Organ-specific regulation of phosphofructokinase during facultative anaerobiosis in the marine whelk *Busycotypus canaliculatum*

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The kinetic properties of 6-phosphofructo-1-kinase (PFK) were assessed in five organs (ventricle, radular retractor muscle, gill, hepatopancreas, and kidney) of aerobic and anoxic (21 h in N_2-bubbled seawater) whelks, *Busycotypus canaliculatum*. The enzyme in all organs showed a stable modification of kinetic parameters as a result of exposure of the animal to anoxic conditions. In most cases these changes were consistent with the conversion of the enzyme to a less active form in the anoxic organ. In ventricle, for example, the anoxic enzyme form showed significant changes, including a 36% increase in the value of the substrate affinity constant ($S_{0.5}$) for Mg · ATP, a 19% increase in $S_{0.5}$ fructose-6-phosphate, a 57% increase in the 50% inhibition value (I_{50}) for phosphoenolpyruvate, a 30% increase in I_{50} citrate, and a fivefold increase in the activator constant (K_a) for fructose-2,6-bisphosphate, as compared with the aerobic enzyme. Analysis of the time course of anoxia-induced modification of PFK showed that changes to the properties of gill PFK were accomplished within 2 h of the exposure to N_2-bubbled seawater, whereas changes to ventricle PFK required up to 8 h. In vitro incubation of ventricle homogenates with Mg · ATP plus protein kinase second messengers or with Mg^{2+} plus added protein phosphatases showed that the aerobic enzyme form was modified by protein kinase action with an increase in K_a fructose-2,6-bisphosphate that mimicked the effect of the aerobic to anoxic transition on the enzyme. Phosphatase action on the anoxic enzyme form had the opposite effect. The data suggest that the modification of PFK properties under anoxia in whelk organs is due to protein phosphorylation of the enzyme. Such covalent modification of PFK and other enzymes, notably pyruvate kinase, coordinates the anoxia-induced glycolytic rate depression and overall metabolic arrest that is a prominent feature of facultative anaerobiosis in marine molluscs.

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Les propriétés cinétiques de la 6-phosphofructo-1-kinase (PFK) ont été mesurées dans cinq organes (ventricule, muscle rétracteur de la radula, branchie, hépatopancréas et rein) de buccins *Busycotypus canaliculatum* en conditions aérobiques et anoxiques (21 h dans de l'eau de mer où barbotait du N_2). Dans tous les organes, l'enzyme opère une modification stable des paramètres cinétiques à la suite de l'exposition de l'animal à l'anoxie. Dans la plupart des cas, ces changements correspondent à la conversion de l'enzyme en une forme moins active dans l'organe anoxique. Dans le ventricule, par exemple, la forme anoxique de l'enzyme montre des changements importants, notamment une augmentation de 36% de la constante d'affinité du substrat ($S_{0.5}$) du Mg · ATP, une augmentation de 19% du $S_{0.5}$ du fructose-6-phosphate, une augmentation de 57% de la valeur d'inhibition à 50% (I_{50}) du phosphoénolpyruvate, une augmentation de 30% de la I_{50} du citrate, et une multiplication par 5 de la constante d'activation (K_a) du fructose-2,6-biphosphate par rapport à l'enzyme aérobique. L'analyse du déroulement des modifications de la PFK provoquées par l'anoxie a démontré que les changements des propriétés de l'enzyme dans la branchie avaient lieu en moins de 2 h après l'exposition à l'eau de mer traitée au N_2, alors que les changements dans le ventricule nécessitaient jusqu'à 8 h. L'incubation in vitro d'homogénats de ventricule dans du Mg · ATP additionné de messagers secondaires de la protéine kinase ou de Mg^{2+} additionné de protéine phosphatases a démontré que la forme aérobique de l'enzyme était modifiée sous l'action de la protéine kinase avec augmentation de la K_a du fructose-2,6-biphosphate, imitant l'effet de la transition de l'enzyme aérobique en enzyme anoxique. L'action de la phosphatase sur la forme anoxique de l'enzyme avait l'effet opposé. Les données indiquent que la modification des propriétés de la PFK dans des organes de buccins en conditions d'anoxie est due à la phosphorylation des protéines de l'enzyme. Une telle modification covalente de la PFK et d'autres enzymes, notamment la pyruvate kinase, contrôle la chute du taux de glycolyse à la suite de l'anoxie et l'arrêt métabolique général, caractéristiques importantes de l'anaérobiose facultative chez les mollusques marins.

