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GLYCOLYTIC ENZYME BINDING AND METABOLIC CONTROL IN ESTIVATION AND ANOXIA IN THE LAND SNAIL *OTALA LACTEA*

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Summary

The mechanisms controlling glycolytic rate were examined in foot muscle of the terrestrial snail *Otala lactea* (Müller) (Pulmonata, Helicidae), during short and long periods of estivation and anoxia. Binding associations between glycolytic enzymes and the particulate fraction of the cell were assessed in both states. The percentage of enzyme activity bound to particulate matter decreased significantly over the short term (4 days estivation and 14.5 h anoxia); significant changes were seen for hexokinase (HK), phosphofructokinase (PFK), aldolase and lactate dehydrogenase (LDH) in estivation and, for these enzymes plus triosephosphate isomerase and pyruvate kinase (PK), in anoxia. Over the longer term in estivation (22 days) and anoxia (45 h), enzyme binding returned to control values. Tissue content of fructose-2,6-bisphosphate, a potent phosphofructokinase activator, decreased under all experimental conditions. Total glycogen phosphorylase activity decreased during short-term anoxia (14.5 h) and during long-term estivation (22 days), but the percentage of the active *a* form decreased significantly during anoxia only. Significant changes in the maximal activities of several enzymes were observed during both estivation and anoxia. Decreases in the maximal activity of HK, PFK, glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate kinase (PGK) and LDH were observed during long-term estivation. Increases in PGK and PK maximal activity in short-term anoxia and aldolase and PGK in long-term anoxia were also observed. These results suggest that changes in glycolytic enzyme binding may be part of an immediate mechanism used to cause a rapid decrease in glycolytic flux and initiate glycolytic rate depression, which also includes a reduction of fructose-2,6-bisphosphate content and decreased glycogen phosphorylase activity. In the long term, however, control of snail glycolytic rate is reorganized, so that enzyme binding associations revert to the control values. In the long term, then, control is mediated by lower fructose-2,6-bisphosphate concentrations and, during estivation, also by a decrease in maximal enzyme activities.

Introduction

Pulmonate land snails are limited in their physical ability to elude environmental

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extremes and so have adapted metabolically to endure environmental stresses. For example, low water availability induces estivation, seasonal cold temperatures induce hibernation, and low oxygen conditions initiate anaerobiosis (Machin, 1975; Livingstone and de Zwaan, 1983). The common strategy underlying all these responses is metabolic rate depression: reversible entry into a hypometabolic state where energy expenditures are reduced to only 5–30% of the corresponding amounts in active snails (Herreid, 1977; Shick *et al.* 1983; Barnhart and McMahon, 1987). The molecular mechanisms of metabolic rate depression identified to date in a variety of animals involve specific controls on key regulatory enzymes to reorganize metabolism and permit a rapid entry into, and arousal from, dormancy (Storey, 1985*a*, 1988*a*; Hochachka and Guppy, 1987).

Studies of facultative anaerobiosis in marine molluscs provided the first indications of the control mechanisms involved in metabolic depression (Storey, 1985*a*, 1988*a*). Anoxia triggers a drop in metabolic rate to a final value which is 5–10% of aerobic basal rates (Shick *et al.* 1983). Despite the dependence on carbohydrate fermentation for anaerobic energy production, glycolytic rate depression parallels the overall metabolic rate depression and, consequently, no Pasteur effect is seen (de Zwaan and Wijsman, 1976; Storey, 1985*a*). The mechanisms modulating this process include: (a) a modification of the activity state of regulatory enzymes by reversible phosphorylation of enzyme subunits, (b) enzyme and pathway control by the reversible association of enzymes with cellular particulate matter, and (c) control of carbohydrate utilization *via* fructose-2,6-bisphosphate regulation of phosphofructokinase (Storey, 1984, 1985*b*, 1988*b*; Plaxton and Storey, 1984, 1985, 1986). These same mechanisms have also been shown to control glycolysis in anoxia-tolerant goldfish and turtles (Rahman and Storey, 1988; Brooks and Storey, 1988*a*, 1989) and studies of hibernating small mammals indicate that these mechanisms also operate to depress aerobic metabolic rate during the transition to the hibernating state (Storey, 1987).

