

Similar to the situation for GP, glycogen synthetase in *L. littorea* tissues also appeared to be controlled by two mechanisms–the *I* and *D* interconversion, as is well known in mammalian systems, and changes in the total activity of the enzyme (Table 2). Both of these mechanisms have been associated with GS control in molluscs previously, with seasonal changes in both total GS activity and the %*I* occurring in *M. edulis* (Gabbott et al. 1979; Gabbott and Whittle 1986). In foot the total activity of the enzyme was again strongly reduced in anoxia, in line with an overall MR depression, but the %*I* increased, resulting in net activities of the active *I* form of 0.106, 0.074 and 0.152 $U \cdot g\,ww^{-1}$ in control, 24 h anoxic and 24 h frozen muscle, respectively. The %*I* was also elevated during freezing and anoxia in hepatopancreas without any large change in total activity. The reason for the apparently greater activities of active GS *I* under stress conditions is not known.

PFK is often considered to be the rate-controlling enzyme of glycolysis and is frequently a target of reversible phosphorylation. Regulation of whelk PFK during anoxia by both reversible phosphorylation and association with subcellular macromolecules has been previously documented (Plaxton and Storey 1986; Whitwam and Storey 1991). However, analysis of both foot muscle and hepatopancreas PFK revealed only a single enzyme form in each tissue whose elution profile was unaltered by either anoxia or freezing exposures in vivo or by in vitro incubations under conditions promoting phosphorylation or dephosphorylation. Furthermore, a preliminary kinetic characterization of foot and hepatopancreas PFK from control, 48 h frozen and 48 h anoxic snails indicated that neither stress significantly affected the K_m for F6P or K_a for AMP or $F2,6P_2$ (S. Greenway and K. Storey, unpublished results). Hence, it appears that PFK in *L. littorea* tissues does not undergo stress-induced covalent modification. However, both the elution profile on DE-52 (indicating different charge characteristics) and the kinetic parameters of foot and hepatopancreas PFK differed substantially suggesting the presence of tissue-specific isozymic forms.

PK is a critical enzyme in the regulation of anaerobic metabolism in marine molluscs. Anoxia-induced phosphorylation strongly reduces enzyme activity, thereby directing the switch from the aerobic flow of glycolytic carbon into the tricarboxylic acid cycle (via PK and PDH) to the anaerobic processing of PEP via phosphoenolpyruvate carboxykinase (PEPCK) and into the reactions of succinate synthesis. Ion exchange chromatography on DE-52 Sephadex revealed the presence of two forms of PK in foot muscle and hepatopancreas and both anoxia and freezing exposures strongly altered their proportions. In both tissues, anoxia exposure led to a complete conversion of the enzyme to the peak I form. Freezing had the same effect on foot muscle PK but did not affect the proportions of the two forms in hepatopancreas. Chromatographic or electrophoretic separation of two forms of PK has also been reported for other marine molluscs (Siebenaller 1979; Holwerda et al. 1983; Plaxton and Storey 1984; DeVooys and Holwerda 1986: Michaelidis et al. 1988) and the effect of anoxia in changing the proportions of these two forms has been documented in these and other species [summarized in Storey (1993)]. Hence, the effect of anoxia on *L. littorea* PK was not unexpected. However, the present data are the first demonstration that extracellular freezing also changes the form of PK implying that freezing, or the ischemia that it induces, triggers many of the same biochemical regulatory responses as does anoxia. The hepatopancreas results also show that not all responses to anoxia are mimicked by freezing exposure. The reasons for this must await further investigation but may be two fold: (1) metabolic needs of freezing-exposed tissues (such as synthesis of end products with cryoprotective actions) may be different than those of anoxic tissues, or (2) freezing imposes a more gradual hypoxia/anoxia on cells than does placement into a N_2 gas atmosphere and metabolic changes during freezing (such as enzyme phosphorylation) may develop more slowly than during anoxia.