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Introduction

Many marine molluscs are excellent facultative anaerobes. One of the keys to long-term survival without oxygen is metabolic rate depression, the ability to lower metabolic rate while anoxic by as much as 10- to 20-fold, compared with the resting normoxic rate (Famme *et al.* 1981; Shick *et al.* 1983; Hochachka and Guppy 1987; Storey 1985, 1988*a*). From this the animal gains a comparable extension of the time that fermentative metabolism can support anaerobiosis. Central to metabolic depression is control over glycolytic rate, because strict regulation of ATP production must be a part of the coordinated reduction of ATP turnover that defines the hypometabolic state. In recent years, the molecular mechanisms that regulate metabolic rate depression have begun to be analyzed. Common features of metabolic arrest recur in a variety of anoxia-tolerant animals (including marine molluscs, freshwater turtles, and goldfish) as well as in species that undergo other forms of facultative metabolic arrest (e.g., hibernation, estivation) (Storey 1988*a*; Storey and Storey 1990). One of these mechanisms is the posttranslational modification of regulatory enzymes via protein phosphorylation or dephosphorylation reactions. Covalent modification frequently causes major changes to the kinetic or regulatory properties of enzymes; the functional result of this can be a change in pathway flux or a change in the direction of carbon flow at a branchpoint. Anoxia-induced covalent modification of pyruvate kinase in marine molluscs is particularly well described. Phosphorylation of the enzyme produces major changes in kinetic and regulatory properties that virtually inactivate the enzyme during long term anoxia (Holwerda *et al.* 1983; Plaxton and Storey 1984*a*, 1984*b*, 1985*a*; Hakim *et al.* 1985; Michaelidis *et al.* 1988; Whitwam and Storey 1990).

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6-Phosphofructo-1-kinase (PFK) is an important regulatory enzyme in glycolysis that frequently appears to catalyze the rate-limiting reaction of the pathway (Uyeda 1979). The enzyme is responsive to numerous regulatory mechanisms, including allosteric control by a wide variety of metabolite effectors, aggregation–disaggregation of subunits and of enzyme polymers, pH effects on enzyme kinetics and polymeric state, and reversible protein phosphorylation (Ramaiah 1974; Uyeda 1979; Pilkis *et al.* 1987). In mammals the effects of protein phosphorylation on PFK are organ specific; for example, glucagon-stimulated phosphorylation of liver PFK produces a less active enzyme form, whereas epinephrine-stimulated phosphorylation activates heart PFK (Sakakibara and Uyeda 1983; Narabayashi *et al.* 1985). Phosphorylation of skeletal muscle PFK by Ca^{2+}-activated, phospholipid-dependent protein kinase C significantly altered enzyme properties (Hofer *et al.* 1985), but phosphorylation by cAMP-dependent protein kinase produced only small changes in enzyme properties (Foe and Kemp 1982). Phosphorylation of skeletal muscle PFK appears to have a major influence, however, on the binding association between PFK and F-actin (Luther and Lee 1986). Among anoxia-tolerant animals we have recently demonstrated that stable modifications of the properties of PFK producing less active enzyme forms are part of the aerobic to anaerobic transition in foot muscle of the marine whelk, brain of the freshwater turtle, and five organs of the goldfish (Storey 1984; Brooks and Storey 1988; Rahman and Storey 1988).

The present study more closely examines the regulation of PFK in five organs of the anoxia-tolerant marine whelk *Busycotypus canaliculatum*, analyzing organ-specific differences in the kinetic and regulatory properties of the enzyme, in the effects of anoxia on the enzyme, and in the time course of enzyme modification during anaerobiosis. Furthermore, in vitro incubations of PFK under conditions that stimulate protein kinase versus protein phosphatase action suggest that the aerobic versus anoxic forms of the enzyme are the low phosphate versus high phosphate forms of PFK, respectively.

Materials and methods

Chemicals and animals

All biochemicals and coupling enzymes were obtained from Sigma Chemical Co., St. Louis, MO, or Boehringer Mannheim, Montreal, Que. Specimens of the channelled whelk *Busycotypus canaliculatum* were obtained from the Marine Biological Laboratory in Woods Hole, MA. They were held until use in recirculating aerated seawater (1000 mOsmol) at 18°C. Aerobic whelks were sampled directly from the tank. Tissues were quickly dissected out, immediately frozen in liquid N_2, and then transferred to −60°C for storage. To impose anoxia, whelks were placed in large sealed tubs of seawater (bathed in 18°C water for temperature control) that had been previously bubbled with N_2 gas for 12 h; N_2 gassing was continued throughout the anoxia exposure. Tissues were then sampled as described above. The standard length of anoxia exposure was 21 h. To follow the time course of changes in PFK kinetic parameters, however, equivalent experiments were carried out but with sampling after a variety of shorter anoxia exposures.

