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STUDIES ON THE REGULATION OF ENZYME BINDING DURING ANOXIA IN ISOLATED TISSUES OF *BUSYCON CANALICULATUM*

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Summary

The role of pH and protein kinase second messengers in triggering or potentiating anoxia-linked changes in enzyme binding to particulate matter were evaluated using *in vitro* incubations of isolated ventricle strips of *Busycon canaliculatum* (L.) (Prosobranchia, Melongenidae). Incubating whelks under anoxic conditions for 4 h reduced the percentage of phosphofructokinase (PFK), aldolase, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and pyruvate kinase (PK) activity associated with cellular particulate matter. Triose phosphate isomerase, phosphoglycerate kinase, phosphoglyceromutase and enolase showed no changes in enzyme binding when whelks were subjected to anoxic stress *in vivo*. Incubating isolated ventricle strips *in vitro* under anoxic conditions simulated the changes seen *in vivo* in whole, anoxic whelks with respect to the percentage of PFK and PK bound during anoxic stress; both whole-animal studies and isolated tissue studies showed reduced PFK and PK binding after 4 h of anoxic incubation. Tissue pH could be artificially changed by incubating isolated ventricle strips in sea water buffered to a desired pH. This permitted an investigation of the effect of intracellular pH on PFK and PK binding *in situ*. PFK and PK responded to altered intracellular pH with increased enzyme binding at lower intracellular pH values and decreased enzyme binding at higher intracellular pH values. These binding patterns were exactly the opposite of those observed during anoxia; during anoxia stress, both intracellular pH and the percentage of PFK and PK associated with particulate matter decreased. Addition of the second messenger compounds dibutyryl cyclic AMP, dibutyryl cyclic GMP or phorbol 12-myristate 13-acetate plus the calcium ionophore A23187 had no effect on the percentage of activity bound to subcellular structures measured under either normoxic or anoxic conditions. This study suggests that enzyme binding *in vivo* is not regulated by changes in intracellular pH or concentrations of protein kinase second messenger compounds during anoxia.

Introduction

Changes in intracellular pH are frequently linked to changes in cellular function.

Key words: *Busycon canaliculatum*, glycolytic enzyme binding, regulatory role of pH, metabolic rate depression.

In particular, a significant correlation between decreased pH and decreased glycolytic rate has been well established using several animal models. In *Artemia* embryos, glycolysis was inhibited during both anaerobic dormancy and aerobic acidosis in gastrula-stage embryos (Carpenter and Hand, 1986*a*; Hand and Gnaiger, 1988); both conditions were associated with large decreases in intracellular pH. In perfused rat heart, lowering the pH of the perfusate from 7.4 to 6.6 resulted in a 71 % reduction in total glycolytic flux (Williamson *et al.* 1975) and a severely reduced developed pressure and cardiac rate (Williamson *et al.* 1976; Watters *et al.* 1987; Jeffrey *et al.* 1987). In white muscle fibers, decreasing pH during anaerobic work gradually led to decreased work output (Roos and Boron, 1981; Metzger and Fitts, 1987; Park *et al.* 1987; Madshus, 1988). A decreased intracellular pH was also associated with dormant states in hibernating mammals (Snapp and Heller, 1981; Tijane *et al.* 1989), hibernating reptiles (Lutz, 1989), estivating pulmonate land snails (Barnhart, 1986; Barnhart and McMahon, 1988) and anaerobiosis in marine invertebrates (Portner *et al.* 1984) and sea molluscs (Ellington, 1983*a*,*b*; Walsh *et al.* 1984).

The decrease in intracellular pH associated with exposure to anoxia stress in molluscs is largely the result of a switch from aerobic to anaerobic metabolism with the production of alternative fermentative end-products such as alanine, succinate and propionate derived from catabolism of glycogen and aspartate (de Zwaan, 1983). This reorganization of metabolism, together with an ability to depress anoxic metabolic rates to levels only 5–10 % of normoxic rates, represents an effective overall strategy for survival during prolonged periods of anoxia. Anoxic survival, therefore, includes strict regulatory mechanisms to control glycolytic flux in these facultative anaerobes, since glycolysis is the central pathway of anaerobic ATP synthesis (Storey, 1985). In these animals, several mechanisms for glycolytic control have been identified, including: (1) covalent modification of regulatory enzymes to alter kinetic parameters, producing less active enzymes (Storey, 1984; Plaxton and Storey, 1985), (2) changes in the concentration of the potent PFK allosteric regulator fructose 2,6-bisphosphate (Storey, 1988*a*), (3) lowering of intracellular pH (Ellington, 1983*a*,*b*; Busa and Nuccitelli, 1984; Somero, 1986; Madshus, 1988) to reduce the activities of enzymes such as phosphofructokinase (PFK; Carpenter and Hand, 1986*a*,*b*; Tijane *et al.* 1989), hexokinase (Carpenter and Hand, 1986*a*; Rees *et al.* 1989) and the enzymes of protein synthesis (Winkler, 1982) and (4) changes in the distribution of cytosolic enzymes between free and subcellular structure-associated states (Plaxton and Storey, 1986) to activate cytosolic enzymes (Srere, 1987; Friedrich, 1988).

