Regulation of cockroach flight muscle phosphofructokinase by fructose 2,6-bisphosphate

Role in the activation of muscle metabolism during flight

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Received 7 June 1983

Fructose 2,6-bisphosphate is a potent activator of cockroach flight muscle 6-phosphofructo-1-kinase; at 5 μM, fructose 2,6-P_2 lowers $S_{0.5}$ for fructose 6-P 36-fold from 16 ± 0.90 to 0.44 ± 0.14 mM. Muscle concentration of fructose 2,6-P_2 is elevated at the initiation of flight suggesting that the compound plays a key role in the activation of muscle phosphofructokinase, and therefore in the activation of glycolytic flux, during the initiation of flight.

Phosphofructokinase *Fructose 2,6-bisphosphate* *Insect flight muscle* *Activation of glycolysis*

1. INTRODUCTION

The initiation of flight in insects is accompanied by an increase in oxygen consumption of up to 100-fold and a rapid activation of flight muscle metabolism [1]. Amongst insects (e.g., flies, bees, cockroaches) which power flight by carbohydrate oxidation, the rate limiting enzyme in the activation of glycolytic flux appears to be 6-phosphofructo-1-kinase (EC 2.7.1.11). Indeed, measured maximal activities of phosphofructokinase in insect flight muscles closely approximate calculated glycolytic flux rates during flight [1]. At physiological levels of substrates, activators and inhibitors, however, enzyme activity would appear to be considerably lower than that required to support glycolytic flux during flight.

Recent studies of mammalian phosphofructokinase have revealed a potent new activator of the enzyme, fructose 2,6-bisphosphate [2–4]. Fructose 2,6-P_2 activates both liver and muscle phosphofructokinases and appears to mediate hormone effects on the enzyme [2]. I here report that fructose 2,6-P_2 is also a potent activator of insect flight muscle phosphofructokinase and demonstrate that levels of the activator are increased during flight. Fructose 2,6-P_2 may be a key effector responsible for producing a maximal activation of phosphofructokinase during the initiation of flight.

2. MATERIALS AND METHODS

Adult male cockroaches, *Periplaneta americana*, were used. Biochemicals and coupling enzymes were from Boehringer Mannheim; Affigel blue was from BioRad.

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 27000 × *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 $A_{280\ nm}$ dropped to a minimum value and then phosphofructokinase activity was

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00145793/83/$3.00

eluted in buffer containing 20 mM ATP + 0.4 mM fructose 1,6-P_2. After dialysis against two changes of the equilibration buffer, the phosphofructokinase preparation was used for kinetic studies and for the assay of fructose 2,6-P_2 levels. The enzyme was purified 8.5-fold removing contaminating activities of ATPase, NADH oxidase, phosphoglucoisomerase and fructose 2,6-bisphosphatase.

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 dialyzed coupling enzymes: 0.2 units aldolase, 1.2 units triosephosphate isomerase and 0.16 units glycerol 3-P dehydrogenase. Assays were performed at 23°C; kinetic constants were determined from Hill plots.

For measurement of muscle metabolite levels cockroaches were flown for 20 s and then immediately frozen in liquid nitrogen. For controls, rested animals in mesh containers were rapidly immersed in liquid nitrogen. Frozen thoraces were ground to a powder under liquid nitrogen and perchloric acid extracts of the tissues were made [5]. Metabolites were measured by enzymatic assay [6], arginine phosphate as in [5]. For the determination of fructose 2,6-P_2 levels, frozen powdered thoraces were extracted in alcoholic KOH as in [7] and fructose 2,6-P_2 was quantitated using a modification of the assay in [3]. Assay conditions were 50 mM imidazole buffer (pH 7.4), 1 mM fructose 6-P, 1 mM ATP, 0.15 mM NADH, 0.2 mM EDTA, 1 mM NH_4Cl, 2.5 mM dithiothreitol, cockroach phosphofructokinase and coupling enzymes as above. Fructose 2,6-P_2 levels in samples were determined by comparison to a standard curve relating fructose 2,6-P_2 (5–50 pmol) to the percentage activation of phosphofructokinase.

3. RESULTS

3.1. *Fructose 2,6-P_2 effects on cockroach phosphofructokinase*

Cockroach phosphofructokinase shows sigmoidal fructose 6-P kinetics at pH 7 with $S_{0.5}$ = 16 ± 0.9 mM and a Hill coefficient, h, of 2.3 ± 0.4. Fructose 2,6-P_2 is a strong positive modulator affecting $S_{0.5}$ for fructose 6-P. The addition of fructose 2,6-P_2 at 0.1, 1.0 or 5.0 μM lowered $S_{0.5}$ to 9.3 ± 0.9 (h = 2.1 ± 0.4), 1.2 ± 0.3 (h = 2.7 ± 0.6) or 0.44 ± 0.14 mM (h = 1.8 ± 0.2) but had no significant effect on h. At 5 μM $S_{0.5}$ for fructose 6-P was lowered 36-fold.