Enzyme preparation

Tissue samples were homogenized in 50 mM imidazole-HCl buffer (pH 7.0) containing 100 mM NaF, 5 mM EDTA, 5 mM EGTA, 15 mM 2-mercaptoethanol, and 0.1 mM phenylmethylsulfonyl fluoride, using a Polytron PT-10 homogenizer. Weight to volume ratios were 1:4 for radula retractor muscle and ventricle, 1:3 for kidney and gill, and 1:2 for hepatopancreas. Polyethylene glycol 8000 (PEG) was added to the homogenates to a final 4% (w/v) for radula retractor muscle and ventricle homogenates, and 1% (w/v) for gill, kidney, and hepatopancreas homogenates. After stirring for 10–15 min at 5°C, homogenates were centrifuged at 25 000 × *g* for 30 min at 5°C. Supernatant fractions were saved and further PEG was added to raise the concentration to 8% in radula retractor muscle and ventricle extracts, and to 6% in gill, kidney, and hepatopancreas. After further stirring, extracts were again centrifuged as above. Supernatant fractions were discarded and the resulting pellets, containing PFK, were resuspended in a volume of homogenization buffer equivalent to that used originally for homogenization. This procedure was used to eliminate high background blank problems in the assay when the crude supernatant was used and to remove low molecular weight effectors, since the enzyme was unstable to dialysis.

Enzyme assay

PFK was assayed by monitoring NADH utilization at 340 nm on a Gilford-240 recording spectrophotometer. Standard assay conditions were 50 mM imidazole-HCl buffer (pH 7.0), 50 mM KCl, 5 mM $MgCl_2$, 0.2 mM NADH, 0.2 U/mL aldolase, 2.5 U/mL glycerol-3-phosphate dehydrogenase, 0.4 U/mL triosephosphate isomerase (all coupling enzymes previously dialyzed), and fructose-6-phosphate (F6P) and Mg · ATP substrates as indicated. Final volume was 1 mL and assays were initiated by the addition of enzyme preparation. Substrate affinity constant ($S_{0.5}$) values for substrates were estimated by fitting the data to the Hill equation using a nonlinear least squares regression program. A modified Hill equation which included a V_0 term (rate at zero activator) was used for determining K_a values. I_{50} values were obtained as described by Job *et al.* (1978). Analysis of all three parameters uses a computer software package developed by S.P.J. Brooks. Tests for significant differences between results were done using Student's *t*-test.

In vitro phosphorylations or dephosphorylations

Samples of whelk ventricle were homogenized 1:3 (w/v) in buffer containing 50 mM imidazole-HCl (pH 7.0), 100 mM NaF, 5 mM EDTA, 5 mM EGTA, 15 mM 2-mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride, 20% glycerol, and 1 mM ATP (low ATP in all steps was needed to stabilize PFK). The homogenate was centrifuged at 20 000 × *g* for 20 min at 5°C. The resulting supernatant was loaded onto a 6-mL column of Sephadex G-25-80 equilibrated in 40 mM imidazole-HCl (pH 7.0), 0.5 mM EDTA, 10 mM potassium phosphate, 1 mM ATP, and 20% glycerol. The column was centrifuged for 1 min at top speed in an IEC benchtop centrifuge and the desalted filtrate was collected. The filtrate was then divided into aliquots and each aliquot was diluted 1:1 with a solution containing 40 mM imidazole-HCl (pH 7.0), 10 mM 2-mercaptoethanol, 20% (v/v) glycerol, and one of the following:

A) 20 mM $MgCl_2$, 40 mM NaF, 2 mM $CaCl_2$, 20 μg/mL phorbol 12-myristate 13-acetate (PMA), and 10 mM ATP (high ATP provides substrate for protein kinase);

B) 20 mM $MgCl_2$, 40 mM NaF, 8 mM cGMP, and 10 mM ATP;

C) 20 mM $MgCl_2$, 40 mM NaF, 4 mM cAMP, 2 mg/mL cAMP-dependent protein kinase (bovine heart, approx. 2 pM/μg protein of phosphorylating activity), and 10 mM ATP;

