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Kinetic and regulatory properties of pyruvate kinase isozymes from flight muscle and fat body of the cockroach, *Periplaneta americana*

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Summary. Pyruvate kinases from flight muscle and fat body of the cockroach, *Periplaneta americana,* were purified to homogeneity. The two tissues contained different forms of the enzyme which were separable by starch gel electrophoresis and isoelectric focusing (pI = 5.75 for flight muscle and 6.15 for fat body). Both enzymes had molecular weights of 235,000 ± 20,000.

Flight muscle pyruvate kinase displayed Michaelis-Menten kinetics with respect to both ADP and P-enolpyruvate with K_m values of 0.27 and 0.04 mM, respectively. K_m for Mg^{2+} was 0.60 mM and K_a for K^+ was 15 mM. The enzyme was weakly inhibited by four compounds, ATP, arginine-P, L-alanine and citrate with apparent K_i values of 3.5, 15, 20 and 24 mM, respectively. Competitive inhibition by 3 mM ATP or 10 mM arginine-P raised the K_m for P-enolpyruvate to 0.067 or 0.057 mM. Fructose-1,6-P_2 did not activate the enzyme but reversed inhibitions by ATP and arginine-P.

Fat body pyruvate kinase showed sigmoidal kinetics with respect to P-enolpyruvate with $S_{0.5}$ = 0.32 mM and $n_H = 1.43$. K_m values for ADP and Mg^{2+} were 0.30 and 0.80 mM, respectively with a K_a for K^+ of 10 mM. ATP and L-alanine were inhibitors of the enzyme; 2 mM ATP raised $S_{0.5}$ for P-enolpyruvate to 0.48 mM while 3 mM L-alanine increased $S_{0.5}$ to 0.84 mM. Neither citrate nor arginine-P inhibited the enzyme but citrate affected the enzyme by reversing L-alanine inhibition. Fat body pyruvate kinase was strongly activated by fructose-1,6-P_2 with an apparent K_a of 1.5 M. Fructose-1,6-P_2 at 0.1 mM reduced $S_{0.5}$ for P-enolpyruvate to 0.05 mM and n_H to 1.0.

Flight muscle and fat body pyruvate kinases from the cockroach show properties analogous to those of the muscle and liver forms of mammalian pyruvate kinase. Fat body pyruvate kinase is suited for on-off function in a tissue with a gluconeogenic capacity. Strong allosteric control with a feed-forward activation by fructose-1,6-P_2 is key to coordinating enzyme function with glycolytic rate. The function of flight muscle pyruvate kinase in energy production during flight is aided by a low K_m for P-enolpyruvate, weak inhibitor effects by high energy phosphates and deinhibition of these effects by fructose-1,6-P_2.

Introduction

Pyruvate kinase (E.C. 2.7.1.40) is a key site in the control of glycolysis in tissues. The enzyme occurs in tissue specific isozymic forms. In mammals liver (L) and muscle (M) have distinct forms with a third form (called M_2 or K) occurring in kidney, lung and various other tissues (Ibsen 1977; Hall and Cottam 1978). Some authors classify erythrocyte pyruvate kinase as a fourth isozyme (Hall and Cottam 1978); others feel it is the L type enzyme (Ibsen 1977). M type pyruvate kinase exhibits Michaelis-Menten kinetics but the L type shows sigmoidal kinetics with respect to P-enolpyruvate, is controlled by allosteric effectors and undergoes covalent modification by phosphorylation and dephosphorylation (Ibsen 1977; Hall and Cottam 1978). The complicated regulatory mechanisms of the L isozyme allow efficient on-off control of pyruvate kinase in tissues which carry out both glycolytic and gluconeogenic functions.

Pyruvate kinase from a number of invertebrate sources has been characterized. In general enzymes analogous to the L or M forms of mammalian pyruvate kinase have been found. Hepatopancreas and hypodermis of crustaceans, both multifunctional gluconeogenic tissues, contain an allosteric pyruvate kinase strongly activated by fructose-1,6-

P_2 (Lesicki 1976; Guderley and Hochachka 1977; Giles et al. 1977). Pyruvate kinase in aerobic muscles (cephalopod mantle muscle, crustacean leg muscle, insect flight muscle) resembles the mammalian M type (Storey and Hochachka 1975; Guderley et al. 1976; Lesicki 1976; Bailey and Walker 1969) but the enzyme from muscle of anoxia-tolerant molluscs shows activation by fructose-1,6-P_2 and inhibition by L-alanine (Mustafa and Hochachka 1971; de Zwaan 1972).

