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PHOSPHOFRUCTOKINASE FROM FLIGHT MUSCLE OF THE COCKROACH, *PERIPLANETA AMERICANA*

CONTROL OF ENZYME ACTIVATION DURING FLIGHT

KENNETH B. STOREY
Institute of Biochemistry and Department of Biology, Carleton University, Ottawa, Ontario, Canada K1S 5B6

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Abstract—Phosphofructokinase (PFK) from the flight muscle of the cockroach, *Periplaneta americana*, was partially purified. Michaelis constants for ATP and Mg^{2+} were 0.05 ± 0.005 and 0.50 ± 0.038 mM while $S_{0.5}$ for fructose-6-P was 16 ± 0.9 mM ($h = 2.3 \pm 0.4$) at pH 7.0 and 9.4 ± 0.3 mM ($h = 1.5 \pm 0.3$) at pH 8.0. ADP, fructose-1,6-P_2 and citrate had no effect on cockroach PFK but ammonium ion, inorganic phosphate, AMP and fructose-2,6-P_2 were all activators of enzyme activity with apparent K_as of 1.0 mM, 0.7 mM, 8.0 μM and 0.1 μM, respectively. Activators increased enzyme affinity for fructose-6-P and reduced enzyme substrate inhibition by high levels of ATP. Effects of activators were additive with strong synergistic interactions seen when AMP and fructose-2,6-P_2 were added together; alone 0.16 mM AMP reduced $S_{0.5}$ by 7-fold and 0.1 μM fructose-2,6-P_2 reduced $S_{0.5}$ by 1.7-fold but when added together $S_{0.5}$ for fructose-6-P decreased by 114-fold to 0.14 mM. Addition of all four activators, at levels close to physiological concentrations in flight muscle (3 mM NH_4^+, 10 mM P_i, 0.4 mM AMP and 1 μM fructose-2,6-P_2), reduced $S_{0.5}$ by 640-fold to 0.025 mM, a value within the physiological range of fructose-6-P concentrations *in vivo*. In combination all four activators also raised I_{50} for ATP to 18 mM compared to I_{50}s of between 1.7 and 6.5 mM in the presence of each activator alone. At the initiation of flight full activation of PFK (allowing a maximal glycolytic rate) in flight muscle is accomplished through the combined effects of activators on PFK, in particular the effects of elevated concentrations of AMP and fructose-2,6-P_2 in working muscle.

Key Word Index: Phosphofructokinase, fructose-2,6-bisphosphate, insect flight muscle, *Periplaneta americana*, activation of glycolysis, flight muscle metabolism

INTRODUCTION

Phosphofructokinase (PFK: EC 2.7.1.11) is a key regulatory enzyme in animal tissues which controls the rate of hexose phosphate utilization by the glycolytic chain. The enzyme in mammalian systems is closely regulated by a variety of effectors including allosteric activation by ammonium ion, inorganic phosphate and AMP, product activation by ADP and fructose-1,6-P_2 and inhibition by citrate and by high levels of the substrate ATP (Ramaiah, 1974; Uyeda, 1979). In addition, recent studies have identified a potent new activator of PFK, fructose-2,6-P_2 (Hers and van Schaftingen, 1982; Uyeda *et al.*, 1981; Claus *et al.*, 1982). Levels of this effector are modulated in mammalian tissues in response to hormonal signals (glucagon, insulin, adrenaline; Hers and van Schaftingen, 1982) providing a mechanism for altering PFK activity in response to hormones.

The transition from resting to flying in insects occurs with an increase in metabolic rate (oxygen consumption) of up to 100-fold in some species (Crabtree and Newsholme, 1975). Amongst insects which fuel flight with carbohydrate reserves this requires a corresponding and almost instantaneous increase in carbon flux through glycolysis. Activation of key regulatory enzymes, notably PFK, is required to allow this increased flux and support the energy demands of flight. The present study examines the kinetics and regulatory properties of cockroach, *Periplaneta americana*, flight muscle phosphofructokinase including the key regulatory role of fructose-2,6-P_2 in enzyme function. A full activation of muscle PFK during flight appears to depend upon the additive and synergistic effects enzyme activators, NH_4^+, P_i, AMP and fructose-2,6-P_2.

