Anaerobiosis and organ-specific regulation of glycolysis in a marine whelk

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Changes in the levels of glycolytic intermediates, adenylates, phosphagen, and substrates and products of anaerobic metabolism were monitored over the initial hours of anaerobiosis in radular retractor muscle, foot muscle, and gill of the marine whelk *Busycotypus canaliculatum*. In all organs, changes in glycolytic intermediates upon exposure to N_2-bubbled seawater indicated a biphasic response by glycolytic rate to anoxia: an immediate activation (within 30 min to 1 h) followed after 1–4 h by glycolytic rate depression. In both cases, regulatory control at phosphofructokinase and pyruvate kinase was indicated. The accumulation of anaerobic end products differed between the three organs. In radular retractor and gill, the ratio alanine:succinate produced was 2:1 (but with higher amounts of both products in radular retractor), whereas in foot the ratio was 12.5:1. Foot muscle showed a large depletion of arginine phosphate reserves early in anoxia but little perturbation of adenylate pools, whereas gill and radular retractor showed a much lower net use of phosphagen but substantial depletion of organ ATP content. The data support several conclusions: (*i*) metabolic rate depression in the whelk is initiated within 1–2 h of anoxia exposure, (*ii*) mechanisms of metabolic depression override allosteric activating effects by phosphagen and adenylates on phosphofructokinase, and (*iii*) inhibition of flux through pyruvate kinase parallels the course of metabolic depression, not changes in carbon partitioning at the phosphoenolpyruvate branch point.

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Les variations de concentration des produits intermédiaires de la glycolyse, adénylates, phosphagène, substrats et produits du métabolisme anaérobie, ont été enregistrées au cours des premières heures de l'anaérobie dans le muscle rétracteur de la radula, le muscle du pied et les branchies chez le buccin marin *Busycotypus canaliculatum*. L'introduction de N_2 dans l'eau de mer déclenche des perturbations des produits intermédiaires de la glycolyse qui indiquent l'existence d'une réaction biphasique du taux de glycolyse à l'anoxie : une activation immédiate (après 30–60 min) suivie d'une diminution (après 1–4 h). Dans les deux cas, la phosphofructokinase et la pyruvate kinase interviennent. L'accumulation des produits terminaux anaérobies diffère dans les trois organes. Dans le muscle rétracteur de la radula et dans les branchies, le rapport alanine:succinate final est de 2:1 (quantités plus élevées des deux produits dans le muscle), alors que, dans le pied, le rapport final est de 12,5:1. Le muscle du pied subit une importante perte de ses réserves de phosphate d'arginine au début de l'anoxie, mais ses réserves d'adénylate sont peu perturbées, alors que les branchies et le rétracteur de la radula subissent une perte nette de phosphagène beaucoup plus faible, mais une perte substantielle de leurs réserves d'ATP. Ces données indiquent que (*i*) la diminution du taux métabolique chez le buccin est déclenchée 1–2 h après le début de l'anoxie, (*ii*) les mécanismes impliqués dans la diminution métabolique enrayent les effets allostériques activateurs du phosphagène et des adénylates sur la phosphofructokinase et (*iii*) l'inhibition du flux par voie de la pyruvate kinase suit la diminution métabolique et non les changements dans l'allocation du carbone au point de jonction phosphoénolpyruvate.

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Introduction

Many marine molluscs, particularly those with intertidal or burrowing life-styles, are excellent facultative anaerobes. Key adaptations supporting long-term survival in the absence of oxygen include (*i*) a sharp reduction in metabolic rate to levels 5–10% aerobic basal rate (Famme et al. 1981; Shick et al. 1983), and (*ii*) the use of alternative pathways of fermentative ATP production (de Zwaan 1983). Typically, the coupled fermentation of glycogen and aspartate as substrates results in the accumulation of alanine and succinate (sometimes also propionate, acetate, and imino acids) as end products. The energetic advantage of such pathways derives from the additional substrate-level phosphorylations of ADP linked to organic acid synthesis.