Previous studies of insect pyruvate kinase have been limited to an examination of selected properties of the unpurified enzymes (Bailey and Walker 1969), to species which apparently maintain only a single form of the enzyme in both muscle and fat body (Hoffmann 1975) and to the larval form (Hoffmann 1977). The present study examines the kinetic and regulatory properties of pyruvate kinase isozymes purified to homogeneity from the flight muscle and fat body of the cockroach, *Periplaneta americana*.

Materials and methods

Chemicals and animals. Biochemicals were purchased from Sigma Chemical Co., Ampholines were from LKB Products, and Sephadex G-200, Sepharose 6B, Sephacryl S-300 and Blue Dextran Sepharose were from Pharmacia Fince Chemicals. Adult male *Periplaneta americana* were obtained from Carolina Biological Supply Co. (Burlington, N.C.) and were held at 23 °C and fed rat chow and water ad lib.

Enzyme assay. Pyruvate kinase activity was measured by coupled enzyme assay using an Pye-Unicam SP 1800 recording spectrophotometer with water jacketed cell holder for maintaining assay temperature constant at 23 °C. Standard assays were performed using 20 mM imidazole buffer, pH 7.5 and optimal conditions were 5 mM $MgCl_2$, 50 mM KCl, 0.10 mM NADH, 2 mM ADP, excess dialyzed lactate dehydrogenase and 1 mM or 5 mM P-enolpyruvate for the flight muscle or fat body enzyme, respectively. In experiments involving enzyme inhibition, the product sampling assay of Newsholme et al. (1970) was used to ensure that metabolite effects were specific for pyruvate kinase and did not affect the coupling enzyme. Kinetic constants were determined from direct linear (Cornish-Bowden 1979), Hill or Dixon plots. All kinetic constants are the means of $n=4$ determinations with reproducibility $\pm 10\%$ using $n=2$ separate preparations of each purified enzyme.

Purification of fat body pyruvate kinase. Fat bodies were homogenized 1:3 w/v in ice cold buffer A: 25 mM imidazole, pH 7.5 (at 23 °C) containing 20 mM 2-mercaptoethanol. The homogenate was centrifuged at 35,000 *g* for 30 min at 4 °C. The supernatant was removed and polyethylene glycol (m.w. 6,000) was added to a concentration of 10% w/v. After stirring for 1 h at 23 °C, the solution was centrifuged as above. The supernatant was discarded and the pellet was resuspended in buffer A and loaded onto a column (1 × 5 cm) of Blue Dextran Sepharose equilibrated in buffer A (all purification steps were performed at 23 °C). The column was washed with buffer until the A_{280} nm of the effluent dropped to near zero. Pyruvate kinase was then eluted from the column with a gradient of KCl (0 to 1 M) in buffer A; the enzyme eluted at 280 mM KCl. Fractions containing pyruvate kinase activity were pooled and dialyzed against buffer A for 2 h. The enzyme was then loaded onto a second column of Blue Dextran Sepharose equilibrated in buffer A and washed as above. Pyruvate kinase was eluted in buffer A containing 20 mM ATP + 2 mM fructose-1,6-P_2. Peak fractions were pooled and applied to a column (1 × 40 cm) of Sephacryl S-300 equilibrated in buffer A. Peak fractions were collected, pooled and used for kinetic study. The enzyme was stable for at least one week at 3 °C.

