Mechanisms of glycolytic control during facultative anaerobiosis in a marine mollusc: tissue-specific analysis of glycogen phosphorylase and fructose-2,6-bisphosphate

KENNETH B. STOREY

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

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Changes in the activity of glycogen phosphorylase and the content of fructose-2,6-bisphosphate (F-2,6-P_2) were monitored in tissues of the whelk, *Busycotypus canaliculatum*, over a 21-h course of environmental anoxia. Tissue-specific responses to anoxia were seen with respect to phosphorylase content: in the radular retractor muscle and foot, the content of phosphorylase *a* expressed rose rapidly over the initial hours of anoxia (maximal increases were 4.3- and 2.5-fold, respectively) while in the gill, content dropped 2-fold during anoxia. Phosphorylase content was modulated by two mechanisms, changes in the percentage of enzyme in the active *a* form and changes in the total amount (*a* + *b*) of enzyme expressed. Anoxia stimulated a dramatic reduction in F-2,6-P_2 content in five tissues. In the ventricle, content fell by 224-fold with a $t_{1/2}$ of only 35 min. Levels in gill, radular retractor, hepatopancreas, and kidney fell to 2.5–3.5% of control values within the first 8 h of anoxia. F-2,6-P_2 content in foot muscle was not altered during anoxia. Changes in glycogen phosphorylase activities and F-2,6-P_2 contents help to produce tissue-specific responses of glycolysis to environmental anoxia that acknowledge competing metabolic demands including metabolic rate depression, changes in fuel use, anaerobic energy needs, and carbohydrate use for anabolic purposes.

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Les changements dans l'activité de la glycogène-phosphorylase et les concentrations de fructose-2,6-diphosphate (F-2,6-P_2) ont été mesurés pendant 21 h dans les tissus du buccin *Busycotypus canaliculatum*, dans des conditions d'anoxie. Les concentrations de phosphorylase ont subi des modifications spécifiques aux tissus à la suite de l'anoxie : dans le muscle retracteur de la radula et dans le pied, le contenu en phosphorylase a augmenté rapidement au cours des premières heures de l'anoxie (par un facteur maximum de 4,3 dans le muscle et de 2,5 dans le pied), alors qu'il a diminué par un facteur de 2 dans les branchies. La concentration de phosphorylase est contrôlée par deux mécanismes, les changements dans le pourcentage d'enzyme sous sa forme active *a* et les changements dans le contenu total (*a* + *b*) d'enzyme décelable. L'anoxie provoque une réduction spectaculaire du contenu en F-2,6-P_2 dans cinq tissus : diminution par un facteur de 224 dans le ventricule, avec un $t_{1/2}$ de seulement 35 min; dans les branchies, le muscle retracteur de la radula, l'hépatopancreas et le rein, la concentration a diminué jusqu'à n'être plus que 2,5–3,5% des valeurs témoins, moins de 8 h après le début de l'anoxie. L'anoxie n'a cependant pas affecté le contenu en F-2,6-P_2 dans le pied. Les fluctuations de l'activité de la glycogène-phosphorylase et des concentrations de F-2,6-P_2 durant une anoxie du milieu permettent à la glycolyse de s'ajuster, dans chaque tissue, en fonction des autres besoins métaboliques, notamment la diminution du taux de métabolisme, les modifications dans l'utilisation des carburants, les exigences énergétiques anaérobies et l'utilisation des hydrates de carbone à des fins anaboliques.