The present study examines the universality of these mechanisms by investigating another form of environmentally induced metabolic rate depression, estivation. During the dry summer months, land snails retreat into their shells, secrete an epiphragm over the shell opening, and enter a state of estivation. The physiology of estivation has been extensively investigated in *Otala lactea* by Barnhart and coworkers (Barnhart, 1986*a*,*b*; Barnhart and McMahon, 1987, 1988). While estivating, metabolic rate is strongly reduced and animals experience hypercapnia, hypoxia and respiratory acidosis due to prolonged periods of apnea (Barnhart, 1986*a*). The most recent studies have implicated hypercapnia as an agent in metabolic rate depression (Barnhart and McMahon, 1988).

The present study analyzes the glycolytic regulation of glycolysis in *O. lactea* foot muscle in response to estivation and anoxia. Tolerance of anoxia is fairly high in *O. lactea* and animals readily survived the 45 h of anoxia exposure used in the present study. We postulated that the same molecular mechanisms that underlie glycolytic rate depression during anaerobiosis in marine molluscs would also support both anoxia tolerance and estivation in the land snails, and that this would

clearly demonstrate that common molecular principles of metabolic arrest can underlie the response to diverse stresses.

Materials and methods

Chemicals

All biochemicals and coupling enzymes were purchased from Sigma Chemical Co., St Louis, MO, or Boehringer Mannheim Corp., Montreal, PQ.

Animals

Snails, *O. lactea*, were provided by Dr C. Barnhart from an introduced population in San Diego County, CA. Animals were held in the laboratory at 22°C in covered plastic containers lined with moist paper towels. Snails were fed cabbage and ground chalk every 12–15 days. Active snails were identified 16 h after the introduction of food and water and were then divided into three groups: (1) control: active snails (sampled immediately); (2) estivation: active snails were removed to a glass jar without food or water but with ample aeration and were allowed to re-enter a dormant state with sampling after 4 or 22 days of estivation; and (3) anoxia: active snails were placed in an air-tight container (with food and moist paper towels) that was flushed with 95 % N_2/5 % CO_2 gas; animals were sampled after 14.5 and 45 h of anoxia. Foot muscle was rapidly excised. For measurements of subcellular enzyme binding, fresh muscle was used immediately, whereas for measurements of glycogen phosphorylase activity or metabolite levels, tissue was frozen in liquid nitrogen and stored at −80°C until used.

Subcellular binding of enzymes

Measurement of glycolytic enzyme binding to the subcellular particulate fraction used the method of Clarke *et al.* (1980), as described by Plaxton and Storey (1986). Briefly, fresh foot muscle was rapidly homogenized (within 20 s of removal) in a 250 mmol l^{-1} sucrose buffer (containing 15 mmol l^{-1} β-mercaptoethanol and 0.1 mmol l^{-1}, phenylmethylsulfonyl fluoride, PMSF) using an Ultraturrax homogenizer at 80 % of full speed. A sample was removed for the determination of total enzyme activity and the remainder of the homogenate was centrifuged at 12 000 *g* for 5 min at 22°C. The supernatant, containing free enzymes, was removed and diluted fivefold with stabilization buffer: 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} β-mercaptoethanol. The pellet fraction was re-extracted twice in stabilization buffer to solubilize particle-bound enzymes; combined supernatants were pooled to produce the fraction representing bound enzyme activity.

Activities of glycolytic enzymes were measured in the three fractions: total, bound and free. Spectrophotometric assay conditions were as described previously (Plaxton and Storey, 1986; Brooks and Storey, 1988*b*). All assays were performed at 22°C.

Glycogen phosphorylase and fructose-2,6-bisphosphate measurements

Tissue homogenates of previously frozen foot muscle were prepared and assayed for phosphorylase as described previously (Storey, 1988*b*). Enzyme activity in settled homogenates was measured in the presence of 5 mmol l^{-1} caffeine (phosphorylase *a*) or in the presence of 1.6 mmol l^{-1} AMP (total phosphorylase). Fructose-2,6-bisphosphate was measured by the method of van Schaftingen (1984).