Purification of flight muscle pyruvate kinase. Flight muscle was homogenized 1:5 w/v in ice-cold buffer A and centrifuged as above. The supernatant was removed and polyethylene glycol (m.w. 6,000) was added to a concentration of 8% w/v. After stirring at 23 °C for 1 h, the preparation was centrifuged as above. The supernatant containing pyruvate kinase activity was saved and was adjusted to 12% w/v with polyethylene glycol. After stirring for 1 h, the solution was centrifuged and the supernatant was discarded. Pyruvate kinase activity in the pellet was resuspended in buffer A and applied to a column (1 × 5 cm) of Blue Dextran Sepharose equilibrated in buffer A. The column was washed with buffer until $A_{280\ nm}$ fell to near zero and the pyruvate kinase activity was eluted with a gradient containing Mg.ATP + fructose-1,6-P_2 (concentration ratio 10:1). Pyruvate kinase eluted at a concentration of 10 mM/Mg.ATP and 1 mM fructose-1,6-P_2. Peak fractions were pooled and run on a column of Sephacryl S-300 as above. Peak fractions of purified enzyme were pooled for kinetic study. The enzyme was stable for at least one week at 3 °C.

Protein concentrations were measured by the method of Waddell (1956).

Covalent modification of pyruvate kinase. Rested cockroaches were compared with those which were flown for 30 s. Animals were quickly frozen by immersion in liquid nitrogen. Frozen flight muscle and fat body were dissected out under liquid nitrogen and were ground to a powder using a mortar and pestle. Powdered tissues were homogenized in ice-cold 100 mM imidazole buffer, pH 7.0 containing 0.1 mM phenylmethylsulphonyl fluoride, 5 mM EDTA, 5 mM EGTA, 100 mM NaF and 15 mM 2-mercaptoethanol using a Polytron homogenizer. The homogenate was centrifuged as above and the supernatant was used as the source of enzyme.

Electrophoresis and isoelectrofocusing. Isoelectrofocusing was carried out using a 110 ml LKB 8101 column and a pH 3.5 to 10 gradient of Ampholines. Both crude supernatant and purified pyruvate kinase were run at 700 V for 18–24 h at 4 °C. Columns were drained, 1 ml fractions were collected and enzyme activity was assayed using optimal assay conditions.

Vertical starch gels of crude enzyme preparations were run by the method of Dando (1970). Eleven percent starch gels were run overnight at 290 V (19 mamp) with Tris-borate, pH 8.7 containing 0.1 mM fructose 1,6-P_2 as the electrode buffer. Staining was performed using 2% agar overlays and the negative stain described by Scopes (1968) with visualization under UV light.

SDS polyacrylamide gel electrophoresis of the purified enzymes was run according to the method of Weber and Osborn (1969).

Molecular weight determination. Molecular weight determinations were performed as described by Storey (1977) on columns of Sephadex G-200 and Sepharose 6B (both 1 × 40 cm) at 4 °C using buffer A as the elution buffer.

Results

Purification and physical properties of pyruvate kinase isozymes from flight muscle and fat body

The activity of pyruvate kinase in cockroach flight muscle was 177 µmol substrate utilized per min·g wet weight (2.6 U/mg soluble protein) while fat body had an activity of 77 U/g wet weight (0.64 U/mg protein). Purification resulted in final specific activities of 400–450 U/mg protein for flight muscle pyruvate kinase and 420 U/mg for the fat body enzyme with yields of 43 and 24%, respectively. Both enzymes were purified to homogeneity as judged by a single band on SDS polyacrylamide gel electrophoresis stained for protein.

Sephadex G-200 and Sepharose 6B chromatography of crude supernatants or purified enzyme showed a molecular weight of 235,000 + 20,000 for both flight muscle and fat body pyruvate kinase.

Isoelectric focusing revealed isoelectric points (pI) of 5.75 ± 0.1 and 6.15 ± 0.15 (both $n = 4$ runs) for the flight muscle and fat body enzymes, respectively, using either crude or purified enzyme. The enzymes from the two tissues were also separated by starch gel electrophoresis. Under the conditions employed both run anodally to a distance from the origin of 6.5 cm for flight muscle and 3.0 cm for fat body pyruvate kinase.

Covalent modification

Pyruvate kinase from a number of sources can undergo phosphorylation/dephosphorylation reactions in response to physiological stress (Ibsen 1977). Covalent modification is reflected in a change in enzyme affinity for P-enolpyruvate, the phosphorylated enzyme having a much lower affinity for this substrate. Pyruvate kinase from flight muscle and fat body of rested versus flown cockroaches was examined. Neither enzyme showed a change in enzyme affinity for P-enolpyruvate in response to 30 s flight.