D) 25 mM KCl, 10 mM $MgCl_2$, 1 mM ATP, and 50 IU alkaline phosphatase (grade 1, calf intestine);

E) 25 mM KCl, 10 mM $MgCl_2$, 1 mM ATP, 50 IU alkaline phosphatase, 2 mg/mL acid phosphatase (type 1, from wheat germ, 0.46 U/mg), and 1 mg/mL spermidine;

F) 25 mM KCl, 10 mM $MgCl_2$, 1 mM ATP, 1 mg/mL spermidine;

G) 40 mM NaF, 5 mM EDTA, and 1 mM ATP. This was the control and is minus Mg^{2+}, the cation needed for both kinase and phosphatase reactions.

Samples were incubated at 22°C for 12 h; shorter incubations, 2 or 6 h, showed the expected trend, but the longer incubation provided statistically significant changes. After incubation, low molecular weight metabolites were removed by passage through 5-mL spun columns equilibrated in 40 mM imidazole-HCl (pH 7.0), 0.5 mM EDTA, 1 mM ATP, 10 mM 2-mercaptoethanol, and 20% glycerol. Filtrates were than assayed for PFK activity.

TABLE 1. Kinetic properties of PFK isolated from ventricle and radular retractor muscle of aerobic and anoxic (21 h) whelks

	Ventricle		Radular retractor muscle	
	Aerobic	Anoxic	Aerobic	Anoxic
$S_{0.5}$ F6P (mM)	0.36±0.01	0.43±0.02*	0.37±0.01	0.28±0.02*
n_H	2.8±0.3	3.1±0.3	2.2±0.2	1.7±0.1
$S_{0.5}$ Mg·ATP (μM)	22.0±2.0	30.0±2.0*	37.0±2.0	28.0±3.0*
n_H	1.4±0.1	1.5±0.1	1.3±0.1	1.4±0.1
I_{50} Mg·ATP (mM)	1.25±0.07	1.44±0.06	2.2±0.2	2.2±0.1
I_{50} PEP (mM)	1.4±0.2	2.2±0.2*	2.7±0.4	3.6±0.3
I_{50} Citrate (mM)	23.0±3.0	30.0±1.0*	24.0±2.0	17.0±2.0
K_a AMP (μM)	3.3±0.3	3.5±0.6	6.8±0.3	6.1±0.4
K_a F2,6P_2 (μM)	0.42±0.02	2.2±0.2**	0.64±0.06	0.67±0.06

NOTE: Data are means ± SEM of determinations on samples from n = 3–6 different animals. Cosubstrate concentrations were 2 mM F6P for $S_{0.5}$ Mg·ATP and 0.2 mM Mg·ATP for $S_{0.5}$ F6P. I_{50} Mg·ATP was determined at 0.8 mM F6P and other I_{50} values were determined at 0.8 mM F6P and 0.2 mM Mg·ATP. K_a AMP and K_a F2,6P_2 were determined at 0.4 mM F6P and 0.2 mM Mg·ATP. Mg·ATP and Mg·citrate were prepared with $MgCl_2$ in 1:1 or 2:1 mixtures, respectively. *, Significantly different from the corresponding value for the aerobic enzyme form at $P < 0.05$; **, $P < 0.005$.

TABLE 2. Kinetic properties of PFK from hepatopancreas and gill of aerobic and anoxic whelks

	Hepatopancreas		Gill	
	Aerobic	Anoxic	Aerobic	Anoxic
$S_{0.5}$ F6P (mM)	0.47±0.03	0.30±0.02*	0.28±0.02	0.35±0.02*
n_H	1.5±0.1	1.2±0.1	1.5±0.1	1.2±0.1
$S_{0.5}$ Mg·ATP (μM)	53.0±6.0	45.0±3.0	23.0±1.0	23.0±2.0
n_H	1.3±0.1	1.7±0.1	1.3±0.1	1.0±0.1
I_{50} Mg·ATP (mM)	6.7±0.3	6.4±0.4	3.9±0.2	1.9±0.1*
I_{50} PEP (mM)	7.6±0.7	7.8±0.7	9.4±0.9	3.3±0.2**
I_{50} Citrate (mM)	10.1±0.4	18.4±0.6**	>20.0	>20.0

NOTE: Data are means ± SEM of determinations on samples from n = 3–6 different animals. Cosubstrate concentrations were 3 mM F6P for $S_{0.5}$ Mg·ATP and 0.3 mM Mg·ATP for $S_{0.5}$ F6P. I_{50} Mg·ATP was determined at 1 mM F6P for hepatopancreas and 0.5 mM F6P for gill PFK; other I_{50} values were determined at 1 mM F6P and 0.3 mM Mg·ATP. *, Significantly different from the corresponding aerobic control value at $P < 0.05$; **, $P < 0.005$.