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Introduction

Marine molluscs have impressive capacities for withstanding environmental anoxia. Two key adaptations support this tolerance: (*i*) an ability to strongly depress metabolic rate, to levels 5–10% of normoxic rates (Famme et al. 1981; Shick et al. 1983; Gnaiger 1983); and (*ii*) an altered energy metabolism involving the coupled fermentation of glycogen and aspartate, the production of alternative end products (e.g., alanine, succinate, propionate), and substrate level phosphorylations of ADP linked to organic acid synthesis (de Zwaan 1983). Regulatory control over glycolysis is a prime requirement for anaerobic survival. Glycolytic rate must accommodate both the dependence on carbohydrate (glycogen) fermentation as the chief mechanism of anaerobic energy production and the generalized metabolic rate depression of anoxia. In anoxia-tolerant animals, however, metabolic depression appears to be the overriding factor and anoxic energy demand is so strongly depressed that the rate of carbohydrate fermentation is also lowered despite the reduced energy yield of glycolysis (5 v. 38 mol ATP/mol glucosyl units oxidized to succinate vs. to CO_2 and H_2O, respectively) (Storey 1985*a*, 1988). Thus, no Pasteur effect is seen (de Zwaan and Wijsman 1976), glucose utilization is depressed (Zaba and Davies 1980), and changes in the levels of fructose-6-phosphate (an increase) and fructose-1,6-bisphosphate (a decrease) in tissues suggest an inhibition of phosphofructokinase with the aerobic–anaerobic transition (Ebberink and de Zwaan 1980).

Anaerobic metabolism has been extensively characterized in marine molluscs, particularly in intertidal bivalves (recent reviews include de Zwaan 1983; Livingstone and de Zwaan 1983; Hochachka and Somero 1984; Kreutzer et al. 1985). Much emphasis has been placed on the biochemical controls regulating carbon flow to alternative end products, particularly carbohydrate partitioning at the phosphoenolpyruvate branch point. Fewer studies have addressed the molecular basis of metabolic rate control (Storey 1985*a*, 1988). Our studies of a marine gastropod, the whelk *Busycotypus canaliculatum*, have identified three levels of metabolic control operating to control enzymes and pathways during anaerobiosis: (*i*) allosteric regulation of enzymes; (*ii*) covalent modification of proteins; and (*iii*) enzyme binding to the subcellular particulate fraction of the cell (Storey 1985*a*, 1988).

The present study continues our investigation of glycolytic rate control and metabolic depression in facultative anaerobes. Two key indices of glycolytic function are examined in the whelk: glycogen phosphorylase as the indicator of anoxia effects on tissue glycogenolysis (carbohydrate catabolism) and fructose-2,6-bisphosphate (F-2,6-P_2) as a predictor of carbohydrate use for anabolic functions in the anoxic state. Both general principles for anaerobic metabolic regulation and specific controls for individual tissue function are indicated.

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TABLE 1. Total glycogen phosphorylase ($a + b$) activities and percentage of the active a form in tissues of the whelk *Busycotypus canaliculatum* over a time course of anoxia

	Heart		Radular retractor		Gill		Kidney		Foot	
	$a + b$	% a	$a + b$	% a	$a + b$	% a	$a + b$	% a	$a + b$	% a
Control	6.3±0.9	34±3.4	3.8±0.6	40±6.9	0.7±0.08	61±6.9	0.9±0.18	33±6.3	1.2±0.25	32±5.2
0.5 h	4.2±1.1	23±3.5								
1 h	5.8±0.7	38±5.2							1.7±0.58	37±4.7
2 h	6.1±0.8	55±2.2*	10.9±1.0*	59±8.9	0.4±0.07*	64±3.9	0.8±0.05	43±2.6	1.7±0.41	43±6.1
4 h			11.2±2.5*	48±7.6	0.5±0.04*	57±6.0			2.4±0.52	42±3.8
8 h	7.2±0.8	41±6.2	11.2±2.2*	33±3.4	0.3±0.02*	43±4.6	0.7±0.08	36±3.8	2.4±0.59	45±7.3
16 h	5.1±0.5	37±5.2	9.8±0.2*	52±5.3						
21 h	4.5±0.7	39±4.9	6.5±1.5*	69±9.0	0.4±0.05*	51±2.2	0.5±0.05	44±3.4	1.4±0.14	45±5.0

NOTE: Activities are μmol glucose-1-phosphate formed · min^{-1} · g wet $weight^{-1}$, means ± SEM, $n = 4$ for heart, $n = 5$ for radular retractor muscle, gill, and kidney, and $n = 6$ for foot muscle.
*Values are significantly different from corresponding controls by the Student's t-test, $p < 0.05$.