Kinetic properties

pH effects. Fig. 1 shows pH profiles for fat body (A) and flight muscle (B) pyruvate kinase. Activities of both enzymes varied with the buffer used with activity in P_i > imidazole > Tris buffer. The pH optimum for both enzymes was 7.0 in P_i buffer, and 7.5 in Tris buffer. The optimum in imidazole buffer was 7.0 for flight muscle and 6.5 for fat body pyruvate kinase. The addition of fructose-1,6-P_2 did not affect the optimum for flight muscle but for fat body pH optimum was shifted to 7.0 in imidazole buffer in the presence of fructose-1,6-P_2. The activity of fat body pyruvate kinase was also greatly increased by addition of fructose-1,6-P_2.

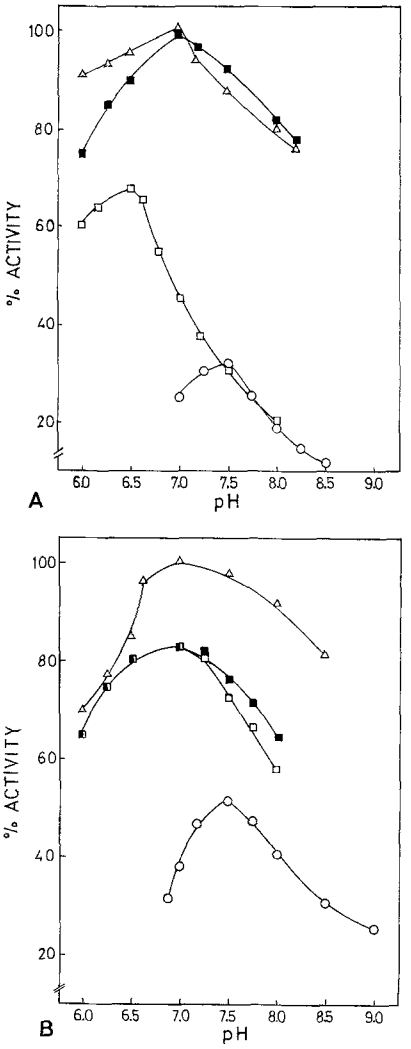


Fig. 1. pH profiles of fat body (**A**) and flight muscle (**B**) pyruvate kinase. Assays used subsaturating levels of substrates, 0.5 mM P-enolpyruvate and 0.4 mM ADP with other conditions as in Materials and methods. Relative activity is plotted for △, 50 mM P_i buffer; ○, 50 mM Tris-HCl buffer; □, 50 mM imidazole-HCl buffer; and ■, 50 mM imidazole-HCl buffer containing 0.02 mM fructose-1,6-P_2

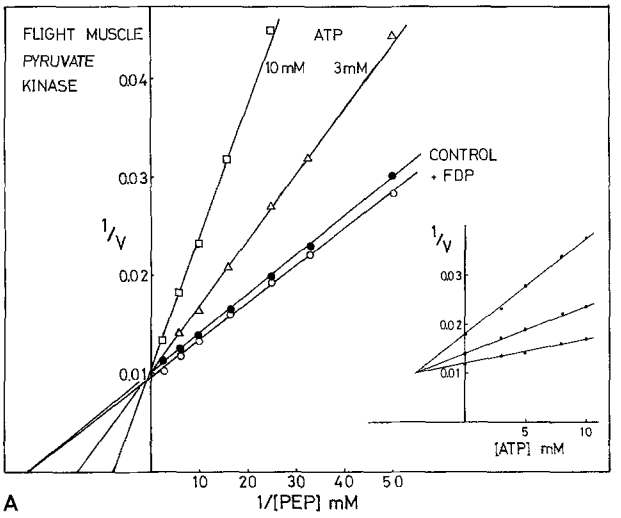
Flight muscle pyruvate kinase – substrate affinities. Enzyme kinetics were hyperbolic with respect to both substrates with K_m's of 0.27 and 0.04 mM for ADP and P-enolpyruvate, respectively. Michaelis constants for divalent cations (all tested as Cl^- salts) were 0.60, 0.12 and 0.35 mM for Mg^{2+}, Mn^{2+} and Co^{2+}, respectively, with relative maximal velocities of 100, 60 and 50 percent for the three cations. Potassium was required for enzyme

activity; the K_a was 15 mM. NH_4^+ could replace K^+ with a K_a of 2 mM but a maximal velocity only 60% of that with K^+.

