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Author(s): Ross E. Whitwam and Kenneth B. Storey
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# Regulation of Phosphofructokinase during Estivation and Anoxia in the Land Snail, *Otala lactea*

**Ross E. Whitwam**
**Kenneth B. Storey***
Institute of Biochemistry and Department of Biology, Carleton University, Ottawa, Ontario, Canada K1S 5B6

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**Abstract**

*The properties of 6-phosphofructo-1-kinase (PFK) were analyzed in foot muscle, mantle, and hepatopancreas of the land snail,* Otala lactea *(Pulmonata, Helicidae). The enzyme in all tissues showed properties common to PFK from other invertebrate sources including sigmoidal fructose 6-phosphate (F6P) kinetics at pH 7, activation by AMP, fructose-2,6-bisphosphate, $NH_4^+$, and inorganic phosphate, and weak inhibition by Mg ATP and citrate. Novel to* O. lactea *PFK, however, was an activation of the enzyme by low levels of phosphoenolpyruvate ($K_a$ = 30–75 μM). The properties of PFK were compared in tissues isolated from three experimental groups of snails: aroused for 2 d (control), estivating for 22 d, or anoxic ($N_2$ gas atmosphere) for 45 h. The PFK in both hypometabolic states showed stable modifications of enzyme properties. In mantle and hepatopancreas the pattern of changes to PFK properties were similar in both estivation and anoxia, and in both cases effects on F6P affinity and inhibitor and activator constants were consistent with the conversion of PFK to a less active enzyme form in the hypometabolic state. In foot muscle anoxia and estivation were similar in their effects on F6P affinity but had different effects on other enzyme parameters. The molecular basis of the changes in enzyme properties with the transition from the aroused to dormant or anoxic states appears to be protein phosphorylation. In vitro incubations of hepatopancreas extracts from control snails with agents promoting the action of cAMP-dependent protein kinase, cGMP-dependent protein kinase, or protein kinase C all mimicked the changes in $K_a$ fructose-2,6-bisphosphate and inhibition by citrate seen with the natural transition from the aroused to estivating state. These agents were without effect, however, on PFK from estivating snails. It appears, then, that a key component of glycolytic rate control in* O. lactea *is a change in the activity state of PFK via phosphorylation of the enzyme and that this mechanism serves metabolic rate depression in either estivation or anoxia.*

* To whom all correspondence should be addressed.

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## Introduction

Estivation is a common survival strategy among animals that inhabit seasonally arid environments. The phenomenon has been described for many pulmonate land snails (Schmidt-Nielsen, Taylor, and Shkolnik 1971; Herreid 1977; Horne 1979; Umezurike and Iheanacho 1983; Vorhaben, Klotz, and Campbell 1984). When water and food supplies disappear, snails retreat into their shells and enter a dormant state characterized by an aerobic metabolic rate that is only 10%–30% of the normal resting rate when they are aroused. Living off endogenous fuel reserves, the snails may continue in such a state for months or even years.

The mechanisms of metabolic rate depression have received considerable attention in recent years (for reviews, see Hochachka and Guppy 1987; Storey 1988*a,* Storey and Storey 1990). At the enzymatic level, regulatory mechanisms include (1) changes in the activity state of regulatory enzymes via protein phosphorylation or dephosphorylation reactions, (2) pathway control by altering the association or dissociation of enzymes from multienzyme complexes bound to subcellular structural elements, and (3) regulation of the use of carbohydrate reserves for anabolic purposes via fructose-2,6-bisphosphate ($F2,6P_2$) control over 6-phosphofructo-1-kinase (PFK) (Storey 1988*a*). These mechanisms, as well as the signals involved in mediating the transition to the hypometabolic state, have been most extensively studied in an anoxia-tolerant marine gastropod *Busycotypus canaliculatum* (Plaxton and Storey 1984*a,* 1984*b,* 1986; Storey 1984, 1988*b;* Brooks and Storey 1989, 1990*a*). However, recent studies have shown that the same mechanisms also support facultative anaerobiosis by vertebrate species (e.g., freshwater turtles and goldfish) and, in addition, are involved in other types of metabolic arrest, namely hibernation and estivation (for review, see Storey and Storey 1990).