The apparent activation constant, K_a, for fructose 2,6-P_2 (at constant substrate concentrations, 0.2 mM fructose 6-P and 0.2 mM ATP) was 0.97 μM (fig.1). At higher fructose 6-P levels (10 mM) this was reduced to 0.1 μM. The addition of other positive modulators of phosphofructokinase to the assay reduced the apparent K_a for fructose 2,6-P_2 (fig.1). AMP and fructose 2,6-P_2 showed a strong synergistic interaction with 0.16 and 0.40 mM AMP reducing the apparent K_a for fructose 2,6-P_2 to 0.019 and 0.0045 μM, respectively. NH_4^+ at 1 and 3 mM lowered the apparent K_a to 0.86 and 0.70 μM, respectively, while 10 mM inorganic phosphate reduced K_a to 0.02 μM but also strongly depressed the maximal velocity of the enzyme.

3.2. *Levels of intermediary metabolites in cockroach flight muscle*

Table 1 shows the concentrations of metabolites in the flight muscle of rested *vs* flown cockroaches. After 20 s of flight, arginine phosphate and ATP levels had decreased by 2.2 and 17.7%, respective-

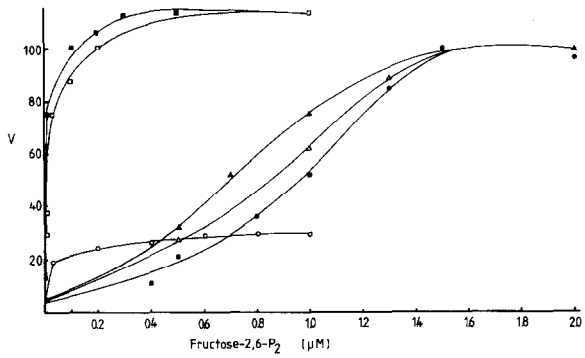

Fig.1. Activation of cockroach phosphofructokinase by fructose 2,6-P_2 and interaction with other positive modulators. Assay conditions are: 50 mM imidazole buffer (pH 7.0), 0.2 mM fructose 6-P, 0.2 mM ATP, 0.1 mM NADH, 5 mM $MgCl_2$, 50 mM KCl and excess dialyzed coupling enzymes. (●) Control (minus other added effectors); (△) plus 1 mM NH_4^+; (▲) plus 3 mM NH_4^+; (○) plus 10 mM inorganic phosphate; (□) plus 0.16 mM AMP; and (■) plus 0.40 mM AMP.

Table 1

Metabolite concentrations in cockroach flight muscle

Metabolite	Concentration (μmol/g wet wt)	
	Rested	Flown
Arginine phosphate	9.2 ± 0.55	9.0 ± 0.45
ATP	5.1 ± 0.20	4.2 ± 0.39
ADP	1.5 ± 0.09	1.85 ± 0.17
AMP	0.17 ± 0.03	0.45 ± 0.05
P_i	9.9 ± 0.57	9.0 ± 0.76
Fructose 6-P	0.11 ± 0.01	0.04 ± 0.006
Fructose 1,6-P_2	0.10 ± 0.012	0.25 ± 0.03
Fructose 2,6-P_2[a]	1.19 ± 0.08	2.69 ± 0.32
NH_4^+	0.9 ± 0.11	2.5 ± 0.22

[a] Fructose 2,6-P_2-values are in nmol/g wet wt

Data are means ± SEM for $n = 6$ rested and $n = 8$ flown cockroaches

ly, with a corresponding rise in ADP and AMP content. Fructose 6-P content was decreased at the initiation of flight while fructose 1,6-P_2 levels increased; this crossover indicates an activation of phosphofructokinase during flight. Fructose 2,6-P_2 content of the muscle rose from 1.19 nmol/g at rest to 2.69 nmol/g in exercised muscle, an increase of 126%. The levels of other activators, AMP and NH_4^+, rose by 165 and 178%, respectively.