It is clear that changes in the binding of enzymes to subcellular structures are related to the metabolic state of the cell. Studies with electrically stimulated bovine skeletal muscle (Clarke *et al.* 1980), exercised trout white muscle (Brooks and Storey, 1988*a*) and ischemic sheep and rat heart (Clarke *et al.* 1984) related increased enzyme binding to increased metabolic demand. This link between changes in enzyme association with particulate matter and changes in metabolic rate was further demonstrated by a decrease in enzyme binding during periods of

decreased metabolic demand in foot and ventricle of anoxic whelks (Plaxton and Storey, 1986).

Several models have been developed to describe completely the binding of cytosolic enzymes to particulate matter *in vivo* (Brooks and Storey, 1988*b*,*c*, 1990*b*; Kurganov, 1986; Liou and Anderson, 1980; Luther and Lee, 1986; Somero, 1986). In these models, filamentous actin (F-actin) was identified as the subcellular structure that binds cytosolic enzymes because of experiments that demonstrated glycolytic enzyme binding to F-actin *in vitro* (Chan *et al.* 1986; Liou and Anderson, 1980; Luther and Lee, 1986). Several of these models included the identification of putative signal compounds responsible for initiating enzyme binding *in vivo*. For example, changes in intracellular calcium concentration (Kurganov, 1986), changes in the degree of enzyme phosphorylation (Liou and Anderson, 1980; Luther and Lee, 1986) or changes in intracellular pH (Brooks and Storey, 1988*c*, 1990*b*; Somero, 1986) have all been indicated as possible signal events responsible for increased enzyme binding *in vivo*. In the case of pH changes, studies with fish muscle showed that the percentage of glycolytic enzyme activity associated with particulate matter *in vivo* increased with decreasing intracellular pH during burst swimming (Brooks and Storey, 1988*a*). *In vitro* studies have also demonstrated an effect of pH on the affinity of PFK (Somero, 1986; Brooks and Storey, 1988*b*), glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Dagher and Hultin, 1975) and lactate dehydrogenase (LDH; Hultin, 1975) for F-actin as well as an effect of pH on the affinity of PFK and GAPDH for erythrocyte membranes (Higashi *et al.* 1979; Kant and Steck, 1973). Both F-actin and erythrocyte membrane systems showed increased enzyme binding at lower pH values. In the case of erythrocyte membranes, PFK (Higashi *et al.* 1979) and GAPDH (Kant and Steck, 1973) bound to a specific plasma membrane protein, suggesting a physiological relevance for this interaction.

The present study investigated intracellular signal compounds which may potentiate changes in enzyme binding that occur as part of facultative anaerobiosis in the anoxia-tolerant whelk *Busycon canaliculatum* (L). In whelk ventricle, both intracellular pH and enzyme binding decreased during anoxia in sharp contrast to the vertebrate systems described above (Plaxton and Storey, 1986). Thus, the whelk ventricle model system allowed a unique opportunity to study the apparent uncoupling between *in vivo* changes in pH and in the degree of enzyme binding. In the present study, whelk ventricle strips were used. Ventricle strips represent a convenient system to study *in vivo* processes because intracellular concentrations of effectors can be altered by changing the composition of the incubating medium in a fashion analogous to that used in the perfusion of vertebrate hearts (Jeffrey *et al.* 1987; Watters *et al.* 1987). Previous studies with isolated molluscan tissue systems indicated that they show equivalent responses to anoxia both *in vitro* and *in vivo* (see Brooks and Storey, 1989) as well as identical patterns of substrate utilization and end-product accumulation (Collicutt and Hochachka, 1977). This system thus presents a useful model system for studying the effect of potential glycolytic complex modulators on enzyme binding *in vivo*.

