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PHOSPHOFRUCTOKINASE FROM THE ANTERIOR BYSSUS RETRACTOR MUSCLE OF *MYTILUS EDULIS*: MODIFICATION OF THE ENZYME IN ANOXIA AND BY ENDOGENOUS PROTEIN KINASES

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Abstract—1. Anoxia exposure resulted in a stable modification of the kinetic properties of 6-phosphofructo-1-kinase (PFK) from the anterior byssus retractor muscle (ABRM) of the sea mussel *Mytilus edulis* L.

2. Compared to the aerobic enzyme, the anoxic form of PFK showed a reduced affinity for both substrates, fructose-6-phosphate (F6P) and ATP, and an increased sensitivity to inhibition by phosphoenolpyruvate.

3. To analyze the involvement of protein kinases in the modification of PFK, extracts from aerobic or anoxic muscle were incubated with ATP and Mg^{2+} plus protein kinase second messengers cyclic 3′,5′-adenosine monophosphate (cAMP), cyclic 3′,5′-guanosine monophosphate (cGMP) or Ca^{2+} plus phorbol 12-myristate 13-acetate (PMA).

4. Both forms of the enzyme responded to the presence of cAMP with a strong increase in affinity for F6P.

5. In response to cGMP affinity of the aerobic enzyme for F6P decreased whereas that of the anoxic enzyme form was not affected (at 0.5 mM ATP) or increased (at 3 mM ATP).

6. Incubation with Ca^{2+} + PMA had only a limited effect on PFK kinetics but appeared to enhance the response to cGMP when the three compounds were given together.

7. Treatment of PFK-aerobic with alkaline phosphatase resulted in a strong decrease in enzyme activity and affinity for F6P; subsequent treatment with cAMP reversed the effect on $S_{0.5}$ F6P.

8. The data indicate that PFK activity is altered during the aerobic–anaerobic transition by a change in the phosphorylation state of the enzyme and that cAMP and cGMP act oppositely to regulate PFK activity, and thereby alter glycolytic rate, during this transition.

INTRODUCTION

Many marine invertebrates, particularly those with intertidal or burrowing lifestyles, are excellent facultative anaerobes. Adaptive strategies for anoxia survival include metabolic rate depression and the use of alternative pathways for fermentive ATP production (de Zwaan, 1983; Storey, 1985a). The glycolytic pathway is central to the anaerobic response and, as such, a variety of regulatory mechanisms have been elaborated that control glycolytic enzymes to regulate glycogenolysis and coordinate glycolytic rate depression during anoxia. These mechanisms include:

(1) the covalent modification of regulatory enzymes via protein phosphorylation or dephosphorylation;
(2) the reversible association of enzymes into complexes bound to subcellular particles; and
(3) control over carbohydrate use for biosynthetic purposes via fructose-2,6-bisphosphate (F2, $6P_2$) regulation of 6-phosphofructo-1-kinase (PFK) (Story, 1985a).

The role of covalent modification in the control of glycolytic enzymes in anoxia has been particularly well analyzed for pyruvate kinase (PK) in marine molluscs. Anoxia-induced phosphorylation of PK via endogenous protein kinase(s) converts the enzyme to a less active form that has a lower maximal activity, reduced substrate affinity for phosphoenolpyruvate (PEP), reduced activation by fructose-1,6-bisphosphate, and greatly enhanced inhibition by L-alanine (Holwerda *et al.*, 1983, 1989; Plaxton and Storey, 1984a,b, 1985; Michaelidis *et al.*, 1988; Michaelidis and Storey, 1990a). The same mechanism also appears

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Abbreviations: ABRM, anterior byssus retractor muscle; PK, pyruvate kinase (EC 2.7.1.40); PFK, 6-phosphofructo-1-kinase (EC 2.7.1.11); PEPCK, phosphoenolpyruvate carboxykinase (EC 4.1.1.32); PMA, phorbol 12-myristate 13-acetate; cAMP, cyclic 3′,5′-adenosine monophosphate; cGMP, cyclic 3′,5′-guanosine monophosphate; F6P, fructose-6-phosphate; $F2,6P_2$, fructose-2,6-bisphosphate; PEP, phosphoenolpyruvate.