The control of glycolysis is central to anaerobiosis. The pathway is the primary ATP-producing route during anoxia and therefore control over glycolysis is key to maintaining homeostasis in the anoxic state. Metabolic rate depression must feature, as a major component, control over glycolytic flux to permit coordinated regulation of ATP-producing, ATP-utilizing, and passive processes in the cell. Recent studies in our laboratory have focused on the enzymatic mechanisms involved in glycolytic control and glycolytic rate depression during anoxia in vertebrates and invertebrates (Storey 1985, 1987, 1988*a*, 1988*b*; Plaxton and Storey 1984*a*, 1984*b*, 1986; Rahman and Storey 1988; Brooks and Storey 1988, 1989). Three molecular mechanisms have been identified that participate in glycolytic rate reduction: (*i*) anoxia-induced covalent modification of regulatory enzymes to produce less active enzyme forms, (*ii*) anoxia-induced disruption of glycolytic enzyme complexes bound to subcellular particulate fractions, and (*iii*) a reduction in levels of fructose-2,6-bisphosphate (a key activator of PFK) to help restrict carbohydrate use for anabolic purposes in the anoxic state (Storey 1985, 1988*a*). All three of these mechanisms are exemplified in the whelk *Busycotypus canaliculatum* (Storey 1984, 1988*b*; Plaxton and Storey 1984*a*, 1984*b*, 1985*a*, 1985*b*, 1986; Brooks and Storey 1989; Whitwam and Storey 1990).

The present study was designed to examine, in an organ-specific manner, the metabolic events underlying the transition

ABBREVIATIONS: PFK, phosphofructokinase (EC 2.7.1.11); PK, pyruvate kinase (EC 2.7.1.40); F6P, fructose-6-phosphate; F1,6BP, fructose-1,6-bisphosphate; G3P, glyceraldehyde-3-phosphate; PEP, phosphoenolpyruvate.

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from the aerobic to the anoxic state in the whelk. The focus is on changes in glycolytic activity over the early hours of anoxia and the roles of PFK and PK as regulatory sites.

Materials and methods

Chemicals

All biochemicals were purchased from Sigma Chemical Company, St. Louis, Missouri, or Boehringer Mannheim Corporation, Montreal. Purified succinyl-CoA synthetase was a gift from Dr. W. Bridger, University of Alberta, Edmonton.

Animals

Whelks (*Busycotypus canaliculatum*) were purchased from the Marine Biological Laboratory, Woods Hole, Massachusetts. Animals were held without feeding in large aerated tanks of seawater at 15°C for 2 weeks before use.

Control aerobic whelks were sampled directly from the seawater tank. To impose anoxia, whelks were placed in containers of seawater (10 L) that had been previously bubbled with nitrogen gas (100%) for 12 h. Containers were closed, and bubbling with N_2 was continued at a low rate. The container was bathed in a larger tank to maintain water temperature at 15°C. Whelks were sampled at timed intervals. Shells were quickly removed and organs were rapidly excised, blotted, and immediately frozen in liquid nitrogen. Tissue samples were stored at −80°C until used.

Preparation of organ extracts

Samples of frozen tissue (up to 350 mg) were ground to a powder under liquid nitrogen, using a mortar and pestle. Samples were then transferred to preweighed polypropylene tubes stored on dry ice. After weighing, tubes were moved one at a time to a dry ice – methanol bath at −8°C. Perchloric acid (6%, containing 1 mM EDTA, chilled at −8°C) was rapidly added (1:5 w/v), and the sample was homogenized using a Polytron PT10 homogenizer. Homogenates were centrifuged at 6000 × *g* for 15 min at 4°C to remove precipitated protein. The acid supernatants were removed, transferred to clean tubes, and then neutralized by addition of a solution containing 3 M KOH, 0.4 M Tris base, and 0.3 M KCl. After a second centrifugation to remove precipitated $KClO_4$, an aliquot of the neutralized extract was removed for immediate assay of pyruvate and PEP; the remainder of the extract was frozen on dry ice and then stored at −20°C.

Metabolite assays

All metabolites were quantified by coupled enzyme assays (Williamson and Corkey 1969; Lowry and Passonneau 1972). Glycolytic intermediates and adenylates were measured fluorometrically; alanine, succinate, and aspartate were determined spectrophotometrically. For succinate, ATP and the ATP-dependent succinyl-CoA synthetase from *E. coli* were substituted in the assay of Williamson and Corkey (1969). Arginine phosphate was assayed using the assay conditions for creatine phosphate described by Lowry and Passonneau (1972), with the substitution of arginine kinase (0.9 U/assay) for creatine kinase.

Statistics

Results are presented as means ± SEM. For significant differences between groups, Student's *t*-test was used.

