J. exp. Biol. 154, 321–337 (1990)
Printed in Great Britain © The Company of Biologists Limited 1990

PYRUVATE KINASE FROM THE LAND SNAIL *OTALA LACTEA*: REGULATION BY REVERSIBLE PHOSPHORYLATION DURING ESTIVATION AND ANOXIA

BY ROSS E. WHITWAM AND KENNETH B. STOREY

Institute of Biochemistry and Department of Biology, Carleton University, Ottawa, Ontario, Canada K1S 5B6

Accepted 7 June 1990

Summary

Pyruvate kinase (PK) from tissues of the desert snail *Otala lactea* (Müller) undergoes a stable modification of its kinetic properties during estivation or in response to anoxia stress. In foot muscle and mantle, the kinetic changes induced by either state were virtually identical and were consistent with a less active enzyme form in estivation or anoxia: $S_{0.5}$ PEP increased, and I_{50} values for Mg–ATP and L-alanine decreased, compared to the enzyme in control (aroused) snails. Estivation and anoxia also changed the properties of PK from hepatopancreas; some changes were consistent with a more active enzyme form ($S_{0.5}$ PEP decreased, I_{50} values for Mg–ATP and L-alanine increased) but the enzyme lost all sensitivity to the potent activator fructose-1,6-bisphosphate. A time course of changes in I_{50} Mg–ATP for foot PK and $S_{0.5}$ PEP for hepatopancreas PK revealed that estivation-induced changes in enzyme properties occurred between 12 and 48 h after snails were deprived of access to food and water, whereas the reversal of these changes occurred within as little as 10 min in foot muscle after arousal was initiated. The molecular basis of the stable modification of PK kinetics appears to be reversible protein phoshorylation. The action of added cyclic-AMP-dependent protein kinase on foot or hepatopancreas PK from control (aroused) snails changed PK kinetic parameters to those characteristic of the enzyme form in estivating animals; the addition of stimulators of endogenous cyclic-GMP-dependent protein kinase or protein kinase C had the same effect. Conversely, treatment with added phosphatases reconverted the properties of foot muscle PK from estivating snails to those characteristic of the control enzyme. The data suggest that reversible phosphorylation control over the activity state of regulatory enzymes of glycolysis is one mechanism contributing to the overall metabolic rate depression of the estivating state.

Introduction

Metabolic rate depression is a common strategy used by animals to survive

Key words: control of glycolysis, metabolic rate depression, covalent modification, gastropod metabolism.

periodic exposures to extreme environmental conditions that are unsuitable for normal life functions. An escape to a hypometabolic or dormant state is the frequent response to temperature extremes, aridity, lack of oxygen or lack of food and underlies such phenomena as estivation, hibernation, facultative anaerobiosis, anhydrobiosis and diapause (Hochachka and Guppy, 1987). The biochemical mechanisms that control metabolic arrest have been the subject of much recent work by our laboratory and we have found that the molecular controls serving anoxia-induced glycolytic rate depression (a reverse Pasteur effect) are conserved across a phylogenetically diverse group of facultative anaerobes including marine molluscs, goldfish and freshwater turtles (reviewed in Storey, 1988*a*). The same metabolic control principles could also apply to other forms of metabolic arrest.

Numerous species of terrestrial snails exploit seasonally arid environments by entering a state of estivation whenever water and food supplies disappear (Schmidt-Nielsen *et al.* 1971; Burky *et al.* 1972; Herreid, 1977; Horne, 1979; Umezurike and Iheanacho, 1983; Vorhaben *et al.* 1984). During estivation aerobic metabolic rate is reduced to 10–30 % of the resting rate when aroused, an adaptation that allows endogenous fuel reserves to be stretched to support months (or even years) of dormancy. The present study examines estivation in the land snail *Otala lactea* (Pulmonata, Helicidae). Physiological aspects of estivation have been well analyzed in this species. Estivation is characterized by hypoxia, hypercapnia, extracellular acidosis and a reduction of aerobic metabolic rate by as much as 85 % (Herreid, 1977; Barnhart, 1983, 1986*a*,*b*; Barnhart and McMahon, 1987, 1988). Carbohydrate reserves appear to be the primary fuel supporting metabolism in estivating snails (Umezurike and Iheanacho, 1983; Livingstone and de Zwaan, 1983; Cedeno-Leon, 1984) and, thus, control over glycolytic rate must play a central role in the transition to and from the dormant state.

One of the key mechanisms involved in metabolic depression in facultative anaerobes is the post-translational modification of regulatory enzymes *via* protein phosphorylation or dephosphorylation reactions to bring about rapid and readily reversible changes in the activity of regulatory enzymes (Storey, 1988*a*). This mechanism was first identified for pyruvate kinase (PK) in marine molluscs (Siebenaller, 1979; Holwerda *et al.* 1983; Plaxton and Storey, 1984*a*) but we now know that anoxia-induced covalent modification coordinates the activities of glycogen phosphorylase, 6-phosphofructo-1-kinase, 6-phosphofructo-2-kinase and PK to create a balanced depression of glycolytic rate (Storey, 1984, 1988*a*,*b*; L. Bosca and K. Storey, unpublished data). The present study examines the regulation of PK in *O. lactea* tissues. This species also has a substantial tolerance for anoxia [snails readily recovered after 4 days under a nitrogen gas atmosphere (Brooks and Storey, 1990*a*; this study)] and so we analyzed the effects of both estivation and anoxia on the properties of PK. The results show that in both states PK undergoes a stable modification that changes enzyme properties to those of a less active enzyme form. The changes in PK properties induced by either estivation or anoxia were virtually identical and could be mimicked by the actions of protein kinases on the enzyme *in vitro*. It is evident, then, that enzyme

regulation *via* covalent modification may be a major control mechanism supporting metabolic rate depression during estivation.

Materials and methods

Chemicals and animals

All biochemicals and coupling enzymes were obtained from Sigma Chemical Co., Boehringer Mannheim Corp., or J. T. Baker Co. Snails, *O. lactea*, were provided by Dr M. C. Barnhart from an introduced population at Playa del Rey in Los Angeles County, California. In the laboratory, estivation is readily induced by the removal of water and food and, conversely, dormancy is broken by the reintroduction of these items. While being held in the laboratory, the snails were usually kept in their dormant state in a dry container and without food. Every 3 weeks they were aroused by the reintroduction of water (a light spraying plus damp paper towels) and the addition of food (cabbage sprinkled with chalk dust). Arousal usually lasted only a few days before the snails once again entered dormancy voluntarily.