to control PFK. The properties of PFK isolated from foot muscle of anaerobic whelks were distinctly different from those of the aerobic enzyme form and were consistent with a less active enzyme form in anoxia (Storey, 1984). The anoxic enzyme form, as compared to the aerobic form of PFK, had reduced affinity for fructose-6-phosphate (F6P), and was more strongly affected by inhibitors and less affected by activators. In contrast to PK, however, the role of covalent modification in regulating PFK for aerobic/anoxic function and the protein kinase/phosphatase involvement in the reversible phosphorylation of PFK are less well understood. Recent data obtained from *in vitro* studies on PFK from the posterior adductor muscle of *Mytilus edulis* revealed that endogenous cyclic nucleotide-dependent and Ca^{2+}/phospholipid-dependent protein kinases are involved in the phosphorylation of PFK in this organ (Michaelidis and Storey, 1990b). Other studies have shown that cyclic 3′,5′-adenosine monophosphate (cAMP) and cyclic 3′,5′-guanosine monophosphate (cGMP) concentrations in posterior adductor muscle drop during the first hours of anaerobiosis (Holwerda *et al.*, 1981); in this same time period, changes in muscle metabolite levels indicate an inhibition of flux through the PFK locus (Ebberink and de Zwaan, 1980). These data suggest, therefore, that the anoxia-induced covalent modification of PFK in mussel tissues may be cyclic nucleotide dependent.

In the anterior byssus retractor muscle (ABRM) of *M. edulis* the concentrations of cAMP and cGMP are affected by the neurotransmitters serotonin and acetylcholine, respectively. The former compound causes an increase in cAMP concentration and a relaxation of the ABRM, whereas the latter compound increases cGMP content and stimulates tonic contraction of the muscle (Kohler and Lindl, 1980). In the catch muscles tonic contraction results in a lower energy expenditure (Baquet and Gillis, 1968) and a depression of glycolytic rate (Ebberink and de Zwaan, 1980; Ebberink *et al.*, 1979), whereas during the first hours of recovery after muscle relaxation glycolysis is activated (de Zwaan, 1983; Lazou *et al.*, 1989).

The present study examines PFK from the ABRM of *M. edulis* analyzing the kinetic parameters of the aerobic vs anoxic enzyme forms and testing, in muscle extracts, the effects of added second messengers, cAMP, cGMP or Ca^{2+} plus phorbol 12-myristate 13-acetate (PMA) (stimulators of protein kinase C), to determine the role of protein kinases in modifying PFK for anoxic function.

MATERIALS AND METHODS

Animals and chemicals

Mussels, *M. edulis*, were obtained from the Marine Biological Laboratory, Woods Hole, and were held without feeding for 2 weeks in aerated, recirculating water at 18–20°C. To impose anoxia, mussels were placed in large jars filled with seawater and the water was continuously bubbled with nitrogen gas for 24 hr at 23°C.

All biochemicals and coupling enzymes were purchased from Boehringer–Mannheim Corporation, Montreal, PQ.

Tissue preparation

ABRM was dissected out of individual mussels within 1 min of opening the valves. Samples were immediately frozen in liquid nitrogen and then stored at −60°C until use. Muscle samples were homogenized 1:3 (w/v) in homogenization buffer: 50 mM imidazole–HCl (pH 7.0) containing 100 mM NaF, 5 mM EDTA, 5 mM EGTA, 0.1 mM phenylmethylsulfonyl fluoride, 30 mM 2-mercaptoethanol, and 40% v/v glycerol. After centrifugation at 25,000*g* for 20 min at 4°C, the supernatant was removed and passed through a 5 ml column of fine Sephadex G-25 equilibrated in 40 mM imidazole–HCl (pH 7.0), 5 mM EDTA, 10 mM 2-mercaptoethanol and 20% v/v glycerol. The column was spun in a desk-top centrifuge at top-speed for 1 min. The filtrate was used as the enzyme source for all studies.