Materials and methods

Animals and chemicals

Whelks, *Busycotypus canaliculatum*, were purchased from the Marine Biological Laboratory, Woods Hole, MA, and were held in aerated, recirculating seawater (1000 mosmol) at 12−15°C without feeding until use. Control, aerobic animals were sampled directly from the seawater tank. To impose anoxia, animals were transferred to sealed plastic buckets (two per bucket) containing 10 L of seawater that had been previously bubbled with a steady stream of nitrogen gas for 12−16 h. Bubbling with N_2 gas was continued throughout the anoxia exposure and containers were bathed in a larger holding tank for temperature control. After a timed anoxia exposure, tissues were rapidly dissected out, blotted, and immediately frozen in liquid nitrogen. For long-term storage, tissues were kept at −80°C.

F-2,6-P_2 determination

Frozen tissue samples (approximately 100 mg) were ground to a powder under liquid nitrogen. The frozen powder was quickly weighed and then homogenized in 10 vol. hot (80°C) 50 mM NaOH using a Tekmar tissuemizer followed by incubation at 80°C for 10 min. Homogenates were centrifuged at 12 000 × g for 10 min at 4°C and supernatants were removed and stored on ice. Assay for F-2,6-P_2 followed the method of van Schaftingen (1984) which relies upon the ability of F-2,6-P_2 to activate potato tuber pyrophosphate-linked phosphofructokinase (PFK). F-2,6-P_2 contents of sample aliquots (1−200 μL) were determined by comparison with a standard curve (0.5−20 pmol) of F-2,6-P_2 activation of PP_i−PFK. That enzyme activation was due to the F-2,6-P_2 content of samples alone was confirmed when subsequent acid treatment of samples eliminated their activating effects.

Glycogen phosphorylase

Frozen tissue samples (approximately 100 mg) were rapidly weighed and homogenized 1:5 w/v in ice-cold 50 mM imidazole buffer, pH 7.0, containing 15 mM 2-mercaptoethanol, 100 mM NaF, 5 mM EDTA, 5 mM EGTA, and 0.1 mM phenylmethylsulfonyl fluoride using a Tekmar tissuemizer. Particulate matter in the homogenate was allowed to settle (without centrifugation) and then aliquots of the supernatant were removed for assay. Assay conditions for phosphorylase a were: 50 mM potassium phosphate buffer, pH 7.0, 2 mg/mL glycogen (previously dialyzed), 0.4 mM NADP, 10 μM glucose-1,6-bisphosphate, 0.25 mM EDTA, 15 mM $MgCl_2$, and excess dialyzed phosphoglucomutase and glucose-6-phosphate dehydrogenase. For measurements of total phosphorylase, assays were conducted in the presence of 1.6 mM AMP. Assays were performed at 23°C using a Pye Unicam SP8-100 recording spectrophotometer.

Results

Glycogen phosphorylase

Table 1 shows the total activities of glycogen phosphorylase ($a + b$) and percentage of the active a form in five tissues of the whelk over a time course of anoxia. The activity of phosphorylase a (units/gram wet weight) in each tissue over the course of anoxia is plotted in Fig. 1; this illustration reflects changes in both total phosphorylase content and percentage of the a form. The response of glycogen phosphorylase to anoxia differed between tissues. As Fig. 1 shows, the amount of active enzyme in radular retractor and foot muscles increased rapidly during the early hours of anoxia; the maximal increase was 4.3-fold in radular retractor and 2.5-fold in foot after 2 and 4 h of anoxia, respectively. In radular muscle, this effect was due to a rise in the total amount of phosphorylase ($a + b$) expressed in the tissue while in foot, alterations to both total phosphorylase content and percentage of the a form were responsible for enzyme activation. Phosphorylase activity in both tissues then declined over the later hours of anoxia but remained significantly elevated above control values in radular muscle after 21 h of anoxia. Phosphorylase a activity in ventricle was significantly increased over control values at a sampling time of 2 h of anoxia (Fig. 1), because of an increase in the percentage of the a form (Table 1), but was not significantly different from control values at any other time over the experiment. Gill response was opposite to that of muscle tissues and included a significant decrease in phosphorylase a content after 8 and 21 h of anoxia (Fig. 1) because of a reduction in the total activity of phosphorylase expressed in the tissue (percent a did not change) (Table 1). Phosphorylase activity in kidney was unaffected over the course of anoxia.