All snails used for experiments were awakened from dormancy and given continuous access to food and regular spraying with water to ensure that they remained aroused for at least 2 days. Control snails (aroused and active) were sampled directly from this population. To impose anoxia, aroused snails were placed in a jar containing damp paper towels and small a piece of cabbage. The jars were then flushed with nitrogen gas (95 % N_2/5 % CO_2) and sealed with a screw-on lid and parafilm. A wire mesh barrier prevented the snails from climbing more than half-way up the jar. Under anoxic conditions the snails distended themselves out of their shells and pressed the foot out flat against the walls of the jar; they never ate under anoxia. To induce estivation, aroused snails were removed and placed in dry containers without food or paper toweling but with mesh lids to permit continuous aeration. In the dry environment the snails withdrew into their shells within about 2 h. For the time course of dormancy and recovery, the length of dormancy was timed from the moment aroused snails (previously active for at least 2 days) were placed in the dry jar. After 22 days of constant dormancy, the length of recovery was timed from the moment that food and water were reintroduced, as described above. Because there was considerable variation in the rate at which individual snails egressed from their shells and resumed movement, we always chose from the experimental pool the four animals that were most active at each time point on the recovery time course.

Tissue preparation

To dissect snails the shell was broken open, the head end was severed from the remainder of the foot, and then foot, mantle and hepatopancreas were rapidly removed (within 1 min). Tissues were immediately frozen in liquid nitrogen and stored at −60 °C until use. For the preparation of enzyme extracts, tissues were homogenized 1:5 (w/v) in homogenization buffer containing 100 mmol l^{-1} NaF (to

inhibit protein phosphatases), 5 mmol l^{-1} EDTA, 5 mmol l^{-1} EGTA (to inhibit protein kinases), 0.1 mmol l^{-1} phenylmethylsulfonyl fluoride (a protease inhibitor), 30 mmol l^{-1} 2-mercaptoethanol, and 40% glycerol (v/v) (an enzyme stabilizer) in 50 mmol l^{-1} imidazole–HCl (pH 7.0); a Polytron PT 10 or an Ultra-Turrax homogenizer was used. 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 (IEC benchtop centrifuge at top speed for 1 min) through a 5 ml column of Sephadex G-25, as described by Helmerhorst and Stokes (1980). The column was equilibrated in 40 mmol l^{-1} imidazole–HCl (pH 7.0) with 5 mmol l^{-1} EDTA, 15 mmol l^{-1} 2-mercaptoethanol and 20% v/v glycerol. The filtrate was removed, stored on ice, and used for the analysis of enzyme kinetic properties.

Enzyme assay

PK activity was monitored by coupled enzyme assay following NADH utilization at 340 nm using a Gilford 240 recording spectrophotometer. Assay conditions for maximal PK activity in all three tissues were 50 mmol l^{-1} imidazole–HCl (pH 7.0) buffer, 3 mmol l^{-1} ADP, 3 mmol l^{-1} phosphoenolpyruvate (PEP), 50 mmol l^{-1} KCl, 5 mmol l^{-1} $MgCl_2$, 0.2 mmol l^{-1} NADH and 1 i.u. ml^{-1} lactate dehydrogenase (previously dialysed when used for hepatopancreas PK assays) in a final cuvette volume of 1 ml. All assays were initiated by the addition of enzyme preparation. I_{50} values (the concentration of inhibitor producing a 50% decrease in enzyme rate) and K_a values (the activator concentration producing half-maximal activation) were determined at suboptimal PEP concentrations, 0.1 and 0.5 mmol l^{-1}, respectively. One unit of enzyme activity is defined as the amount of enzyme that catalyzes the utilization of 1 μmol of PEP per min at 20°C.

In vitro *phosphorylation or dephosphorylation*

Snails for these experiments were sampled from a second group of animals collected about 16 months after the animals that were used for all other experiments. The preparation of enzyme extracts from control *versus* estivating (4 weeks) animals followed the same protocol as that given above up to the point of centrifugation, except that tissues from freshly killed animals were used and tissues were homogenized in a 1:2.5 (w/v) ratio with buffer. After centrifugation, the supernatant was passed through a Sephadex G-25 spun-column (as described above) but with the column equilibrated in 40 mmol l^{-1} imidazole–HCl (pH 7.0), 20% v/v glycerol, 10 mmol l^{-1} potassium phosphate, 10 mmol l^{-1} 2-mercaptoethanol and 0.1 mmol l^{-1} EDTA. The filtrate was collected, divided into seven samples, and each sample was diluted 1:1 in a solution containing 40 mmol l^{-1} imidazole–HCl (pH 7.0), 10 mmol l^{-1} 2-mercaptoethanol and 20% v/v glycerol and one of the following to stimulate phosphorylation or dephosphorylation of the enzyme. (a) For cyclic-AMP-dependent phosphorylation; 20 mmol l^{-1} $MgCl_2$, 40 mmol l^{-1} NaF, 4 mmol l^{-1} cyclic 3′5′-adenosine monophosphate (cyclic AMP), 2 mg ml^{-1} cyclic-AMP-dependent protein kinase (from

bovine heart, 2.0 pmol l^{-1} phosphate per μg of protein phosphorylating activity) and 10 mmol l^{-1} ATP. (b) For cyclic-GMP-dependent phosphorylation: 20 mmol l^{-1} $MgCl_2$, 40 mmol l^{-1} NaF, 8 mmol l^{-1} cyclic 3′5′-guanosine monophosphate (cyclic GMP) and 10 mmol l^{-1} ATP. (c) To stimulate phosphorylation by protein kinase C: 20 mmol l^{-1} $MgCl_2$, 40 mmol l^{-1} NaF, 2 mmol l^{-1} $CaCl_2$, 20 μg ml^{-1} phorbol 12-myristate 13-acetate, 80 μg ml^{-1} brain extract (phospholipid source; Type 1, Sigma Chemical Co. no. 1502) and 10 mmol l^{-1} ATP. (d) For protein dephosphorylation by alkaline phosphatases: 25 mmol l^{-1} KCl, 5 mmol l^{-1} EDTA, 15 mmol l^{-1} $MgCl_2$ and 50 i.u. alkaline phosphatase (grade 1, from calf intestine). (e) For protein dephosphorylation by alkaline and acid phosphatases: 25 mmol l^{-1} KCl, 5 mmol l^{-1} EDTA, 15 mmol l^{-1} $MgCl_2$, 50 i.u. alkaline phosphatase (grade 1, from calf intestine), 2 mg ml^{-1} acid phosphatase (type 1, from wheat germ, 0.46 i.u. mg^{-1} activity) and 1 mg ml^{-1} spermidine. (f) For stimulation of endogenous protein phosphatases: 25 mmol l^{-1} KCl, 5 mmol l^{-1} EDTA, 15 mmol l^{-1} $MgCl_2$, and 1 mg ml^{-1} spermidine (a protein phosphatase activator). (g) For the control: 40 mmol l^{-1} NaF and 5 mmol l^{-1} EDTA.