Enzyme assay and kinetics studies

PFK activity was monitored by following NADH oxidation at 340 nm using a Gilford recording spectrophotometer attached to a circulating water bath for temperature control of cuvettes. Temperature for all assays was maintained at 20°C. Optimal assay conditions were 100 mM imidazole–HCl buffer, pH 7.0, 10 mM F6P, 0.5 mM ATP, 0.1 mM NADH, 50 mM KCl, 5 mM $MgCl_2$ and excess (1 U each) dialyzed coupling enzymes aldolase, triosephosphate isomerase and glycerol-3-P dehydrogenase. Substrate affinity constants ($S_{0.5}$) and Hill coefficients were calculated from Hill plots. Activation constants (K_a) were determined using double-reciprocal plots of $(1/V - V_0)$ vs 1/[activator]). I_{50} values (inhibitor concentration producing 50% inhibition of enzyme activity) were determined by the method of Job *et al.* (1978). All kinetic parameters are means ± SEM for $n = 3$ separate determinations on different preparations of enzyme.

In vitro phosphorylation and dephosphorylation

The preparation of muscle extracts for these experiments followed the same protocol as above to the point of G-25 filtration, except that tissue from freshly killed animals was used and homogenized 1:2.5 (w/v) in homogenization buffer. After centrifugation, the supernatant was passed through a 5 ml column of Sephadex G-25 equilibrated in 40 mM imidazole–HCl (pH 7.0), 20% v/v glycerol, 10 mM potassium phosphate, 10 mM 2-mercaptoethanol and 5 mM EDTA [glycerol and inorganic phosphate (P_i) are added to stabilize PFK activity]. The columns were spun in a desk-top centrifuge at top-speed for 1.5 min. The filtrate was collected and divided into aliquots of 100 μl. Each aliquot was diluted with 100 μl of a solution containing 40 mM imidazole–HCl (pH 7.0), 20 mM $MgCl_2$, 40 mM NaF, 10 mM 2-mercaptoethanol, 20% v/v glycerol, ATP (0.5 or 3.0 mM) and one of the following:

(a) 5 μM $CaCl_2$, and 10 μg/ml PMA;
(b) 3 mM cAMP;
(c) 3 mM cGMP;
(d) 3 mM cAMP and 3 mM cGMP;
(e) 3 mM cAMP, 2.5 μM $CaCl_2$, 10 μg/ml PMA; or
(f) 3 mM cGMP, 2.5 μM $CaCl_2$, 10 μg/ml PMA.

Filtrates that did not contain cAMP, cGMP and $MgCl_2$, but did include 0.5 or 3.0 mM ATP and 5.0 mM F6P were used as controls. Incubations were carried out for 2 hr at 30°C, then desalted by passage through spun columns as described above, and PFK kinetics were assessed.

For experiments involving *in vitro* dephosphorylation and subsequent phosphorylation of PFK-aerobic, filtrates were prepared as described above. Samples were then incubated at 30°C for 2 hr in the presence of 0.3 U/ml of alkaline phosphatase and 20 mM $MgCl_2$. After incubation the mixtures were dialyzed by centrifugation through 2 ml columns of Sephadex G-25 equilibrated with a buffer containing 40 mM imidazole–HCl (pH 7.0), 10 mM 2-mercaptoethanol, 20% v/v glycerol, 10 mM potassium phosphate and 0.1 mM EDTA. The filtrates were then incubated at 30°C for 2 hr in the presence of 3.0 mM cAMP, 20 mM $MgCl_2$, 40 mM NaF and 3.0 mM ATP. After incubation the samples were again

Table 1. Kinetic constants of aerobic and anoxic forms of phosphofructokinase from anterior byssus retractor muscle of *M. edulis*