Effectors. Flight muscle pyruvate kinase was only weakly affected by metabolite modulators. ATP and arginine-P were non-competitive inhibitors with respect to ADP but were competitive with respect to P-enolpyruvate. ATP at 3 and 10 mM raised the K_m for P-enolpyruvate from 0.04 to 0.067 and 0.13 mM, respectively, while arginine-P at 10 and 20 mM raised K_m to 0.057 and 0.063 mM, respectively (Fig. 2). Alanine and citrate were weak non-competitive inhibitors with respect to P-enolpyruvate. Apparent K_i's for the inhibitors, with respect to P-enolpyruvate, were 3.5, 15, 20 and 24 mM for ATP, arginine-P, alanine and citrate, respectively. These inhibitors showed no synergistic interactions in their effects on the enzyme. Ca^{2+} inhibited the flight muscle enzyme with a K_i of 0.03 mM with respect to Mg^{2+} and 0.8 mM with respect to Mn^{2+}. Phenylalanine (1 mM) did not inhibit the enzyme nor did several other amino acids including isoleucine (1 mM), glutamate, valine and serine (all 5 mM). Fructose-1,6-P_2, at levels of up to 0.8 mM, did not alter the K_m's for either ADP or P-enolpyruvate. But while not having a direct activating effect on the enzyme, the compound effectively reversed enzyme inhibition by ATP or arginine-P (Fig. 2). Inorganic phosphate also activated the enzyme with a K_a of 3 mM.

Fat body pyruvate kinase – substrate affinities. Kinetics with respect to ADP were hyperbolic with a K_m of 0.30 mM but in contrast to the flight muscle enzyme, P-enolpyruvate kinetics for the fat body enzyme were sigmoidal with an $S_{0.5}$ of 0.32 mM and a Hill coefficient, n_H, of 1.43. K_m values for Mg^{2+}, Mn^{2+} and Co^{2+} (all tested as Cl^- salts) were 0.8, 0.2 and 0.9 mM, respectively, with relative maximal velocities of 100, 75 and 40 for the three cations. K_a for K^+ was 10 mM and for NH_4^+ was 2 mM.

Effectors. Unlike flight muscle pyruvate kinase, the fat body enzyme was strongly affected by a variety of metabolite modulators. Alanine and ATP inhibited the enzyme with effects on the $S_{0.5}$ and n_H for P-enolpyruvate (Fig. 3). At 3 mM alanine, $S_{0.5}$ was increased by nearly 3 fold while 2 mM ATP raised $S_{0.5}$ by 1.5 fold (Table 1). Effects of the two inhibitors were additive. Ca^{2+} inhibited with a K_i of 0.45 mM with respect to Mg^{2+}. Fructose-1,6-P_2 was an effective activator of the fat body