MATERIALS AND METHODS

Animals and chemicals

Adult male cockroaches, *Periplaneta americana*, were used. Biochemicals and coupling enzymes were purchased from Boehringer Mannheim Corp.; fructose-2,6-P_2 was from Sigma Chemical Co.; Affigel Blue was from BioRad Laboratories and ampholines were from LKB Ltd.

Purification of PFK

Cockroach thoraces containing flight muscle (with gut removed) were homogenized in 10 vol ice-cold 20 mM imidazole buffer, pH 7.0 (all buffers adjusted to pH at 25°C) containing 30 mM 2-mercaptoethanol, 5 mM $MgCl_2$ and 1 mM EDTA using a Polytron PT-10 homogenizer. After centrifugation at 27,000 *g* for 30 min at 4°C, the supernatant was removed and layered onto a column of Affigel Blue (5 × 1.5 cm) equilibrated in 10 mM imidazole buffer, pH 7.0 containing 30 mM 2-mercaptoethanol. The column was washed with buffer until A_{280nm} dropped to a minimum value

and then PFK activity was eluted in buffer containing 20 mM ATP and 0.4 mM fructose-1,6-P_2. After dialysis against two changes of the equilibration buffer, the PFK preparation was used for kinetic studies. The enzyme preparation was stable for several days at 4°C. The enzyme was purified 8.5-fold removing contaminating activities of ATPase, NADH oxidase, phosphoglucoisomerase, fructose-1,6-diphosphatase and fructose-1,6-diphosphatase and fructose-2,6-diphosphatase.

Enzyme assay and kinetics

PFK activity was monitored by following NADH utilization at 340 nm using a Pye Unicam SP 8-100 recording spectrophotometer with water jacketed cell holder for constant temperature control at 23°C. Optimal assay conditions were 50 mM imidazole buffer, pH 7.0 containing 30 mM 2-mercaptoethanol, 5 mM $MgCl_2$, 50 mM KCl, 40 mM fructose-6-P (in the absence of effectors), 0.2 mM ATP, 0.1 mM NADH and dialysed coupling enzymes: 0.2 U aldolase, 1.2 U triosephosphate isomerase and 0.16 U glycerol-3-P dehydrogenase in a final volume of 1 ml. For examination of fructose-1,6-P_2 effects on the enzyme, the pyruvate kinase/lactate dehydrogenase coupled assay was used. Kinetic constants, $S_{0.5}$, were determined from Hill plots

Isoelectrofocusing

Isoelectrofocusing was carried out using a 110 ml LKB 8101 column with a pH 3.5 to 10 gradient for 12 hr at 600 V.

RESULTS

Isoelectrofocusing, pH and salt effects

The pI of cockroach PFK was determined to be 4.9 ± 0.2, $n = 3$.

The pH optimum of cockroach PFK was 7.5 in both Tris and imidazole buffers at saturating fructose-6-P concentrations. At subsaturating fructose-6-P, the optimum shifted to a higher pH, pH 7.8 and 8.35 at 20 and 10 mM fructose-6-P, respectively; addition of positive effectors reversed this shift and returned the pH optimum to 7.5.

Addition of monovalent cations, K^+ and Na^+, produced an activation of PFK, maximal activation being 33% at 40–50 mM K^+ or 20 mM Na^+ compared to assays run in the absence of added monovalent salts. Effects were similar for Cl^- and SO_4^{2-} salts. At higher concentrations these salts became inhibitory. Ammonium chloride and ammonium sulfate also activated PFK with maximal activation at 10 mM of the monovalent salt and 5 mM of the divalent salt; again anion did not influence enzyme activity.

Substrate affinities

Michaelis constants for ATP, Mg^{2+} and Mn^{2+} were 0.05 ± 0.005, 0.50 ± 0.038 and 0.1 ± 0.009 mM, respectively. Like PFK from other sources, cockroach PFK showed sigmoidal fructose-6-P kinetics at pH 7.0 with a substrate affinity constant, $S_{0.5}$ of 16.0 ± 0.9 mM and a Hill coefficient, h, of 2.3 ± 0.4. At pH 8.0 fructose-6-P kinetics were more hyperbolic and enzyme substrate affinity was higher with $S_{0.5} = 9.4 \pm 0.3$ mM and $h = 1.5 \pm 0.3$.

