J. exp. Biol. **145**, 31–43 (1989)
Printed in Great Britain © *The Company of Biologists Limited 1989*

INFLUENCE OF HORMONES, SECOND MESSENGERS AND pH ON THE EXPRESSION OF METABOLIC RESPONSES TO ANOXIA IN A MARINE WHELK

BY STEPHEN P. J. BROOKS AND KENNETH B. STOREY
Institute of Biochemistry and Department of Biology, Carleton University, Ottawa, Ontario, Canada, K1S 5B6

Accepted 24 April 1989

Summary

The roles of hormones, second messengers and pH in triggering or potentiating biochemical responses to anaerobiosis were evaluated using *in vitro* incubations of isolated muscle tissues (foot, radular retractor, ventricle) from the marine whelk *Busycon canaliculatum* (L.). Incubating tissues *in vitro* under anoxic conditions stimulated changes in muscle fructose-2,6-bisphosphate levels and pyruvate kinase kinetics (K_m values for phosphoenolpyruvate, I_{50} values for L-alanine) that were virtually equivalent to those that occur *in vivo*. Additions of hormones (epinephrine, norepinephrine, octopamine, serotonin, glucagon, insulin) or inhibitors of prostaglandin synthesis (dexamethasone, aspirin) had no effect on these metabolic responses to anoxia. The second messenger compounds, dibutyryl cyclic AMP and Ca^{2+} + ionophore A23187 + phorbol myristate acetate, produced isolated and tissue-specific responses in muscles incubated under aerobic conditions, but the magnitude and pattern of these responses differed from those seen in anoxia. Second messengers also had no effect on the development of biochemical responses in anoxic muscles. Tissue pH was artificially altered in order to evaluate the role of pH change (acidification occurs during anoxia *in vivo*) in the control of metabolic responses to anoxia. In all cases, the changes in the kinetic properties of pyruvate kinase (PK) correlated with the state of oxygenation of the tissue and not with the measured tissue pH value; higher tissue pH did not prevent anoxia-induced phosphorylation of PK and lower tissue pH did not alter the kinetic patterns of the aerobic enzyme. Overall, the study indicates that cells and tissues of the whelk respond individually to anoxia and that coordination of the action of protein kinases during anoxia is not mediated by pH or by common second-messenger mechanisms.

Introduction

Many marine molluscs are excellent facultative anaerobes and can live for days or weeks in the absence of environmental oxygen (Hochachka, 1980). The biochemical adaptations supporting anoxic tolerance have been extensively studied in several species of marine molluscs (de Zwaan, 1983; Livingstone & de

Key words: *Busycon canaliculatum*, fructose-2,6-bisphosphate, protein kinases, metabolic rate depression, pyruvate kinase, covalent modification, anaerobiosis, regulatory role of pH.

Zwann, 1983; Kreutzer *et al.* 1985; Storey, 1985, 1988*b*). Two adaptations are fundamental to anoxic survival: (1) metabolic rate depression, i.e. the ability to lower anoxic metabolic rate to a level only 5–10 % of normoxic rate, and (2) an altered energy metabolism involving the coupled catabolism of glycogen and aspartate, the production of alternative end-products (e.g. alanine, succinate, propionate), and additional sites for substrate-level phosphorylation of ADP linked to organic acid synthesis.

Since glycolysis is the central pathway of anaerobic ATP synthesis, facultative anaerobes have elaborated strict regulatory controls over glycolytic rate. In these animals, no Pasteur effect is observed during anoxia, and glycolytic rate is actually depressed compared to that in the aerobic state, as part of the anoxia-induced metabolic rate depression (de Zwaan & Wijsman, 1976; Ebberink & de Zwaan, 1980; Storey, 1985, 1988*a*; K. B. Storey, J. D. Duncan & D. A. Kelly, in preparation). The molecular mechanisms controlling glycolytic rate depression, first investigated in studies of the marine whelk *Busycon canaliculatum* (Storey, 1984, 1985; Plaxton & Storey, 1984*a*,*b*, 1985, 1986), are proving to be universal among anoxia-tolerant vertebrates and invertebrates (Storey, 1985, 1987, 1988*b*; Rahman & Storey, 1988; Brooks & Storey, 1988). These controls include: (1) covalent modification of regulatory enzymes (*via* reversible protein phosphorylation), (2) reversible associations of enzymes into complexes bound to the subcellular particulate fraction, and (3) allosteric regulation, in particular fructose-2,6-bisphosphate (fructose-2,6-P_2) control over phosphofructokinase. The regulation of pyruvate kinase (PK) by enzyme phosphorylation and dephosphorylation has additional importance for marine molluscs. Control of PK regulates carbon flux through the 'phosphoenolpyruvate branchpoint', directing the flow of glycogen carbon into alanine *versus* succinate as end-products (Holwerda *et al.* 1983; Plaxton & Storey, 1984*a*).

Although the molecular mechanisms involved in regulating glycolytic rate (and overall metabolic rate) during anoxia are now fairly well understood, the method of signal transmission remains unknown. Clearly, the actions of protein kinases and phosphatases are central to the anaerobic response; these enzymes are typically regulated in the cell by low molecular weight effectors (e.g. cyclic AMP, Ca^{2+}), often in response to hormone stimulation (Krebs & Beavo, 1979; Mieskes *et al.* 1987; Pallen *et al.* 1987; Connelly *et al.* 1987; Lincoln, 1983). Furthermore, anaerobiosis is always accompanied *in vivo* by intracellular acidification (Ellington, 1983*a*,*b*; Walsh *et al.* 1984; Portner *et al.* 1984). The present study uses an *in vitro* system of isolated muscle tissues to investigate the roles of hormones, second messengers and tissue pH in triggering or potentiating the metabolic responses to anoxia in the whelk.