All samples were then incubated for 16 h at 30 °C for the foot muscle enzyme or 20 °C for the hepatopancreas enzyme (hepatopancreas PK was not stable at the higher incubation temperature). Samples were then passed through Sephadex G-25 spun-columns equilibrated in 45 mmol l^{-1} imidazole–HCl (pH 7.0), 0.5 mmol l^{-1} EDTA, 10 mmol l^{-1} mercaptoethanol and 20 % v/v glycerol. The filtrates were used for subsequent kinetic analyses.

Calculations and statistics

$S_{0.5}$ values (the substrate concentration producing half-maximal enzyme velocity), n_H (the Hill coefficient) and V_{max} (enzyme maximal velocity) values were determined by fitting the data to the Hill equation using a nonlinear least squares regression program for the computer. A modified Hill equation which included a V_0 term (rate at zero activator) was used for determining K_a values. I_{50} values were estimated from plots of reaction velocity *vs* [inhibitor]. Results are presented as means±S.E.M. Tests for significant differences between values used the Student's *t*-test.

Results

The kinetic properties of PK from *O. lactea* were analyzed in three tissues, foot muscle, mantle and hepatopancreas. In all tissues the enzyme underwent a stable modification (i.e. the modification was retained after gel filtration had removed low molecular weight metabolites) that changed enzyme properties during both estivation and anoxia.

Foot muscle pyruvate kinase

The properties of PK from foot muscle of control (2 days aroused with food and water), estivating (22 days) and anoxic (45 h of nitrogen gas exposure) *O. lactea*

Table 1. *Kinetic properties of pyruvate kinase from the foot muscle of control (aroused 2 days), estivating (22 days) and anoxic (45 h)* Otala lactea

	Control	Estivating	Anoxic
Units g^{-1} wet mass	13±1	17.6±0.7*	16±2
$S_{0.5}$ PEP (mmol l^{-1})	0.062±0.001	0.089±0.008†	0.094±0.007†
n_H	1.62±0.06	1.43±0.06	1.38±0.07
$S_{0.5}$ ADP (mmol l^{-1})	0.34±0.01	0.40±0.03*	0.35±0.03
n_H	1.30±0.02	1.28±0.07	1.14±0.07
I_{50} alanine (mmol l^{-1})	5.0±0.5	2.1±0.2†	3.3±0.3*
I_{50} Mg–ATP (mmol l^{-1})	13.9±0.7	8.0±0.4†	8.2±0.6†

Results are means±S.E.M. for determinations on enzyme preparations from N=4–6 different animals for each treatment group.

*Significantly different from the corresponding control value using the Student's t-test, $P<0.05$; † $P<0.005$.

are shown in Table 1. PK was modified during both estivation and anoxia; the changes induced by these states were very similar both qualitatively and quantitatively. In both situations, enzyme affinity for PEP decreased ($S_{0.5}$ increased by about 50 %) and inhibition by L-alanine and Mg–ATP increased (I_{50} values decreased). PK from estivating snails also showed small, but significant, increases in $S_{0.5}$ ADP and in the activity of the enzyme in the tissue. Foot muscle PK was not activated by fructose-1,6-bisphosphate (F1,6P_2); levels up to 1 mmol l^{-1} F1,6P_2 were tested, compared to physiological levels of less than 0.030 mmol l^{-1} (Churchill and Storey, 1989).

Mantle pyruvate kinase

The properties of PK from *O. lactea* mantle are shown in Table 2. Overall, the kinetic properties of the mantle enzyme were very similar to those of foot muscle

Table 2. *Kinetic properties of pyruvate kinase from the mantle tissue of control (aroused 2 days), estivating (22 days) and anoxic (45 h)* Otala lactea

	Control	Estivating	Anoxic
Units g^{-1} wet mass	8±1	12±2	10±2
$S_{0.5}$ PEP (mmol l^{-1})	0.041±0.001	0.075±0.001*	0.100±0.010*
n_H	1.08±0.04	1.47±0.11*	1.40±0.09*
$S_{0.5}$ ADP (mmol l^{-1})	0.34±0.01	0.42±0.02*	0.44±0.02*
n_H	1.22±0.04	1.22±0.08	1.54±0.03*
I_{50} alanine (mmol l^{-1})	4.6±0.4	8.0±0.6*	2.5±0.2*
I_{50} Mg–ATP (mmol l^{-1})	15.0±0.6	10.0±0.9*	5.3±0.3*

Results are means±S.E.M. for determinations on enzyme preparations from N=4–6 different animals for each treatment group.

*Significantly different from the corresponding control value using the Student's t-test, $P<0.005$.

PK, particularly the properties of the enzyme from control (aroused) snails. Again, F1,6P_2 did not activate the enzyme. All the kinetic properties tested, with the exception of V_{max} (and n_H for $S_{0.5}$ ADP in estivation), were altered significantly during both estivation and anoxia. Affinity for both substrates decreased; $S_{0.5}$ for ADP increased about 25 % whereas $S_{0.5}$ PEP increased 1.8-fold in dormant and 2.4-fold in anoxic snails. I_{50} for Mg–ATP decreased significantly under both experimental conditions but the change was again more pronounced in anoxia. The I_{50} for L-alanine responded differently in the two experimental states, increasing by 74 % in estivating animals but decreasing by 46 % in anoxic snails.

Hepatopancreas pyruvate kinase

The properties of PK from hepatopancreas of *O. lactea* are shown in Table 3. The properties of the enzyme in control (aroused) hepatopancreas were substantially different from those of the enzyme in foot or mantle. Enzyme affinity for PEP was much lower ($S_{0.5}$ about 10-fold higher) and inhibition by alanine was stronger (I_{50} five-fold lower). In addition, the enzyme responded to F1,6P_2 activation with a K_a of only 0.004 mmol l^{-1}. All the properties of hepatopancreas PK were changed significantly in estivation and anoxia and the changes induced by either hypometabolic state were the same in both direction and magnitude.