Fructose-2,6-bisphosphate

Figure 2 shows the content of F-2,6-P_2 in six tissues of the whelk over the 21-h course of anoxia. Content in five tissues fell dramatically during anoxia; in foot muscle, however, F-2,6-P_2 content was unaffected. Ventricle showed the largest changes in F-2,6-P_2 concentration during anoxia; content fell from 1230 ± 300 pmol/g wet weight in controls to an average of 5.5 ± 1.5 pmol/g between 12 and 21 h of anoxia, a 224-fold decrease with a $t_{1/2}$ (estimated from Fig. 2) of only about 35 min. Contents of F-2,6-P_2 in hepatopancreas and kidney remained constant over the initial 1 or 2 h of anoxia and then dropped rapidly to new steady-state values averaging 17 and 23% of control values, respectively, between 4 and 12 h of anoxia. After 21 h of anoxia, F-2,6-P_2 contents in the two tissues were 3.4 and 2.5% of control values, respectively. F-2,6-P_2 contents in radular retractor muscle and gill also fell during anoxia with estimated $t_{1/2}$ values of about 75 and 200

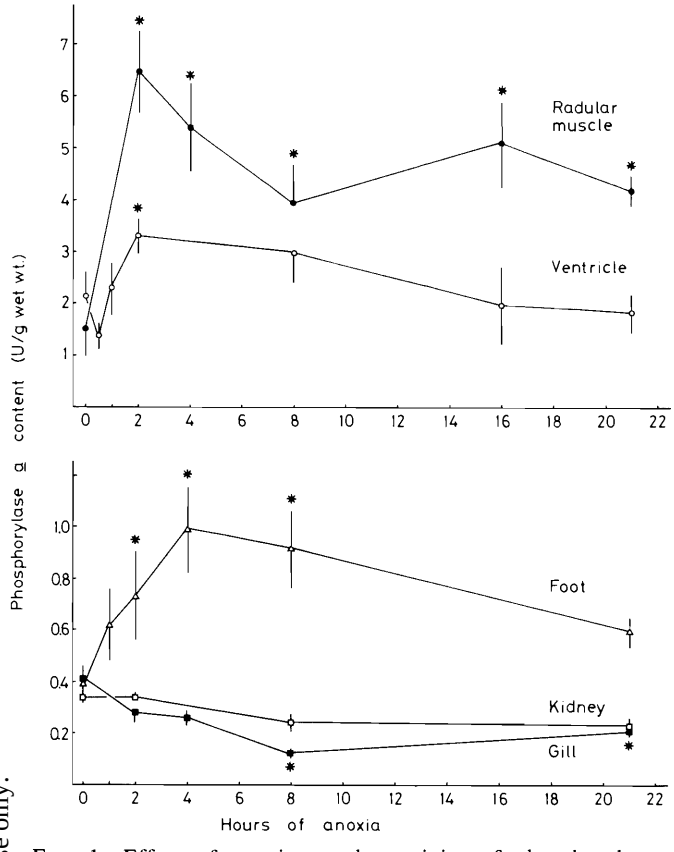

FIG. 1. Effect of anoxia on the activity of phosphorylase *a* expressed in tissues of the channeled whelk, *Busycotypus canaliculatum*. Activities are units (i.e., μmol glucose-1-phosphate formed · min^{-1} · g wet $weight^{-1}$ (± SEM); $n = 6$ for radular retractor muscle, kidney, and foot muscle, $n = 5$ for gill, and $n = 4$ for heart. *, values are significantly different from corresponding control values, $p < 0.05$.