Materials and methods

Chemicals and animals

All chemicals were purchased from Sigma Chemical Co. (St Louis, MO) or

Boehringer-Mannheim (Montréal, PQ), and were of the highest quality available. Whelks (*Busycon canaliculatum*) were obtained from the Marine Biological Laboratory at Woods Hole (MA) and were held in aerated artificial sea water (1000 mosmol l^{-1}) at 8–10 °C until use.

Enzyme assays and fructose-2,6-bisphosphate determinations

Kinetic properties of PK were determined in a cuvette containing 1 ml of buffer A (50 mmol l^{-1} imidazole, pH 7·0, containing 50 mmol l^{-1} KCl and 5 mmol l^{-1} $MgCl_2$), 2 mmol l^{-1} Mg–ADP, 0·2 mmol l^{-1} NADH and excess dialysed lactate dehydrogenase. For the determination of I_{50} values (the inhibitor concentration that reduces enzyme velocity by 50 %), phosphoenolpyruvate (PEP) concentrations were 0·5 mmol l^{-1} for radular retractor muscle and 0·3 mmol l^{-1} for foot muscle. Initial velocities were measured at 340 nm and 22 °C, and the reaction was started by the addition of sample homogenate (see below). Fructose-2,6-P_2 was extracted and assayed by the method of van Schaftingen (1984).

Preparation of samples

Tissues from aerobic whelks were rapidly dissected out and separated into small pieces; ventricles were divided into six pieces (approximately 0·08 g each), radular retractor muscles were separated into bundles of strands (approximately 0·3 g each), and foot muscle was cut into thin (1–2 mm) slices (approximately 0·5 g). Muscle samples were then placed in vials containing 10 ml of sea water previously bubbled for 1 h with either air (O_2, aerobic), a mixture of 95 % N_2 and 5 % CO_2 (anoxic) or 100 % N_2 (anoxic). The sea water contained the appropriate concentrations of effector(s), or was adjusted to the appropriate pH value at 22 °C. After 4 h of incubation at 22 °C (with continuous bubbling), each tissue sample was divided in half. One half was immediately frozen in liquid nitrogen and then transferred to −80 °C for long-term storage; this sample was used for the assay of fructose-2,6-P_2 and PK activity. The other half was quickly rinsed in distilled water and homogenized immediately in distilled water (1:2 w/v). The pH of this homogenate was quickly measured to obtain an estimate of the tissue pH value. Although this method does not provide an absolute measurement of the cytosolic pH value, the results obtained using this method are similar to values previously reported using n.m.r. (Ellington, 1983*a*, 1985; Wiseman & Ellington, 1987) and can be used to monitor relative changes in cellular pH. For the analysis of pyruvate kinase kinetic constants, frozen muscle samples were homogenized 1:4 (v/w) in buffer B (50 mmol l^{-1} imidazole–HCl, pH 7·5, containing 0·1 mmol l^{-1} phenylmethylsulphonyl fluoride, 30 mmol l^{-1} β-mercaptoethanol, 100 mmol l^{-1} NaF, 5 mmol l^{-1} EDTA and 5 mmol l^{-1} EGTA) for 20 s using a Polytron PT10 homogenizer. Homogenates were centrifuged at 15 000 ***g*** for 10 min at 10 °C, and the supernatant was desalted by centrifugation through a Sephadex G-25 'spun column' (Helmerhorst & Stokes, 1980) previously equilibrated with buffer C [40 mmol l^{-1} imidazole, pH 7·0, containing 20 % (v/v) glycerol, 10 mmol l^{-1} potassium phosphate, 0·1 mmol l^{-1} EDTA and 25 mmol l^{-1} β-mercaptoethanol].

This procedure rapidly removes all low molecular weight species ($M_r < 5000$) from the homogenate with greater than 95 % recovery of PK activity; the spun column procedure effectively acts to dialyse PK into buffer C. Fructose-2,6-P_2 samples were prepared by grinding frozen tissue in 50 mmol l^{-1} NaOH (at 80 °C) at a 1:20 dilution. Fructose-2,6-P_2 was then measured according to van Schaftingen (1984).

In vitro *phosphorylation of pyruvate kinase*

Tissues were excised from freshly killed aerobic whelks and homogenized 1:2 (w/v) in buffer B plus 25 % glycerol. Low molecular weight species were removed by centrifugation through a Sephadex G-25 spun column pre-equilibrated with buffer C. The eluant was then mixed 1:2 (v/v) with 50 mmol l^{-1} imidazole buffer, pH 7·0, containing 30 mmol l^{-1} NaF, 20 % glycerol, 0·1 mmol l^{-1} EDTA, 10 mmol l^{-1} potassium phosphate and 10 mmol l^{-1} β-mercaptoethanol. Samples were divided in half and incubated for 16 h at 22 °C in the presence of 10 mmol l^{-1} ATP plus 20 mmol l^{-1} EDTA (control) or 10 mmol l^{-1} ATP plus 20 mmol l^{-1} $MgCl_2$ (experimental). Changes in PK kinetic parameters depended on the presence of exogenously added $MgCl_2$. Immediately before assaying PK activity, these samples were centrifuged through a spun column to remove NaF, ATP, EDTA and magnesium (Helmerhorst & Stokes, 1980).