Our studies of the biochemistry of metabolic arrest in estivation have used the pulmonate land snail, *Otala lactea.* The physiology of dormancy in this species has been well studied. Estivation is characterized by hypoxia, hypercapnia, extracellular acidosis, and a reduction of metabolic rate to as little as 15% of the resting rate of the aroused animal (Herreid 1977; Barnhart 1986*a,* 1986*b;* Barnhart and McMahon 1987, 1988). The aerobic oxidation of carbohydrate reserves appears to support metabolism in estivating snails (Umezurike and Iheanacho 1983; Livingstone and de Zwaan 1983; Cedeno-Leon 1984), and, as such, mechanisms of glycolytic rate depression must be central to the transition to or from the dormant state. Indeed, we have found strong evidence of changes in glycolysis in the estivating state. Entry into dormancy was accompanied by the phosphorylation of pyruvate kinase

(PK), a regulatory enzyme of glycolysis, with the effect of the posttranslational modification being to create a less active enzyme form (Whitwam and Storey 1991*b*). Estivation also resulted in changes in the percentages of glycolytic enzymes associated with subcellular particulate matter and in a reduced tissue F2,6$P_2$ content (Brooks and Storey 1990*b*). Measurements of changes in the concentrations of glycolytic intermediates in tissues of *O. lactea* over time suggested that early in estivation (after 3 d) glycolytic rate was increased but subsequently glycolysis decreased as estivation became prolonged (22 d) (Churchill and Storey 1989). Analysis of these changes via crossover plots indicated that PFK, aldolase, and PK were regulatory sites acting in the response of glycolysis to estivation or anoxia in two organs, foot muscle and hepatopancreas (Churchill and Storey 1989).

This study analyzes the control of PFK (ATP:D-fructose-6-phosphate-1-phosphotransferase, EC 2.7.1.11), an important regulatory enzyme of glycolysis, in estivation and in response to anoxia stress in *O. lactea.* Like marine gastropods, land snails have a considerable tolerance for anoxia; *O. lactea* readily survives 2 d of anoxia (Brooks and Storey 1990*b*). The species provides an excellent model system, therefore, in which to compare the control of glycolysis in estivation versus anoxia and to determine whether the mechanisms of metabolic arrest used in facultative anaerobiosis by marine gastropods are conserved for use in another form of inducible dormancy, estivation by land snails.

## Material and Methods

### *Chemicals and Animals*

All biochemicals and coupling enzymes were obtained from Sigma Chemical, Saint Louis, Missouri, or Boehringer Mannheim, Montreal, Quebec. Pulmonate land snails, *Otala lactea,* were kindly provided by M. C. Barnhart from an introduced population at Playa del Rey in Los Angeles County, California. Care, feeding, and experimental manipulation of the snails were as described in Whitwam and Storey (1991*b*). In brief, experimental design was as follows. All snails were awakened from dormancy and given continuous access to food and regular spraying with water to ensure that arousal was maintained for at least 2 d. Control snails were then sampled directly. To impose anoxia, we placed other aroused snails in jars that were then flushed with 95% $N_2$ and 5% $CO_2$ (this level of $CO_2$ creates a hypercapnia similar to the natural hypercapnia experienced in the estivating state; M. C. Barnhart, personal communication). The jars were sealed with a screw-on lid and Parafilm. To induce estivation, aroused snails were removed and

placed in dry containers with no food but with mesh lids to permit continuous aeration. Snails were sampled after 45 h of anoxia or 22 d of estivation; tissues were rapidly dissected out, frozen in liquid nitrogen, and then transferred to −60°C for storage.

*Preparation of Enzyme Extracts*

Tissues were homogenized 1:5 (wt/vol) in homogenization buffer containing 100 mM NaF, 5 mM ethylenediaminetetraacetic acid (EDTA), 5 mM ethylene glycol-bis (B-aminoethylether) *N,N,N′,N′*-tetraacetic acid (EGTA), 0.1 mM phenylmethylsulfonyl flouride (PMSF), 30 mM 2-mercaptoethanol, and 40% glycerol (vol/vol) in 50 mM imidazole-HCl (pH 7.0); a Polytron PT 10 or an Ultra-Turrax homogenizer was used. Homogenates were centrifuged for 20 min at 25,000 *g* in a Sorvall RC-5B refrigerated centrifuge at 5°C. The supernatant was removed and passed through a 5-mL column of Sephadex G-25 equilibrated in 40 mM imidazole-HCl (pH 7.0) with 5 mM EDTA, 15 mM 2-mercaptoethanol, and 20% vol/vol glycerol. Columns were spun in a desktop centrifuge at top speed for 1 min. The filtrate was removed, stored on ice, and used for the analysis of enzyme kinetic properties.