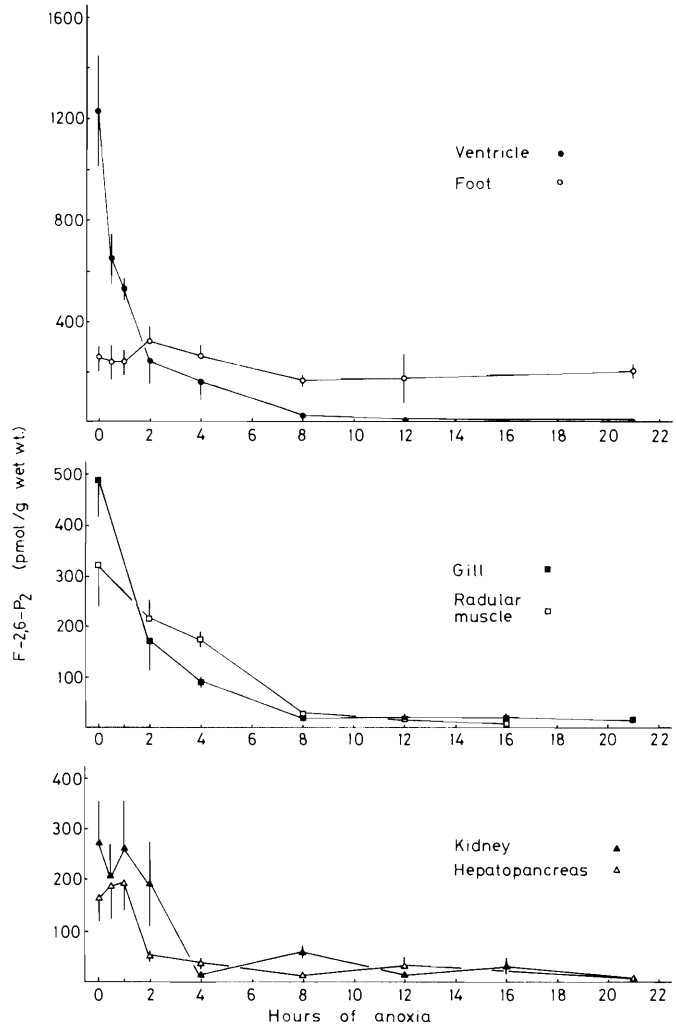

FIG. 2. Effect of anoxia on the contents of fructose-2,6-bisphosphate (F-2,6-P_2) in tissues of the channeled whelk, *Busycotypus canaliculatum*. Data are pmol/g wet weight (± SEM) for $n = 5$ or 6 samples from individual animals.

min, respectively, and final levels of 2.6 and 3.5% of control values.

Discussion

The key biochemical adaptations supporting facultative anaerobiosis in marine molluscs are metabolic rate depression and the use of alternative routes of substrate fermentation to enhance anoxic energy production. It is increasingly apparent, however, that there are tissue-specific variations in both of these responses, designed to accommodate the specific metabolic functions of each tissue in the aerobic versus anoxic states and to create a balanced reorganization of whole-animal function for long-term survival of anoxia. Tissue-specific differences in anoxic metabolic rate (as judged by total end product accumulation), in the capacity for glycogen fermentation to succinate, and in the effects of anoxia-induced phosphorylation on pyruvate kinase kinetics have all been reported (Korycan and Storey 1983; de Zwaan 1983; Plaxton and Storey 1984, 1985*a*, 1985*b*). The present study shows additional tissue-specific differences in glycolytic function during facultative anaerobiosis that govern the catabolism of glycogen and the control of glycolytic rate at the phosphofructokinase locus.

Anaerobic metabolism in marine molluscs is fueled chiefly by endogenous substrate reserves, glycogen and aspartate, with little or no contribution by blood-borne fuels. Thus, control over anaerobic carbohydrate utilization is vested in glycogen phosphorylase in each individual tissue. The enzyme must respond to the aerobic–anaerobic transition with a modification of activity that reflects three pressures: (*i*) anoxia-induced depression of metabolic rate; (*ii*) anoxic ATP requirements of individual tissues; and (*iii*) in some instances, a switch to anoxic glycogen fermentation from a aerobic dependence on an alternative fuel (e.g., lipids). The relative influence of each of these factors determines the net effect on glycogen phosphorylase activity in the anoxic state. Not unexpectedly, marked tissue differences in the responses of phospohorylase were seen. Changes in the activity of phosphorylase in gill (a reduction in phosphorylase *a* content) and kidney (no change in phosphorylase activity) were consistent with an overriding influence of metabolic rate depression that dramatically reduced ATP needs, and therefore glycogenolysis, during anoxia. Similarly, no activation of phosphorylase occurred during anoxia in bivalve adductor muscle (Ebberink and Salimans 1982). The response of radular retractor muscle, ventricle, and foot muscle of the whelk differed, however; all showed increased phosphorylase *a* activity within the first 2–4 h of anoxia. This persisted throughout anoxia in radular