Results

The properties of PFK were assessed in five organs of the whelk *B. canaliculatum*: ventricle and radular retractor muscle (Table 1), hepatopancreas and gill (Table 2), and kidney (Table 3). Some distinct differences in enzyme properties were found between organs. Substrate affinity for ATP was high in all organs. ($S_{0.5}$ = 22–53 μM), but F6P kinetics were more variable. Affinity for F6P was very high in kidney ($S_{0.5} \approx 0.1$ mM) and somewhat lower in other organs, whereas F6P saturation curves were strongly sigmoidal in muscles and kidney (n_H = 2.2–2.8 for the aerobic form) but less so in other tissues (n_H = 1.5 in hepatopancreas and gill). The enzyme in all organs was inhibited by higher levels of the substrate ATP; in the muscles and kidney inhibition was strong (I_{50} = 1.2–2.2 mM), but hepatopancreas and gill showed less ATP inhibition. Whelk PFK was also inhibited by phosphoenolpyruvate (PEP) (more strongly in muscles, weakly in soft tissues) and weak inhibition by citrate was found in muscles and hepatopancreas. AMP and fructose 2,6-bisphosphate (F2,6P_2) were strong activators of PFK in ventricle, radular retractor, and kidney. Overall, the properties of ventricle and radular retractor muscle were fairly similar, as were those of hepatopancreas and gill, but the properties of kidney PFK were quite distinctly different from either of these two groups. This suggest the probable presence of at least three organ-specific isozymic forms of PFK in the whelk, similar to the situation in vertebrates (Pilkis *et al.* 1987).

Exposure to anoxia resulted in a modification of the properties of PFK in all five organs of the whelk; this modification was stable after PEG fractionation, indicating that anoxia caused an alteration to the enzyme protein rather than a change in the levels of low molecular weight effectors of the enzyme. In ventricle, 21 h of anoxia exposure produced statistically significant increases in the $S_{0.5}$ values for ATP and F6P, I_{50} values for PEP and citrate, and the K_a for F2,6P_2, the five-fold increase in K_a being the most substantial change in the properties of the ventricle enzyme in anoxia (Table 1). In radular retractor muscle, however, enzyme properties changed only slightly in anoxia; $S_{0.5}$ values for both substrates decreased by 25% in anoxic muscle (Table 1).

In gill and hepatopancreas, 21 h of anoxia exposure produced a significant change in $S_{0.5}$ F6P but did not affect ATP affinity (Table 2). Inhibitor effects changed significantly in anoxia. In hepatopancreas, I_{50} citrate increased by 82%, whereas in gill I_{50} values for Mg·ATP and PEP decreased by 50 and 65%, respectively. The effect of anoxia exposure on the properties of kidney PFK were limited to changes in the I_{50} values for Mg·ATP and

TABLE 3. Kinetic properties of PFK from kidney of aerobic and anoxic whelks

	Aerobic	Anoxic
$S_{0.5}$ F6P (mM)	0.092±0.003	0.108±0.004
n_H	2.13±0.21	2.71±0.08**
$S_{0.5}$ Mg·ATP (μM)	25.0±1.0	22.0±1.0
n_H	1.3±0.07	1.4±0.1
I_{50} Mg·ATP (mM)	1.16±0.05	0.83±0.03*
I_{50} PEP (mM)	12.5±1.0	6.5±0.5*
I_{50} Citrate (mM)	>20.0	>20.0
K_a AMP (μM)	33±3	29±2
K_a F2,6P_2 (μM)	1.6±0.3	1.5±0.3

NOTE: Data are means ± SEM of determinations on tissue from n = 3–6 different animals. Cosubstrate concentrations were 2 mM F6P for $S_{0.5}$ Mg·ATP and 0.2 mM Mg·ATP for $S_{0.5}$ F6P. I_{50} Mg·ATP was determined at 0.1 mM F6P; other I_{50} values were determined at 1 mM F6P and 0.3 mM Mg·ATP. K_a values were determined at 0.5 mM F6P and 0.3 mM Mg·ATP. *, Significantly different from the corresponding aerobic value at $P < 0.005$; **, $P < 0.025$.