Results

Figure 1 shows the effect of anoxia exposure on the contents of arginine phosphate and adenylates in three organs of the whelk. In all three organs, arginine phosphate reserves are mobilized early in anoxia, within the first 1–4 h, with no significant changes in phosphagen content thereafter. Use of phosphagen was greatest in foot muscle, with a net decrease of 4.3 μmol/g wet weight over the first hour of anoxia. Phosphagen in radular retractor muscle and gill, by contrast, decreased by only 1.2 and 0.4 μmol/g, respectively, a net loss of about 40% of the total pool size in each organ. Probably as a result of a contribution by phosphagen to the maintenance of the ATP pool, levels of the adenylates in foot muscle remained virtually constant over the first 8 h of anoxia, with energy charge averaging 0.78. However, after 12 h some energy stress became apparent: energy charge decreased to 0.70 and total adenylate content declined by 15%. Radular retractor muscle and gill both showed an early drop in ATP content, with corresponding changes in ADP and AMP contents. Energy charge fell rapidly in gill, from 0.84 in the aerobic control organ to an average of 0.62 which was maintained between 1 and 12 h of anoxia. Total adenylate content in gill decreased by 20% between 2 and 4 h and remained constant thereafter. In radular retractor, energy charge reached a low of 0.73 (compared with control, 0.86) after 2 h of anoxia but increased slightly thereafter (an average of 0.78) following a 23% decrease in the total adenylate pool.

Figure 2 shows the effects of anoxia on organ contents of the anaerobic substrate, aspartate, and the end products, succinate and alanine. In radular retractor, levels of succinate and alanine rose in parallel over the 12 h, net accumulations being 2.7 and 3.4 μmol/g, respectively. Inversely, aspartate content declined by 2.8 μmol/g. Foot muscle accumulated substantial amounts of alanine, the net amount being 6.7 μmol/g over the 12 h, but very little succinate, the net amount being only 0.8 μmol/g over the same time period. Aspartate depletion and alanine accumulation in foot were inversely correlated between 4 and 12 h of anoxia, both changing by 2.6 μmol/g; however, early in anoxia there was no significant change in aspartate content in foot despite a rapid accumulation of alanine. In gill, anoxia exposure had no effect on the levels of aspartate, succinate, or alanine over the first hour, but all three changed significantly thereafter. Aspartate content decreased by 3 μmol/g between 1 and 12 h of anoxia, whereas succinate increased by 1.6 μmol/g. Alanine content increased by 2.7 μmol/g between 1 and 4 h, but subsequently declined as anoxia progressed.

Figure 3 shows the effects of anoxia on the contents of glycolytic intermediates in whelk organs. Activation or inhibition of a metabolic pathway produces characteristic changes in the levels of pathway intermediates, particularly in the substrate(s) and product(s) of the regulatory (nonequilibrium) enzymes. These changes can be used diagnostically to identify the regulatory enzyme(s) participating in flux control in response to an external stress on the system. For example, enzyme activation typically results in a drop in the substrate content and a rise in the product content of the reaction. Applied to a time course of anoxia exposure in the whelk, this analysis indicated time-dependent changes in glycolytic activity as anoxia progresses.

In the radular muscle, levels of F6P and F1,6BP changed in an inverse pattern over the early hours of anoxia (Fig. 3A). For F6P, significant changes were as follows: a decrease compared with control values after 30 min, a subsequent increase in content at 1 h, and then a large decrease in content by 4 h of anoxia. Coupled with the opposite changes in F1,6BP content, this indicated an increase in flux through the PFK locus at 30 min, a relative decrease a 1 h, and a subsequent increase in flux again in the 4- to 8-h range. G3P content largely paralleled that of F1,6BP throughout. Levels of both PEP and pyruvate declined over the early hours of anoxia, substantially reducing the total content of glycolytic intermediates in the anoxic muscle. Between 2 and 4 h, an increase in PEP, coupled with a further drop in pyruvate content, indicated inhibitory action at the PK locus.