Enzyme constants	Aerobic muscle	Anoxic muscle
$S_{0.5}$ F6P (mM)	1.34 ± 0.077	2.62 ± 0.12[a]
n_H	1.38 ± 0.23	1.11 ± 0.012
K_m Mg·ATP (μM)	16 ± 0.3	21 ± 0.7[a]
K_a AMP (mM)	0.12 ± 0.014	0.12 ± 0.016
K_a F2,6P$_2$ (μM)	8.3 ± 0.96	8.5 ± 1.0
K_a P$_i$ (mM)	1.42 ± 0.20	1.22 ± 0.11[b]
I_{50} PEP (mM)	1.18 ± 0.057	0.81 ± 0.049[a]
I_{50} Mg·ATP (mM)	2.06 ± 0.042	2.54 ± 0.046[a]

Values are means ± SEM, $n = 3$ determinations on separate preparations of enzyme from different animals. Effector constants were determined at 0.2 mM F6P for K_a values for both enzyme forms and 3.0 mM F6P for I_{50} values.
[a]Significantly different from the corresponding values for the aerobic enzyme form as determined by the Student's t-test, $P < 0.005$.
[b]$P < 0.1$.

passed through spun columns and the filtrates were used for measurement of PFK activity. Control samples were treated identically except that alkaline phosphatase was not added.

RESULTS

Table 1 shows the kinetic properties of PFK from the ABRM of *M. edulis* sampled from aerobic vs 21 hr anoxic mussels. Both aerobic and anoxic forms of PFK showed Michaelis–Menten behavior with respect to F6P as the substrate. The affinity for both substrates decreased significantly in anoxia: $S_{0.5}$ (F6P) increased by 2-fold whereas K_m ATP increased by 30% in anoxic, compared to aerobic, muscle.

ABRM PFK was activated by AMP, F2,6P$_2$ and P$_i$ (Table 1); the enzyme was not activated by ammonium ion. Anoxia exposure resulted in a small change in the K_a for P$_i$ but did not alter K_a values for AMP or F2,6P$_2$. ATP and PEP inhibited ABRM PFK but the enzyme was not inhibited by citrate. Inhibition constants for ATP and PEP changed significantly in anoxia; I_{50} for PEP decreased by 30% whereas I_{50} for ATP increased by 23% (Table 1).

In vitro phosphorylation

Table 2 shows the $S_{0.5}$ values for F6P of the aerobic vs anoxic forms of ABRM PFK incubated *in vitro* without or with different second messengers. Both forms of PFK lost substantial amounts of their activity during incubation of the extracts at 30°C for 2 hr, even though stabilizers, F6P (5.0 mM), ATP (0.5 or 3.0 mM) and glycerol (20% v/v), were present in the incubation mixture. In addition, the affinity for F6P decreased ($S_{0.5}$ increased by 50–80%) over the incubation time for the control samples (compare Tables 1 and 2). In the presence of high ATP (3.0 mM) as a stabilizer, this change in F6P affinity was even more pronounced.

The presence of various second messengers in the incubations had marked effects on the resulting $S_{0.5}$ values of both forms of PFK.

cAMP

In the presence of cAMP the $S_{0.5}$ value for F6P decreased as compared to the control enzyme for both the aerobic and anoxic forms of PFK. In both cases, the presence of cAMP had a greater effect on the $S_{0.5}$ of the anoxic enzyme form. In the presence of 0.5 mM ATP, incubation with cAMP decreased the $S_{0.5}$ value for the aerobic enzyme to 82% of the aerobic control value vs a decrease to 62% of the anoxic control for the anoxic enzyme form. At 3 mM ATP, the effect of the presence of cAMP was much greater with $S_{0.5}$ reduced to 30 vs 21% of the corresponding controls.

cGMP

In contrast to the effect of cAMP, the presence of cGMP in the incubation (at 0.5 mM ATP) increased the $S_{0.5}$ of PFK-aerobic significantly (by 54%) as compared to the control enzyme. Under the same conditions the $S_{0.5}$ value of PFK-anoxic did not change. At high ATP, however, the presence of cGMP did not alter the apparent $S_{0.5}$ for the aerobic enzyme form but did reduce the value for the anoxic form.

cAMP + cGMP

In the presence of both cyclic nucleotides, the effects on $S_{0.5}$ values of the aerobic enzyme form were much the same as were seen in the presence of cAMP alone. For the anoxic enzyme form, however, the results were similar to the effects of cGMP alone: no change in $S_{0.5}$ F6P at low ATP and a decrease in $S_{0.5}$ to *ca* 35% of the control value at high ATP.