Materials and methods

Chemicals and animals

All chemicals were purchased from Sigma Chemical Co. (St Louis, MO) or Boehringer–Mannheim (Montreal, 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 at 6–9°C until use.

Enzyme assays

Maximal enzyme activities were determined in a 1 cm path length cuvette using a Gilford model 240 spectrophotometer at 340 nm following NADH oxidation. All enzyme assays were performed at room temperature (21±1°C). Enzyme activities were optimized with respect to pH and substrate concentrations. PFK, aldolase (ALD) and PK were assayed according to Plaxton and Storey (1986). GAPDH and phosphoglycerate kinase (PGK) were measured according to Brooks and Storey (1988*a*). Enolase, triosephosphate isomerase (TPI) and phosphoglyceromutase (PGM) were assayed according to Storey and Bailey (1978). The reaction velocity was measured before and after the addition of substrate to allow quantification of a small contaminating background activity.

Whole-animal experiments

Control animals were sampled directly from the holding tank. To impose anoxia, whelks were placed in artificial sea water that had previously been bubbled with N_2 gas overnight. Animals were held under anoxic conditions for 4 h with continuous bubbling with N_2 gas.

Isolated tissue experiments

Tissue incubations were performed according to Brooks and Storey (1989). Ventricles from control whelks were rapidly dissected out and separated into small pieces of approximately 80 mg each. These tissue strips were immediately placed in vials containing 10 ml of artificial sea water previously bubbled for 10 min with either air (O_2, aerobic) or N_2 gas (anoxic). Samples were incubated for 4 h with continuous bubbling. When required, the appropriate concentrations of effectors were added to the incubating sea water.

Sample preparation

Measurement of bound *versus* free enzyme activities was performed according to Plaxton and Storey (1986). Ventricles from whole animals or ventricle strips from *in vitro* incubations were quickly rinsed twice in distilled water and homogenized (1:4 w/v) at 0–4°C in 800 mmol l^{-1} sucrose containing 10 mmol l^{-1} β-mercaptoethanol and 0.1 mmol l^{-1} phenylmethylsulfonyl fluoride for 15 s using an Ultraturrax homogenizer at 80 % of full speed. The pH of the homogenates was quickly measured using a pH meter to obtain an estimate of the tissue pH value

Although this method does not provide an absolute measure of the tissue pH, the results obtained using this method are similar to values previously reported for ventricle using ^{31}P nuclear magnetic resonance (n.m.r.) (Ellington, 1983*a*, 1985; Wiseman and Ellington, 1987) and to those obtained using a modification of this method (Brooks and Storey, 1989). A 50 μl sample was removed and diluted 1:4 (v:v) with 100 mmol l^{-1} potassium phosphate (pH 7.5), 1 mmol l^{-1} EDTA, 1 mmol l^{-1} EGTA, 25 mmol l^{-1} NaF, 0.1 mmol l^{-1} fructose 1,6-bisphosphate, 0.1 mmol l^{-1} ATP and 10 mmol l^{-1} 2-mercaptoethanol (Stabilization Buffer, SB). This sample served as a control to check recovery in the supernatant and pellet fractions. Another 50 μl sample was centrifuged at 12 000 *g* for 5 min, and the supernatant was removed and diluted 1:4 with SB; this fraction represented the free enzyme. The pellet was twice extracted with 125 μl of SB; these extracts were combined to give the bound enzyme fraction. These two pellet extractions solubilized more than 97 % of the activity associated with the pellet.

Results

The procedure of Clarke *et al.* (1984) offers a rapid, reproducible method for measuring the distribution of glycolytic enzymes between soluble and particulate-associated cytosolic fractions. In this procedure, homogenization in iso-osmotic buffer ruptures plasma membranes without disrupting electrostatic associations between enzymes and subcellular structures. The results of the present paper were obtained using a modification of the original procedure of Clarke *et al.* (1984), which involved increasing the sucrose concentration of the homogenization buffer from 250 to 800 mmol l^{-1}. This followed from the experiments of Plaxton and Storey (1986), who showed that both PFK and ALD were sensitive to the osmotic strength of the homogenization buffer. For marine invertebrates that maintain body fluids iso-osmotic with sea water, the higher sucrose concentration represented the more physiological osmotic strength.