For foot muscle, the pattern of changes in F6P and F1,6BP was similar to that seen in radular retractor (Fig. 3B). Changes

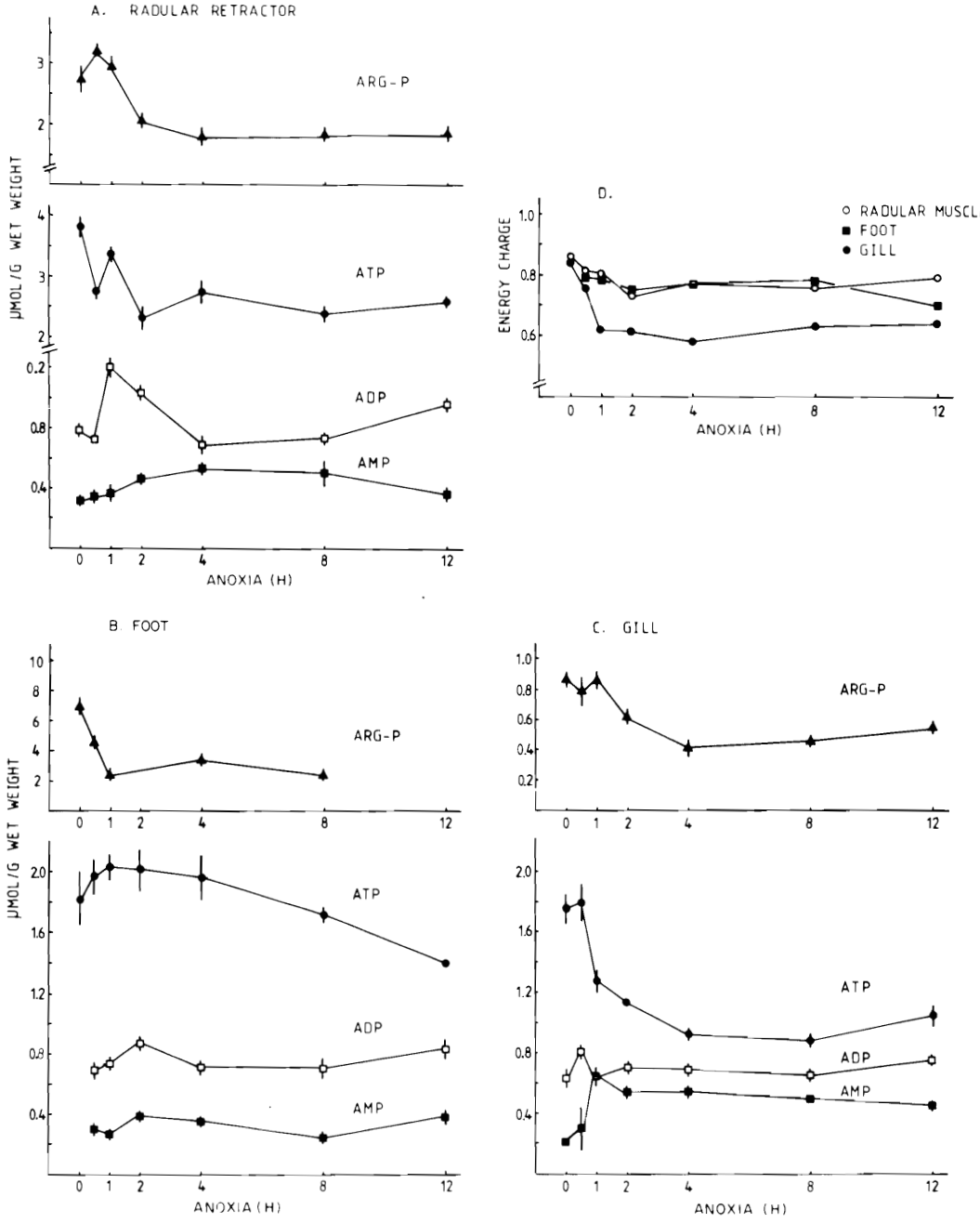

FIG. 1. Mean arginine phosphate (ARG-P) and adenylate levels and energy charge in three organs of the whelk *B. canaliculatum* over 12 h of anoxia exposure. Energy charge is defined as ([ATP] + 1/2[ADP])/([ATP] + [ADP] + [AMP]). (A) Radular retractor muscle. (B) Foot muscle. (C) Gill. (D) Energy charge. Vertical bars indicate standard error (n = 4 animals at each point).

in F6P (a significant decrease) and F1,6BP (a significant increase) between 30 min and 1 h indicated an activation of PFK early in anoxia. Subsequently, between 2 and 4 h, flux appears to be inhibited: F6P rose, whereas F1,6BP content remained the same. Between 8 and 12 h, F6P content again fell and F1,6BP content rose. G3P content again paralleled that of F1,6BP throughout. PEP and pyruvate showed a similar pattern of changes. Pyruvate content doubled between 30 min and 1 h, with a small increase in PEP, indicating activation of PK at this time. Subsequently, however, content of both compounds dropped (again as in radular retractor). Between 2 and 4 h, PEP content rose whereas pyruvate content fell, indicating PK inhibition during the intermediate hours of anoxia. A reversal was apparent, however, as anoxia exposure stretched to 12 h.