Results

The method of Clarke *et al.* (1980) offers a rapid and reproducible technique for measuring enzyme binding to the particulate fraction of the cell. In this method, the tissue is homogenized in a high-sucrose buffer that stabilizes enzyme–particle associations during the homogenization and centrifugation steps. The free enzyme fraction (supernatant) is removed and the particle-bound enzymes are subsequently released by extraction of the pellet in a high-salt buffer. The partitioning of eight different glycolytic enzymes between bound and free states was analyzed in *O. lactea* foot muscle and the results are presented in Figs 1 and 2. The recovery

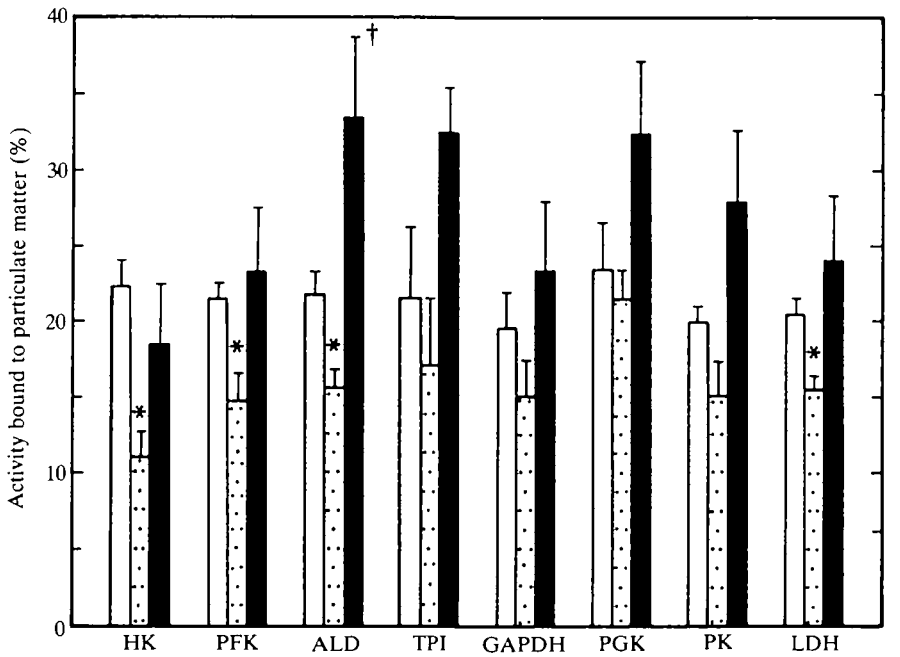

Fig. 1. Effect of estivation on the binding of glycolytic enzymes to the subcellular particulate fraction in *Otala lactea* foot muscle. Values are means±S.E.M., $N=4$ for controls (□), 4 days of estivation (⊡) and 22 days of estivation (■). Significantly different from control values by the Student's *t*-test: *, $P<0.05$; †, $P<0.01$. HK, hexokinase; PFK, phosphofructokinase; ALD, aldolase; TPI, triosephosphate isomerase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PGK, phosphoglycerate kinase; PK, pyruvate kinase; LDH, lactate dehydrogenase.