The time course for decreases in the percentage of PFK, ALD, GAPDH and PK associated with particulate matter during anoxia are presented in Fig. 1. After 4 h of anoxia, binding of all four enzymes had decreased maximally and no further changes occurred beyond this time. The results of Fig. 1, coupled with previous studies showing that other anoxia-associated cellular responses in isolated tissues were also maximally expressed after 4 h of incubation (Brooks and Storey, 1989), indicated that this time was optimal for studying cellular changes associated with anoxia in whelk ventricle tissue strips.

The distribution of enzyme activities between free and particulate-associated fractions after 4 h of anoxia was examined as a function of oxygen lack for eight glycolytic enzymes: PFK, ALD, TPI, GAPDH, PGK, PGM, enolase and PK. These enzymes were chosen because previous experiments had shown that changes in glycolytic enzyme binding may play a role in regulating glycolytic flux during anoxia stress in whelk ventricle (Plaxton and Storey, 1986). The maximal

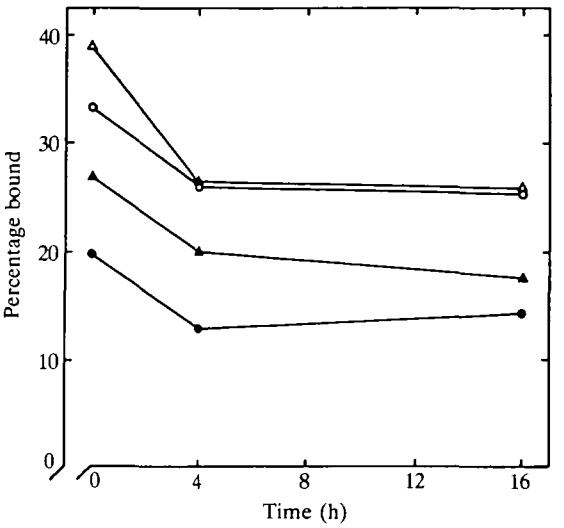

Fig. 1. Time course of changes in enzyme binding to the particulate fraction of whelk ventricle during anoxia *in vivo*. Percentage of total phosphofructokinase (●), aldolase (○), glyceraldehyde phosphate dehydrogenase (▲) and pyruvate kinase (△) activities associated with particulate matter in *Busycon canaliculatum* ventricle measured at various times after immersing whole animals in N_2-bubbled sea water.

Table 1. *Maximal activities of glycolytic enzymes in whelk ventricle*

Enzyme	Activity (units g^{-1} wet mass)
Phosphofructokinase	7.0±0.5
Aldolase	7.5±0.5
Triose phosphate isomerase	332.0±19.2
Glyceraldehyde 3-phosphate dehydrogenase	100.2±6.5
Phosphoglycerate kinase	54.6±3.9
Phosphoglyceromutase	23.3±0.6
Enolase	4.5±0.6
Pyruvate kinase	42.9±1.4

Values are means±S.E.M. (N=6).

Values from aerobic and 4 h anoxic animals were combined, since a comparison of aerobic *versus* anoxic values revealed no significant differences using Student's t-test.

activities of these enzymes, reported in Table 1, were not significantly different for aerobic and 4 h anoxic animals.

The data in Table 2 show the fraction of whelk ventricle enzyme activities associated with particulate matter after 4 h of anoxic exposure of either the whole animal or isolated ventricle strips. Typically, recovery of enzyme activity approximated 100 % when free and particulate-bound activities were compared with tota

Table 2. *Effect of anoxia on the distribution of enzymes between soluble and particulate fractions of ventricle: comparison of anoxia* in vivo versus in vitro *incubation of isolated ventricle strips*

	Percentage bound			
	Whole animal		Isolated tissue	
Enzyme	O_2	N_2	O_2	N_2
PFK	19.8±2.3	12.9±0.5[a]	19.2±0.9	15.3±1.0[b]
ALD	33.0±2.0	26.1±0.2[a]	24.4±1.1[a]	22.3±1.5
TPI	21.1±1.2	19.8±1.6	ND	ND
GAPDH	26.9±1.5	19.9±0.8[a]	18.3±0.9[a]	19.1±0.8
PGK	23.3±0.6	21.6±1.3	ND	ND
PGM	12.7±1.1	15.0±2.1	ND	ND
Enolase	23.4±0.8	20.1±1.2	ND	ND
PK	39.0±1.2	26.4±3.0[a]	33.6±1.0[a]	21.4±1.4[b]

Ventricle samples were incubated in unbuffered artificial sea water, pH value approximately 8.

Data are means±S.E.M. (N=4 for whole-animal studies, N=8 for isolated tissue studies).