In gill, F6P content was low and constant over the first hour of anoxia (Fig. 3C). F1,6BP content, however, rose by 40% within the first 30 min. G3P paralleled this response with a sharp increase in content over the first hour. These responses suggested increased flux through PFK over the first hour of anoxia. Subsequently, however, F6P content increased sharply, whereas G3P content dropped by 50% (F1,6BP remained constant); this indicated restricted flux through PFK between 1 and 2 h. The same pattern occurs at the PK locus. Pyruvate content rose sharply over the first 30 min of anoxia, suggesting

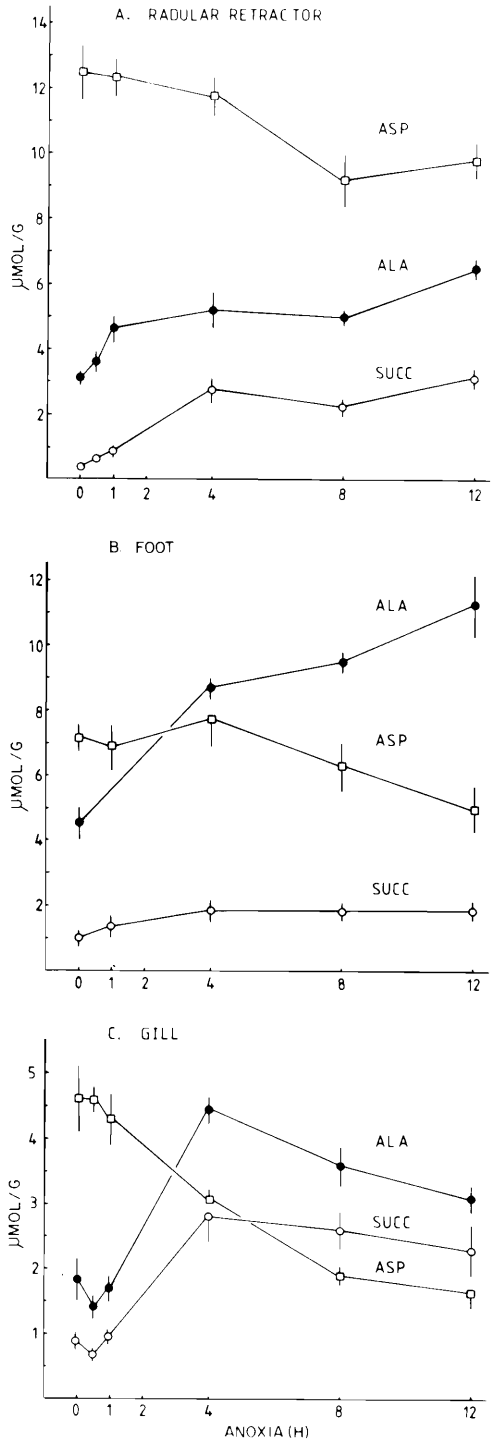

FIG. 2. Mean levels of aspartate (ASP), succinate (SUCC), and alanine (ALA) in three organs of the whelk *B. canaliculatum* over 12 h of anoxia exposure. (A) Radular retractor muscle. (B) Foot muscle. (C) Gill. Vertical bars indicate standard error (n = 4 animals at 0.5 and 1 h; n = 5 at remaining time points).

PK activation. Between 1 and 2 h, however, PEP content rose significantly with no change in pyruvate, indicating an inhibition of PK at this time.