Table 2. The effect of second messengers on the $S_{0.5}$ value for F6P of aerobic and anoxic forms of PFK from ABRM in the presence of different concentrations of ATP

	$S_{0.5}$ F6P (mM)			
	Incubation with			
	0.5 mM ATP		3.0 mM ATP	
Conditions	Aerobic	Anoxic	Aerobic	Anoxic
Control	2.16 ± 0.024	4.08 ± 0.024	6.60 ± 0.71	9.51 ± 0.74
cAMP	1.77 ± 0.010[a]	2.54 ± 0.16[a]	1.98 ± 0.060[a]	2.00 ± 0.070[a]
cGMP	3.32 ± 0.15[a]	4.23 ± 0.19	6.52 ± 0.44	3.03 ± 0.37[a]
cAMP + cGMP	1.72 ± 0.038[a]	4.19 ± 0.077	2.47 ± 0.11[a]	3.57 ± 0.18[a]
Ca^{2+} + PMA	2.52 ± 0.028	4.36 ± 0.24	3.98 ± 0.049[b]	10.35 ± 1.65
cAMP + Ca^{2+} + PMA	2.16 ± 0.020	2.85 ± 0.067[a]	2.04 ± 0.042[a]	3.10 ± 0.28[a]
cGMP + Ca^{2+} + PMA	3.54 ± 0.11[a]	4.20 ± 0.053	12.12 ± 1.37[b]	3.40 ± 0.28[a]

Values are means ± SEM, $n = 3$ determinations on separate preparations of enzyme from different animals. Conditions for enzyme incubation with second messengers and the determination of $S_{0.5}$ values are described in the Materials and Methods.
[a]Significantly different from the control values as determined by the Student's t-test, $P < 0.005$.
[b]$P < 0.025$.

Ca^{2+} + PMA

The stimulators of protein kinase C action, Ca^{2+} and PMA had an effect only on the $S_{0.5}$ of PFK-aerobic in the presence of 3.0 mM ATP ($S_{0.5}$ decreased significantly) and did not affect enzyme affinity for F6P under any of the other conditions.

cAMP + Ca^{2+} + PMA

The effect of these three compounds added together was very similar to the effects of cAMP alone ($S_{0.5}$ decreased significantly) with the exception that the $S_{0.5}$ value of PFK-aerobic at 0.5 mM ATP was not altered by the experimental treatment.

cGMP + Ca^{2+} + PMA

In three out of four conditions, the effect of these compounds was essentially the same as the effect of cGMP alone on enzyme $S_{0.5}$. Thus, at low ATP, $S_{0.5}$ of the aerobic form increased whereas that of the anoxic form was not affected. By contrast with the effects of cGMP alone, however, $S_{0.5}$ of the aerobic enzyme, assayed at high ATP, also increased (by 2-fold).

In vitro dephosphorylation

The effect of treatment of PFK-aerobic with alkaline phosphatase + $MgCl_2$ (2 hr at 30°C) followed by incubation with cAMP + ATP + $MgCl_2$ + NaF (2 hr at 30°C) on enzyme activity and affinity for F6P is shown in Fig. 1. Treatment with alkaline phosphatase reduced the measurable activity of PFK; calculated V_{max} decreased from 3.81 ± 0.050 to 2.42 ± 0.08 U/g fresh wt. The treatment also strongly reduced enzyme affinity for F6P; $S_{0.5}$ increased from 1.47 ± 0.03 to 9.52 ± 0.15 mM. The subsequent treatment of the alkaline phosphate-treated enzyme with cAMP + ATP + $MgCl_2$ reversed the change in enzyme $S_{0.5}$, reducing $S_{0.5}$ to 2.35 ± 0.015 mM, a value very similar to the untreated control value (Fig. 1). The treatment did not significantly change enzyme V_{max}, however.