Samples are significantly different at the $P<0.05$ level compared with either (a) whole animal O_2 or (b) isolated tissue O_2 as measured by the one-tailed Student's t-test.

Tissue N_2 values were not significantly different from whole animal N_2 values as determined by the one-tailed Student's t-test.

ND, not determined.

PKF, phosphofructokinase; ALD, aldolase; TPI, triose phosphate isomerase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; PGK, phosphoglycerate kinase; PGM, phosphoglyceromutase; PK, pyruvate kinase.

activity, showing that the procedure accurately accounted for the enzyme activity in all fractions. In whole-animal studies, anoxic stress produced a significant decrease in the binding of four of the eight glycolytic enzymes assayed: PFK, ALD, GAPDH and PK. These results are consistent with previous studies on whelk ventricle enzyme binding during anoxia (Plaxton and Storey, 1986). The decrease in PFK and PK binding was also observed in isolated ventricle strips incubated for 4 h under anoxic conditions (Table 2). In the case of PFK, the response in isolated ventricle strips was equivalent to that in whole animals; PFK binding changed in the same direction and by a similar amount in either whole animals or in isolated strips. PK binding was also similar when the isolated tissue response was compared to that of whole animals: aerobic tissues had a slightly lower percentage of PK bound to particulate matter when compared to whole animals, but binding decreased by the same extent in both isolated ventricle strips and whole animals during anoxia.

Measurement of ALD and GAPDH binding in isolated ventricle strips showed no change in binding after 4 h of anoxic incubation, even though the percentage of these enzymes associated with particulate matter was reduced in whole-animal

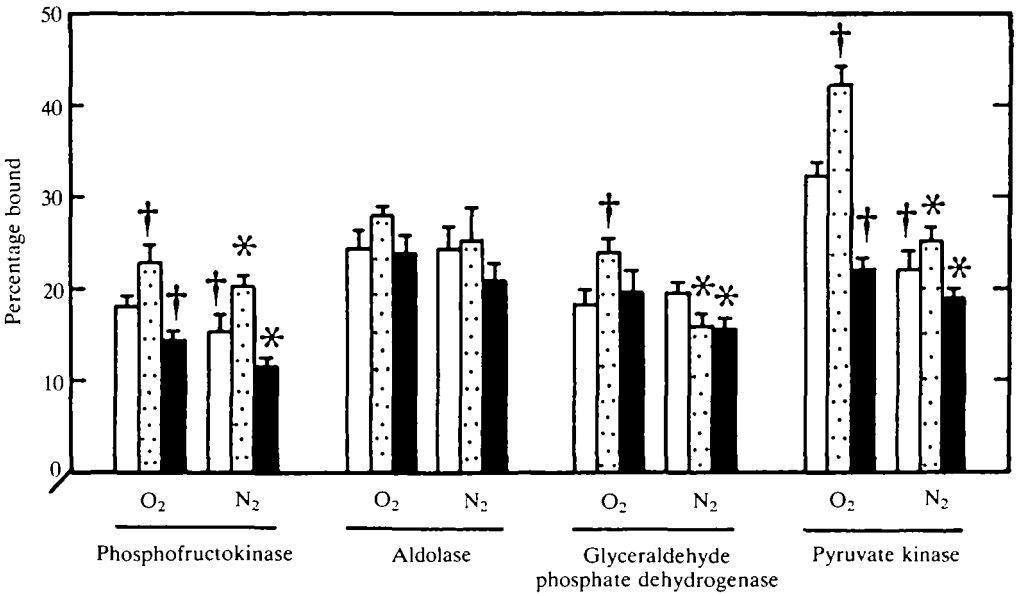

Fig. 2. Effect of varying pH on glycolytic enzyme binding in isolated ventricle strips. Ventricle samples were incubated in (unbuffered) sea water, pH approximately 8 (□) or sea water adjusted to a specific pH value after the addition of either 20 mmol l^{-1} potassium succinate, pH 5.5 (⁘), or 100 mmol l^{-1} Taps, pH 9.5 (■). Data are means +S.E.M. (N=4). Intracellular pH values are reported in Table 3. Data were analysed by the one-tailed paired Student's t-test. Samples are significantly different at the $P<0.05$ level compared with O_2 control (†) or N_2 control (*).

ventricle (Table 2). This lack of response was apparently due to reduced enzyme binding in aerobic isolated tissues; the level of enzyme binding in aerobic isolated tissues was identical to that in anoxic animal ventricle and to that in anoxic isolated ventricle strips. These preliminary studies show that isolated tissues are a good model for studying anoxia-associated changes in PFK and PK binding to cellular particulate matter.