Discussion

The present study examines organ-specific glycolytic control over the early hours of anaerobiosis in the whelk. Three organs were chosen as representatives of distinct tissue types: red muscle (radular retractor), white muscle (foot), and soft tissue (gill). These three organ types differ in isozyme patterns of glycolytic enzymes (Plaxton and Storey 1982; 1985*a*) and have distinct metabolic designs. The present data show that the organs also have distinct biochemical responses to anoxia. For example, foot muscle contained much larger phosphagen pools than did either of the other organs and showed a rapid depletion of phosphagen over the early minutes of anoxia (Fig. 1). Phosphagen appeared to be much less important to the energetics of the other two organs, however. As a consequence, anoxia had very little effect on energy charge and total adenylate content in foot. This same pattern characterized the adductor muscle, another white muscle type tissue, in bivalve species (de Zwaan et al. 1982; Gade 1983). The red muscle (radular retractor), on the other hand, showed a lesser dependence on phosphagen (and a delayed response in mobilizing phosphagen) and a somewhat greater effect of anoxia on energy charge and total adenylate pools; the same pattern was found in another red muscle of the whelk, the ventricle (Ellington 1981). Gill showed a third pattern: a limited store of arginine phosphate, a large drop in energy charge and ATP content very early in anoxia, and a corresponding rise in AMP.

The patterns of anaerobic end product accumulation also differed between organs. Alanine and succinate accumulated in a 2:1 ratio in radular retractor and gill, but the ratio was 12.5:1 in foot. Aspartate depletion and alanine accumulation were balanced in radular retractor and gill (and in foot between 4 and 12 h), indicating, as has been documented in other species, that the two are linked by amino group transfer (Collicutt and Hochachka 1977; de Zwaan et al. 1982; Kluytmans and Zandee 1983; Gade 1983; Gade and Ellington 1983). Early in anoxia, however, foot rapidly accumulated alanine without aspartate depletion (or succinate production), suggesting that the first response of foot to anoxia is a reliance on the routes of energy production commonly used during work (functional anoxia) in this white muscle (i.e., arginine phosphate hydrolysis and fermentative glycolysis ending in alanine accumulation). Succinate accumulation in all three organs could be fully accounted for by aspartate depletion, indicating that for the initial 12 h of anoxia, metabolism in these organs utilizes the coupled conversion of glycogen to alanine and aspartate to succinate. Indeed, even during longer-term anoxia (24 h), this relationship held for most organs of the whelk; only ventricle showed a continuing accumulation of high amounts of succinate after aspartate reserves had been depleted (and alanine levels had reached a plateau) (Eberlee and Storey 1988). This suggests that the anoxia-induced phosphorylation of PK that is seen in all organs of the whelk (Plaxton and Storey 1984*a*, 1985*a*, 1985*b*; Brooks and Storey 1989) and is well developed within 4 h of anoxia exposure (Whitwam and Storey 1990) has as its primary function the control of glycolytic rate depression. A role in facilitating the conversion of PEP to succinate appears to be secondary in this species and is used only in long-term anoxia in selected organs.

Changes in the contents of glycolytic intermediates over the anoxic excursion provide information about the regulatory control of glycolysis during anoxia and changes in glycolytic flux over the course of anoxia. In all three organs it is apparent that the first response to immersion in N_2-bubbled seawater is an activation of glycolysis. Significant changes in substrate (a decrease) and product (an increase) levels, creating positive