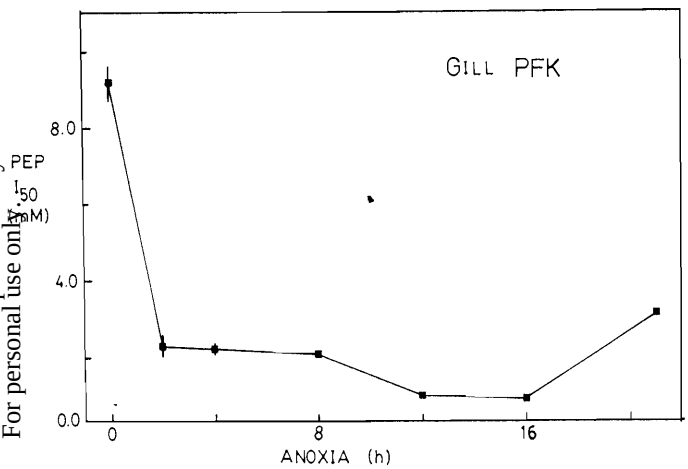

FIG. 1. Time course of the change in I_{50} PEP of PFK from *B. canaliculatum* gill during anoxia exposure. Data points are means ± SEM for gill PFK from n = 3 different animals; where error bars are not shown, these are within the dimensions of the symbol used. I_{50} PEP was measured at 1 mM F6P and 0.3 mM Mg·ATP.

citrate, I_{50} Mg·ATP decreasing by 28% and I_{50} citrate decreasing by 50% (Table 3).

Figures 1 and 2 show time courses of the changes in kinetic properties of PFK during anoxia, following K_a F2,6P_2 for ventricle PFK and I_{50} PEP for gill PFK, respectively. In gill, the change in I_{50} PEP was very rapid, dropping more than fourfold within the first 2 h of exposure of the animal to anoxic seawater. Over the subsequent hours of anoxia, the I_{50} was largely unchanged. Changes to the K_a F2,6P_2 of ventricle PFK, on the other hand, were slower. After 2 h of anoxia, the K_a had risen significantly, but the maximal effect was not seen until 6 h of anoxia.

The molecular basis of anoxia-induced changes in the kinetic properties of pyruvate kinase in whelks has proven to be protein phosphorylation (Plaxton and Storey 1984*a*, 1984*b*; Brooks and Storey 1989, 1990). To assess whether the same mechanism was responsible for the changes in PFK kinetic properties, we incubated PFK from aerobic versus anoxic ventricle with agents that promote protein phosphorylation or dephosphorylation. Data in Table 1 indicated that the change in K_a F2,6P_2 was the most dramatic alteration induced by anoxia and so the effect of

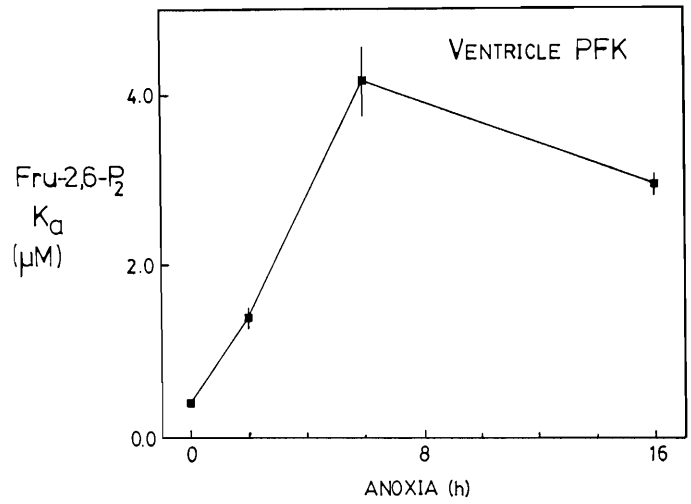

FIG. 2. Time course of the change in K_a F2,6P_2 for PFK from *B. canaliculatum* ventricle during anoxia exposure. Data points are means ± SEM for ventricle PFK from n = 3 different animals; where error bars are not shown, these are within the dimensions of the symbol used. K_a F2,6P_2 was measured at 0.4 mM F6P and 0.2 mM Mg·ATP.