The effect of changing intracellular pH on the degree of enzyme binding in isolated tissues is presented in Fig. 2. For these experiments, intracellular pH was altered by incubating isolated tissues in artificial sea water buffered with either 20 mmol l^{-1} succinate (pH 5.5) or 100 mmol l^{-1} Taps (pH 9.5). Both succinate and Taps may permeate the plasma membrane (see Walsh, 1990) so that the intracellular pH of tissues incubated with these buffers, and measured in homogenates using a pH meter, was significantly different from that of tissues incubated in unbuffered sea water (Table 3). Although the homogenization/pH meter procedure used in the present paper may not measure the true intracellular pH, the results obtained with this procedure are in excellent agreement with the values determined for *Busycon contrarium in vivo* using ^{31}P n.m.r. Using the n.m.r. technique, Ellington (1983) measured aerobic *Busycon contrarium* ventricle intracellular pH values of 7.14±0.05, which decreased to 6.85±0.04

Table 3. *Effect of incubation conditions on ventricle intracellular pH measured in tissue homogenates using a pH meter*

Condition	pH
Whole animals	
O_2	6.78±0.03
N_2	6.59±0.06[a]
Isolated tissues	
O_2/SW	7.00±0.13
O_2/5.5	6.47±0.15[a]
O_2/9.5	8.01±0.02[a]
N_2/SW	6.69±0.13[a]
N_2/5.5	6.40±0.15[b]
N_2/9.5	7.81±0.09[b]

All incubations were performed for 4 h.

In isolated tissue experiments, ventricle samples were incubated in unbuffered artificial sea water, pH approximately 8 (SW), or sea water adjusted to a specific pH value after the addition of either 20 mmol l^{-1} potassium succinate (pH 5.5) or 100 mmol l^{-1} Taps (pH 9.5).

Data are means±S.E.M. (N=4).

Data were analysed by the one-tailed paired Student's t-test (isolated tissues) or the one-tailed Student's t-test (whole animals). Samples are significantly different at the $P<0.05$ level as compared to the O_2/SW (a) or the N_2/SW (b) condition.

(means±S.E.M.) after 4 h of anoxia plus ischemia. These values are not significantly different from the values measured in isolated tissues reported in Table 3, as determined by the one-tailed Student's t-test. The decrease in pH after 4 h of anoxia plus ischemia in both whole animals (Ellington, 1983; Table 3) and in isolated tissues (Table 3) is approximately 0.3 units.

As shown in Fig. 2, the effect of pH on enzyme binding to particulate matter in isolated ventricle strips was identical for PFK and PK: lower pH increased enzyme binding and higher pH decreased enzyme binding under both anoxic and normoxic conditions. Note, however, that the effect of pH on enzyme binding measured under anoxic conditions was less pronounced than that observed under aerobic conditions. For example, the percentage of PK associated with particulate matter under aerobic conditions was 32.5±1.5 at neutral pH values (7.00±0.13), 42.3±2.1 at low pH values (6.47±0.15) and 22.0±1.4 at high pH values (8.01±0.02), whereas under anaerobic conditions PK enzyme binding was significantly reduced: values were 22.0±2.0 at pH 6.69±0.13, 25.3±1.4 at low pH values (6.40±0.15) and 18.8±1.2 at high pH values (7.81±0.09).

In contrast to the responses observed with PFK and PK, ALD showed no response to changing pH values: the percentage of bound ALD did not vary with changing pH in either aerobic or anoxic tissues. Binding of GAPDH was significantly increased by lowering the incubation pH of aerobic tissues, but was significantly decreased by both higher and lower incubation pH under anoxic conditions.

Fig. 2 also shows that the presence or absence of oxygen is the overriding factor in determining the percentage of PFK and PK binding in isolated ventricle strips. For example, when isolated tissues were incubated in the presence of succinate buffer, pH 5.5, exposure of tissues to anoxia stress reduced the percentage of PK binding from 42.2±2.1 (aerobic values) to 25.3±1.4 % (anoxic values). This effect was also apparent when isolated strips were incubated in Taps-buffered sea water (pH 9.5). In this case, the percentage of PK binding decreased from an aerobic value of 22.0±1.4 to an anoxic value of 18.8±1.2. These patterns were observed for PFK binding to particulate matter as well.