*In Vitro Phosphorylation or Dephosphorylation*

The preparation of enzyme extracts for these experiments followed the same protocol as described above except that fresh tissues were always used, tissues were homogenized in 1:2.5 (wt/vol), and 1 mM ATP was added to the homogenization buffer as a stabilizer of PFK activity. After centrifugation, the supernatant was passed through a Sephadex G-25 spun column (as described above) but with the column equilibrated in 40 mM imidazole-HCl (pH 7.0), 20% vol/vol glycerol, 10 mM phosphate, 10 mM 2-mercaptoethanol, 1 mM ATP, and 0.1 mM EDTA. The filtrate was collected and divided into seven aliquots, and each aliquot was diluted 1:1 with a solution containing 40 mM imidazole-HCl (pH 7.0), 10 mM 2-mercaptoethanol, and 20% vol/vol glycerol (to stabilize the enzyme) and one of the following specific additions:

1. 20 mM $MgCl_2$, 40 mM NaF, 2 mM $CaCl_2$, 20 μg/mL phorbol 12-myristate 13-acetate, 80 μg/mL brain extract (phospholipid source; type 1, Sigma Chemical no. 1502), and 10 mM ATP;
2. 20 mM $MgCl_2$, 40 mM NaF, 8 mM cGMP, and 10 mM ATP;
3. 20 mM $MgCl_2$, 40 mM NaF, 4 mM cAMP, 2 mg/mL cAMP-dependent protein kinase (from bovine heart, 2.0 pM phosphate/μg protein phosphorylating activity), and 10 mM ATP;

4. 25 mM KCl, 5 mM EDTA, 15 mM $MgCl_2$, 1 mM ATP, and 50 IU alkaline phosphatase (grade 1, from calf intestine);
5. 25 mM KCl, 5 mM EDTA, 15 mM $MgCl_2$, 1 mM ATP, 50 IU alkaline phosphatase (grade 1, from calf intestine), 2 mg/mL acid phosphatase (type 1, from wheat germ, 0.46 U/mg activity), and 1 mg/mL spermidine;
6. 25 mM KCl, 5 mM EDTA, 15 mM $MgCl_2$, 1 mM ATP, and 1 mg/mL spermidine;
7. 40 mM NaF, 5 mM EDTA, and 1 mM ATP. Solution (7) was the control.

All samples were then incubated for 12 h at 20°C. Samples were then passed through Sephadex G-25 spun columns equilibrated in 40 mM imidazole-HCl (pH 7.0), 1 mM ATP, 0.5 mM EDTA, 10 mM 2-mercaptoethanol, and 20% vol/vol glycerol. The filtrates were used for subsequent kinetic analyses.

### *Enzyme Assays*

We measured PFK activity at 20°C using a coupled enzyme assay at 340 nm and a Gilford 240 recording spectrophotometer. We assayed PFK from foot muscle and mantle in the presence of 50 mM imidazole-HCl buffer (pH 7.0), 50 mM KCl, 5 mM $MgCl_2$, 0.1 mM NADH, 0.5 U/mL aldolase, 0.5 U/mL triosephosphate isomerase, 2 U/mL glycerol-3-phosphate dehydrogenase (all coupling enzymes were dialyzed before use), and fructose-6-phosphate (F6P) and Mg ATP (1:1 $MgCl_2$:ATP; this $Mg^{2+}$ is in addition to the constant 5 mM cited above) as indicated in the results. We assayed PFK from hepatopancreas under the same conditions except that 30 mM KCl and 3 mM $MgCl_2$ were used. All assays were initiated by the addition of enzyme homogenates.

### *Calculations and Statistics*

We determined substrate affinity constant ($S_{0.5}$), Hill coefficients ($n_H$), and maximal velocity ($V_{max}$) values by fitting the data to the Hill equation through a nonlinear least squares regression program for the computer. A modified Hill equation that included a $V_0$ term (rate at zero activator) was used for determining $K_a$ values. We determined the inhibitor constant ($I_{50}$) values from plots of vol. versus [inhibitor]. We determined $K_a$ and $I_{50}$ values at subsaturating concentrations of F6P as given in tables 1 and 3. Results are presented as means ± SEM. Tests for significant differences between values used the Student's *t*-test.

## Results

The properties of PFK from *Otala lactea* were assessed in three tissues: foot muscle, mantle, and hepatopancreas (tables 1–3). The enzyme from control, active snails showed low affinity for F6P in foot and hepatopancreas ($S_{0.5}$ F6P 1.5–3.1 mM), such that enzyme function in vivo may rely closely on the effects of activators. Mantle PFK from control snails had a much greater affinity for F6P ($S_{0.5}$ about 0.3 mM). The enzyme in all three tissues showed sigmoidal kinetics with respect to F6P saturation; $n_H$ ranged from 1.5–2.3. Enzyme activators reduced $S_{0.5}$ and lowered $n_H$. Adenosine 5′-monophosphate and F2,6$P_2$ activated *O. lactea* PFK as occurs for the enzyme from other animal sources. Perhaps unique to *O. lactea* PFK, however, was the identification of phosphoenolpyruvate (*P*-enolpyruvate) as an activator of the enzyme, at quite low levels ($K_a$ values ranged between 30 and 75 μM). The Mg ATP was a weak inhibitor of the foot and mantle enzymes but $I_{50}$ values were less than 2 mM for hepatopancreas PFK. Citrate was a weak