DISCUSSION

PFK from ABRM showed similar properties to the muscle enzyme from other invertebrate sources, including PFK from the posterior adductor muscle of *M. edulis* (Ebberink, 1982; Hofer *et al.*, 1982; Storey, 1976, 1984, 1985b; Michaelidis and Storey, 1990b). The ABRM enzyme was inhibited by ATP, P_i and PEP, and activated by AMP and $F2,6P_2$. The enzyme was not affected by NH_4^+ or citrate however. Lack of citrate inhibition contrasts with the inhibitory effects of this compound on vertebrate PFK but is consistent with the findings for PFK from most invertebrate sources. ABRM PFK had a fairly low affinity for F6P ($S_{0.5}$ is probably *ca* 10-fold higher than physiological F6P concentrations) and was strongly inhibited at physiological ATP concentrations such that enzyme function *in vivo* is undoubtedly dependent on the actions of allosteric effectors, particularly activators.

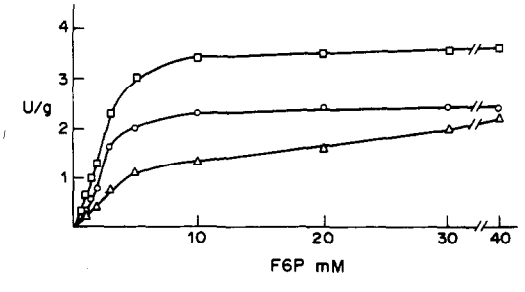

Fig. 1. F6P saturation curves for PFK-aerobic from ABRM of *M. edulis* after *in vitro* dephosphorylation and subsequent rephosphorylation of the enzyme as described in Materials and Methods. *Conditions* are: □, control incubated without alkaline phosphatase or cAMP; △, treatment with alkaline phosphatase + $MgCl_2$; ○, treatment with alkaline phosphatase followed by treatment with cAMP, ATP + $MgCl_2$ + NaF.

The inhibition of ABRM PFK by PEP may have special significance for PFK control in facultative anaerobes. PEP concentration rises during anoxia, presumably due to the phosphorylation inactivation of the PK locus (Ebberink and de Zwaan, 1980). Feedback inhibition of PFK by the substrate of the PK reaction would help to coordinate the activities of these regulatory enzymes. It is noteworthy that the anoxic enzyme form of ABRM PFK is significantly more sensitive to PEP inhibition than is the aerobic enzyme.

ABRM PFK undergoes a stable modification that changes its kinetic properties in response to anoxia exposure. Affinity for both substrates decreased in anoxia and the kinetic constants for P_i activation and for PEP and ATP inhibition changed significantly. The net effect is probably to convert the enzyme to a less active enzyme form in anoxia. These anoxia-induced alterations to ABRM PFK are similar to those reported for PFK from foot muscle of the gastropod whelk *Busycotypus canaliculatum* (Storey, 1984) although ABRM PFK showed fewer alterations to activator constants as a result of anoxia. Activator actions on the enzyme can be modified in another way, however. Thus, although the K_a for $F2,6P_2$ did not change in anoxia, tissue content of the bisphosphate is frequently strongly depressed in the organs of marine molluscs during anoxia and levels of $F2,6P_2$ in *M. edulis* muscles (0.1–0.15 μM) (Storey, 1985c) are far below the apparent activation constant for ABRM PFK (8.3–8.5 μM). This suggests that the enzyme could be highly sensitive to small changes in $F2,6P_2$ concentrations during anaerobiosis.

For mammalian PFK, stable changes in the kinetic properties of PFK, as a response to physiological stress or hormone action, are typically the result of covalent modification of the enzyme protein via phosphorylation or dephosphorylation reactions (Hofer, 1985; Pilkis *et al.*, 1987). The enzyme from parasitic helminths is also regulated by reversible phosphorylation; the response to serotonin, for example, is phosphorylation and activation of PFK (Daum *et al.*, 1986; Kamemoto *et al.*, 1987). The results of the *in vitro* incubations of ABRM PFK preparations with protein kinase second messengers (+ATP + Mg^{2+}) indicate that changes in the phosphorylation state of the enzyme are also responsible for the anoxia-induced modification of the ABRM enzyme.