TABLE 4. Effect of in vitro incubations with agents that stimulate protein phosphorylation or dephosphorylation on PFK from *B. canaliculatum* ventricle

	K_a F2,6P_2 (μM)	
	Aerobic PFK	Anoxic PFK
Control	3.6±0.5	4.5±0.4
+ Ca^{2+} + PMA + Mg·ATP	6.2±0.6*	3.9±0.6
+ cGMP + Mg·ATP	5.7±0.1*	3.4±0.4
+ cAMP + Mg·ATP + cAMP protein kinase	5.6±0.1*	3.0±0.2*
+ alkaline phosphatase	1.3±0.2*	1.5±0.4*
+ alkaline and acid phosphatases + spermidine	nd	2.2±0.4*

NOTE: Data are means ± SEM of two determinations. K_a values were determined at 0.4 mM F6P and 0.2 mM Mg·ATP. Control incubations contained ATP, EDTA, and NaF but no Mg^{2+}. PMA is phorbol 12-myristate 13-acetate; spermidine is a phosphatase activator. *, Significantly different from the corresponding control incubation at $P < 0.05$; nd, not determined.

in vitro incubation on this property was assessed. As Table 4 shows, treatment of the aerobic enzyme form with Mg·ATP plus protein kinase second messengers significantly increased the K_a value, the same qualitative change as occurred with the aerobic to anoxia transition. The response was virtually the same when stimulators of protein kinase C (Ca^{2+} + PMA) or of cGMP-dependent protein kinase were used, or when the enzyme was incubated with cAMP + added cAMP-dependent protein kinase. Treatments that stimulated protein kinase action did not, however, affect PFK from anoxic ventricle, except that treatment with cAMP-dependent protein kinase resulted in a significant decrease in the K_a value, an effect opposite to the action of the enzyme on aerobic PFK. Contrary to the effects of protein kinases, treatment with alkaline phosphatase reduced the K_a value for F2,6P_2, affecting the enzyme from both aerobic and anoxic organs. Treatment with a mixture of alkaline and acid phosphatases plus the phosphatase stimulator spermidine also significantly lowered K_a F2,6P_2 of the anoxic enzyme form. The action of phosphatase on aerobic PFK indicated that the enzyme was partially phosphorylated in this state.

Discussion

PFK from whelk organs show a variety of properties characteristic of the enzyme from most sources, including sigmoidal F6P kinetics at pH 7, substrate inhibition by higher levels of ATP, sensitivity to AMP and $F2,6P_2$ as activators, and inhibition by PEP and citrate (Pilkis *et al.* 1987). Inhibition by citrate was weak and likely of little or no physiological relevance; this is proving to be typical of PFK from invertebrate sources as compared with the mammalian enzyme, which is much more sensitive to citrate (Newsholme *et al.* 1977; Storey 1976, 1984; Ebberink 1982). Inhibition by PEP was also very weak in the soft organs (I_{50} = 7–12 mM) but less so in the muscles (Table 1; Storey 1984). However, the physiological relevance of PEP effects are debatable, since PEP content in vivo is low compared with I_{50} values for PFK; over the aerobic-anoxic transition in radular retractor muscle, for example, PEP levels were 0.15 μmol/g wet weight or less (Storey *et al.* 1990), whereas I_{50} PEP values for PFK were 2.7–3.6 mM in the same muscle.

PFK from the whelk showed both organ-specific differences in kinetic and regulatory properties as well as organ-specific alterations to kinetic properties as a result of anoxia exposure. This indicates the probable presence of organ-specific isozymic forms of PFK as are known to occur in mammals (Pilkis *et al.* 1987). In general, there were distinct differences between PFK from red muscles (ventricle and radular retractor) versus two soft tissues (hepatopancreas, gill) of the whelk; these included larger Hill coefficients for F6P substrate affinity, substantially lower K_a values for activators, and lower I_{50} values for PEP. Kidney PFK showed properties distinctly different from both of these groups. The characteristics of the muscle forms are also similar to those previously reported for whelk foot muscle PFK (Storey 1984). For a muscle enzyme, the sigmoidicity of F6P binding plus the high sensitivity to AMP activation are features that would favour a rapid increase in enzyme activity under conditions of increased muscle work load.