TABLE 1
*Kinetic and regulatory properties of phosphofructokinase from foot muscle of* Otala lactea

| | Active | Dormant | Anoxic |
|---|---|---|---|
| U/g wet wt | .81 ± .05 | .99 ± .03* | 1.55 ± .07* |
| $S_{0.5}$ F6P, mM | 3.3 ± .2 | 2.4 ± .4* | 2.3 ± .2* |
| $n_H$ | 2.2 ± .1 | 1.8 ± .3 | 1.7 ± .2* |
| $S_{0.5}$ F6P, mM + 6 mM $NH_4^+$ | 1.5 ± .1 | .9 ± .2* | 1.2 ± .1* |
| $n_H$ | 1.9 ± .1 | 1.6 ± .2* | 2.1 ± .1 |
| $I_{50}$ Mg ATP, mM | 7.0 ± .3 | 6.5 ± .3 | 6.2 ± .5 |
| $I_{50}$ Mg Citrate, mM | 11.4 ± .7 | 11.4 ± .8 | 8.4 ± .8* |
| $K_a$ AMP, mM | .42 ± .03 | .28 ± .01* | .68 ± .02* |
| $K_a$ F2, 6$P_2$, μM | 3.4 ± .2 | 2.2 ± .3* | 3.8 ± .1* |
| $K_a$ *P*-enolpyruvate, μM | 49 ± 3 | 74 ± 2* | 59 ± 2 |

Note. Snails were active for 2 d, dormant for 22 d, or anoxic for 45 h. Data are means ± SEM of determinations on muscle preparations from 3–6 individual animals in each treatment group. One unit of PFK activity is defined as the amount that uses 1 μmol F6P per min at 20 °C. Values for $S_{0.5}$ were determined at 3 mM Mg ATP. Citrate $I_{50}$ values were determined at 2 mM F6P and 3 m*M* Mg ATP with citrate added in a 2:1 molar ratio with $MgCl_2$. Values for $K_a$ were determined at 0.4 mM F6P and 3 mM Mg ATP.
* Significantly different from the value for the enzyme in control, active animals, $P < 0.05$.

TABLE 2
*Kinetic and regulatory properties of phosphofructokinase from mantle of* Otala lactea

| | Active | Dormant | Anoxic |
|---|---|---|---|
| U/g wet wt | .44 ± .07 | .46 ± .03 | .37 ± .03 |
| $S_{0.5}$ F6P, mM | .31 ± .03 | 1.9 ± .2[a] | 1.7 ± .3[a] |
| $n_H$ | 1.5 ± .1 | 1.7 ± .1 | 1.8 ± .1 |
| $S_{0.5}$ F6P, mM + 6 mM $P_i$ | .30 ± .01 | 1.6 ± .03[a] | .52 ± .05[a] |
| $n_H$ | 1.4 ± .1 | 2.4 ± .4** | 2.0 ± .1** |
| $S_{0.5}$ F6P, mM + 6 mM $NH_4^+$ | .22 ± .01 | 1.1 ± .1[a] | 1.4 ± .2[a] |
| $n_H$ | 1.4 ± .1 | 1.6 ± .2 | 1.5 ± .2 |
| $S_{0.5}$ F6P, mM + 5 μM F2, $6P_2$ | .17 ± .02 | .39 ± .04[a] | .25 ± .01[a] |
| $n_H$ | 1.28 ± .08 | 1.54 ± .19 | 1.31 ± .05 |
| $I_{50}$ Mg ATP, mM | 12.7 ± .7 | 7.6 ± .6[a] | 7.9 ± .2[a] |
| $I_{50}$ Mg citrate, mM | N.E. | N.E. | N.E. |
| $K_a$ *P*-enolpyruvate, μM | 67 ± 5 | 67 ± 6 | 72 ± 5 |
| $K_a$ F2, $6P_2$, μM | 2.2 ± .5 | 1.8 ± .4 | 4.2 ± .2[a] |

Note. Conditions are as in table 1; N.E. = no effect of citrate at levels up to 15 mM.
[a] Significantly different from the value for the enzyme in control, active animals, $P < 0.005$.
** $P < 0.01$.

inhibitor of foot muscle and hepatopancreas PFK but had no effect at levels up to 15 mM on the mantle enzyme.