Table 3. *Kinetic properties of pyruvate kinase from the hepatopancreas of control (aroused 2 days), estivating (22 days) and anoxic (45 h)* Otala lactea

	Control	Estivating	Anoxic
Units g^{-1} wet mass	3.5±0.6	5.7±0.7*	8.0±0.8†
$S_{0.5}$ PEP (mmol l^{-1})	0.57±0.03	0.20±0.03†	0.17±0.02†
n_H	1.84±0.15	1.41±0.05*	1.37±0.11*
$S_{0.5}$ ADP (mmol l^{-1})	0.25±0.01	0.38±0.01†	0.34±0.02†
n_H	1.94±0.13	1.29±0.08†	1.51±0.05*
I_{50} Mg–ATP (mmol l^{-1})	4.7±0.5	13±1†	11.8±0.8†
I_{50} alanine (mmol l^{-1})	0.97±0.05	3.9±0.7†	4.3±0.7†
K_a F1,6P_2 (mmol l^{-1})	0.004±0.0004	≫5	≫5
Relative activities			
no addition	100	100	100
+10 mmol l^{-1} Pi	123±7	130±12	106±11
+10 mmol l^{-1} NH_4^+	113±2	111±5	108±3
+5 mmol l^{-1} proline	55±2‡	74±8	116±8
+5 mmol l^{-1} glycerol-3-phosphate	82±2	88±5	118±5
+5 mmol l^{-1} phenylalanine	98±2	155±14	92±3

Results are means±S.E.M. for determinations on enzyme preparations from N=3–6 different animals for each treatment group.

Relative activities are expressed compared to activities with no additions, set at 100.

* Significantly different from the corresponding control value using the Student's t-test, $P<0.05$; † $P<0.005$.

‡ Significantly different from the activity with no addition, $P<0.05$.

F1,6P_2, fructose-1,6-bisphosphate; Pi, inorganic phosphate.

Estivation or anoxia resulted in an increase in enzyme V_{max}, a decrease in $S_{0.5}$ PEP, increases in $S_{0.5}$ ADP, I_{50} Mg–ATP and I_{50} alanine, and a change from high sensitivity to F1,6P_2 activation to no effect of F1,6P_2 at levels up to 5 mmol l^{-1}.

A number of other metabolites were tested for their effects on hepatopancreas PK (Table 3). Most had little effect on enzyme velocity, including phenylalanine, which is a well known inhibitor of mammalian liver PK. Proline (at 5 mmol l^{-1}) reduced the activity of PK from active snails by 45 % but had little effect on the enzyme from estivating or anoxic snails.

Time course of changes in pyruvate kinase properties during estivation

To analyze the progress of PK modification during estivation and subsequent arousal, selected properties of PK from foot and hepatopancreas were monitored over the course of 22 days estivation followed by 2 days of arousal. The parameters chosen were those that showed a large change between control and estivating states, namely the I_{50} value for Mg–ATP for foot muscle PK and the $S_{0.5}$ for PEP for hepatopancreas PK (Tables 1 and 3). Fig. 1 shows the time course of changes in I_{50} Mg–ATP of foot muscle PK. This parameter remained constant for the first 12 h after snails were moved to a dry container without food and water but dropped to the low value, characteristic of dormancy, within 48 h. The I_{50} remained at this level for the subsequent 20 days of estivation. When food and water were reintroduced, however, the I_{50} value for Mg–ATP of foot PK rebounded to control levels within 10 min. At about this time snails began to emerge from their shells and within 30 min they were actively crawling and feeding.

The time course of changes in $S_{0.5}$ PEP for hepatopancreas PK is similar during entry into dormancy. As Fig. 2 shows, $S_{0.5}$ remains high over the first 12 h but was

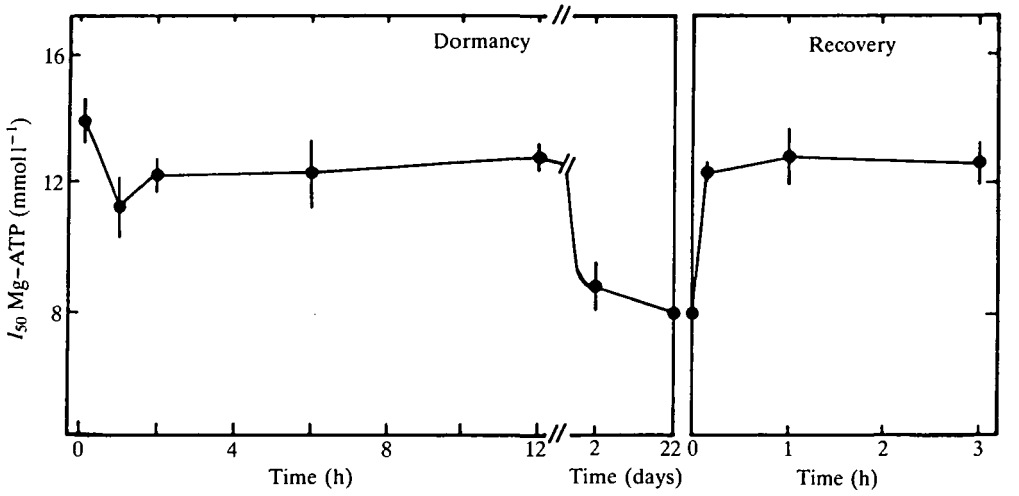

Fig. 1. Time course of the change in the I_{50} value for Mg–ATP of pyruvate kinase from *Otala lactea* foot muscle during entry into and arousal (recovery) from estivation. Data are means±S.E.M., N=4 animals at each time point.

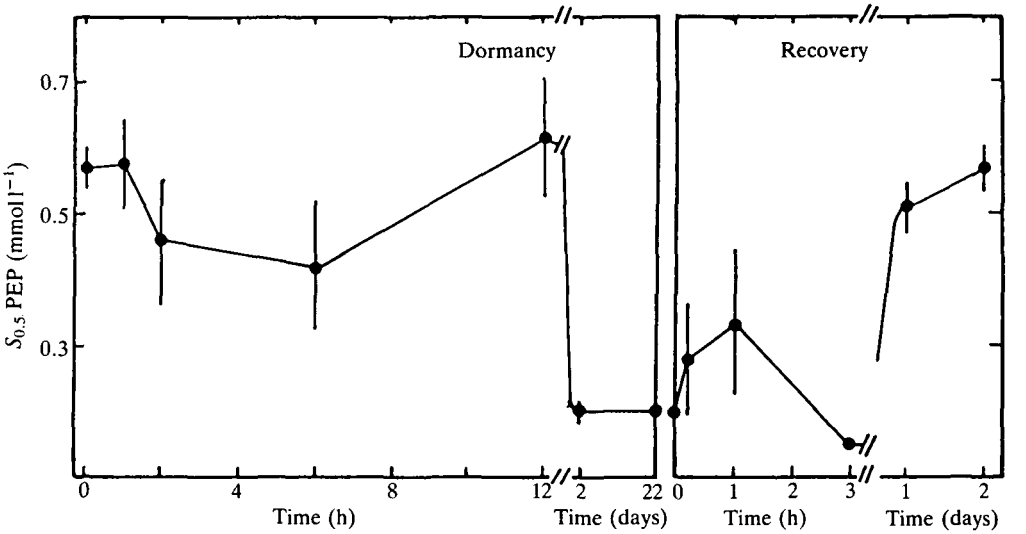

Fig. 2. Time course of the change in the $S_{0.5}$ value for phosphoenolpyruvate (PEP) of pyruvate kinase from *Otala lactea* hepatopancreas during entry into and arousal (recovery) from estivation. Data are means±S.E.M., N=4 animals at each time point.

reduced to a value characteristic of the estivating state within 48 h after placing snails into a dry environment. Changes in hepatopancreas PK during arousal, however, took a longer time than was seen for foot PK, occurring between 3 and 24 h after the reintroduction of food and water.