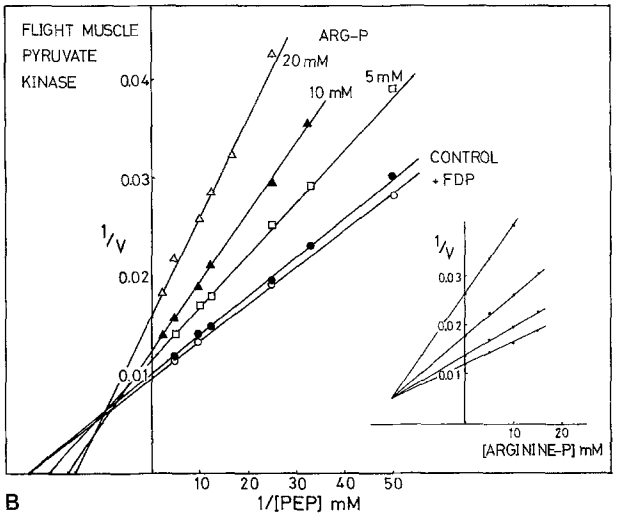


Fig. 2A, B. Double reciprocal plots of the reaction velocity of purified flight muscle pyruvate kinase versus P-enolpyruvate concentration and effects of modulators. Assays used subsaturating levels (0.4 mM) of ADP, varying concentrations of P-enolpyruvate with other conditions as in Materials and Methods. **A** Effects of ATP. ●, control; △, plus 3 mM Mg.ATP; □, plus 10 mM Mg.ATP; ○, all preceding conditions in the presence of 0.02 mM fructose-1,6-P_2. Inset is a Dixon plot of ATP inhibition data with P-enolpyruvate at 0.05, 0.10 and 0.20 mM. **B** Effects of arginine-P. ●, control; □, plus 5 mM arginine-P; ▲, plus 10 mM arginine-P; △, plus 20 mM arginine-P; ○, all preceding conditions in the presence of 0.02 mM fructose-1,6-P_2. Inset is a Dixon plot of the arginine-P inhibition data with P-enolpyruvate at 0.025, 0.05, 0.10 and 0.20 mM

enzyme; at 0.1 mM fructose-1,6-P_2 $S_{0.5}$ for P-enolpyruvate was lowered by 6 fold and n_H was reduced to 1.0 (Table 1). The apparent K_a for fructose-1,6-P_2 (at 2 mM ADP and 0.3 mM P-enolpyruvate) was 1.5 μM. Fructose-1,6-P_2 (at 0.02 mM) also completely overrode inhibitions by ATP and alanine (Fig. 3). P_i activated the fat body enzyme with a K_a of 7 mM. Citrate had no effect on the enzyme

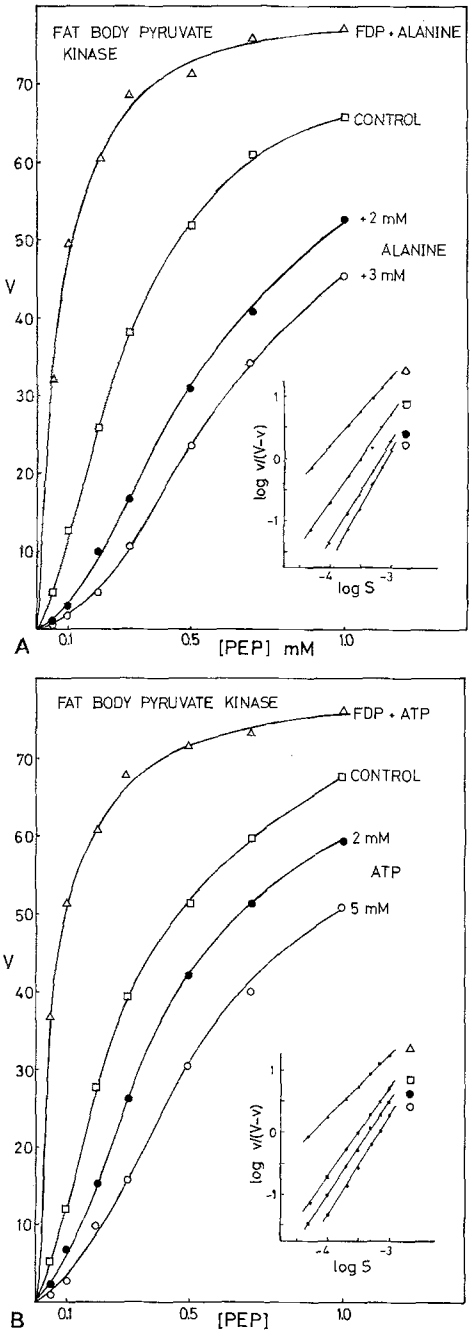


Fig. 3A, B. Effects of L-alanine, ATP, and fructose-1,6-P_2 on P-enolpyruvate saturation kinetics for purified fat body pyruvate kinase. Reaction conditions are as in Fig. 2. **A** Effects of L-alanine. □, control; ●, plus 2 mM alanine; ○, plus 3 mM alanine; △, all preceding conditions in the presence of 0.02 mM fructose-1,6-P_2. **B** Effects of ATP. Symbols are: □, control; ●, plus 2 mM Mg.ATP; ○, plus 5 mM Mg.ATP; △, all preceding conditions plus 0.02 mM fructose-1,6-P_2. Insets are Hill plots of the data

itself but selectively reversed alanine inhibition (but not ATP inhibition) of the enzyme (Table 1). Arginine-P did not inhibit the fat body enzyme at levels up to 20 mM nor was the enzyme affected by glutamate, valine or serine (all 5 mM), isoleucine (1 mM) or phenylalanine (0.1 mM).