The data in tables 1–3 show that PFK from all three tissues of *O. lactea* underwent a stable modification of enzyme kinetic properties during estivation or in response to anoxia.

### *Foot Muscle*

The PFK from foot muscle showed an increase in affinity for F6P ($S_{0.5}$ decreased by about 30%) in both estivation and anoxia, and the effect was maintained in the presence of ammonium ion, an activator of the enzyme (table 1). However, with regard to other kinetic parameters (and except for $I_{50}$ Mg ATP, which was not altered by either experimental treatment), estivation and anoxia did not have the same effects on the enzyme. The $I_{50}$

TABLE 3

*Kinetic and regulatory properties of phosphofructokinase from the hepatopancreas of* Otala lactea

| | Active | Dormant | Anoxic |
|---|---|---|---|
| U/g wet wt | .49 ± .04 | .78 ± .05*** | .60 ± .04 |
| $S_{0.5}$ F6P, mM | 2.4 ± .2 | 3.2 ± .1* | 3.1 ± .3* |
| $n_H$ | 2.3 ± .2 | 2.3 ± .1 | 2.6 ± .1 |
| $S_{0.5}$ F6P, mM + 5 mM $NH_4^+$ | 1.56 ± .09 | 1.81 ± .10 | 1.67 ± .02 |
| $n_H$ | 2.1 ± .1 | 2.8 ± .4* | 2.8 ± .2* |
| $I_{50}$ Mg ATP, mM | 1.9 ± .2 | 1.9 ± .1 | 1.6 ± .2 |
| $I_{50}$ Mg citrate, mM | 46 ± 3.5 | 24 ± .5*** | 7.4 ± .4*** |
| $K_a$ $P_i$, mM | .21 ± .03 | .30 ± .01* | .26 ± .02 |
| $K_a$ AMP, mM | .30 ± .01 | .23 ± .01*** | .27 ± .02 |
| $K_a$ F2, $6P_2$, μM | .70 ± .09 | 2.3 ± .4*** | 2.3 ± .3*** |
| $K_a$ *P*-enolpyruvate, μM | 42 ± 3 | 30 ± 1* | 30 ± 3* |

Note. Data are means ± SEM of 4–10 determinations on tissue from individual animals. Values for $S_{0.5}$ F6P were determined at 0.3 mM Mg ATP. Values for $I_{50}$ were determined at 4 mM F6P and 0.3 mM Mg ATP. Values for $K_a$ were determined at 0.5 mM F6P and 0.3 mM Mg ATP, except for $K_a$ $P_i$, which was determined at 2 mM F6P and 0.3 mM Mg ATP.

* Significantly different from the value for the enzyme in control, active animals, $P < 0.05$.

*** $P < 0.005$.

for citrate inhibition decreased by 25% in anoxia compared with the enzyme from control, active snails but was unchanged in estivation; on the other hand, the $K_a$ *P*-enolpyruvate increased significantly in estivation but was unchanged in anoxia. With respect to activators, the $K_a$ values for AMP and F2,$6P_2$ decreased significantly for PFK from foot muscle of estivating snails but increased for the enzyme from anoxic tissue.

### *Mantle*

The response of mantle PFK to estivation and anoxia was somewhat different from that seen for foot. With one exception (the $K_a$ for F2,$6P_2$), the effects of estivation and anoxia on PFK were qualitatively the same (table 2). Under both conditions, $S_{0.5}$ F6P increased dramatically by 5.5–6.1-fold. Such a large decrease in enzyme substrate affinity would have major consequences for enzyme function at physiological F6P levels; hence, the role of activators

in reducing $S_{0.5}$ could become very important. Indeed, the addition of 5 μM $F2,6P_2$ strongly reduced the $S_{0.5}$ F6P for PFK from both estivating or anoxia snails. Ammonium ion and inorganic phosphate were less effective in this function although $P_i$ sharply reduced $S_{0.5}$ F6P of the anoxic enzyme form. In both estivation and anoxia, enzyme inhibition by ATP increased, $I_{50}$ Mg ATP decreasing by about 40% in both cases. Citrate (at levels up to 15 mM) did not inhibit mantle PFK under any condition. Estivation and anoxia did not affect enzyme activation by *P*-enolpyruvate but the anoxic enzyme form showed a twofold increase in $K_a$ $F2,6P_2$ compared with the control enzyme.