The addition of cAMP to ABRM muscle homogenates had a marked effect on PFK from both aerobic and anoxic tissues, in both cases strongly reducing $S_{0.5}$ for F6P (Table 2). Thus, a cAMP-dependent protein kinase appears to be present in ABRM homogenates that can phosphorylate PFK and, in doing so, activate the enzyme by increasing affinity for F6P substrate. The action of this protein kinase reverses the effect of anoxia on enzyme $S_{0.5}$. These effects of cAMP stimulation on ABRM PFK were further studied after initial treatment of the aerobic enzyme form with alkaline phosphatase. Alkaline phosphatase decreased both the maximal activity of the enzyme and its affinity for F6P ($S_{0.5}$ increased 6.5-fold) (Fig. 1). When this preparation was further incubated in the presence of cAMP, however, the effect on $S_{0.5}$ was largely reversed. These results, therefore, strongly indicate that cAMP-dependent protein kinase phosphorylates ABRM PFK and that the action of this phosphorylation is enzyme activation by increasing affinity for F6P. Furthermore, the results suggest that the aerobic vs anoxic forms of ABRM PFK are the phosphorylated vs dephosphorylated enzymes, respectively.

Other workers have studied the role of cAMP in the regulation of the contraction–relaxation cycle in ABRM. Application of the neurotransmitter serotonin, which activates adenylate cyclase, increases the concentration of cAMP in ABRM (Cole and Twarog, 1972; Kohler and Lindl, 1980). Relaxation of ABRM after tonic contraction can be directly induced by cAMP, probably through the activation of cAMP-dependent protein kinase (Castelanni and Cohen, 1987a,b; Cornelius, 1982). Catch muscles, such as ABRM, show an increased demand for energy during the first hours of recovery after valve closure and this is met by an activation of glycolysis (de Zwaan, 1983; Lazou *et al.*, 1989). Thus, it seems reasonable to conclude that one of the cAMP-mediated components of the relaxation of ABRM, and of the accompanying anoxic to aerobic transition associated with valve opening, is the phosphorylation of PFK *in vivo*. This appears to function to activate the enzyme and promote increased glycolytic flux in catch muscles during recovery.

This effect of cAMP on muscle PFK resembles the situation described for the parasitic helminths. Here serotonin also stimulates an increase in intracellular cAMP content (Mansour *et al.*, 1960; Donahue *et al.*, 1981) and the administration of either serotonin or cAMP increases the activity of PFK when added directly to a *Fasciola hepatica* homogenate (Mansour and Mansour, 1962). Recent studies have shown that serotonin stimulates the phosphorylation of PFK in both *F. hepatica* and *Ascaris suum* (Kamemoto *et al.*, 1987; Harris *et al.*, 1982) and that the phosphorylated form of the enzyme has a lower $S_{0.5}$ value for F6P than the dephosphorylated enzyme (Kamemoto and Mansour, 1986; Kamemoto *et al.*, 1987; Daum *et al.*, 1986; Hofer *et al.*, 1982). Furthermore, PFK from mammalian heart and adipose tissue also responds similarly to catecholamine stimulation (Narabayashi *et al.*, 1985; Sale and Denton, 1985). For example, epinephrine stimulates a cAMP-mediated phosphorylation of PFK that increases affinity for F6P, increases sensitivity to activators, and decreases sensitivity to inhibitors (Narabayashi *et al.*, 1985).