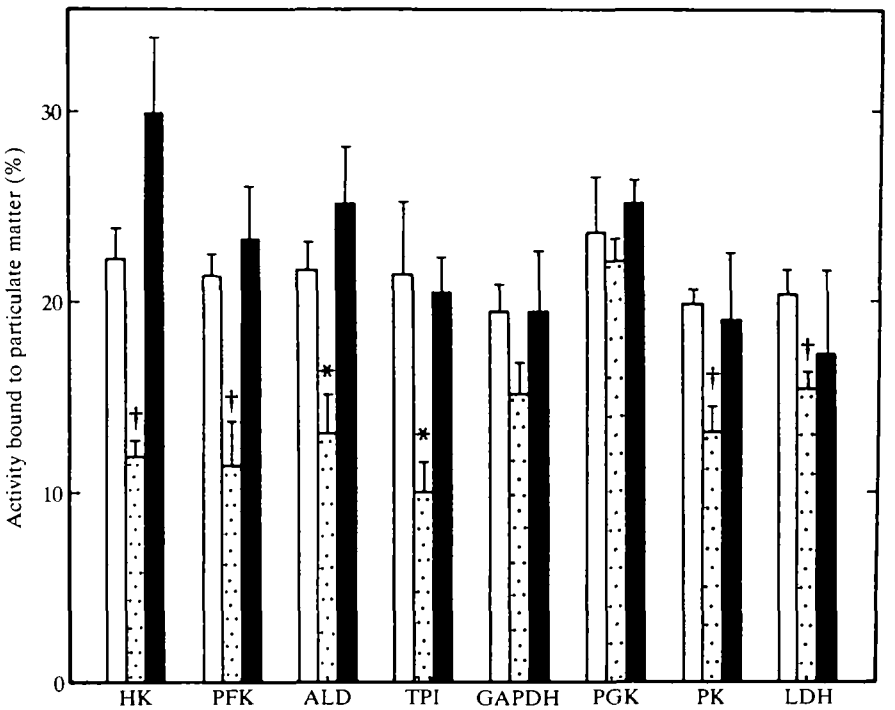

Fig. 2. Effect of anoxia on the binding of glycolytic enzymes to the subcellular particulate fraction in *Otala lactea* foot muscle. Values are means±S.E.M., $N=4$ for controls (□), 14.5 h of anoxia (⊡) and 45 h of anoxia (■). Abbreviations are as in Fig. 1. Significantly different from control: *, $P<0.05$; †, $P<0.01$.

of enzymes (total of free+bound activities) averaged 120% (range 110–145%) compared to the total enzyme activities measured in the crude homogenate.

The changes in glycolytic enzyme binding in foot muscle during estivation in *O. lactea* are shown in Fig. 1. After 4 days of dormancy, the percentage of the total activity bound to particulate matter had decreased significantly for four glycolytic enzymes: hexokinase (HK), 6-phosphofructo-1-kinase (PFK), aldolase (ALD) and D-lactate dehydrogenase (LDH). The most pronounced effect was on HK; the percentage of bound enzyme decreased to a value approximately 50% of control (22.3±1.6% in control foot to 10.9±2.8% in estivating animals). However, when the period of estivation was extended to 22 days, the initial trends largely reversed themselves. The percentages of enzyme activity associated with particulate matter at 22 days were not significantly different from those found in the muscle of control animals (except for ALD) and were significantly increased compared to the percentages bound after 4 days of estivation.

The effects of anoxia on glycolytic enzyme binding to the particulate fraction in *O. lactea* foot muscle are shown in Fig. 2. Anoxia exposure for 14.5 h significantly reduced enzyme binding for six glycolytic enzymes, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and phosphoglycerate kinase (PGK) being the excep-

Table 1. *Maximal activities (per gram dry mass) of glycolytic enzymes in foot muscle of* Otala lactea*: effect of estivation and anoxia*

		Estivation		Anoxia	
	Control	4 days	22 days	14.5 h	45 h
Hexokinase	2.50±0.52	2.08±0.03	1.15±0.23**	2.86±0.39	3.12±0.26
Phosphofructokinase	25.2±3.90	22.1±1.70	10.6±2.29**	24.2±1.43	32.5±1.30
Aldolase	19.6±2.60	17.8±0.81	12.9±2.29	22.5±2.08	25.3±1.30*
Triosephosphate isomerase	1779±649	2504±396	1832±435	2454±182**	2831±442
Glyceraldehyde-3-phosphate dehydrogenase	375±52.0	245±28.7*	77.1±13.7**	281±20.8	373±33.8
Phosphoglycerate kinase	87.0±5.20	49.5±18.8	45.0±6.90*	158±6.45**	131±13.0*
Pyruvate kinase	97.4±9.10	118±15.8	100±16.8	168±6.51**	108±9.10
D-Lactate dehydrogenase	96.1±23.4	114±19.8	38.9±7.63*	110±7.86	103±10.4

Data are in i.u. g^{-1} dry mass±S.E.M., N=4 separate preparations from individual animals. Significantly different from control value by Student's t-test, * $P<0.05$, ** $P<0.01$.

tions. Binding by HK, PFK and triosephosphate isomerase (TPI) was strongly affected, the amount of enzyme bound decreasing by about 50 % in each case. In a pattern similar to that seen in long-term estivation, when anoxia was prolonged to 45 h, the percentages of enzymes bound to the particulate fraction again reverted to values not significantly different from those of the controls.