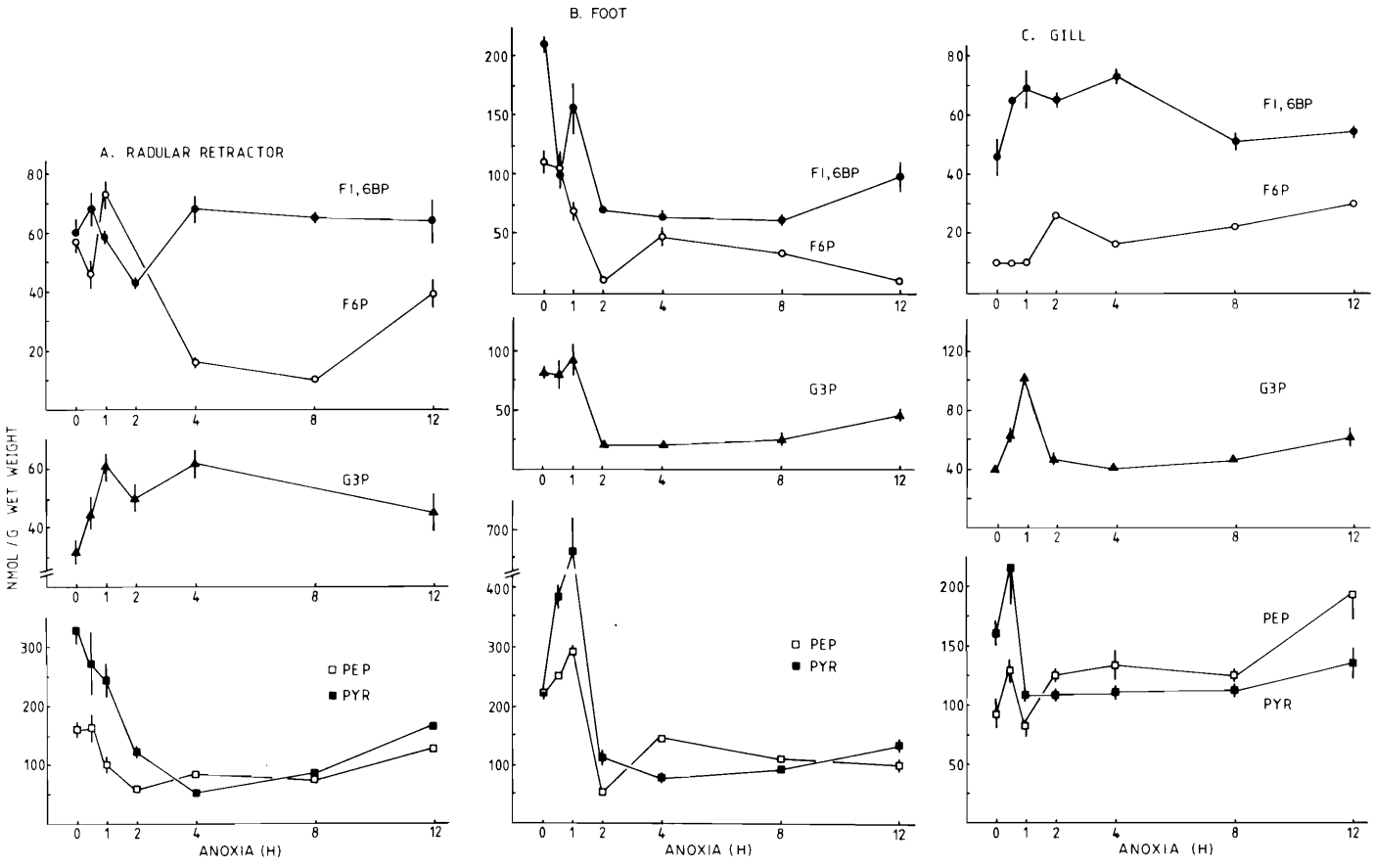

FIG. 3. Mean levels of some glycolytic intermediates in three organs of the whelk *B. canaliculatum* over 12 h of anoxia exposure. (A) Radular retractor muscle. (B) Foot muscle. (C) Gill. Vertical bars indicate standard error (n = 4 animals at each point). PYR, pyruvate.

crossovers as defined by Williamson (1970), indicate an activation of PFK in radular retractor within 30 min of exposure to anoxic water and activations of both PFK and PK in gill and foot within 30–60 min. Glycogen phosphorylase *a* content also increased rapidly in the two muscles early in anoxia (Storey 1988*b*). These enzyme changes correlated with periods of substantial alanine accumulation (foot, radular retractor) and arginine phosphate (foot, gill) or ATP (gill, radular retractor) depletion. These are undoubtedly responses to hypoxia; a constant ATP demand is met by increased glycolytic ATP production as oxygen tension in tissues declines and the ATP contribution from oxidative phosphorylation decreases. This initial response may be regulated by simple allosteric effects of adenylates (ATP, AMP) and phosphagen on the enzymes involved (such effects on PFK are the basis of the Pasteur effect (Storey 1985)). This response, in fact, resembles the Pasteur effect but does not fully meet its definition, for (*i*) the effect does not persist throughout anoxia, and (*ii*) the response seen is probably elicited by hypoxia and not by anoxia.

Indeed, animals that tolerate wide variation in environmental oxygen availability typically have two strategies for dealing with changing oxygen tensions. The response to hypoxia is compensation, a strategy that permits normal physiological activity to be maintained over a wide range of low oxygen tensions (Lutz et al. 1985; Hochachaka 1988). Two mechanisms are involved: (*i*) the rates of oxidative and nonoxidative (fermentative glycolysis + phosphagen breakdown) ATP production are varied inversely over a range of low oxygen tensions, and (*ii*) if hypoxia is long term, steps are taken to improve oxygen delivery to organs. Below a critical lower limit, however, a conservation strategy is brought into play as the response to anoxia. A profound metabolic depression is initiated. This reduces anaerobic ATP requirements to a level that can be sustained over the long term by fermentative ATP production alone. Typically, anoxic metabolic rate in facultative anaerobes is only 5–20% of aerobic resting rate (Famme et al. 1981; Shick et al. 1983; Storey 1985; Herbert and Jackson 1985).