The role of cGMP in regulation of biological processes is not yet well understood. It has been suggested that cAMP and cGMP may play opposing roles in cell function (Tremblay *et al.*, 1988) and the results presented here tend to confirm this suggestion. In most cases, incubation with cGMP ($+ATP + Mg^{2+}$) had the opposite effect on $S_{0.5}$ F6P of ABRM PFK compared to that seen in the presence of cAMP. At 0.5 mM ATP cGMP did not affect the anoxic enzyme form but was the only second messenger that increased the $S_{0.5}$ of the aerobic enzyme form and mimicked the effect of anoxia on the enzyme. When cAMP and cGMP were given together, cAMP effects appeared to dominate when enzyme from aerobic ABRM was used whereas cGMP effects seemed dominant in the anoxic enzyme preparation. It appears, therefore, that cGMP stimulated effects may be responsible for the anoxia-induced changes in ABRM PFK. Indeed, levels of cGMP increase in ABRM in response to the catch-inducing neurotransmitter acetylcholine (Kohler and Lindl, 1980), and, as discussed above, during tonic contraction of the ABRM glycolytic rate is reduced. Thus, it appears that the action of a cGMP-dependent protein kinase could be responsible for reducing the activity of PFK in muscle and by this action (and probably others as well) slow glycolytic rate during tonic contraction.

However, since the effect of cGMP action on ABRM PFK is similar to the effect of alkaline phosphatase on the enzyme it appears that cGMP control of PFK is more complicated than cAMP effects. Two possible actions of cGMP could be proposed:

(1) cGMP-dependent protein kinase may phosphorylate ABRM PFK at a different site than that affected by cAMP-dependent protein kinase and phosphorylation at this site would reduce enzyme affinity for F6P; or
(2) cGMP stimulation may set up a cascade reaction which ultimately stimulates the action of a PFK phosphatase.

The second suggestion is perhaps the more reasonable but both require investigation. Other recent studies have also implicated cGMP in enzyme control during the aerobic–anoxic transition. In *B. canaliculatum* cGMP-dependent protein kinase mediates the anoxia signal in stimulating the phosphorylation and inactivation of pyruvate kinase (Brooks and Storey, 1990). Thus, it is apparent that cGMP-dependent protein kinase has critical roles in the regulation of anaerobiosis that may include both the reorganization of metabolic pathways for anaerobic function and the regulation of anoxia-induced metabolic rate depression.

Incubation with Ca^{2+} and PMA was used to assess the ability of protein kinase C to phosphorylate ABRM PFK. Phospholipid-dependent, Ca^{2+}-activated protein kinase is widely distributed in organs of vertebrates and invertebrates (Kuo *et al.*, 1980) and has a broad protein substrate specificity, including phosphorylation of contractile proteins, membrane proteins and enzymes (Kikkawa and Nishizuka, 1986). The presence of stimulators of protein kinase

C in extracts of ABRM changed the affinity for F6P of the aerobic enzyme form but did not affect the anoxic form. The greatest effect was on PFK-aerobic incubated with 3 mM ATP where $S_{0.5}$ was reduced by 40%. Hofer *et al.* (1985) have reported that rabbit muscle PFK can be phosphorylated by protein kinase C and this resulted in an increased affinity for F6P. The addition of Ca^{2+} + PMA along with cGMP had a synergistic effect on the aerobic form of ABRM PFK (at 3 mM ATP only) but along with cAMP there was no difference in the response compared to cAMP alone. Overall, ABRM PFK showed limited responses to incubation with stimulators of protein kinase C and the responses showed no clear trend. Furthermore, measurements of Ca^{2+} concentration *in vivo* in ABRM have shown no significant change in Ca^{2+} levels over the contraction–relaxation cycle (Ishii *et al.*, 1989). Therefore, the role of protein kinase C in regulating ABRM PFK can not yet be postulated.

In summary, then, we have presented evidence that PFK from *M. edulis* ABRM undergoes a stable modification with the aerobic–anaerobic transition, we have shown that the modification appears to be due to covalent modification via protein phosphorylation or dephosphorylation reactions, and we have demonstrated the opposing actions of cAMP and cGMP dependent protein kinases in altering PFK kinetic properties. The data indicate that cAMP dependent protein kinase action activates the enzyme by increasing enzyme affinity for F6P and that the enzyme in aerobic muscle is the high phosphate form. The data are consistent with the known effects of these second messengers on the contraction–relaxation cycle in ABRM and the changes in glycolytic rate that occur during valve closure and opening.

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