Table 1 shows the maximal activities of the eight glycolytic enzymes in foot muscle from control, estivating and anoxic *O. lactea*. Because *O. lactea* partially dehydrates during estivation, the percentage water in the foot tissue decreases from 92.3±1.0 % (control, anoxic tissues) to 89.9±0.3 % after 4 days of estivation, and to 86.9±0.2 % after 22 days of estivation (N=3 for all cases). Thus, the maximal activities in Table 1 are reported on a per gram dry mass basis. The activity of TPI was not affected under any experimental situation, but the activities of HK, PFK, ALD, GAPDH, PGK, PK and LDH were significantly different from the corresponding control values in certain instances. The total activity of GAPDH increased after a short period of estivation (4 days) and, by 22 days of estivation, HK, PFK, PGK and LDH had also decreased in comparison with control activities. Specific increases in total enzyme activity were also noted for PGK and PK in short-term anoxia (14.5 h), but during long-term anoxia (45 h) PK had returned to control levels whereas ALD had increased slightly.

The effects of estivation and anoxia on glycogen phosphorylase in foot muscle are shown in Table 2. During short-term estivation there was no significant change in either the total activity ($a+b$) of phosphorylase or the percentage of the enzyme in the active a form, but animals estivating for 22 days had an overall glycogen phosphorylase activity that was 42 % of the control value. Under short-term anoxic conditions both parameters were strongly depressed, resulting in a drop in the overall activity of phosphorylase a to 31 % (from 0.41 to 0.13 i.u. g^{-1}) of that in control foot muscle.

Table 2. *Glycogen phosphorylase in foot muscle of estivating and anoxic* Otala lactea

	Total phosphorylase (i.u. g^{-1} dry mass)	Phosphorylase *a* (%)
Control	9.35±1.56	56.6±4.6
Estivation		
4 days	9.90±2.98	58.6±3.8
22 days	3.98±1.30*	56.9±3.4
Anoxia		
14.5 h	4.16±1.95*	39.5±2.5*

Values are means±S.E.M., N=4.
* Significantly different from control, $P<0.05$.

Table 3. *Fructose-2,6-bisphosphate levels in foot muscle of estivating and anoxic* Otala lactea

	Fructose-2,6-bisphosphate (nmol g^{-1} dry mass)
Control	23.4±4.2
Estivation	
4 days	1.19±0.26*
22 days	1.22±0.80*
Anoxia	
14.5 h	1.17±0.20*
45 h	1.43±0.13*

Values are means±S.E.M., N=4.
Significantly different from control, * $P<0.01$.

Table 3 shows the levels of fructose-2,6-bisphosphate in foot muscle of estivating and anoxic *O. lactea*. Both experimental states were characterized by a large decrease in fructose-2,6-bisphosphate concentration to levels that were only 5–9 % of control values.

Discussion

The mechanisms underlying glycolytic control in *O. lactea* foot muscle are best understood when compared with previous measurements of respiratory quotients (RQ) and of changing patterns of glycolytic intermediate and end-products over the course of estivation or anoxia in this species. RQ values during estivation in *O. lactea* range between 0.9 and 1.0 (Barnhart 1986*b*; Barnhart and McMahon, 1987), indicating that this species relies primarily on carbohydrate oxidation