Effectors of PFK

ADP and fructose-1,6-P_2 are product activators of mammalian PFK. However neither affected cockroach PFK when tested at levels of up to 1 mM and in the presence of both saturating and subsaturating levels of fructose-6-P. Similarly citrate, an inhibitor of mammalian PFK, did not affect the cockroach enzyme at levels of up to 25 mM with a 2:1 ratio of Mg:citrate. Alanine had a slight inhibitory effect on cockroach PFK but proline did not affect enzyme activity.

Ammonium ion, inorganic phosphate and AMP were all strong activators of cockroach PFK as they are for PFK from other sources. In addition, the newly discovered regulatory metabolite, fructose-2,6-P_2, was also a potent activator of cockroach PFK. All four activators affect enzyme activity by increasing affinity for fructose-6-P and by relieving inhibition by high levels of ATP. Apparent activator constants, K_a (determined at 10 mM fructose-6-P and 0.2 mM ATP), were 1.0 and 0.7 mM for NH_4^+ and P_i and 8.0 and 0.1 μM for AMP and fructose-2,6-P_2.

Effect of activators on fructose-6-P kinetics

Table 1 shows the effects of positive modulators on $S_{0.5}$ and h for fructose-6-P for cockroach PFK at pH 7.0. Activators were tested at concentrations within their physiological ranges *in vivo*; flight muscle concentrations of P_i, NH_4^+, AMP and fructose-2,6-P_2 were measured as 9.9, 0.9, 0.17 mM and 1.2 μM in

Table 1. Effects of modulators on enzyme affinity ($S_{0.5}$) for fructose-6-P and Hill coefficient (h) of cockroach phosphofructokinase at pH 7.0

			Plus fructose-2,6-P_2 at			
			0.1 μM		1.0 μM	
	$S_{0.5}$	h	$S_{0.5}$	h	$S_{0.5}$	h
Control	16.0 ± 0.90	2.3 ± 0.4	9.30 ± 0.90	2.1 ± 0.4	1.20 ± 0.30	2.7 ± 0.6
+1 mM NH_4^+	15.0 ± 2.00	2.0 ± 0.3	6.60 ± 1.20	2.5 ± 0.6	1.00 ± 0.20	2.5 ± 0.4
+3 mM NH_4^+	11.0 ± 1.00	2.2 ± 0.2	5.50 ± 0.70	2.6 ± 0.7	0.80 ± 0.10	2.7 ± 0.5
+10 mM P_i	1.30 ± 0.10	3.2 ± 0.4	0.76 ± 0.14	2.5 ± 0.2	0.16 ± 0.03	2.9 ± 0.4
+0.16 mM AMP	2.30 ± 0.10	2.1 ± 0.2	0.14 ± 0.013	2.8 ± 0.2	0.036 ± 0.004	1.8 ± 0.3
+0.4 mM AMP	1.70 ± 0.10	2.2 ± 0.2	0.083 ± 0.010	1.8 ± 0.2	0.038 ± 0.003	1.5 ± 0.1
+1 mM NH_4^+ and 0.16 mM AMP	1.60 ± 0.20	3.4 ± 0.4	0.13 ± 0.015	2.4 ± 0.2	0.032 ± 0.003	1.8 ± 0.3
+3 mM NH_4^+ and 0.40 mM AMP	1.20 ± 0.30	2.3 ± 0.3	0.080 ± 0.010	1.8 ± 0.1	0.025 ± 0.004	2.1 ± 0.6
+1 mM NH_4^+, 10 mM P_i and 0.16 mM AMP	0.072 ± 0.016	1.2 ± 0.4	0.031 ± 0.002	1.2 ± 0.1	0.028 ± 0.007	1.9 ± 0.3
+3 mM NH_4^+, 10 mM P_i and 0.40 mM AMP	0.042 ± 0.015	1.2 ± 0.2	0.027 ± 0.002	1.4 ± 0.2	0.025 ± 0.008	1.2 ± 0.1