The effect of protein kinase activators on the degree of enzyme binding in *B. canaliculatum* ventricle was examined to test whether the degree of enzyme phosphorylation had an effect on enzyme binding. Addition of either 0.4 mmol l^{-1} dibutyryl cyclic AMP (which activates protein kinase A), 0.4 mmol l^{-1} dibutyryl cyclic GMP (which activates cyclic-GMP-dependent protein kinase) or 30 μmol l^{-1} calcium ionophore A23187 plus 10 μg ml^{-1} phorbol 12-myristate 13-acetate (which activates protein kinase C and increases intracellular calcium concentrations) had no effect on the extent of enzyme binding in isolated ventricle tissues incubated under aerobic or anoxic conditions. In all cases the percentage of enzyme activity associated with the particulate matter was well within the mean±S.E.M. of either the normoxic or anoxic control values given in Table 2 (data not shown), indicating that the binding of PFK, ALD, GAPDH and PK was unaffected by common protein kinase second messenger compounds and by increases in intracellular calcium concentration.

Discussion

The present study investigated the effects of changing intracellular pH, increasing intracellular calcium ion concentrations and increasing second messenger concentrations on the degree of PFK and PK binding to particulate matter in isolated whelk ventricle strips. These effectors were chosen because they represent compounds thought to mediate changes in glycolytic enzyme binding resulting from changes in glycolytic demand (Brooks and Storey, 1988*b*,*c*, 1990*b*; Kurganov, 1986; Liou and Anderson, 1980; Luther and Lee, 1986; Somero, 1986).

Whelks, and whelk ventricles in particular, represent an ideal experimental model for this study because of (1) a demonstrated correlation between anaerobiosis and reduced tissue pH (Ellington, 1983*a*,*b*), (2) a reduced enzyme binding during anoxia (Plaxton and Storey, 1986) and (3) an equivalent response of *in vitro* isolated ventricle strips when compared to *in vivo* studies using whole animals (Brooks and Storey, 1989). The results obtained with isolated strips showed that lack of oxygen was the overriding factor that determined the degree of PFK and PK binding in whelk ventricle during anoxic stress. This was best illustrated by the data of Fig. 2, which show that the effect of changing pH on the binding of PFK and PK was exactly opposite to that expected from changes in intracellular pH during anoxia stress. For example, in our experiments, intracellular pH values fe

from 7.00±0.13 to 6.69±0.22 during anoxic stress in isolated tissues whereas PFK and PK binding decreased by 30%. This was exactly opposite to the response obtained when intracellular pH values were artificially altered: enzyme binding increased with decreasing pH and decreased with increasing pH under both normoxic and anoxic conditions. This demonstrates that intracellular pH did not regulate enzyme binding during anoxia in whelk ventricle. It also indicates that other anoxia-associated cellular changes must be responsible for determining the percentage of glycolytic enzyme binding in *B. canaliculatum* ventricle.

In an attempt to identify the intracellular signal responsible for mediating the changes in enzyme binding that characterize the metabolic response to anoxia in these facultative anaerobes, isolated tissues were also incubated in the presence of protein kinase activators (dibutyryl cyclic AMP, dibutyryl cyclic GMP and phorbol 12-myristate 13-acetate plus A23187). Previous studies with whole animals have demonstrated the importance of enzyme phosphorylation during anoxia (see Storey, 1988*b*). Anoxia-induced covalent modification of PK (Plaxton and Storey, 1985) and PFK (Storey, 1984) have been documented in *B. canaliculatum*. These modifications result in stable changes in enzyme kinetic patterns to produce enzymes with lower activity during anoxia. Reduced glycolytic flux is a key part of an overall strategy to conserve glycogen stores and to limit the accumulation of often toxic end-products during anoxia (Storey, 1988*b*). Previous studies have also demonstrated a correlation between increased PFK binding to F-actin *in vivo* and increased enzyme phosphorylation using rabbit muscle proteins (Luther and Lee, 1986). Because of the importance of enzyme phosphorylation during anoxia in whelks (Plaxton and Storey, 1985; Storey, 1984), we added known protein kinase second messengers in an attempt to alter the phosphorylation state of the enzymes *in vivo* to determine if an effect similar to that observed with mammalian PFK was operating. In all cases, addition of dibutyryl cyclic AMP, dibutyryl cyclic GMP or phorbol 12-myristate 13-acetate plus the calcium ionophore A23187 had no affect on the degree of enzyme binding in either anoxic or normoxic tissues. These second messenger derivatives are known to permeate membranes and increase cyclic AMP and Ca^{2+} levels *in vivo*. This has been directly shown for dibutyryl cyclic AMP and Ca^{2+} plus phorbol 12-myristate 13-acetate in isolated whelk tissues, where addition of these compounds changed selected PFK kinetic patterns and fructose 2,6-bisphosphate concentrations in individual tissues (Brooks and Storey, 1989). Cyclic GMP is known to stimulate PK phosphorylation in isolated whelk homogenates (Brooks and Storey, 1990*a*) and cyclic AMP is a well-known promoter of protein kinases (Edelman, 1987). The lack of an effect of these compounds on enzyme binding in both aerobic and anoxic isolated tissue incubations suggests that enzyme phosphorylation does not play a role in reducing enzyme binding during anoxia. Addition of the calcium ionophore A23187 illustrated that increasing Ca^{2+} concentrations do not alter the degree of enzyme binding in isolated whelk ventricle strips. These results suggest that increases in free Ca^{2+} concentrations do not regulate enzyme binding *in vivo*.