during estivation. Fermentative products (D-lactate, succinate, L-alanine) do not accumulate during estivation (Barnhart, 1986*a*; Churchill and Storey, 1989). Anaerobic metabolism in *O. lactea*, in contrast, is primarily based on glycogenolysis with D-lactate as the primary product (levels rose 50-fold to 14 μmol g^{-1} wet mass in foot muscle after 14 h of anoxia) as well as small accumulations of succinate and L-alanine (Churchill and Storey, 1989). Changes in the concentrations of glycolytic intermediates (analyzed *via* cross-over plots) indicated an activation of glycolysis after 2 h of expsoure to anoxia (representing the hypoxic transition period) but a subsequent strong inhibition of glycolysis (glycolytic rate depression) by 14 h of exposure to anoxia (Churchill and Storey, 1989). Comparable measurements in estivating snails indicated glycolytic rate depression in snails dormant for 22 days. Regulatory control of glycolysis in both estivation and anoxia was indicated at the PFK, ALD and PK loci (Churchill and Storey, 1989).

The response to anoxia by intertidal marine molluscs is a rapid depression of overall metabolic rate coupled with an initiation of fermentative pathways of ATP production. The response to anoxia by the land snail *O. lactea* appears to be the same. Although a glycolytic activation occurs briefly during the hypoxic transition period, a glycolytic rate depression with inhibitory control at the PFK and PK loci is firmly in place within 14 h of exposure to anoxia (Churchill and Storey, 1989). This glycolytic rate depression can be related to the results of the present study. The percentage of enzyme activity associated with the particulate fraction decreased for six of the glycolytic enzymes assayed in anoxic foot muscle. Reduced enzyme binding was also associated with anoxia-induced metabolic rate depression in the marine gastropod, *Busycotypus canaliculatum*, with the most pronounced changes seen for HK and ALD (percentage of bound enzyme decreased from 40–45 % to 9–10 % in anoxia), similar to the situation in *O. lactea* (Plaxton and Storey, 1986). The opposite response, increased binding of enzymes to the particulate fraction, has been documented in several systems during glycolytic rate activation, including ischemia in rat and sheep hearts (Clarke *et al.* 1984), contraction in skeletal muscle (Clarke *et al.* 1980) and burst exercise in trout white muscle (Brooks and Storey, 1988*b*). In all these instances, decreased enzyme binding correlated with a decreased glycolytic rate, whereas increased enzyme binding correlated with an increased glycolytic rate. This mechanism appears, therefore, to be a simple and fast way to change the overall flux through glycolysis in a readily reversible fashion. Bound enzymes are thought to organize themselves into catalytically efficient complexes that can channel substrates and products between consecutive enzymes and accomplish the conversion of hexose phosphates to pyruvate at a faster rate than in a comparable system of soluble enzymes. This complex responds quickly to changing energy needs (Brooks and Storey, 1988*b*) and thus may represent a rapid, short-term, glycolytic control mechanism.

Depressed glycolytic rate in anoxia was also related to a decrease in the activity of glycogen phosphorylase. In anoxic foot muscle, the overall glycogen phosphorylase activity dropped to 31 % of that found in control snails, whereas in long-term estivation, the overall rate decreased to 42 % of the control value. Anoxia also

strongly reduced the content of fructose-2,6-bisphosphate in *O. lactea* foot to only 5–6% of the control value. Fructose-2,6-bisphosphate is an extremely potent activator of PFK and a reduction in the levels of this compound in anoxia would clearly contribute to a restriction of flux through the PFK locus. Typically, high fructose-2,6-bisphosphate levels mediate the use of carbohydrate reserves for anabolic purposes. In conditions where carbohydrate use must be spared or restricted (e.g. starvation, anoxia), however, fructose-2,6-bisphosphate content is reduced, leading to restricted flux through the PFK locus (Hue and Rider, 1987). A sharp drop in fructose-2,6-bisphosphate content also characterizes the response to environmental anoxia by the organs of marine molluscs (Storey, 1985*b*, 1988*b*); in the most pronounced case, fructose-2,6-bisphosphate content dropped by 224-fold in whelk ventricle under anoxia with a half-time of only 35 min. Overall, then, the early response (seen at 14.5 h) to environmental anoxia by the land snail is qualitatively the same as that which occurs for both marine molluscs as well as vertebrate facultative anaerobes (Storey, 1985*a*, 1988*a*), namely a decrease in phosphorylase *a* content, sharply reduced fructose-2,6-bisphosphate levels, and a reduction in glycolytic enzyme binding to the particulate fraction of the cell, all mechanisms designed to lower glycolytic rate and contribute to metabolic rate depression during anaerobiosis.