In vitro *phosphorylation and dephosphorylation of snail pyruvate kinase*

To determine whether the changes in PK kinetic properties occurring during estivation were the result of a post-translational modification of the enzyme *via* protein phosphorylation or dephosphorylation, PK from foot or hepatopancreas was incubated under various regimens to stimulate protein kinase or protein phosphatase actions on the enzyme. When PK from the foot muscle of control, active snails (2 days aroused) was incubated for 16 h in the presence of Mg–ATP, cyclic AMP and cyclic-AMP-dependent protein kinase, the I_{50} value for alanine dropped to the value seen in either dormant or anoxic snails (Table 4). A similar significant decrease in I_{50} was seen when the enzyme was incubated with cyclic GMP plus Mg–ATP to stimulate the activity of endogenous cyclic-GMP-dependent protein kinase or with Mg–ATP, Ca^{2+}, phorbol 12-myristate 13-acetate and brain phospholipid fraction to stimulate protein kinase C. However, when the enzyme from active snails was incubated with alkaline phosphatase or with alkaline phosphatase plus acid phosphatase and the phosphatase stimulator spermidine, no change in the I_{50} value was seen. PK from foot muscle of estivating (4 weeks) snails responded in the opposite direction. Phosphatase treatments significantly increased the I_{50} value for alanine, restoring it to the value seen for PK from active snails. Stimulators of endogenous cyclic-GMP-dependent protein kinase or protein kinase C had no effect on foot muscle PK from dormant snails,

Table 4. *Changes to the I_{50} for L-alanine of foot muscle pyruvate kinase from* Otala *brought about by* in vitro *treatment with protein phosphorylating or dephosphorylating agents: pyruvate kinase from aroused (2 days)* versus *estivating (4 weeks) snails*

	I_{50} alanine (mmol l^{-1})	
	Aroused	Estivating
Control	7.27 (6.89, 7.65)	3.10 (2.86, 3.34)
+Ca^{2+}+PMA+Mg–ATP	5.32 (4.78, 5.86)*	4.27 (4.91, 3.63)
+cyclic GMP+Mg–ATP	3.92 (3.46, 4.37)*	3.36 (3.49, 3.23)
+cyclic AMP+Mg–ATP+ cyclic-AMP-protein kinase	3.41 (3.40, 3.42)†	2.15 (2.04, 2.25)*
+alkaline phosphatase	6.78 (6.29, 7.27)	7.20 (5.99, 8.41)*
+alkaline phosphatase+ acid phosphatase+spermidine	6.82 (6.34, 7.29)	7.65 (8.58, 6.72)*

Conditions for incubations are given in Materials and methods. Results are means of $N=2$ determinations with actual values in parenthesis.

PMA, phorbol 12-myristate 13-acetate.

* Significantly different from the corresponding value for the control incubation, $P<0.05$; † $P<0.005$.

but treatment with added cyclic-AMP-dependent protein kinase further lowered the I_{50} value of the enzyme.

Similar incubations were carried out with PK from hepatopancreas. When PK from the hepatopancreas of active snails was given treatments that promote protein kinase action, the enzyme showed a fourfold decrease in $S_{0.5}$ PEP, similar to the effect of estivation on the enzyme (Table 3). The effect was similar for PK treated with added cyclic-AMP-dependent protein kinase or with the stimulators of endogenous cyclic-GMP-dependent protein kinase or protein kinase C (Table 5). In addition, the activating effect of $F1,6P_2$ was lost after protein kinase action; 0.05 mmol l^{-1} $F1,6P_2$ produced a 3.8-fold activation of PK from control hepatopancreas but the ratio $(V+F1,6P_2)/V$ was not significantly different from 1.0 after protein kinase treatments. However, treatments of the control enzyme with phosphatases or stimulation of endogenous phosphatases by spermidine had no effect on the properties of PK from control hepatopancreas. Equivalent treatments were carried out with hepatopancreas PK from estivating snails but neither $S_{0.5}$ PEP nor enzyme activation by $F1,6P_2$ was changed by any of the *in vitro* incubations. Parenthetically we note that the values for $S_{0.5}$ PEP for the enzyme in untreated incubations of extracts from active *versus* estivating snails differed somewhat from the comparable values reported in Table 3; we were unable to find a reason for this behavior except that a different population of snail was used for the *in vitro* incubations (see Materials and methods). However, in both cases the effect of estivation was the same, a strong decrease in $S_{0.5}$.

Table 5. *Changes in kinetic parameters of hepatopancreas pyruvate kinase* Otala lactea *brought about by* in vitro *treatments with protein phosphorylating or dephosphorylating agents; pyruvate kinase from aroused (2 days)* versus *estivating (4 weeks) snails*

	$S_{0.5}$ PEP (mmol l^{-1})		$(V+F1,6P_2)/V$	
	Active	Estivating	Active	Estivating
Control	1.70 (1.55, 1.86)	0.074 (0.070, 0.078)	3.38 (3.27, 3.50)	1.03 (1.00, 1.05)
+Ca^{2+}+PMA+Mg–ATP	0.39 (0.33, 0.45)†	0.052 (0.056, 0.061)	1.18 (1.14, 1.22)†	1.04 (1.04, 1.03)
+cyclic GMP+Mg–ATP	0.45 (0.37, 0.54)*	0.054 (0.048, 0.059)	1.31 (1.28, 1.33)†	0.98 (0.93, 1.02)
+cyclic AMP+Mg–ATP+ cyclic AMP-protein kinase	0.35 (0.34, 0.36)†	0.061 (0.056, 0.065)	1.11 (1.00, 1.23)†	1.00 (0.98, 1.03)
+spermidine	1.67 (1.54, 1.81)	0.050 (0.044, 0.056)	3.8 (2.4, 5.2)	0.99 (0.98, 0.100)
+alkaline phosphatase	1.60	0.058 (0.053, 0.062)	ND	1.01 (1.00, 1.03)
+alkaline phosphatase+acid phosphatase+spermidine	1.58 (1.52, 1.64)	0.063 (0.059, 0.066)	3.0 (2.7, 3.3)	1.23 (1.03, 1.42)

Results are means of $N=2$ determinations with actual values in parentheses, except where indicated.
Enzyme velocity ratios $(V+F1,6P_2)/V$ were performed with 1 mmol l^{-1} PEP, 3 mmol l^{-1} Mg–ADP and ±0.05 mmol l^{-1} $F1,6P_2$.
PMA, phorbol 12-myristate 13-acetate. ND, not determined.
* Significantly different from the corresponding control treatment, $P<0.05$; † $P<0.005$.