It is clear that the reversible binding of enzymes to subcellular structures is

related to the metabolic state of the cell, as demonstrated by the correlation between enzyme binding and glycolytic flux *in vivo* (Clarke *et al.* 1980, 1984; Plaxton and Storey, 1986; Brooks and Storey, 1988*b*). The importance of changes in enzyme binding to overall glycolytic flux can be inferred from a consideration of enzyme activities in the cytosol. In the cytosol, enzyme activity is reduced by three different effects: (i) a low free concentration of substrate (Srivastava and Bernhard, 1986), (ii) a relatively long time required for free diffusion of product from a primary enzyme to the next enzyme in the sequence (Kurganov *et al.* 1985), and (iii) a relatively high concentration of allosteric inhibitors such as ATP (Srivastava and Bernhard, 1986). The formation of multi-enzyme complexes *via* increased enzyme association with F-actin (particulate matter, see Kurganov *et al.* 1985) could greatly increase individual enzyme activities by effectively increasing the concentration of enzyme active sites. This would serve (1) to increase the relative concentration of enzyme substrates and (2) to reduce the distance between active sites. Enzymes may also be allosterically activated when bound to F-actin to negate the effect of inhibitors (such as occurs for PFK). Thus, kinetic studies of PFK have demonstrated an increased activity when bound to F-actin *in vitro* (Choate *et al.* 1985; Luther and Lee, 1986). *In vitro* binding studies have also demonstrated a relationship between pH and PFK binding (Choate *et al.* 1985; Brooks and Storey, 1988*b*), GAPDH binding (Dagher and Hultin, 1975) and LDH binding (Hultin, 1975) and a relationship between increased PFK phosphorylation and increased enzyme binding (Luther and Lee, 1986). The combined weight of these studies suggested to several authors that pH and enzyme phosphorylation played a central role in regulating glycolytic flux by directly influencing the bound/free status of cytosolic glycolytic enzymes (Ovadi, 1988; Brooks and Storey, 1990*b*). The results of the present study, however, suggest that neither changes in intracellular pH nor changes in the degree of enzyme phosphorylation could reproduce the changes in enzyme binding observed during anoxia *in vivo*. A comparison with *in vivo* and *in vitro* data from other laboratories (using mammalian and fish muscle enzymes) demonstrates that these results are not unexpected. For example, *in vitro* studies with mammalian muscle PFK and F-actin show that lower pH and increased enzyme phosphorylation increased PFK binding. Since whelk ventricle PFK appears to bind particulate matter in a similar fashion to vertebrate PFK (lower pH increases the degree of enzyme association *in vivo*, Fig. 2), anoxia-related pH and phosphorylation changes would be expected to increase the extent of PFK binding. In the present study, relative changes in intracellular pH did not alter enzyme binding during anoxia in isolated whelk ventricle strips. These results imply that pH may not regulate enzyme binding in vertebrate muscle tissues during periods of increased metabolic demand. This conclusion agrees with previous data on the time course of enzyme binding and pH changes in trout white muscle: enzyme binding increased prior to decreases in intracellular pH (Brooks and Storey, 1988*a*). The results of the present study, therefore, suggest that a re-examination of the intracellular signal(s) that mediate

enzyme binding is required to elucidate the exact role of enzyme binding in controlling metabolic rates during periods of altered metabolism.

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