Results

Cell-free phosphorylation of pyruvate kinase

Table 1 demonstrates the dependence of PK kinetic parameters on the phosphorylation state of the enzyme. Both the K_m value for PEP (expressed as the ratio $V_5/V_{0.5}$) and the I_{50} value for alanine (expressed as the ratio $V_{0.5}/V_{+ala}$)

Table 1. *Phosphorylation of pyruvate kinase in cell-free extracts of whelk muscles*

Parameter	Tissue	+20 mmol l^{-1} EDTA	+20 mmol l^{-1} $MgCl_2$
$V_5/V_{0.5}$	Foot	1·55 ± 0·16	2·97 ± 0·53*
	Radular retractor	1·11 ± 0·03	2·38 ± 0·33†
	Ventricle	1·20 ± 0·15	9·45 ± 2·35*
$V_{0.5}/V_{+ala}$	Foot	1·42 ± 0·18	5·79 ± 1·10†
	Radular retractor	1·17 ± 0·11	6·02 ± 1·12†
	Ventricle	1·44 ± 0·32	11·15 ± 3·60*

The value $V_5/V_{0.5}$ represents the ratio of PK activity measured at 5 mmol l^{-1} PEP to that at 0·5 mmol l^{-1} PEP (both at 2 mmol l^{-1} ADP).

The value $V_{0.5}/V_{+ala}$ represents the ratio of PK activity measured at 0·5 mmol l^{-1} PEP in the absence *versus* the presence of 0·8 mmol l^{-1} L-alanine (foot) or 2 mmol l^{-1} L-alanine (other tissues) determined at 2 mmol l^{-1} ADP.

Data are means ± S.E.M., $N = 3$ (ventricle) or $N = 4$ (other tissues).

Values are significantly different from the corresponding values in the presence of 20 mmol l^{-1} EDTA as determined by the one-tailed Student's *t*-test; * $P < 0·05$, † $P < 0·005$.

increase only when the tissue is incubated in the presence of Mg^{2+} and ATP. In these experiments, the cell-free extracts were passed through a Sephadex G-25 spun column to remove low molecular weight metabolites and salts, prior to incubation in the presence of ATP with (+$MgCl_2$) or without (+EDTA) divalent ion. This procedure ensures that the reaction is dependent on externally added magnesium ions and ATP. Note that the kinetic parameters changed only when magnesium ions were present (protein kinase is active only in the presence of Mg^{2+}: ATP substrate) and not when EDTA was added to the mixture, despite the inclusion of ATP in both experimental cases. Table 1 shows that phosphorylation of PK resulted in an enzyme with a lower affinity for PEP and a higher sensitivity to alanine (measured at 0·5 mmol l^{-1} PEP). This agrees with previous studies on radular retractor muscle PK; anoxia stimulated the incorporation of ^{32}P into the enzyme and produced similar changes in enzyme kinetic parameters (Plaxton & Storey, 1984*a*,*b*). Table 1 also shows that the extent of PK modification was not altered by the addition of either 4 mmol l^{-1} cyclic AMP or 2 mmol l^{-1} $CaCl_2$ plus 50 μg ml^{-1} phorbol 12-myristate 13-acetate (PMA). The PK kinetic parameters were, however, measured after a long (16 h) incubation which corresponds to the maximal change observed under these conditions. These experiments demonstrate the importance of phosphorylation in determining the functional PK kinetic parameters, and imply that effectors influencing protein kinases and phosphatases may play a pivotal role in the cellular anoxic response.

Isolated tissues and anoxia in vitro

The effects of anoxia *in vitro* on metabolic parameters in whelk foot muscle, radular retractor and ventricle are shown in Table 2 for fructose-2,6-P_2 and Table 3 for the kinetic properties of PK. Anaerobic incubation had no effect on the content of the bisphosphate in foot or ventricle, but fructose-2,6-P_2 in anoxic radular retractor muscle was significantly reduced to 63 % of the aerobic value. The effects of anoxia on PK kinetics were consistent in all three tissues. PK from anoxic tissues showed a greatly reduced affinity for PEP as a substrate (K_m increased 6·6- to 17·2-fold over control values) and an increased inhibition by L-alanine (I_{50} dropped to 17–60 % of control). These results are virtually identical to the metabolic responses of these tissues during anoxia *in vivo* (Storey, 1988*a*; Plaxton & Storey, 1984*a*,*b*, 1985) and we feel that this validates the use of an isolated muscle preparation as the model system for studying hormone, second messenger and pH control over anaerobic metabolism. The only exception noted was the fructose-2,6-P_2 content of the ventricle after aerobic incubation; this was 10-fold lower than the level in freshly excised tissue from an aerobic whelk (Storey, 1988*b*).