Discussion

The properties of foot and mantle PK from *O. lactea* were very similar and not unlike the muscle form of PK from many species (Storey and Hochachka, 1975; Berglund and Humble, 1979; Storey, 1985). Thus, affinity for PEP was high ($S_{0.5}$ 0.040–0.090 mmol l^{-1}) and well within the range of PEP concentrations in *O. lactea* muscle (0.190–0.330 mmol kg^{-1} wet mass) (Churchill and Storey, 1989), the enzyme was unaffected by F1,6P_2, and Mg–ATP inhibition was weak. Like PK in most aerobic muscles, the enzyme in foot or mantle of *O. lactea* appears to be poised to be highly responsive to changes in glycolytic flux associated with muscular work. However, the enzyme displayed a significant sensitivity to inhibition by L-alanine with an I_{50} of 5 mmol l^{-1} and, as is the case for anoxia-tolerant marine molluscs (Hochachka and Mustafa, 1972; Plaxton and Storey, 1984*a*), such alanine sensitivity may be the key to enzyme inactivation in the hypometabolic state (see below).

The enzyme from hepatopancreas, however, showed very different kinetic properties, and probably represents a different isozymic form of PK, as also occurs in marine gastropods (Plaxton and Storey, 1985*a*). PK from *O. lactea* hepatopancreas showed a much lower affinity for PEP ($S_{0.5}$=0.570 mmol l^{-1}) compared to foot and mantle PK and also compared to PEP levels in hepatopancreas *in vivo* (0.09–0.15 mmol l^{-1}) (Churchill and Storey, 1989). The enzyme was also highly sensitive to F1,6P_2 activation and alanine inhibition. These properties are characteristic of PK in liver, or liver-like organs, from other species (Storey, 1985; Plaxton and Storey, 1985*b*; Engstrom *et al.* 1987) and they allow PK activity to be modulated *in vivo* in a multifunctional organ that includes both glycolytic and gluconeogenic carbon flow.

PK in all three tissues of *O. lactea* undergoes a stable modification of the enzyme protein upon entry into estivation or in response to anoxia. In all three organs this resulted in significant alterations to enzyme kinetic properties. The mechanism of this stable modification appears to be enzyme phosphorylation. Thus, the *in vitro* incubation studies revealed that kinetic changes characteristic of the control to estivating transition were mimicked by the actions of protein kinases on the control enzyme. Phosphatase treatments, however, did not affect the enzyme from control (aroused) snails but reconverted the kinetic parameters of PK from foot muscle of estivating animals to those characteristic of the control enzyme. These studies suggest that PK in tissues of control (aroused) *O. lactea* is a low-phosphate form of the enzyme whereas the enzyme in tissues from estivating/anoxic animals is a high-phosphate form. The effect of anoxia on PK in tissues of marine molluscs is the same. Anoxia induces a phosphorylation of PK and creates a much less active enzyme form (Plaxton and Storey, 1984*a*,*b*, 1985*b*). In the marine whelk, the effect is not mediated by cyclic-AMP-dependent protein kinase or the Ca^{2+}- and phospholipid-dependent protein kinase C (Brooks and Storey, 1989*a*) but is due to the action of a cyclic-GMP-dependent protein kinase (Brooks and Storey, 1990*b*). The present results suggest that all three protein kinases could phosphorylate *O. lactea* PK *in vitro* but are also consistent with the presence of a second-

messenger-independent protein kinase in *O. lactea* extracts. The protein kinase that mediates PK phosphorylation during the natural transition to an estivating state remains to be determined.

The changes in PK kinetic properties brought about by estivation or anoxia for the *O. lactea* foot and mantle enzymes are consistent with less active enzyme forms in the dormant or anoxic states. Thus, the affinity of foot muscle PK for its PEP substrate decreased in these states and inhibition by alanine or ATP increased. Similar results were seen for mantle (except for alanine inhibition in estivation), although here the changes in enzyme properties were more pronounced in anoxia than in estivation. These results are qualitatively the same as the effects of anoxia on PK from tissues of marine molluscs. In marine molluscs anoxia typically reduces enzyme V_{max}, lowers affinity for PEP, reduces F1,6P_2 activation and enhances alanine inhibition of the enzyme (Holwerda *et al.* 1983; Plaxton and Storey, 1984*a*; Hakim *et al.* 1985; de Vooys and Holwerda, 1986; Whitwam and Storey, 1990). Changes to PK that produced a less active enzyme form in foot muscle after long-term anoxia (45 h) also correlated well with changes in metabolic rate (measured as changes in rates of lactate accumulation in anoxia) and with changes in glycolytic rate inferred from measurements of the changes in concentrations of glycolytic intermediates. Thus, the rate of lactate accumulation declined rapidly under anoxia; the average was 1.90 μmol g^{-1} h^{-1} between 0 and 2 h of anoxia and only 0.81 μmol g^{-1} h^{-1} between 2 and 14 h (Churchill and Storey, 1989). Furthermore, changes in the levels of PEP and pyruvate in foot muscle, when assessed *via* crossover analysis, indicated a relative activation at the PK locus during the initial stage of anoxia (2 h), but subsequently showed strong metabolic inhibition at PK with longer exposure (14 h anoxia) (Churchill and Storey, 1989). During estivation, rates of oxygen uptake decline to about 70 % of the rate in active snails within 6–8 h (Barnhart and McMahon, 1988) and average only 15 % of the active rate over the long term (2–4 weeks of estivation) (Barnhart and McMahon, 1987). PK inactivation occurs between 12 and 48 h after estivation begins, suggesting that enzyme phosphorylation is not the first triggering event in the metabolic slow-down but is certainly one of the early events in establishing a stable hypometabolic state. Changes in metabolite levels in foot muscle were also consistent with metabolic inhibition at the PK locus between 3 and 22 days of estivation (Churchill and Storey, 1989). It appears, then, that enzyme phosphorylation, creating a less active enzyme form of PK, is an integral part of the transition from an active to a hypometabolic state in molluscs and that the response can be triggered by different stimuli (low water availability, lack of oxygen) in different species. The same use of post-translational modification to reduce the activity of glycolytic enzymes has also been identified in anaerobiosis in goldfish and turtles and hibernation in small mammals (Rahman and Storey, 1988; Brooks and Storey, 1988, 1989*b*; Storey, 1989).