Table 1. Effect of modulators on $S_{0.5}$ (mM) and n_H for phosphoenolpyruvate of purified fat body pyruvate kinase

	$S_{0.5}$	n_H
Control	0.32	1.43
+0.1 mM fructose-1,6-P_2	0.05	1.02
+2 mM ATP	0.48	1.51
+3 mM L-alanine	0.84	1.82
+3 mM L-alanine and 5 mM citrate	0.32	1.43

Citrate was added with Mg^{2+} in a ratio of 1:2

Discussion

Flight muscle and fat body of the cockroach contain distinct forms of pyruvate kinase. The two isozymes are distinguishable by physical means (gel electrophoresis, isoelectrofocusing) and have distinct kinetic differences. Flight muscle pyruvate kinase displays properties resembling those of the mammalian muscle isozyme (Hall and Cottam 1978), in particular the occurrence of Michaelis-Menten kinetics with respect to both substrates. Fat body pyruvate kinase, however, shows many of the properties of the mammalian liver type enzyme (Hall and Cottam 1978), including sigmoidal P-enolpyruvate kinetics and strong allosteric regulation by metabolite modulators. In a previous study of insect pyruvate kinase, Bailey and Walker (1969) also reported kinetic differences between the partially purified enzymes from flight muscle and fat body of the locust, *Schistocerca gregaria* including strong allosteric activation of the fat body enzyme by fructose-1,6-P_2 but no activation of the flight muscle enzyme. Pyruvate kinase from the house cricket, *Acheta domesticus*, (thorax muscle and fat body combined) showed activation by fructose-1,6-P_2 and inhibition by L-alanine and ATP (Hoffmann 1975). The apparent presence of only a single form of the enzyme in the tissues of the cricket is at odds with the findings of the present study and those of Bailey and Walker (1969). However crickets are non-flying insects and the properties of muscle pyruvate kinase in these animals may differ from those of the cockroach and locust.

Flight in insects is powered by aerobic oxidation of substrates with a 50–100 fold increase in oxygen consumption observed during take-off. In the cockroach the principal fuel for flight is the glycogen contained in the flight musculature (Downer and Parker 1979). The initiation of flight demands a rapid activation of glycogenolysis. Con-

trol of this metabolic activation rests at three key non-equilibrium enzyme loci (phosphorylase, phosphofructokinase, and pyruvate kinase). Activation of phosphorylase during take-off is probably the result of a Ca^{2+} mediated conversion of phosphorylase *b* to *a* (Steele 1981). Phosphofructokinase is activated by the effects of allosteric modulators, particularly the actions of AMP and fructose-2,6-P_2 (Storey 1983). Increased flux through the pyruvate kinase locus during the initiation of flight probably results largely from changes in substrate availability as the present study suggests that allosteric regulatory controls on the enzyme are relatively weak. The enzyme has low K_m's for both substrates and the K_m's are well within the physiological range of ADP and P-enolpyruvate levels in flight muscle in vivo (Beis and Newsholme 1975; Storey 1983). The initiation of flight elevates ADP levels in muscle (Storey 1983) while activation of glycogenolysis would increase the availability of P-enolpyruvate. These changes coupled with the high activities of pyruvate kinase in flight muscle would readily allow increased flux through this locus. Regulatory effects by metabolite modulators may also promote the activation of pyruvate kinase. ATP and arginine-P inhibit cockroach flight muscle pyruvate kinase with K_i's of 3.5 and 15 mM, respectively, compared to levels of these two compounds in resting flight muscle of 5.1 and 9.2 μmol/g wet weight (Storey 1983). Levels of both compounds decrease somewhat during the initiation of flight (ATP to 4.2 and arginine-P to 9.0 μmol/g (Storey 1983)) and would result in a decrease in the inhibitory effects on pyruvate kinase. In addition, fructose-1,6-P_2 levels are typically elevated at the initiation of muscle work (Storey 1983) and this would help override the inhibitory effects of the high energy phosphates.