Hormone, prostaglandin synthesis inhibitors and second-messenger effects on anaerobic responses

Experiments were undertaken to determine whether inhibitors of prostaglandin synthesis, hormones or second messengers could mimic or modulate the metabolic

Table 2. *Effect of anoxia and hormone second messengers on fructose 2,6-bisphosphate concentrations during* in vitro *incubation of whelk muscles*

Tissue	Condition	Fructose-2,6-bisphosphate (nmol g^{-1} wet mass) Aerobic	Anoxic
Foot	Control	0·367 ± 0·060	0·361 ± 0·107
	db-cAMP	0·243 ± 0·017	0·369 ± 0·052
	C+A+P	0·416 ± 0·074	0·464 ± 0·052
Radular retractor	Control	0·461 ± 0·040	0·292 ± 0·048*
	db-cAMP	0·211 ± 0·014*	0·317 ± 0·060
	C+A+P	0·768 ± 0·288	0·250 ± 0·095
Ventricle	Control	0·156 ± 0·023	0·197 ± 0·036
	db-cAMP	0·218 ± 0·045	0·127 ± 0·009†
	C+A+P	0·149 ± 0·036	0·101 ± 0·036†

Aerobic *versus* anoxic incubations (4 h at 22 °C) of isolated tissues were carried out as described in Materials and methods with experimental conditions: no addition, control; 10^{-4} mol l^{-1} dibutyryl cyclic AMP added, db-cAMP; 2 mmol l^{-1} $CaCl_2$, 30 μmol l^{-1} ionophore A23187, and 10 μg ml^{-1} phorbol myristate acetate added, C+A+P. Data are means ± S.E.M., $N = 4$.

Values are significantly different at the $P < 0{\cdot}05$ level determined by one-tailed paired Student's *t*-test as compared to aerobic control (*) or anoxic control (†).

Anoxia was imposed by continuous bubbling with a mixture of 95 % N_2, 5 % CO_2.

Table 3. *Effect of anoxia and hormone second messengers on the kinetic properties of pyruvate kinase during* in vitro *incubation of whelk muscles*

Tissue	Condition	K_m PEP (mmol l^{-1}) Aerobic	Anoxic*	I_{50} L-alanine (mmol l^{-1}) Aerobic	Anoxic*
Foot	Control	0·21 ± 0·03	1·38 ± 0·24	2·32 ± 0·20	0·83 ± 0·18
	db-cAMP	0·41 ± 0·13	1·22 ± 0·14	2·56 ± 0·32	0·69 ± 0·21
	C+A+P	0·56 ± 0·08†	1·15 ± 0·15	1·54 ± 0·31‡	0·89 ± 0·16
Radular retractor	Control	0·20 ± 0·04	1·82 ± 0·41	5·43 ± 1·20	0·95 ± 0·23
	db-cAMP	0·15 ± 0·05	1·77 ± 0·12	4·40 ± 0·60	1·68 ± 0·27‡
	C+A+P	0·09 ± 0·01†	1·61 ± 0·15	5·73 ± 1·10	0·89 ± 0·25
Ventricle	Control	0·15 ± 0·01	2·58 ± 0·25	1·13 ± 0·20	0·68 ± 0·08
	db-cAMP	0·18 ± 0·03	2·23 ± 0·23	1·22 ± 0·14	1·17 ± 0·30
	C+A+P	0·37 ± 0·05†	2·33 ± 0·12	0·85 ± 0·11	0·99 ± 0·14

Experimental details are as in Table 1 and abbreviations as in Table 2; PEP, phosphoenolpyruvate.

* All values for anoxic tissues are significantly different from the corresponding aerobic treatments as determined by the one-tailed paired Student's *t*-test at the $P < 0{\cdot}05$ level.

† Significantly different from aerobic control at the $P < 0{\cdot}05$ level.

‡ Significantly different from anoxic control at the $P < 0{\cdot}05$ level.

Anoxia was imposed by continuous bubbling with a mixture of 95 % N_2, 5 % CO_2.

responses to anoxia by isolated whelk tissues. Muscle samples were incubated under aerobic and anoxic conditions in the presence of added hormones: 10^{-7} mol l^{-1} bovine glucagon, 10^{-7} mol l^{-1} bovine insulin, 10^{-6} mol l^{-1} epinephrine, 10^{-6} mol l^{-1} norepinephrine, 10^{-6} mol l^{-1} octopamine and 10^{-5} mol l^{-1} serotonin. No effects of hormones were found; neither levels of fructose-2,6-P_2 nor kinetic parameters of PK (K_m for PEP, I_{50} for L-alanine) were altered by the presence of added hormones in either aerobic or anoxic incubations of any of the muscle tissues (data not shown).

The response to anoxia observed in the individual tissues (Table 3) could also result from alterations in the prostaglandin synthesis pathway present in the plasma membranes. This could arise through one of two different mechanisms that combine to alter plasmalemma nucleotide cyclase activity by changes in membrane fluidity. These changes could result from differences in either phospholipase A_2 activity or plasma membrane cyclo-oxygenase activity (through limited O_2 availability), since both enzymes are involved in the breakdown of phospholipids in the membrane, and changes in their activity are often associated with cyclic nucleotide-mediated responses (Saintsing & Dietz, 1983). Addition of either 20 μmol l^{-1} dexamethasone (phospholipase A_2 inhibitor) or 0·4 mmol l^{-1} aspirin (cyclo-oxygenase inhibitor) to tissues incubated under aerobic or anoxic conditions did not result in aerobic enzyme when added to anoxic incubations, nor did it result in anoxic enzyme when added to aerobic incubations.