The present data indicate that glycolytic rate depression (as well as overall metabolic rate depression) is in place within 1–4 h after anoxia exposure is initiated in whelk gill and foot muscle. Reduced flux through both PFK and PK loci was indicated within 2 h in gill and within 2–4 h in foot. This correlates well with the pattern of PK inactivation via enzyme phosphorylation (Whitwam and Storey 1990). Phosphorylase *a* activity also decreased in these organs as anoxia progressed (Storey 1988*b*). Reduced flux through PFK and PK occurred despite conditions of depleted phosphagen and adenylate pools which would be expected to keep the glycolytic rate high. This indicates that mechanisms of metabolic depression override allosteric control by high-energy intermediates (hence the absence of a Pasteur effect) (Storey 1985, 1988*a*).

Radular retractor muscle showed a slightly different pattern. This is perhaps related to changes in fuel use with the aerobic–anoxic transition that may not occur in the gill and foot. Red muscles often oxidise lipid under normal aerobic conditions,

and in anoxia must switch to the catabolism of carbohydrate instead. Anoxia exposure, therefore, elicits an exaggerated response by glycolysis which begins with an immediate rapid activation of glycolysis (phosphorylase (Storey 1988*b*) and PFK are activated in radular muscle). The subsequent transition to the hypometabolic state involves a reduction in glycolytic rate compared with the initial response, but glycolysis still appears activated relative to the aerobic control situation. The behaviour of phosphorylase illustrates this; the immediate response to anoxia is a 4.3-fold increase in phosphorylase *a* activity, but this drops to a level 2.5-fold greater than the aerobic control value within a few hours (Storey 1988*b*).

Analysis of changes in glycolytic intermediates in response to anoxia has also been carried out for the bivalve *Mytilus edulis*. This species showed an immediate inhibition of glycolysis, with negative crossovers at both the PFK and PK loci within the first hour of aerial exposure (Ebberink and de Zwaan 1980). No short-term activation of glycolysis, as occurred in *B. canaliculatum*, was seen. Explanations for the differing responses can readily be proposed, however: (*i*) the hypoxic transition period may be much longer in the whelk than in the bivalve because of greater oxygen reserves in the body fluids of the much larger whelk, and (*ii*) the metabolic depression response may be much more rapidly triggered by the intertidal bivalve, an excellent strategy for a species that routinely experiences twice-daily aerial exposures.

The molecular mechanisms that control glycolytic rate depression (and overall metabolic rate depression) in response to anoxia are becoming well understood. For PFK, for example, these mechanisms include enzyme phosphorylation (resulting in altered kinetic properties), changes in enzyme binding to the particulate fraction of the cell, and greatly reduced levels of the activator, fructose-2,6-biphosphate (Storey 1984, 1988*b*). Together these obviously produce an enzyme that is much less sensitive to adenylate and phosphagen controls and permit sustained glycolytic rate depression despite a reduced energy status in the anoxic state. Anoxia-induced enzyme phosphorylation and changes in the distribution of bound versus free enzyme are also key to PK control in all organs of the whelk (Plaxton and Storey 1984*a*, 1984*b*, 1985*a*, 1985*b*, 1986; Whitwam and Storey 1990). Indeed, our analysis of the time courses of changes in the phosphorylation states of glycogen phosphorylase, PFK, PK, and 6-phosphofructo-2-kinase, as well as in the levels of fructose-2,6-biphosphate (as a result of covalent modification of 6-phosphofructo-2-kinase), in whelk tissues indicates that anoxia-induced enzyme phosphorylation closely parallels the development of glycolytic rate depression as indicated by the data of this study (Storey 1988*b*; Whitwam and Storey 1989; L. Bosca and K. Storey, submitted for publication). This adds further weight to the suggestion that covalent modification of PK serves a primary function in glycolytic rate control. The rates of the key regulatory enzymes of glycolysis can be coordinated via covalent modification for the transition to the hypometabolic state. Covalent modification may also be the mechanism for widespread and coordinated control over numerous other cellular proteins during anoxia; for example, ion channel proteins may be regulated in this manner.

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