The present data also revealed a intriguing aspect of PK control in *L. littorea*. Previous studies of PK in anoxia-tolerant molluscs were consistent with an active, dephosphorylated enzyme form in aerobic tissues (with a low K_m PEP and little inhibition by L-alanine)

which was phosphorylated in anoxia to produce a subactive form with a high K_m PEP and that showed strong inhibition by L-alanine (Holwerda et al. 1983; Plaxton and Storey 1984; Michaelidis et al. 1988; Whitwam and Storey 1990a, 1990b). The data for *L. littorea* show a very different result. The in vitro studies with extracts of control tissues clearly show that incubation under conditions that promoted protein kinase action shifted PK into the peak II form, whereas incubations that promoted in vitro protein phosphatase action increased the proportion of the peak I form (Figs. 3, 4). Hence, peak I appears to be the low phosphate enzyme form and peak II the high phosphate enzyme form. These results are consistent with the anion exchange data: the more negatively charged protein (phosphorylated) would be eluted at the higher salt concentration. The effect of anoxia or freezing exposures, then, is to stimulate the dephosphorylation of *L. littorea* PK. Furthermore, the kinetic analysis given in Table 4 shows that the low-phosphate, peak I form of muscle PK is clearly the less active form. Compared with the peak II enzyme, peak I had a reduced affinity for PEP and ADP, increased sensitivity to K^+ inhibition, and was highly sensitive to L-alanine inhibition. With in vivo levels of alanine of about 12 $mmol \cdot l^{-1}$ in foot (T.A. Churchill and K.B. Storey, unpublished data), the peak I enzyme (I_{50} alanine = 0.38 $mmol \cdot l^{-1}$) would be strongly inhibited in anoxic or frozen tissue, whereas the peak II enzyme in aerobic tissues (showing no inhibition even at 40 $mmol \cdot l^{-1}$ alanine) would be insensitive to the cellular alanine content. Thus, like other mollusc species, PK is interconverted between an active, aerobic form and a subactive, anoxic form that is functionally inactive under in vivo alanine concentrations. What is different about the *L. littorea* situation, however, is that the active, aerobic form is phosphorylated PK and the inactive, anoxic form is dephosphorylated PK. How such a situation arose and whether it also occurs in other species remains to be investigated.

Under aerobic conditions, pyruvate derived from glycolysis enters the mitochondria where it is committed, via the action of PDH, to oxidation by the TCA cycle. During anoxia the TCA cycle and oxidative phosphorylation cannot function and it would seem logical, therefore, to inhibit pyruvate entry into the system. Although inhibition of PK would largely prevent glycolytic pyruvate from being available to the mitochondria, pyruvate is also made intramitochondrially during anoxia by the action of malic enzyme (de Zwaan 1983). Some of the pyruvate produced is directed into alanine synthesis (via transamination) and in some species a portion is converted to acetate which does require PDH function to produce acetyl-CoA. Hence, both the net metabolic suppression and regulation of the intramitochondrial fate of pyruvate can be achieved by control over PDH. PDH activity was very low in *L. littorea* tissues, possibly suggesting low mitochondrial numbers, and the effects of both anoxia and freezing reduced the percentage of active PDH. PDH is controlled by reversible phosphorylation in other animals, the dephosphorylated form of the enzyme being the active form (Woodford et al. 1992; Brooks and Storey 1992). Thus, it appears that anoxia or freezing stimulated the phosphorylation of PDH to reduce the percentage of active enzyme as part of the overall metabolic suppression induced by either stress.

Acknowledgements Thanks to J.M. Storey for editorial commentary on the manuscript. Supported by a research grant from the N.S.E.R.C. Canada to K.B.S.

References

Aarset AV (1982) Freezing tolerance in intertidal invertebrates (a review). Comp Biochem Physiol 73A: 571–580

Brooks SPJ (1992) A simple computer program with statistical tests for the analysis of enzyme kinetics. Biotechniques 13: 906–911

Brooks SPJ, Storey KB (1992) Properties of pyruvate dehydrogenase from the land snail, *Otala lactea*: control of enzyme activation during estivation. Physiol Zool 63: 620–633

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

De Vooys CGN, Holwerda DA (1986) Anaerobic metabolism in sublittoral living *Mytilus galloprovincialis* Lam. in the Mediterranean–III. The effect of anoxia and osmotic stress on some kinetic parameters of adductor muscle pyruvate kinase. Comp Biochem Physiol 85B: 217–221

Ebberink RHM, Salimans M (1982) Control of glycogen phosphorylase activity in the posterior adductor sea mussel *Mytilus edulis*. J Comp Physiol B 148: 27–33