Equivalent experiments were subsequently carried out to test the effects of second messenger compounds: dibutyryl 3′,5′-cyclic AMP (db-cAMP, to stimulate protein kinase A) or Ca^{2+} + ionophore A23187 + phorbol myristate acetate (C+A+P, to stimulate Ca^{2+}/calmodulin protein kinase and/or Ca^{2+}/phospholipid-dependent protein kinase C). Table 2 (fructose-2,6-P_2) and Table 3 (PK kinetic parameters) show the effects of these additions. Although no consistent pattern of second-messenger effects was observed, some responses were obtained. db-cAMP reduced fructose-2,6-P_2 content in aerobic radular retractor and in ventricle to a level equivalent to that in anoxic tissues. Calcium plus PMA also reduced the fructose-2,6-P_2 level in anoxic ventricle. Addition of C+A+P significantly altered the PK K_m value for PEP in all three tissues under aerobic incubation conditions. C+A+P also altered the I_{50} value for alanine in foot. In foot and ventricle, the direction of change in the K_m was the same as that found in anoxia, but the magnitude of the change was much smaller (a twofold increase only). Addition of db-cAMP affected only the I_{50} value for alanine of radular retractor PK; the value was significantly higher than in the anoxic control.

Effect of pH on metabolic responses to anoxia

To analyse the role of pH in the development of biochemical responses to anoxia, tissues were incubated under aerobic or anoxic conditions in sea water adjusted to altered pH values. Although the technique used for measurement of tissue pH was extremely simple, the values generated were highly reproducible within treatment groups and, for ventricle and radular retractor, the data agree

Table 4. *Effect of altering the pH of the incubation medium on tissue pH and the kinetic properties of pyruvate kinase during aerobic* versus *anoxic incubations of isolated whelk muscle*

Tissue	Condition	Final tissue pH	K_m PEP ($mmol\,l^{-1}$)	I_{50} L-alanine ($mmol\,l^{-1}$)
Foot	O_2/SW	7·53 ± 0·17	0·16 ± 0·04	6·1 ± 1·1
	O_2/6·5	6·97 ± 0·05*	0·13 ± 0·03†	5·3 ± 0·7†
	N_2/SW	7·04 ± 0·13*	0·43 ± 0·02*	2·7 ± 0·4*
	N_2/9·5	7·74 ± 0·10†	0·39 ± 0·02*	2·2 ± 0·3*
Radular retractor	O_2/SW	7·64 ± 0·26	0·12 ± 0·04	18·3 ± 1·9
	O_2/5·5	6·39 ± 0·07*	0·29 ± 0·04*,†	7·8 ± 1·4*,†
	N_2/SW	6·64 ± 0·11*	1·80 ± 0·22*	1·0 ± 0·2*
	N_2/9·5	7·35 ± 0·28†	1·62 ± 0·35*	0·7 ± 0·2*
Ventricle	O_2/SW	7·14 ± 0·10	0·54 ± 0·09	ND
	O_2/5·0	6·11 ± 0·05*,†	0·74 ± 0·11†	ND
	N_2/SW	6·40 ± 0·04*	1·56 ± 0·31*	ND
	N_2/9·5	7·48 ± 0·07†	1·80 ± 0·32*	ND

Muscle samples were incubated in (unbuffered) artificial sea water (SW), pH 8, or sea water adjusted to a specific pH after the addition of either: 50 $mmol\,l^{-1}$ Hepes (pH 8·5 and pH 6·5), 100 $mmol\,l^{-1}$ Taps (pH 9·5) or 20 $mmol\,l^{-1}$ potassium succinate (pH 5·0 and 5·5).

Data are means ± S.E.M., $N = 4$.

* Values are significantly different from the O_2/SW condition at the $P < 0·025$ level.

† Values are significantly different from the N_2/SW condition at the $P < 0·025$ level.

All statistical tests were performed using the one-tailed paired Student's t-test.

ND, not determined.

Anoxia was imposed by continuous bubbling with a mixture of 95 % N_2, 5 % CO_2.

closely with pH determinations made by other methods (Ellington, 1983*a*, 1985; Wiseman & Ellington, 1987) and thus serve as a good guide to changes in cellular pH values. Anoxia alone led to a significant drop ($P < 0·03$) in pH of the isolated muscles, the decreases being 0·49, 1·0 and 0·74 pH units for foot, radular retractor and ventricle, respectively, over the 4 h incubation period (Table 4). However, the separate influences of pH and anoxia on the kinetic properties of PK could be evaluated by altering the pH of the sea water (with added buffers) so that aerobic tissues had a pH value equivalent to that of anoxic tissues and *vice versa*. Table 4 clearly shows that phosphorylation-mediated changes in the kinetic properties of PK (increased K_m for PEP, decreased I_{50} for alanine) were correlated with anoxia and not with measured tissue pH. In all cases, when the pH of an anoxic tissue was similar to that of the corresponding aerobic tissue, anoxia-induced changes in either the K_m or the I_{50} value for PK were still apparent; the kinetic parameters for the N_2/9·5 condition were not different from the values for N_2/SW. Furthermore, acidification under aerobic conditions did not produce the large changes in enzyme kinetic parameters characteristic of anoxia. Thus, the kinetic properties of PK in aerobic radular retractor, foot and ventricle samples, incubated at low pH, were not different from aerobic control values.