Results are mean ± SEM for $n = 3$ determinations on separate enzyme preparations. Assay conditions are 50 mM imidazole buffer, pH 7.0, 50 mM KCl, 5 mM $MgCl_2$, 0.2 mM ATP, 0.1 mM NADH and excess coupling enzymes.

resting muscle and 9.0, 2.5, 0.45 mM and 2.7 μM in muscle after 20 sec of flight (Storey, 1983). Effects of ammonium ion alone on fructose-2,6-P_2 kinetics were moderate; at 3 mM NH_4^+, $S_{0.5}$ was reduced by only 31%. Other activators had stronger effects, 10 mM P_i, 0.4 mM AMP or 1 μM fructose-2,6-P_2 each reduced $S_{0.5}$ by about 10-fold to 1.2 to 1.7 mM.

Effects of NH_4^+ were additive when added in combination with any of the other three activators. P_i and fructose-2,6-P_2 effects also appeared to be additive but P_i and AMP showed a synergistic interaction in their activations of the enzyme. AMP and fructose-2,6-P_2 also showed strong synergistic interactions, in combination the two effectors lowering the $S_{0.5}$ to a much greater extent than would be expected from the additive effects of the two activators alone. Thus AMP at 0.16 mM lowered $S_{0.5}$ by 7-fold to 2.3 mM while fructose-2,6-P_2 at 0.1 μM lowered $S_{0.5}$ by 1.7-fold to 9.3 mM; in combination, however, these two activators reduced $S_{0.5}$ by 114-fold to 0.14 mM. Similar interactions were noted at other AMP and fructose-2,6-P_2 levels. When all four activators were added at levels simulating the concentrations in working flight muscle $S_{0.5}$ was decreased to 0.025 mM, a 640-fold reduction in $S_{0.5}$ over the control situation. The Hill coefficient was also reduced indicating that fructose-6-P kinetics are much more hyperbolic in the presence of multiple activators.

Activators had similar effects in lowering $S_{0.5}$ for fructose-6-P at pH 8.0. In the absence of activators $S_{0.5}$ was 9.4 ± 0.3 mM. Addition of 3 mM NH_4^+, 10 mM P_i 0.4 mM AMP or 1 μM fructose-2,6-P_2 reduced $S_{0.5}$ to 6.95 ± 1.05, 0.38 ± 0.05, 2.25 ± 0.75 or 0.54 ± 0.11 mM, respectively. In the presence of all four activators $S_{0.5}$ decreased to 0.028 ± 0.003 mM ($h = 1.1 \pm 0.2$).

ATP inhibition of PFK

At levels above the optimal (0.2 mM) ATP is a substrate inhibitor of PFK. Figure 1 shows the effects of activators in reversing ATP inhibition of cockroach PFK. In these studies, PFK activity was assayed at 5 mM fructose-6-P; in the absence of activators, enzyme activity at this substrate concentration is less than 0.5% of V_{max}. The inhibitor constant, I_{50}, for ATP in the absence of effectors could not, therefore, be quantitated except to determine that I_{50} was less than 1.7 mM. Figure 1 shows the effects of ATP substrate inhibition on PFK in the presence of various activators. The I_{50} for ATP was determined to be 1.7, 4.7, 2.1, 3.1 and 6.3 mM at 0.1 and 1.0 μM fructose-2,6-P_2, 0.16 and 0.40 mM AMP and 10 mM P_i, respectively. Effects of activators were again additive. At 0.1 μM fructose-2,6-P_2 and 0.16 mM AMP, I_{50} was raised to 4.5 mM. When the four activators were combined, I_{50} was increased to 15.3 mM under conditions of 1 mM NH_4^+, 10 mM P_i, 0.16 mM AMP and 0.1 μM fructose-2,6-P_2 and to 18 mM in the presence of 3 mM NH_4^+, 10 mM P_i, 0.40 mM AMP and 0.1 μM fructose-2,6-P_2. At concentrations of fructose-6-P closer to physiological levels (0.2 mM) inhibition by ATP was increased but was again reversed by activators. In the presence of NH_4^+ (1 or 3 mM) + P_i (10 mM) + AMP (0.16 or 0.40 mM) I_{50} for ATP was 4.5 to 5.5 mM similar to physiological ATP concentrations in flight muscle. Addition of 1 μM fructose-2,6-P_2, however, raised I_{50} to 9.2 mM in the presence of 1 mM NH_4^+, 10 mM P_i and 0.16 mM AMP and to 11.6 mM in the presence of 3 mM NH_4^+, 10 mM P_i and 0.40 mM AMP.