*Hepatopancreas*

The effects of estivation and anoxia on hepatopancreas PFK were similar in some ways. Compared with the enzyme from active, control snails, $S_{0.5}$ F6P increased by about 30%, $I_{50}$ Mg ATP was unchanged, $K_a$ $F2,6P_2$ increased 3.3-fold, and $K_a$ *P*-enolpyruvate decreased by 30% under both experimental treatments (table 3). The $I_{50}$ citrate also decreased under both conditions, to 50% in estivation and to 16% in anoxia of the control value. The effects of estivation and anoxic on hepatopancreas PFK differed only in the effects on $K_a$ values for $P_i$ and AMP; these values changed in estivation but not in anoxia.

*In Vitro Phosphorylation and Dephosphorylation of Hepatopancreas PFK*

Table 4 shows the effects on PFK of in vitro incubations of hepatopancreas extracts with agents that promote protein phosphorylation or dephosphorylation. Two kinetic parameters, $K_a$ $F2,6P_2$ and $I_{50}$ citrate, that were changed by estivation or anoxia in vivo were monitored as indicators of enzyme modification.

Incubation of PFK from active, control snails with cAMP-dependent protein kinase plus cAMP and Mg ATP resulted in a more than twofold increase in the average $K_a$ for $F2,6P_2$. The enzyme also showed enhanced inhibition by citrate after this treatment; the activity ratio in the absence versus presence of 15 mM Mg citrate rose to an average of 1.75. Similar significant increases in $K_a$ $F2,6P_2$ and inhibition by citrate occurred when the enzyme was incubated with cGMP plus Mg ATP, stimulators of endogenous cGMP-dependent protein kinase, or with $Ca^{2+}$ plus phorbol 12-myristate 13-acetate plus Mg ATP, stimulators of endogenous protein kinase C. However, the equivalent treatments did not alter the kinetic properties of PFK in hepatopancreas extracts from estivating snails.

TABLE 4

*Effect of in vitro treatments that promote protein phosphorylation or dephosphorylation on some kinetic properties of phosphofructokinase from hepatopancreas of active versus estivating* Otala lactea

| | $K_a$ F2, $6P_2$ (μM) | | Activity Ratio (minus/plus citrate) | |
|---|---|---|---|---|
| | Active | Dormant | Active | Dormant |
| Control | .56, .76 | 1.7, 2.9 | 1.11, 1.17 | 2.75 |
| $Ca^{2+}$ + PMA + Mg ATP | .99 | 1.9, 3.3 | 1.91 | 3.0 |
| cGMP + Mg ATP | 1.09, 1.25 | 1.7, 2.7 | 1.85, 1.97 | 2.21 |
| cAMP + Mg ATP + cAMP protein kinase | 1.4, 1.8 | 2.1, 3.1 | 1.72, 1.78 | 2.89 |
| spermidine | N.A. | .53 | N.A. | 1.0 |

Note. Data show the results of one or two determinations. Values for $K_a$ F2, $6P_2$ were determined at 0.5 mM F6P and 0.3 mM Mg ATP. Activity ratios, without or with 15 mM Mg citrate, were determined at 4 mM F6P and 0.3 mM Mg ATP. PMA is phorbol 12-myristate 13-acetate. N.A. = no data available.

Treatments to promote in vitro dephosphorylation of PFK from hepatopancreas of active and dormant *O. lactea* were generally unsuccessful. The enzyme proved to be very unstable in incubations containing alkaline phosphatase alone or alkaline phosphatase plus acid phosphatase and the polyamine spermidine, a phosphatase stimulator spermidine. Only in one instance (out of several trials) was enzyme activity recovered after a 12-h incubation in the presence of spermidine; in this instance incubation of PFK from hepatopancreas of estivating snails with spermidine resulted in a large decrease in $K_a$ F2,$6P_2$ and eliminated enzyme inhibition by 15 mM Mg citrate.

Attempts to phosphorylate and dephosphorylate *O. lactea* foot muscle PFK in vitro were all unsuccessful. The enzyme proved to be too unstable to retain sufficient measurable activity after the standard 12-h incubation time. Shorter incubation times suffered from the same high loss of enzyme activity plus insufficient time for distinct changes to be made to PFK kinetics by protein kinase or phosphatase action.

## Discussion

The PFK from the tissues of the land snail *Otala lactea* showed a variety of properties common to the enzyme from other animal sources including sigmoidal F6P kinetics at pH 7, inhibition by high concentrations of the substrate ATP, and activation by AMP, $F2,6P_2$, inorganic phosphate, and ammonium ion (Uyeda 1979; Pilkis et al. 1987). As is also common for the enzyme from invertebrate sources, citrate was only a weak inhibitor of *O. lactea* PFK (Newsholme, Sugden, and Williams 1977; Storey 1984). An unusual property of *O. lactea* PFK, however, was the activation of the enzyme by *P*-enolpyruvate. Phosphoenolpyruvate has been reported to inhibit PFK from a number of sources, including the enzyme from muscles of marine molluscs (Ramaiah 1974; Colombo et al. 1975; Storey 1976, 1984). *Otala lactea* PFK was activated, however, by very low concentrations of *P*-enolpyruvate, with $K_a$ values of 30–70 μM. Phosphoenolpyruvate binding to mammalian muscle PFK affects the enzyme by increasing the affinity for Mg ATP at the ATP inhibitory site (Colombo et al. 1975). Neither the molecular mechanism nor a physiological role of *P*-enolpyruvate activation is apparent from our data.