Table 5. *Effect of varying CO_2 concentrations in anoxia on tissue pyruvate kinase covalent modifications and on tissue pH*

Tissue	Condition	Final tissue pH	K_m PEP ($mmol\,l^{-1}$)	I_{50} L-alanine ($mmol\,l^{-1}$)
Foot	O_2/SW	7·53 ± 0·17	0·16 ± 0·04	6·1 ± 1·1
	95 % N_2, 5 % CO_2/SW	7·04 ± 0·13*	0·43 ± 0·02*	2·7 ± 0·4*
	100 % N_2/SW	8·17 ± 0·06†	0·30 ± 0·07*,†	ND
Radula retractor	O_2/SW	7·64 ± 0·26	0·12 ± 0·04	18·3 ± 1·9
	95 % N_2, 5 % CO_2/SW	6·64 ± 0·11*	1·80 ± 0·22*	1·0 ± 0·2*
	100 % N_2/SW	6·97 ± 0·06*,†	0·54 ± 0·06*,†	3·2 ± 1·1*,†
Ventricle	O_2/SW	7·14 ± 0·10	0·54 ± 0·09	1·13 ± 0·2
	95 % N_2, 5 % CO_2/SW	6·4 ± 0·04*	1·56 ± 0·31*	0·68 ± 0·08*
	100 % N_2/SW	6·67 ± 0·03*,†	1·22 ± 0·15*,†	0·4 ± 0·05*,†

All muscle samples were incubated in (unbuffered) artificial sea water.
Values for O_2/SW and 95 % N_2, 5 % CO_2/SW are those from Table 4.
Values for 100 % N_2/SW were obtained by incubating muscles with continuous bubbling of 100 % N_2 gas.
Data are means ± S.E.M., $N = 4$.
* Significantly different from the O_2/SW condition at the $P < 0·025$ level.
† Significantly different from the 95 % N_2, 5 % CO_2/SW condition at the $P < 0·025$ level.
All statistical tests were performed using the one-tailed paired Student's *t*-test.

Effect of CO_2 on metabolic responses to anoxia

To determine whether CO_2 levels were critical in controlling enzyme phosphorylation in isolated whelk tissues, the effect of incubating tissues in the absence of CO_2 (bubbling tissues with 100 % N_2) was compared with incubations performed at high CO_2 levels (95 % N_2, 5 % CO_2, see Table 4). The results of Table 5 show that PK kinetic parameters change from aerobic values to anoxic values regardless of the CO_2 content of the incubating atmosphere. Note that the tissue pH in anoxic heart and radular retractor dropped significantly both in the absence (100 % N_2) and in the presence (95 % N_2, 5 % CO_2) of CO_2. This demonstrates that atmospheric CO_2 is not important in altering tissue pH in these tissues under the conditions of the incubation. The measured pH of anoxic foot muscle, however, did depend on the CO_2 content of the incubation atmosphere: no tissue acidification was observed in the absence of CO_2, whereas a pronounced acidification occurred when foot was incubated in 95 % N_2, 5 % CO_2. These results show that, although tissue pH was apparently altered by changing the CO_2 concentration of the incubation atmosphere, the tissue response to anoxia (shown by an increase in PK covalent modification) was not affected by the changes in CO_2 concentration.

Discussion

The metabolic responses that characterize anaerobiosis may result from: (1) a

direct response of enzyme(s) to changing oxygen tension, (2) the detection of changing O_2 levels by specific receptor(s) in each cell followed by triggering of second messenger release to coordinate intracellular responses, or (3) the selective detection of changing oxygen levels by a specialized receptor body followed by nervous or hormonal transmission to spread the signal to all tissues. The third possibility can undoubtedly be discounted, at least for marine invertebrates. The present study demonstrates that neither added hormones nor prostaglandin pathway inhibitors affect the biochemical responses to anoxia and, moreover, isolated tissues respond to anoxia in a manner identical (changes in fructose-2,6-P_2, phosphorylation of PK) to that seen *in vivo* (Plaxton & Storey, 1984*a*, 1985; Storey, 1988*b*). The same conclusion can be drawn from earlier studies; the patterns of substrate utilization and end-product accumulation were essentially equivalent when measured after anoxia *in vivo* or *in vitro* (Collicutt & Hochachka, 1977).

It is clear that the regulation of protein kinases and protein phosphatases is central to glycolytic control in anoxia. First, anoxia-induced covalent modifications of PK, phosphofructokinase and glycogen phosphorylase have been documented in marine molluscs (Holwerda *et al.* 1983; Plaxton & Storey, 1984*a*,*b*, 1985; Storey, 1984, 1988*b*, see also Table 1). Second, changes in fructose-2,6-P_2 levels predict that the phosphorylation state of 6-phosphofructo-2-kinase, an enzyme regulated by reversible phosphorylation in other animals, should be altered during anoxia (Hue & Rider, 1987). Third, an analysis of ^{32}P labelling patterns showed widespread phosphorylation of cellular proteins in whelk radular retractor muscle during anoxia (Plaxton & Storey, 1984*b*). It is probable, therefore, that protein phosphorylation is a key to glycolytic regulation during anaerobiosis and also has a major role in coordinating the depression of metabolic rate overall. The anoxia signal may be mediated through the actions of one of the known second messenger compounds in the cell, to stimulate the action of the appropriate protein kinase.