Fat body is the major organ carrying out the synthesis, interconversion and storage of carbohydrate, lipid and protein reserves of the insect (Bailey 1975). The organ carries out many of the functions associated with liver and adipose tissue in mammals and as such has a pyruvate kinase isozyme, resembling the mammalian L form, which appears suited for on-off function in a biosynthetic tissue. The metabolic pathways of fat body are multifunctional with some operations requiring an active pyruvate kinase (ex. lipid synthesis from carbohydrate) and some requiring that pyruvate kinase be inactivated (ex. gluconeogenesis). Enzyme activity is therefore tightly regulated by allosteric effectors. In the absence of effectors, fat body pyruvate kinase has a very low affinity for P-enolpyruvate ($S_{0.5} = 0.32$ mM), the affinity constant being 8 fold higher than that of the flight muscle enzyme. When glycolytic activity is required, however, the enzyme can be potently activated by feed-forward activation by fructose-1,6-P_2, the product of the phosphofructokinase reaction. Allosteric activation of the enzyme by fructose-1,6-P_2 is also a key property of the L type mammalian pyruvate kinase (Hall and Cottam 1978), of fat body pyruvate kinase from other insects (Bailey and Walker 1969) and of pyruvate kinase from gluconeogenic tissues of other invertebrates (Guderley and Hochachka 1977). Fructose-1,6-P_2 activation of pyruvate kinase in fat body would facilitate glycolytic energy production and the synthesis of lipid reserves from carbohydrate. To allow gluconeogenesis, however, the glycolytic pathway must be inhibited to prevent futile cycling of carbon. At the pyruvate kinase site this inhibition is achieved by strong inhibition of the enzyme by L-alanine as is also seen for the mammalian L type enzyme. Citrate reversal of alanine inhibition would aid in enzyme activation for lipid biosynthesis as citrate levels are typically elevated during fatty acid synthesis.

The liver isozyme of pyruvate kinase in mammals is a substrate for phosphorylation by cAMP-dependent protein kinase. Phosphorylation decreases enzyme activity in vivo by decreasing enzyme affinity for P-enolpyruvate. In liver this occurs in response to starvation or to stimulation with glucagon. The present study found no evidence (as determined by enzyme affinity for P-enolpyruvate) to suggest that phosphorylation/dephosphorylation changes were involved in the activation of muscle pyruvate kinase during the initiation of flight or that fat body pyruvate kinase underwent covalent modification during the rest-to-flight transition. Fat body pyruvate kinase may, however, undergo phosphorylation/dephosphorylation modifications in response to other physiological stresses such as starvation.

The validity of phosphagen effects on pyruvate kinase have been questioned recently. Creatine phosphate inhibition of mammalian muscle pyruvate kinase has been reported (Kemp 1973) while arginine phosphate inhibits pyruvate kinase from a number of invertebrate sources (Guderley et al. 1976; Storey 1981). However, de Zwaan und Ebberink (1978) demonstrated that an apparent arginine-P inhibition of partially purified pyruvate kinase was due to contamination of the enzyme preparation by arginine kinase and subsequent enzyme inhibition by the ATP generated in the cuvette when ADP and arginine-P were combined. Wieser and Lackner (1977) reported that purified pyruvate kinase from *Helix pomatia* foot muscle was not

inhibited by arginine-P directly but inhibition was mediated by the addition of a protein fraction separated from pyruvate kinase by the purification procedure. Hoffmann (1981) suggested that arginine-P inhibition of *Tubifex* pyruvate kinase was not due to the presence of arginine kinase but did require an additional factor which could be separated from the enzyme by ultracentrifugation. The arginine-P inhibitions observed in the present study cannot be the result of contamination of the preparation with arginine kinase or other proteins as the preparations used were purified to homogeneity. In addition the differential effects of arginine-P on the two isozymes, inhibition of the flight muscle enzyme and no inhibition of the fat body enzyme, suggest that it is not a contaminant of the arginine-P which is responsible for the inhibition. Arginine-P inhibition of flight muscle pyruvate kinase appears, therefore, to be a real regulatory effect although, as discussed earlier, the compound probably does not play an important role in enzyme regulation in vivo.

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