3.3. *Rest* vs *flight simulation and the effects on phosphofructokinase in vitro*

The importance of fructose 2,6-P_2 to the activation of phosphofructokinase in vivo was estimated in vitro by using assay conditions of substrates and positive modulators which mimicked the intracellular conditions in which the enzyme functions. Table 2 shows the effects of simulated rest *vs* flight conditions on the $S_{0.5}$ for fructose 6-P. Under resting conditions, in the absence of fructose 2,6-P_2, $S_{0.5}$ was 0.27 mM. This fell to 0.20 mM under flight assay conditions, the decrease probably due to elevated AMP levels. These affinity constants are 2.5-times greater under resting and 5-times greater under flight conditions than the measured levels of fructose 6-P in flight muscle (table 1). Addition of physiological concentrations of fructose 2,6-P_2, however, lowered the $S_{0.5}$ to 0.047–0.049 mM under resting conditions and to 0.034–0.036 mM under flight conditions. Under the flown assay conditions, this

Table 2

Effect of simulated rest *vs* flight assay conditions on phosphofructokinase affinity for fructose 6-P ($S_{0.5}$, mM)

Assay conditions	Fructose 2,6-P_2 concentration (μM)		
	0	1.0	5.0
Rest 5 mM ATP 1 mM NH_4^+ 0.16 mM AMP 10 mM P_i	0.27 ± 0.04 ($h = 1.7$)	0.047 ± 0.004 ($h = 1.6$)	0.049 ± 0.006 ($h = 2.0$)
Flight 5 mM ATP 3 mM NH_4^+ 0.4 mM AMP 10 mM P_i	0.20 ± 0.06 ($h = 1.6$)	0.036 ± 0.002 ($h = 1.2$)	0.034 ± 0.005 ($h = 1.2$)

Assays were performed in 50 mM imidazole buffer, pH 7.0. Results are means ± SEM for $n = 3$ determinations

lowered the $S_{0.5}$ for fructose 6-P into the range of the measured concentrations of fructose 6-P in flown flight muscle.

4. DISCUSSION

Fructose 2,6-P_2 activates phosphofructokinase from a number of mammalian tissues including liver, skeletal muscle and pancreatic islets and also affects the enzyme from yeast [2–4]. This study provides the first demonstration that fructose 2,6-P_2 is also a potent activator of phosphofructokinase from an invertebrate, specifically the insect flight muscle enzyme. The effects of fructose 2,6-P_2 on flight muscle phosphofructokinase include a lowering of the $S_{0.5}$ for fructose 6-P, synergistic interactions with the effects of other activators (most notably AMP) on the enzyme and a release of enzyme inhibition by high levels of ATP (not shown). These effects have also been shown for the mammalian enzyme [3].

The metabolic rate observed during flight in the cockroach (15 μmol $C_6.min^{-1}.g$ $muscle^{-1}$ at 25°C) requires essentially maximal activation of phosphofructokinase activity (19 units/g) to support the required glycolytic flux [1]. However, physiological concentrations of substrates (ATP, fructose 6-P) and effectors (AMP, NH_4^+, P_i) in flight muscle (table 1) would seem insufficient to achieve a full activation of phosphofructokinase in vivo during flight. Similar conclusions were reached in [8] for mammalian liver phosphofructokinase; they concluded that under physiological ATP concentrations, the levels of positive effectors of phosphofructokinase in the cell were insufficient to account for the required enzyme activity at the levels of fructose 6-P found in vivo. Fructose 2,6-P_2 appears to be the missing activator required for maximal activation of phosphofructokinase in vivo.

In insect flight muscle fructose 2,6-P_2 acting along with AMP are likely the key effectors producing the activation of phosphofructokinase at the initiation of flight. The levels of both effectors increase rapidly in flight and both effectors would be required to fully activate phosphofructokinase and allow maximal glycolytic flux. The experiments simulating intracellular substrate and effector concentrations demonstrate that in the absence of fructose 2,6-P_2, $S_{0.5}$ for fructose 6-P is several times higher than measured levels of the substrate in vivo. With the addition of physiological concentrations of fructose 2,6-P_2, however, enzyme affinity for fructose 6-P is increased with $S_{0.5}$ for fructose 6-P lowered into the range of fructose 6-P concentrations in vivo in flight muscle.

Studies of fructose 2,6-P_2 metabolism in mammalian systems have shown that levels of the compound are modulated in response to hormonal stimulation, glucagon producing a decrease in fructose 2,6-P_2 levels while insulin and adrenaline increase the concentrations of the activator [2]. Electrical stimulation of rat skeletal muscle has been shown to decrease levels of fructose 2,6-P_2 [2]. This study links fructose 2,6-P_2 levels and muscle work demonstrating that fructose 2,6-P_2 content of muscle can be altered within seconds of the initiation of flight. This suggests that, at least in insect muscle, hormone-independent mechanisms could alter the concentration of this activator.

ACKNOWLEDGEMENTS

The excellent technical assistance of Keith B. Male is gratefully acknowledged. Cockroaches were kindly provided by Ms C.I. McGregor Smith, Research Centre, Agriculture Canada, London, Ontario. Supported by an NSERC operating grant.

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