Four types of protein kinases are known which are responsive to: (a) cyclic AMP (protein kinase A), (b) cytoplasmic free Ca^{2+} (Ca^{2+}/calmodulin-dependent protein kinase), (c) free Ca^{2+}, diglycerides and phospholipids (protein kinase C), or (d) cyclic GMP (cyclic GMP-activated protein kinase) (see Krebs & Beavo, 1979; Lincoln, 1983; Mieskes *et al.* 1987; Pallen *et al.* 1987; Connelly *et al.* 1987; Ochs, 1988). The present experiments show selected effects of cyclic AMP and Ca^{2+}/phospholipid stimulation on the kinetic properties of PK in whelk muscles. In particular, PK in aerobic muscles showed Ca^{2+}-mediated modifications, indicating a role for Ca^{2+}/calmodulin protein kinase or protein kinase C in the regulation of the muscle enzyme. However, none of the second-messenger-mediated effects on PK in aerobic muscles were of a magnitude equivalent to those stimulated by anoxia and second messengers did not enhance or alter the anoxia-induced modifications of PK. It is apparent, therefore, that the link between the anoxia signal and the coordinated covalent modification of cellular proteins is not a simple function of one of the common second messengers used in this study.

Numerous studies have shown a correlation between decreased tissue pH and anaerobiosis in marine invertebrates (Ellington, 1983*a*,*b*; Portner *et al.* 1984; Walsh *et al.* 1984). These and other studies (Busa & Nuccitelli, 1984) devoted considerable attention to the potential role(s) of changing intracellular pH in metabolic regulation. The influence of pH in transitions to, and from, dormancy is now well documented for several systems (Busa & Nuccitelli, 1984), but no previous studies have attempted to dissociate the influence of pH from that of anoxia in the expression of the metabolic responses of anaerobiosis. In the present paper, the effects of anoxia and pH are separated by manipulating tissue pH. The results demonstrate (Table 4) that the kinetic changes in PK, which characterize anaerobiosis and anoxia-induced metabolic rate depression, correlate with anoxia and not with altered pH values. High tissue pH (equal to or greater than the aerobic, control value) could not prevent the changes in PK kinetic properties induced by anoxia. Acidosis under aerobic conditions produced only small alterations in PK properties of a magnitude much less than that seen during anoxia, even when tissue pH was lowered well below the value found in anoxic tissues. Since pH changes develop slowly over the course of anoxia (Ellington 1983*a*,*b*; Portner *et al.* 1984), and since anoxia-tolerant animals take steps to minimize pH changes during anoxia (metabolic rate depression, alternative products to lactic acid), it is not surprising that pH does not trigger the biochemical responses of metabolic depression. However, tissue acidosis is a real consequence of anoxia in all animal systems and a low-pH environment may create a 'metabolic context' (Busa & Nuccitelli, 1984) that favours the induction of metabolic rate depression. Changing cellular pH may similarly have other facilitating effects, such as altering relative activities of protein kinases *versus* protein phosphatases.

Experiments to determine the effects of anoxia on pyruvate kinase phosphorylation were also performed in the presence and absence of CO_2 in the incubating atmosphere (Table 5). These experiments clearly show that, although using CO_2-rich mixtures had some effect in acidifying foot tissue, phosphorylation of PK occurred regardless of the CO_2 content of the incubating atmosphere. These results show that the regulation of enzyme phosphorylation by CO_2 levels is unlikely in this animal, and agree with the results showing that enzyme phosphorylation is largely independent of the tissue incubation pH. These results also indicate that increased CO_2 concentrations are not the cellular signal mediating the changes in PK phosphorylation but, rather, the absence of O_2 is the universal factor which triggers anoxia responses in whelk tissues.

Anoxia-induced metabolic rate depression is widespread throughout the animal kingdom, and the biochemical mechanisms that form the basis of this phenomenon are expressed in numerous invertebrate and vertebrate facultative anaerobes (Storey, 1985, 1988*a*). Enzyme/protein control *via* reversible phosphorylation is a particularly well-documented mechanism of metabolic depression. The present study demonstrates, however, that common routes of protein kinase control (*via* hormones and/or cyclic AMP, Ca^{2+} or Ca^{2+}/phospholipid second messengers) are not the means by which the anoxic signal is transmitted. Alternative

mechanisms must be sought. These could include: (1) a requirement for a different type of second messenger or for synergistic action by cyclic AMP and Ca^{2+}, (2) direct triggering by oxygen itself of cellular protein kinases or phosphatases (perhaps *via* an O_2-binding protein), or (3) an 'anoxic shock' response that modifies transcription/translation in whelk tissues to induce rapid synthesis of specific protein kinase(s).

The authors thank J. M. Storey for reading the manuscript critically. Supported by an NSERC operating grant to KBS and an NSERC postdoctoral fellowship to SPJB.

References

BROOKS, S. P. J. & STOREY, K. B. (1988). Anoxic brain function: molecular mechanisms of metabolic depression. *FEBS Lett.* **232**, 214–219.

BUSA, W. B. & NUCCITELLI, R. (1984). Metabolic regulation via intracellular pH. *Am. J. Physiol.* **246**, R409–R438.

COLLICUTT, J. M. & HOCHACHKA, P. W. (1977). The anaerobic oyster heart: coupling of glucose and aspartate fermentation. *J. comp. Physiol.* **115**, 147–157.

CONNELLY, P. A., SISK, R. B., SCHULMAN, H. & GARRISON, J. C. (1987). Evidence for the activation of the multifunctional Ca^{2+}/calmodulin-dependent protein kinase in response to hormones that increase intracellular Ca^{2+}. *J. biol. Chem.* **262**, 10 154–10 163.