In *O. lactea* organs, PFK was modified in response to either estivation or anoxia. That the modification was a stable alteration of the enzyme protein was shown by the persistence of the modified properties after the removal of low molecular weight effectors of PFK via passage through a G-25 spun column.

Changes in the properties of PFK from all three organs were largely consistent with less active enzyme forms in dormancy or anoxia. Under either condition in the hepatopancreas, affinity for F6P decreased ($S_{0.5}$ F6P increased), sensitivity to $F2,6P_2$ activation decreased ($K_a$ $F2,6P_2$ increased), and significant inhibition by citrate became apparent (tables 3 and 4). These data are consistent with the results from studies of metabolite changes during either long-term estivation (22 d) or long-term anoxia (14 h) (Churchill and Storey 1989). Thus, changes in the levels of substrate (F6P) and product ($F1,6P_2$) of PFK seen at these times, when analyzed via crossover plots, were consistent with an inhibition of PFK in hepatopancreas compared with either the control state or the state after short-term estivation (3 d) or anoxia (2 h) (Churchill and Storey 1989). It appears, then, that the stable modification of PFK properties that is induced during the transition to an arrested metabolic state may help to regulate the concomitant depression of glycolytic flux. In a liver-like organ, a major consequence of this control would be a restriction of the use of the carbohydrate reserves of the organ for biosynthesis while the organism is estivating or anaerobic. In this regard, $F2,6P_2$

effects on hepatopancreas PFK are also very important. In general, $F2,6P_2$ is regarded as mediating a "high glucose" signal that facilitates the use of carbohydrates for anabolic purposes. However, when carbohydrate reserves must be conserved (as in starvation or anoxia), $F2,6P_2$ content is typically rapidly depressed (Hue and Rider 1987). In *O. lactea* not only is the effect of $F2,6P_2$ on PFK much reduced in estivation or anoxia ($K_a$ increases fivefold from 0.7 to 2.3 μM), but $F2,6P_2$ content in hepatopancreas is also dramatically reduced in vivo in these states. Levels of $F2,6P_2$ drop from 0.56 ± 0.2 nmol/g wet weight in hepatopancreas of control *O. lactea* to 0.08 ± 0.02 or 0.045 ± 0.009 nmol/g after 4 or 22 d of estivation and to 0.094 ± 0.011 or 0.079 ± 0.011 nmol/g after 14 or 45 h of anoxia, respectively (S. P. J. Brooks, personal communication). Overall, then, $F2,6P_2$ effects on hepatopancreas PFK would be strongly reduced in the estivating or anoxic states, and again this would favor a restriction of the use of carbohydrate reserves keyed to the basal energy demands of these hypometabolic states.

In mantle the transition to either estivation or anoxia had similar effects on PFK, and these were again consistent overall with a less active form of the enzyme in both cases. Thus, $S_{0.5}$ F6P was increased sixfold and remained significantly elevated compared with the control enzyme, even in the presence of metabolite activators. Mantle PFK also showed increased inhibition by Mg ATP and reduced activation by $F2,6P_2$ (in anoxia only) in the hypometabolic states.

The effects of estivation or anoxia on the properties of PFK in foot muscle were not extensive and, except for the common decrease in $S_{0.5}$ F6P, enzyme properties were affected differently by estivation versus anoxia. After 2 d of anoxia exposure, the changes in PFK properties were generally consistent with a less active enzyme form: citrate inhibition was enhanced ($I_{50}$ decreased), and activation by AMP or $F2,6P_2$ was slightly reduced ($K_a$ values increased). Such a modification could create a less active form of PFK in anoxia, and this agrees well with the data on changes in the levels of glycolytic intermediates during anoxia in foot. These show a short-term (after 2 h) activation of glycolysis during the hypoxia transition period, but, by 14 h of anoxia exposure, changes in F6P levels (an increase) and fructose-1,6-bisphosphate ($F1,6P_2$) levels (a decrease) indicated inhibition of glycolytic flux at the PFK locus (Churchill and Storey 1989). Furthermore, levels of the potent PFK activator, $F2,6P_2$, dropped dramatically in anoxia in foot of this species; $F2,6P_2$ was 0.11 ± 0.01 nmol/g wet weight after 45 h of anoxia compared with control levels of 1.8 ± 0.55 nmol/g (Brooks and Storey 1990*b*). Compared with the $K_a$ values of 2.2–3.8 μM (or nmol/mL), the natural drop in $F2,6P_2$ content in foot clearly results in reduced activity of PFK during prolonged anoxia. The same dramatic depression of $F2,6P_2$ con-

tent also occurs in response to anoxia in tissues of marine molluscs (Storey 1985, 1988*b*).