Famme P, Knudsen J, Hansen ES (1981) The effect of oxygen on the aerobic-anaerobic metabolism of the marine bivalve, *Mytilus edulis*. Mar Biol Lett 2: 345–351

Gabbott PA, Cook PA, Whittle MA (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 PA, Whittle MA (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

Gilles R (ed) (1979) Mechanisms of osmoregulation in animals. Wiley-Interscience, New York

Holwerda DA, Veenhof PR, Van Heugten HAA, Zandee DI (1983) Modification of mussel pyruvate kinase during anaerobiosis and after temperature acclimation. Molec Physiol 3: 225–234

Kanwisher JW (1955) Freezing in intertidal animals. Biol Bull 109: 56–63

Klutymans JH, de Bont AMT, Kruitwagen ECJ, Ravestein HJL, Veenhof PR (1983) Anaerobic capacities and anaerobic energy production of some Mediterranean bivalves. Comp Biochem Physiol 75B: 171–179

Livingstone DR, Zwaan A de (1983) Carbohydrate metabolism in gastropods. In: Wilbur KM (ed) The Mollusca. Academic Press, New York, pp 177–242

Loomis SH (1987) Freezing in intertidal invertebrates: an update. Cryo-Lett 8: 186–195.

Michaelidis B, Gaitanki C, Beis I (1988) Modification of pyruvate kinase from the foot muscle of *Patella caerulea* (L) during anaerobiosis. J Exp Zool 248: 264–271

Murphy DJ (1983) Freezing resistance in intertidal invertebrates. Annu Rev Physiol 45: 289–299

Murphy DJ, Johnson LC (1980) Physical and temporal factors influencing the freezing tolerance of the marine snail *Littorina littorea* (L.). Biol Bull 158: 220–232

Plaxton WC, Storey KB (1984) Purification and properties of pyruvate kinase from red muscle tissue of the channelled whelk, *Busycotypus canaliculatum*. Eur J Biochem 143: 257–265

Plaxton WC, Storey KB (1986) Glycolytic enzyme binding and metabolic control in anaerobiosis. J Comp Physiol 156B: 635–640

Seibenaller JF (1979) Regulation of pyruvate kinase in *Mytilus edulis* by phosphorylation-dephosphorylation. Mar Biol Lett 1: 105–110

Shick JM, Zwaan A de, de Bont AM (1983) Anoxic metabolic rate in the mussel *Mytilus edulis* L. estimated by simultaneous direct calorimetry and biochemical analysis. Physiol Zool 56: 56–63

Storey KB (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

Storey KB (1993) Molecular mechanisms of metabolic arrest in mollusks. In: Hochachka PW et al (eds) Surviving hypoxia: mechanisms of control and adaptation. CRC Press, Boca Raton, pp 253–269

Storey KB, Storey JM (1990) Facultative metabolic rate depression: molecular regulation and biochemical adaptation in anaerobiosis, hibernation, and estivation. Q Rev Biol 65: 145–174

Vaquez-Baanante I, Rosell-Perez M (1979) In vitro studies of *Pecten maximus* glycogen phosphorylase and the interconversion of their forms. Comp Biochem Physiol 62B: 381–387

Wieser W (1980) Metabolic end products in three species of marine gastropods. J Mar Biol Assoc UK 60: 175–180

Whitwam RE, Storey KB (1990a) Organ-specific analysis of the time-course of covalent modification of pyruvate kinase during anaerobiosis in a marine whelk. Physiol Zool 63: 222–234

Whitwam RE, Storey KB (1990b) Pyruvate kinase from the land snail *Otala lactea*: regulation by reversible phosphorylation during estivation and anoxia. J Exp Biol 154: 321–337

Whitwam RE, Storey KB (1991) Regulation of phosphofructokinase during estivation and anoxia in the land snail, *Otala lactea*. Physiol Zool 64: 595–610

Woodford TA, Taylor SJ, Corbin JD (1992) The biological functions of protein phosphorylation and dephosphorylation. In: Bittar E (ed) Fundamentals of medical cell biology, vol 3B. JAI Press, Greenwich, CT, pp 453–607

Zwaan A de (1983) Carbohydrate catabolism in bivalves. In: Wilbur KM (ed) The Mollusca. Academic Press, New York, pp 137–175

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

Communicated by L.C.-H. Wang