DE ZWAAN, A. (1983). Carbohydrate metabolism in bivalves. In *The Mollusca*, vol. 1 (ed. K. M. Wilbur), pp. 137–175. New York: Academic Press.

DE ZWAAN, A. & WIJSMAN, H. E. (1976). Anaerobic metabolism in Bivalvia (Mollusca): characteristics of anaerobic metabolism. *Comp. Biochem. Physiol.* **54B**, 313–324.

EBBERINK, R. & DE ZWAAN, A. (1980). Control of glycolysis in the posterior adductor muscle of the sea mussel *Mytilus edulis*. *J. comp. Physiol.* **137**, 165–171.

ELLINGTON, W. R. (1983*a*). Phosphorus nuclear magnetic resonance studies of energy metabolism in molluscan tissues. *J. comp. Physiol.* **153**, 159–166.

ELLINGTON, W. R. (1983*b*). The extent of intracellular acidification during anoxia in the catch muscles of two bivalve molluscs. *J. exp. Zool.* **227**, 313–317.

ELLINGTON, W. R. (1985). Metabolic impact of experimental reductions of intracellular pH in molluscan cardiac muscle. *Molec. Physiol.* **7**, 155–164.

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

HOCHACHKA, P. W. (1980). *Living Without Oxygen*. Cambridge, MA: Harvard University Press.

HOLWERDA, D. A., VEENHOF, P. R., VAN HEUGTEN, H. A. & ZANDEE, D. I. (1983). Regulation of mussel pyruvate kinase during anaerobiosis and in temperature acclimation by covalent modification. *Molec. Physiol.* **3**, 225–234.

HUE, L. & RIDER, M. H. (1987). Role of fructose-2,6-bisphosphate in the control of glycolysis in mammalian tissues. *Biochem. J.* **245**, 313–324.

KREBS, E. & BEAVO, J. (1979). Phosphorylation–dephosphorylation of enzymes. *A. Rev. Biochem.* **48**, 923–959.

KREUTZER, U., SIEGMUND, B. & GRIESHABER, M. K. (1985). Role of coupled substrates and alternative end products during hypoxia in marine invertebrates. *Molec. Physiol.* **8**, 371–392.

LINCOLN, T. M. (1983). cGMP-dependent protein kinases. *Comp. Biochem. Physiol.* **99**, 62–71.

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

MIESKES, G., KUDUZ, J. & SÖLING, H. D. (1987). Are calcium-dependent protein kinases involved in the regulation of glycolytic/gluconeogenetic enzymes? *Eur. J. Biochem.* **167**, 383–389.

OCHS, R. (1988). Does calcium regulate pyruvate kinase? *Trends Biochem. Sci.* **13**, 1–2.

PALLEN, C. J., SHARMA, R. K. & WANG, J. H. (1987). Modulation of cAMP effects by Ca^{2+}/calmodulin. *BioEssays* **2**, 113–117.

PLAXTON, W. C. & STOREY, K. B. (1984*a*). Phosphorylation *in vivo* of red muscle pyruvate kinase from the channeled whelk, *Busycotypus canaliculatum*, in response to anoxic stress. *Eur. J. Biochem.* **143**, 267–272.

PLAXTON, W. C. & STOREY, K. B. (1984*b*). 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. & STOREY, K. B. (1985). Tissue specific isozymes of pyruvate kinase in the channeled whelk *Busycotypus canaliculatum*: enzyme modification in response to environmental anoxia. *J. comp. Physiol.* B **155**, 291–296.

PLAXTON, W. C. & STOREY, K. B. (1986). Glycolytic enzyme binding and metabolic control in anaerobiosis. *J. comp. Physiol.* B **156**, 635–640.

PORTNER, H. O., GRIESHABER, M. K. & HEISLER, N. (1984). Anaerobiosis and acid–base status in marine invertebrates: effect of environmental hypoxia on extracellular and intracellular pH in *Sipunculus nudus* L. *J. comp. Physiol.* **155**, 13–20.

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

SAINTSING, D. G. & DIETZ, T. H. (1983). Modification of sodium transport in freshwater mussels by prostaglandins, cyclic AMP and 5-hydroxytryptamine: effects of inhibitors of prostaglandin synthesis. *Comp. Biochem. Physiol.* **76**C, 285–290.

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). A re-evaluation of the Pasteur effects: new mechanisms in anaerobic metabolism. *Molec. Physiol.* **8**, 439–461.

STOREY, K. B. (1987). Tissue-specific controls on carbohydrate catabolism during anoxia in goldfish. *Physiol. Zool.* **60**, 601–607.

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.

VAN SCHAFTINGEN, E. (1984). D-Fructose-2,6-bisphosphate. In *Methods of Enzymatic Analysis*, 3rd edn (ed. H. U. Bergmeyer), pp. 335–341. Weinheim: Chemie-Verlag.

WALSH, P. J., MCDONALD, D. G. & BOOTH, C. E. (1984). Acid–base balance in the sea mussel, *Mytilus edulis*. II. Effects of hypoxia and air exposure on intracellular acid–base status. *Mar. Biol. Lett.* **5**, 359–369.

WISEMAN, R. W. & ELLINGTON, W. R. (1987). Phosphorus magnetic resonance studies of the radula protractor muscles of the whelk *Busycotypus contrarium*. *Am. Zool.* **27**, abstract no. 279.