ATP inhibition of cockroach PFK was also apparent at pH 8.0 but inhibition was weaker. I_{50} for ATP was 10.4, 8.2 or 12.6 mM in the presence of 1 μM fructose-2,6-P_2, 0.4 mM AMP or 10 mM P_i, respectively.

DISCUSSION

Cockroach flight muscle phosphofructokinase resembles the enzyme from other sources in many of its kinetic properties including: (a) sigmoidal fructose-6-P kinetics at pH 7 versus hyperbolic kinetics at pH 8, (b) substrate inhibition by elevated levels of ATP and (c) allosteric activation by AMP, NH_4^+ and P_i and synergism in the effects of activators (Ramaiah, 1974; Uyeda, 1979). Like PFK from other insects (Walker and Bailey, 1969; Newsholme *et al.*, 1977), but unlike the enzyme from vertebrate sources, cockroach PFK is not inhibited by citrate; in insects citrate control over glycolytic rate appears to be located in the aldolase reaction (Storey, 1980). Mammalian PFK shows product activation by ADP and fructose-1,6-P_2 (Ramaiah, 1974; Uyeda, 1979) but the cockroach enzyme was not affected by these compounds. These results contrast with those of Walker and Bailey (1969) who reported that fructose-1,6-P_2 inhibited PFK from both flight muscle and fat body of the locust. The present results also differ from those of Walker and Bailey (1969) with regard to the effects of

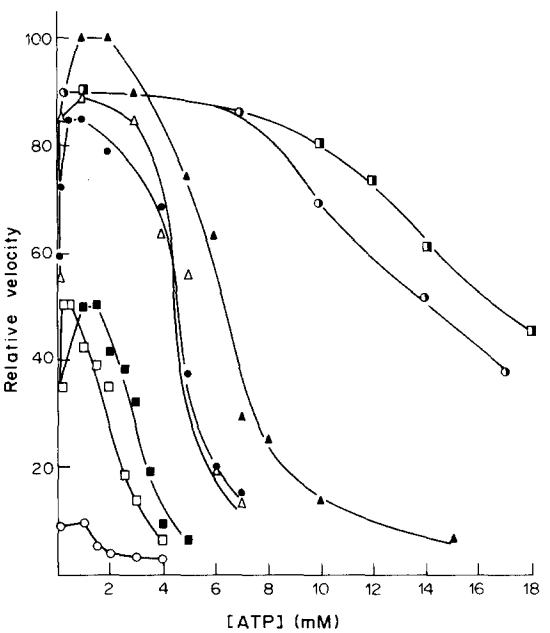

Fig. 1. Effect of ATP substrate concentration on the activity of PFK from cockroach flight muscle in the presence of enzyme activators. Assay conditions are as in Materials and Methods with 5 mM fructose-6-P and pH 7.0. Added activators are: (○) 0.1 μM fructose-2,6-P_2; (□) 0.16 mM AMP; (■) 0.40 mM AMP; (●) 1 μM fructose-2,6-P_2; (▲) 10 mM P_i; (△) 0.1 mM fructose-2,6-P_2 + 0.16 mM AMP; (◑) 1 mM NH_4^+ + 10 mM P_i + 0.16 mM AMP + 0.1 μM fructose-2,6-P_2; (◨) 3 mM NH_4^+, 10 mM P_i, 0.40 mM AMP + 0.1 μM fructose-2,6-P_2.

ammonium ion on insect PFK; their study revealed no effect of NH_4^+ on locust PFK. Cockroach PFK, however, is activated by NH_4^+ although ammonium ion, at physiological levels, is the least effective of the activators studied.