The transition from aerobic to anoxia function resulted in significant alterations to the properties of PFK in all five organs. A similar effect of anoxia on various properties of whelk foot PFK has also been reported (Storey 1984). It appears, then, that anoxia results in a modification of the properties of this rate-limiting enzyme of glycolysis in all organs of the whelk. The quantitative effects of anoxia-induced modifications of PFK were greatest in ventricle, affecting five of the properties measured and including a fivefold increase in the K_a for $F2,6P_2$. In the other organs anoxia resulted in significant changes to two or three of the measured kinetic parameters. In ventricle, gill, and kidney, the anoxia-induced alterations were clearly consistent with the conversion of the enzyme to a less active form in anoxia; compared with the corresponding aerobic enzyme form, anoxic PFK showed changes such as decreased substrate affinity ($S_{0.5}$ values increased), enhanced effects of inhibitors (I_{50} values decreased), and reduced effects of activators (K_a $F2,6P_2$ increased). In radular retractor muscle and hepatopancreas, anoxia-induced changes were of an opposite nature: an increase in affinity for both substrates for radular retractor PFK, and an increase in F6P affinity and decrease in citrate inhibition for hepatopancreas PFK. Nonetheless, the data clearly show that PFK undergoes a stable modification in all organs in anoxia.

Furthermore, as Figs. 1 and 2 show, the progress of PFK modification occurs with distinct time courses in different organs. In gill, the change in enzyme properties is complete within 2 h from the start of anoxia exposure, whereas the modification of ventricle PFK is partial after 2 h and complete after 8 h of anoxia exposure. Organ-specific differences in the process of anoxia-induced enzyme modification were similarly noted for pyruvate kinase in the whelk (Whitwam and Storey 1990); interestingly, in that study the half-times for the change in kinetic parameters of muscle forms of pyruvate kinase (ventricle and foot) were also longer (by two to nine times) than the corresponding values for pyruvate kinase modification in soft tissues (hepatopancreas, kidney). It appears, therefore, that the processes involved in anoxia-induced modification of glycolytic enzymes are more rapidly initiated in non muscular versus muscular tissues. However, other factors may also affect the control of PFK during anoxia in vivo. Key among these are changes in the concentration of the potent PFK activator, $F2,6P_2$, which drops rapidly in anoxia (Storey 1988*b*). In ventricle, for example, content of $F2,6P_2$ drops by 224-fold (from 1.23 to 0.005 μM) with a half-time of only 35 min. This would have a profound effect on PFK activity in vivo well ahead of the subsequent anoxia-induced modification of enzyme properties. Since $F2,6P_2$ action on PFK mediates signals that promote the use of carbohydrate reserves for anabolic purposes, the rapid drop in $F2,6P_2$ content in anoxia helps to limit the use of carbohydrate reserves to fermentative ATP production alone. Glycolytic rate becomes largely responsive, therefore, to cellular energy status and this is mediated primarily through ATP and AMP control over PFK.

The molecular mechanism of the stable modification of PFK during anoxia in whelk organs appears to be enzyme phosphorylation. Analysis of the effects of protein kinases versus protein phosphatases on PFK from aerobic versus anoxic ventricle (Table 4) showed that agents that promoted protein phosphorylation affected the aerobic enzyme form and mimicked the increase in K_a $F2,6P_2$ seen with the aerobic to anoxic transition. The anoxic enzyme form, however, showed little response to protein kinase action, but phosphatases reduced the K_a $F2,6P_2$ of the anoxic enzyme as would occur in the anoxic to aerobic transition in vivo. Treatment with alkaline phosphatase had a similar effect on the properties of the anoxic enzyme form of PFK in whelk foot muscle, decreasing the K_a $F2,6P_2$ from 6.2 to 1.2 μM (Storey 1984). It appears, then, that the aerobic versus anoxic forms of PFK in whelk organs are the low-phosphate versus high-phosphate forms of the enzyme, respectively, and that anoxia exposure stimulates the action of an endogenous protein kinase(s) that phosphorylates PFK. The data in Table 4 show ample evidence of the presence in ventricle homogenates of a protein kinase capable of phosphorylating PFK when incubated with added Mg^{2+} and ATP. However, the natural second messenger associated this PFK-phosphorylating action was not discernible, since there was no significant difference in the response by PFK to incubation with Ca^{2+} + PMA (stimulators of protein kinase C), cGMP, or cAMP + mammalian cAMP-dependent protein kinase. Indeed, the present data could be indicative of the action of a second messenger independent protein kinase. For pyruvate kinase, however, recent studies have clearly linked anoxia-induced phosphorylation of the enzyme in whelk organs to the action of a cGMP-dependent protein kinase (Brooks and Storey 1989, 1990). Further studies will be needed to determine if the same protein kinase coordinates the anoxia-induced phosphorylation of PFK and of other enzymes (e.g., glycogen phosphorylase, 6-phosphofructo-2-kinase) whose activities are modified in response to anoxia stress in the whelk (Storey 1988*b*; L. Bosca and K. Storey, unpublished data).

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