The mechanisms controlling glycolytic flux during short-term estivation are less clear-cut. The present data for snails that had been estivating for 4 days showed no change in the activity of glycogen phosphorylase *a*, a strong depression of fructose-2,6-bisphosphate levels, and a decrease in the percentages of glycolytic enzymes bound to the particulate fraction. All these data suggest a depression of glycolytic rate at this time. These results agree with the observations of R. E. Whitwam and K. B. Storey (in preparation), who found a modification of the kinetic properties of PK from *O. lactea* foot muscle, consistent with an enzyme inactivation *via* protein phosphorylation, which developed within 48 h of entry into dormancy. These results also correspond with an overall metabolic rate depression during dormancy (Herreid, 1977; Barnhart and McMahon, 1987). Thus, the various regulatory mechanisms that we have studied are consistent with a glycolytic rate depression within the early days of dormancy. Nevertheless, these results are at odds with the cross-over analysis taken from measurements of changes in glycolytic intermediates, which suggest a relative activation of glycolysis, compared to the control situation, after 3 days of estivation (Churchill and Storey, 1989). It is difficult to resolve this paradigm completely. However, it is possible that the cross-over analysis after 3 days of estivation reflects an intermediate stage between a highly activated glycolytic state, which may occur very early during estivation (similar to that seen during short-term anoxia), and the depressed metabolic state apparent after 22 days of estivation. In this model, mechanisms to depress glycolytic rates are observable after 4 days of estivation (decreased enzyme binding, lower fructose-2,6-bisphosphate concentrations, lower PK activity) but the glycolytic flux has not yet reached the new, lower, steady-state value. Testing this hypothesis would require a greater frequency of sampling

during the early stages of estivation, specifically involving measurements of glycolytic rate and metabolic cross-overs.

Changes in enzyme binding to the particulate fraction characterized the short-term response in the transition to a new metabolic state (14.5 h anoxia or 4 days of estivation), but over the longer term (45 h anoxia, 22 days of estivation) the percentages of glycolytic enzymes bound to the particulate fraction returned to values virtually equivalent to those found in control snails. For ALD in long-term estivating snails, the percentage bound was actually greater than in the control situation. These data suggest that changes in 'enzyme-complex' formation are involved primarily in mediating initial responses in anaerobiosis and dormancy, perhaps facilitating the transition to the hypometabolic state. Other mechanisms, such as the changes in kinetic and regulatory properties of regulatory enzymes (glycogen phosphorylase, PFK, PK) brought about by reversible protein phosphorylation (Table 2; R. E. Whitwam and K. B. Storey, in preparation) and the decrease in fructose-2,6-bisphosphate concentration (Table 3), may maintain the stable hypometabolic state over the long term. It is apparent that other influences are also important as stress is prolonged. For example, the maximal activities of several enzymes decreased in prolonged estivation. GAPDH activity fell to only 20 % of the control value after 22 days of estivation, and, at this time, HK, PFK, PGK, and LDH fell to approximately 45 % of their control values. Thus, although the percentage of these enzymes bound to particulate matter was not significantly different from control values in snails that had been estivating for 22 days, the total amount of bound activity was sharply reduced. This could significantly alter the make-up and function of glycolytic complexes. Furthermore, intracellular pH decreases during both estivation and anoxia in the tissues of gastropods (Ellington, 1983; Barnhart and McMahon, 1988) and pH change has a marked influence on the kinetic parameters of enzymes, the association–dissociation of enzyme subunits, and enzyme binding associations with subcellular particulate matter. For PFK, for example, decreasing pH (over the range 7.5–6.8) increases the sigmoidicity of fructose-6-phosphate kinetics, enhances inhibition by ATP and citrate, promotes the dissociation of the active tetramer to the inactive dimer, and decreases enzyme binding to particulate matter (Hand and Somero, 1984; Brooks and Storey, 1988*c*). It is clear, then, that metabolic depression is the result of an interplay between a variety of regulatory mechanisms and influencing factors.

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