PFK from cockroach flight muscle is the first PFK from an invertebrate animal to be shown to be activated by fructose-2,6-P_2 (Storey, 1983) and with an apparent K_a of 0.1 μM this effector is the most potent of the activators of the enzyme. Fructose-2,6-P_2 and its role as an activator of PFK was first discovered in 1980 (for review see Hers and van Schaftingen, 1982) and has since been shown to activate PFK from various mammalian tissues as well as the enzyme from yeast (Hers *et al.*, 1982; Hers and van Schaftingen, 1982).

PFK function *in vivo* in cockroach flight muscle is probably strongly dependent upon the action of activators to achieve significant enzyme activity. In the absence of activators an $S_{0.5}$ for fructose-6-P (at pH 7) of 16 mM would render the enzyme essentially inactive at physiological levels of fructose-6-P which range from 0.04 to 0.11 μmol/g wet weight in flight muscle (Storey, 1983). In the absence of activators, PFK activity *in vivo* would also be strongly limited by ATP inhibition [physiological concentration = 5 μmol/g wet weight (Storey, 1983)] occurring above optimal (0.2 mM) ATP concentrations. The action of activators, however, both lowered the $S_{0.5}$ for fructose-6-P and decreased substrate inhibition by ATP as has been reported for PFK from other sources (Ramaiah, 1974). Particularly important to PFK function are the additive and synergistic effects of multiple activators; alone individual activators lowered $S_{0.5}$ for fructose-6-P by only 1.5 to 13-fold while in combination the four activators produced a 640-fold decrease in $S_{0.5}$. Synergistic effects in lowering the $S_{0.5}$ for fructose-6-P were noted in the interactions between AMP and fructose-2,6-P_2 and between AMP and P_i. Synergism between activators is well known for PFK from mammalian sources (Ramaiah, 1974; Uyeda *et al.*, 1981) and appears to be a key property for the rapid activation of PFK activity in working muscle. Synergistic effects between AMP and fructose-2,6-P_2 also occurred with respect to the release of ATP inhibition of PFK.

The initiation of flight in cockroaches requires a rapid activation of glycolytic flux to supply carbohydrate for oxidation by the Krebs cycle. As the key control point regulating the rate of use of hexose phosphates (derived from glycogen or from blood sugars), PFK must be rapidly activated to allow glycolytic flux to meet the energy demands of flight. Crabtree and Newsholme (1975) have determined that the metabolic rate of flying insects (calculated from oxygen consumption data) closely approximates the measured maximal activities of key regulatory enzymes (phosphorylase, hexokinase, phosphofructokinase); in mammalian systems enzyme capacity generally exceeds the maximal metabolic rate by about 10-fold. For cockroaches they determined a metabolic rate of 15 μmol C_6 min^{-1} g $muscle^{-1}$ at 25°C compared to a maximal PFK activity of 19 μmol fructose-6-P utilized min^{-1} g^{-1}. Thus flight requires maximal activity from the full PFK complement of the flight muscle. This can only be achieved through the combined actions of activators in lowering $S_{0.5}$ for fructose-6-P into the physiological range of fructose-6-P concentrations and relieving inhibition by physiological levels of ATP. Table 1 shows that the physiological levels of activators found in resting muscle [1 mM NH_4^+, 10 mM P_i and 0.16 mM AMP (Storey, 1983)] can reduce $S_{0.5}$ for fructose-6-P to 0.072 mM while at activator concentrations found in working muscle [3 mM NH_4^+, 10 mM P_i, 0.40 mM AMP (Storey, 1983)] $S_{0.5}$ is reduced to 0.042 mM. Addition of fructose-2,6-P_2 in either case lowers $S_{0.5}$ to 0.025–0.028 mM. Similarly the combined effects of the four activators override inhibition by ATP by raising the I_{50} for ATP to 15.3 mM under activator conditions found in resting muscle and to 18 mM under the conditions in flown muscle (Fig. 1).

Thus PFK function in cockroach flight muscle is very strongly, almost obligately, linked to the actions of activators. At the initiation of flight muscle concentrations of activators rise rapidly, AMP, fructose-2,6-P_2 and NH_4^+ levels increasing by 2.6, 2.3 and 2.8-fold, respectively (Storey, 1983). This results in a rapid and full activation of PFK and a subsequent rapid increase in glycolytic rate.

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