muscle but phosphorylase *a* content returned to control levels over the long term in the other two organs. For foot muscle, the early activation of phosphorylase may have been due to increased locomotion in an attempt to escape the anoxic water. For radular retractor and ventricle, enzyme activation during anoxia appears contrary to expectations when metabolic rate depression prevails and there is no increase in work load (e.g., bradycardia is well known in anoxia). The response, however, may be due to an anoxia-induced metabolic reorganization, a switch from aerobic lipid oxidation by these red muscles to anoxic carbohydrate fermentation, necessitating a relative activation of glycolysis. In support of this hypothesis, we have also observed changes in fructose-6-phosphate and fructose-1,6-bisphosphate levels during the early hours of anoxia in radular retractor that suggest activation of phosphofructokinase, the rate-limiting enzyme of glycolysis (D. Kelly and K. Storey, unpublished data); this reverses when anoxia becomes prolonged.

The molecular mechanism underlying changes in phosphorylase *a* content in the whelk is unusual. Modulation of the total activity of phosphorylase (content of $a + b$) expressed accounts for some or all of the changes in enzyme activity in radular retractor, gill, and foot. The typical situation in mammalians, by contrast, is a change only in the percentage of the active phosphorylated *a* form with total phosphorylase activity remaining constant (Cohen 1980). The unusual mechanism of phosphorylase control seen in the whelk is not restricted to this species but also occurs as the basis of phosphorylase activation during cryoprotectant synthesis in freeze-tolerant frogs (Storey and Storey 1988). Clearly, the regulatory mechanisms of phosphorylase control may differ throughout the animal kingdom, presumably in an adaptive manner. Uncommon properties of phosphorylase from marine molluscs have been noted previously including unusual AMP activation kinetics for phosphorylases *b* from *Mytilus edulis* adductor muscle (Ebberink and Salimans 1982) and the apparent presence in *Pecten maximus* of an inactive *c* form of the enzyme whose activity was expressed only at AMP concentrations 20-fold higher than those required for optimal phosphorylase *b* activity (Vazquez-Baanante and Rosell-Perez 1979).

F-2,6-P_2 is a potent activator of phosphofructokinase. Synthesis and degradation of the compound are the responsibility of a single enzyme protein displaying both 6-phosphofructo-2-kinase and fructose-2,6-bisphosphatase activities; forward (kinase) versus reverse (phosphatase) functioning is regulated via covalent modification under the influence of extracellular signals (e.g., hormones, dietary state, anoxia or ischemia) (Hue and Rider 1987). In general, high F-2,6-P_2 content signals an abundance of glucose to the cell and potentiates the use of carbohydrate for biosynthetic purposes. F-2,6-P_2 levels drop rapidly when carbohydrate reserves must be spared (e.g., starvation) or conserved for use in ATP production alone (e.g., anoxia) (Hue and Rider 1987). When F-2,6-P_2 control is withdrawn, regulation of phosphofructokinase comes largely from the adenylates, AMP activation, and ATP inhibition; this allows glycolytic rate to be closely matched to basal ATP needs. The present study shows a rapid and strong reduction in F-2,6-P_2 content in five tissues of the whelk during the early minutes and hours of anaerobiosis. The most dramatic change was a 224-fold drop in F-2,6-P_2 content in ventricle with a $t_{1/2}$ of only 35 min. A similar depression of F-2,6-P_2 content occurs during anoxia in the nonmuscle tissues of *M. edulis* and *Ostrea edule* (Storey 1985*b*). The overall result, in all cases, is the removal of F-2,6-P_2 (and the extracellular signals it represents) as an influence on glycolytic rate during anoxia. This facilitates metabolic depression and leaves glycolytic rate primarily responsive to basal ATP demands. Foot muscle of whelk deviates from this pattern, as does bivalve adductor (Storey 1985*b*), but these tissues are apparently subject to an alternative mechanism of phosphofructokinase control that probably accomplishes the same result: enzyme inactivation via anoxia-induced covalent modification (Storey 1984).

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