The response of hepatopancreas PK to estivation or anoxia is more difficult to interpret. Changes in V_{max}, $S_{0.5}$ PEP and I_{50} values for ATP and alanine would suggest a more active enzyme form in the dormant or anoxic state. This is opposite

to the effect of anoxia on the kinetic properties of *B. canaliculatum* hepatopancreas PK (Plaxton and Storey, 1985*b*). However, one dramatic change in the properties of *O. lactea* hepatopancreas PK occurred in anoxia or estivation and this was the loss of $F1,6P_2$ activation. Hepatopancreas PK changed from showing a high sensitivity to $F1,6P_2$ (K_a=0.004 mmol l^{-1}) in control snails to being unaffected by the bisphosphate in estivation or anoxia. The action of $F1,6P_2$ on PK in liver-like tissues is frequently critical to enzyme function (Engstrom *et al.* 1987). PK in liver or hepatopancreas typically has a low affinity for PEP ($S_{0.5}$ is high relative to organ PEP levels) and feedforward activation of the enzyme by $F1,6P_2$ (the product of 6-phosphofructo-1-kinase), which lowers $S_{0.5}$ and overrides the effects of inhibitors, is necessary for substantial glycolytic flux. Thus, the loss of sensitivity to $F1,6P_2$ as the result of enzyme covalent modification may be all that is needed to produce the changes in hepatopancreas PK function that are required in the estivating or anoxic state.

Anoxia-induced phosphorylation of PK, when first described in marine molluscs, was proposed to be the mechanism responsible for regulating the PEP branchpoint and determining the aerobic (*via* PK and into mitochondrial oxidations) *versus* anoxic (*via* PEP carboxykinase and fermented to succinate or proprionate) fate of PEP in these animals. While this is obviously one very important consequence of PK phosphorylation in marine molluscs, the present study suggests that it may not be the primary role of PK phosphorylation. The fact that PK is phosphorylated in estivating or anoxic land snails suggests that the mechanism may have developed first as a method of glycolytic rate control. Thus, PK phosphorylation has no apparent function in redirecting glycolytic flux in *O. lactea*. The enzyme is covalently modified in estivation, even though this is an aerobic state where no fermentative end-products accumulate (Churchill and Storey, 1989) and glycolytic carbon continues to be fed *via* PK into the tricarboxylic acid cycle. PK is also modified in anoxia in *O. lactea* even though this modification does little to redirect carbon flow at the PEP branchpoint; thus, the ratio D-lactate:L-alanine:succinate was 8:2:1 over 14 h of anoxia (Churchill and Storey, 1989). Furthermore, our recent studies with marine whelks have shown that at least three enzymes (PK, 6-phosphofructo-1-kinase and 6-phosphofructo-2-kinase) are phosphorylated and inactivated on parallel time courses during anoxia (Storey, 1988*b*; Whitwam and Storey, 1990; L. Bosca and K. Storey, unpublished data). Together these points suggest that PK phosphorylation functions primarily as a mechanism of overall glycolytic rate control and secondarily, in anoxia-tolerant marine molluscs, as a means of redirecting flux at the PEP branchpoint.

A time course for the changes in kinetic properties of foot muscle and hepatopancreas PK during estivation revealed that the transition of enzyme properties from those characteristic of the aroused state to those characteristic of estivation occurred between 12 and 48 h after the removal of food and water from the snails. As noted above, this indicates that PK modification is one of the early events associated with declining metabolic rate during entry into estivation. At 22

days of estivation the properties of PK were still the same as at 2 days, suggesting that the metabolic transitions associated with entry into estivation are made fairly quickly and abruptly and then maintained at a new steady state over the subsequent period of dormancy. The transition upon arousal from estivation was even faster. For foot muscle, the restoration of PK properties to those of the aroused (control) form, presumably *via* enzyme dephosphorylation, occurred within 10 min of the reintroduction of food and water. This corresponded with our observations that snails were beginning to emerge from their shells by this time. The restoration of PK to the control (aroused) form is apparently associated, therefore, with renewed muscular work. For hepatopancreas PK the time required for recovery was somewhat longer, between 3 and 24 h (although the high variation in the data points at 10 min and 1 h resulted from one animal at each point that showed a high $S_{0.5}$ PEP of 0.55 mmol l^{-1}). Somewhat surprisingly, the time course studies revealed no intermediate values for the kinetic parameters during the transition times; PK from individual animals always showed kinetics characteristic of the active or the dormant states. This is unlike the situation seen in marine molluscs, where there is a gradation of change in kinetic parameters over the aerobic to anoxic transition (Holwerda *et al.* 1983; Whitwam and Storey, 1990). For whelk PK, however, this appears to be due to the presence of two phosphorylation sites per subunit of PK and differential rates of phosphorylation of these sites.

We are very grateful to Dr M. C. Barnhart for supplying the snails as well as information on their care and continued discussions on the physiology of estivation. Supported by an operating grant from the NSERC Canada to KBS.

References

Barnhart, M. C. (1983). Gas permeability of the epiphragm of a terrestrial snail, *Otala lactea*. *Physiol. Zool.* **56**, 436–444.

Barnhart, M. C. (1986*a*). Control of acid–base status in active and dormant land snails, *Otala lactea* (Pulmonata, Helicidae). *J. comp. Physiol.* **156**, 347–354.

Barnhart, M. C. (1986*b*). Respiratory gas tensions and gas exchange in active and dormant land snails, *Otala lactea*. *Physiol. Zool.* **59**, 733–745.

Barnhart, M. C. and McMahon, B. R. (1987). Discontinuous carbon dioxide release and metabolic depression in dormant land snails. *J. exp. Biol.* **128**, 123–138.

Barnhart, M. C. and McMahon, B. R. (1988). Depression of aerobic metabolism and intracellular pH by hypercapnia in land snails, *Otala lactea*. *J. exp. Biol.* **138**, 289–299.

Berlund, L. and Humble, E. (1979). Kinetic properties of pyruvate kinase type A from kidney and type M from muscle. *Archs Biochem. Biophys.* **195**, 347–361.

Brooks, S. P. J. and Storey, K. B. (1988). Anoxic brain function: molecular mechanisms of metabolic depression. *FEBS Lett.* **232**, 214–216.