In estivation, however, the kinetic changes in foot muscle PFK seem to indicate a somewhat more active form of the enzyme after 22 d of estivation; $S_{0.5}$ F6P, $K_a$ AMP, and $K_a$ F2,6$P_2$ were all significantly reduced compared with the control enzyme (table 1). The physiological consequences of such a PFK modification are not yet apparent. When glycolytic control was assessed by monitoring changes in the levels of glycolytic metabolites during long-term estivation, the crossover plot analysis also gave conflicting results (Churchill and Storey 1989). The data suggested a relative activation of PFK (F6P decreased, F1,6$P_2$ increased) after 22 d of estivation compared with aroused, resting control animals, but the plots also indicated concomitant inhibition of glycolysis at the aldolase and PK reactions (Churchill and Storey 1989). Which of these sites takes precedence in regulating overall glycolytic flux in estivation has yet to be established. However, in a parallel study, we found that estivation-induced phosphorylation of PK in foot muscle clearly produced a less active form of this enzyme (Whitwam and Storey 1991*b*). Furthermore, F2,6$P_2$ levels are strongly reduced in foot muscle of estivating snails (by 11-fold to 0.16 ± 0.01 nmol/g after 22 d), and this could produce a significant decrease in PFK activity and in the anabolic use of carbohydrate in foot muscle during estivation. Further studies will be necessary to resolve this conflict.

The molecular basis of the stable modification of PFK in estivation or anoxia in *O. lactea* organs is probably protein phosphorylation. The data in table 4 are quite conclusive for hepatopancreas PFK. Thus, incubation of the enzyme from control animals under conditions promoting protein kinase action increased $K_a$ F2,6$P_2$ and increased inhibition by citrate just as the natural transition from the aroused to the estivating state did. Protein kinase action did not affect the enzyme from estivating snails, but incubation with the phosphatase stimulator, spermidine, reconverted the properties of PFK from estivating snails to those similar to the control enzyme. By analogy, these effects of protein kinases versus protein phosphatases probably extend to the foot or mantle enzymes as well. Indeed, studies of both PFK and PK in *Busycotypus canaliculatum* organs have shown that in vitro conditions that promote enzyme phosphorylation mimic the action of anoxia in converting both enzymes to less active forms (Storey 1984; Whitwam and Storey 1990, 1991*a*). The same also occurred for PK from foot, mantle, and hepatopancreas of *O. lactea.* Protein kinase treatments mimicked the effects of either estivation or anoxia on PK whereas conditions promoting phosphatase action had the opposite effect, reconverting the anoxic or estivating

enzyme form to one with properties similar to the control enzyme (Whitwam and Storey 1991*b*).

The particular intracellular protein kinase responsible for the modification of PFK in vivo under either estivation or anoxia is not apparent from the present results. Hepatopancreas PFK from aroused snails was altered by incubation with ATP and $Mg^{2+}$ and the stimulators of protein kinase C ($Ca^{2+}$ + phorbol 12-myristate 13-acetate), cGMP-dependent protein kinase, or cAMP plus cAMP-dependent protein kinase. These results suggest, in fact, the presence of a second messenger-independent protein kinase. The PFK from *B. canaliculatum* also responded similarly to all three protein kinase treatments (Whitwam and Storey 1991*a*), as did *O. lactea* PK (Whitwam and Storey 1991*b*). Anoxia-induced phosphorylation of *B. canaliculatum* PK, however, was specifically linked to the action of an endogenous cGMP-dependent protein kinase (Brooks and Storey 1990*a*).

In summary, then, the present data on PFK as well as our previous analysis of PK (Whitwam and Storey 1991*b*) in *O. lactea* indicate that stable modifications to enzyme properties, brought about by reversible protein phosphorylation, are an integral part of glycolytic rate control during the transition to an arrested metabolic state. This mechanism of enzyme regulation is expressed during both estivation and anaerobiosis, and this indicates that a common principle of metabolic control can be conserved to create the same glycolytic rate response to different environmental signals.

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