Brooks, S. P. J. and Storey, K. B. (1989*a*). Influence of hormones, second messengers, and pH on the expression of metabolic responses to anoxia in a marine whelk. *J. exp. Biol.* **145**, 31–43.

Brooks, S. P. J. and Storey, K. B. (1989*b*). Anoxia tolerance in the turtle: enzyme regulation by reversible phosphorylation. *Am. J. Physiol.* **257**, R278–R283.

Brooks, S. P. J. and Storey, K. B. (1990*a*). Glycolytic enzyme binding and metabolic control in estivation and anoxia in the land snail, *Otala lactea*. *J. exp. Biol.* **151**, 193–204.

BROOKS, S. P. J. AND STOREY, K. B. (1990*b*). cGMP stimulated protein kinase phosphorylates pyruvate kinase in an anoxia tolerant marine mollusc. *J. comp. Physiol. B* (in press).

BURKY, A. J., PACHECO, J. AND PEREYRA, E. (1972). Temperature, water, and respiratory regimens of an amphibious snail, *Pomacea urceus* (Muller), for the Venezuelan savannah. *Biol. Bull. mar. biol. Lab., Woods Hole* **143**, 304–316.

CEDENO-LEON, A. (1984). Carbohydrate reserves during aestivation of *Pomacea urceus* (Muller) (Gastropoda, Prosobranchia). *Comp. Biochem. Physiol.* A **78**, 553–557.

CHURCHILL, T. A. AND STOREY, K. B. (1989). Intermediary energy metabolism during dormancy and anoxia in the land snail *Otala lactea*. *Physiol. Zool.* **62**, 1015–1030.

DE VOOYS, C. G. N. AND HOLWERDA, D. A. (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.* B **85**, 217–221.

ENGSTROM, L., EKMAN, P., HUMBLE, E. AND ZETTERQVIST, O. (1987). Pyruvate kinase. In *The Enzymes* (ed. P. D. Boyer and E. G. Krebs), pp. 47–75. New York: Academic Press.

HAKIM, G., CARPENE, E., CORTES, P. AND HSANI, G. (1985). Regulation by phosphorylation–dephosphorylation of pyruvate kinase in *Venus gallina* and *Scapharca inequivalvis*. *Comp. Biochem. Physiol.* B **80**, 109–112.

HELMERHORST, E. AND STOKES, G. B. (1980). Microcentrifuge desalting: a rapid, quantitative method for desalting small amounts of protein. *Analyt. Biochem.* **104**, 130–135.

HERREID, C. F. (1977). Metabolism of land snails (*Otala lactea*) during dormancy, arousal, and activity. *Comp. Biochem. Physiol.* A **56**, 211–215.

HOCHACHKA, P. W. AND GUPPY, M. (1987). *Metabolic Arrest and the Control of Biological Time*. Cambridge: Harvard University Press.

HOCHACHKA, P. W. AND MUSTAFA, T. (1972). Invertebrate facultative anaerobiosis. *Science* **178**, 1056–1060.

HOLWERDA, D. A., VEENHOF, P. R. AND DE ZWAAN, A. (1983). 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.* **5**, 185–190.

HORNE, F. R. (1979). Comparative aspects of estivating metabolism in the gastropod, *Marisa*. *Comp. Biochem. Physiol.* A **64**, 309–311.

LIVINGSTONE, D. R. AND DE ZWAAN, A. (1983). Carbohydrate metabolism in gastropods. In *The Mollusca*, vol. 1 (ed. K. M. Wilbur), pp. 177–242. New York: Academic Press.

PLAXTON, W. C. AND STOREY, K. B. (1984*a*). 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.

PLAXTON, W. C. AND STOREY, K. B. (1984*b*) Phosphorylation *in vivo* of red-muscle pyruvate kinase from the channelled whelk, *Busycotypus canaliculatum*. *Eur. J. Biochem.* **143**, 267–272.

PLAXTON, W. C. AND STOREY, K. B. (1985*a*). Tissue-specific isozymes of pyruvate kinase in the channelled whelk *Busycotypus canaliculatum*: enzyme modification in response to environmental anoxia. *J. comp. Physiol.* B **155**, 291–296.

PLAXTON, W. C. AND STOREY, K. B. (1985*b*). Purification and properties of aerobic and anoxic forms of pyruvate kinase from the hepatopancreas of the channelled whelk, *Busycotypus canaliculatum*. *Archs Biochem. Biophys.* **243**, 195–205.

RAHMAN, M. S. AND STOREY, K. B. (1988). Role of covalent modification in the control of glycolytic enzymes in response to environmental anoxia in goldfish. *J. comp. Physiol.* B **157**, 813–820.

SCHMIDT-NEILSEN, K., TAYLOR, C. R. AND SHKOLNIK, A. (1971). Desert snails: problems of heat, water and food. *J. exp. Biol.* **55**, 385–398.

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

STOREY, K. B. (1984). Phosphofructokinase from foot muscle of the whelk, *Busycotypus canaliculatum*: Evidence for covalent modification of the enzyme during anaerobiosis. *Archs Biochem. Biophys.* **235**, 665–672.

STOREY, K. B. (1985). Kinetic and regulatory properties of pyruvate kinase isozymes from flight

muscle and fat body of the cockroach, *Periplaneta americana*. *J. comp. Physiol.* B **155**, 339–345.

STOREY, K. B. (1988*a*). Suspended animation: The molecular basis of metabolic depression. *Can. J. Zool.* **66**, 124–132.

STOREY, K. B. (1988*b*). 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, K. B. (1989). Integrated control of metabolic rate depression *via* reversible phosphorylation of enzymes in hibernating mammals. In *Living in the Cold*, 2nd International Symposium (ed. A. Malan and B. Canguilhem), pp. 309–319. Montrouge: John Libbey Eurotext Ltd.

STOREY, K. B. AND HOCHACHKA, P. W. (1975). Squid muscle pyruvate kinase: control properties in a tissue with an active alpha-glycerophosphate cycle. *Comp. Biochem. Physiol.* B **52**, 187–192.

UMEZURIKE, G. M. AND IHEANACHO, E. N. (1983). Metabolic adaptations in aestivating giant African snail (*Achatina achatina*). *Comp. Biochem. Physiol.* B **74**, 493–498.

VORHABEN, J. E., KLOTZ, A. V. AND CAMPBELL, J. W. (1984). Activity and oxidative metabolism of the land snail, *Helix aspersa*. *Physiol. Zool.* **57**, 357–365.

WHITWAM, R. E. AND STOREY, K. B. (1990). Organ-specific analysis of the time course of covalent modification of pyruvate kinase during anaerobiosis in a marine whelk. *Physiol. Zool.* **63**, 222–234.