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A Comparison of the Properties of the Pyruvate Kinases of the Fat Body and Flight Muscle of the Adult Male Desert Locust

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1. The pyruvate kinases of the desert locust fat body and flight muscle were partially purified by ammonium sulphate fractionation. 2. The fat-body enzyme is allosterically activated by very low (1 μM) concentrations of fructose 1,6-diphosphate, whereas the flight-muscle enzyme is unaffected by this metabolite at physiological pH. 3. Flight-muscle pyruvate kinase is activated by preincubation at 25° for 5 min., whereas the fat-body enzyme is unaffected by such treatment. 4. Both enzymes require 1–2 mM-ADP for maximal activity and are inhibited at higher concentrations. With the fat-body enzyme inhibition by ADP is prevented by the presence of fructose 1,6-diphosphate. 5. Both enzymes are inhibited by ATP, half-maximal inhibition occurring at about 5 mM-ATP. With the fat-body enzyme ATP inhibition can be reversed by fructose 1,6-diphosphate. 6. The fat-body enzyme exhibits maximal activity at about pH 7·2 and the activity decreases rapidly above this pH. This inactivation at high pH is not observed in the presence of fructose 1,6-diphosphate, i.e. maximum stimulating effects of fructose 1,6-diphosphate are observed at high pH. The flight-muscle enzyme exhibits two optima, one at about pH 7·2 as with the fat-body enzyme and the other at about pH 8·5. Stimulation of the enzyme activity by fructose 1,6-diphosphate was observed at pH 8·5 and above.

It has been reported by Hess, Haeckel & Brand (1966) that yeast PyK* is stimulated by FDP. Enzyme preparations from rat liver (Taylor & Bailey, 1967; Tanaka, Sue & Morimura, 1967*b*), mouse liver (Passeron, de Asua & Carminatti, 1967) and rat adipose tissue (Pogson, 1968) are similarly affected by FDP. Rat skeletal-muscle PyK is unaffected by FDP (Taylor & Bailey, 1967; Tanaka *et al.* 1967*b*) and rat liver has been shown to contain a PyK with similar properties (Tanaka, Harano, Sue & Morimura, 1967*a*; Bailey, Stirpe & Taylor, 1968). PyK preparations from some sources have been found to be activated by other metabolites as well as FDP. Thus the enzyme of *Escherichia coli* is also activated by AMP (Maeba & Sanwal, 1968) and that from loach (*Misgurnus fossilis*) embryo by 3′,5′-(cyclic)-AMP (Milman & Yurowitzki, 1967).

Since the properties of PyK from various rat tissues differ and since the properties of an insect PyK had not hitherto been reported, it was decided to compare the properties of the enzymes isolated from desert locust (*Schistocerca gregaria*) fat body and flight muscle. We now report the stimulation of fat-body PyK but not flight-muscle PyK by FDP. No other activator of the fat-body enzyme was found.

* Abbreviations: PyK, pyruvate kinase (ATP–pyruvate phosphotransferase, EC 2.7.1.40); FDP, fructose 1,6-diphosphate; PEP, phosphoenolpyruvate.

EXPERIMENTAL

Materials. All reagents used were of analytical grade or the purest available. The following chemicals were obtained from Boehringer Corp. (London) Ltd. (London, W. 5): ADP, NADH, FDP, PEP (tricyclohexylammonium salt), AMP, 3′,5′-(cyclic)-AMP, 2-phosphoglycerate, glucose 6-phosphate, glucose 1-phosphate, 6-phosphogluconate, 3-phosphoglycerate, glyceraldehyde 3-phosphate and lactate dehydrogenase. Other chemicals were supplied by British Drug Houses Ltd. (Poole, Dorset).

Insects. Mature adult male *Schistocerca gregaria* were used in all experiments. The insects were reared under crowded conditions at a constant temperature of 30° and a photoperiod of 12 hr. The insects were fed on fresh lettuce and bran *ad libitum*. Groups of insects were taken about 20 days after emergence of adults and used to make fresh enzyme preparations daily.

Preparation of PyK from flight muscle and fat body. Fat body from the abdomen and thorax and flight muscle from the thorax were dissected from 20–30 locusts to give about 2 g. of each tissue. Each tissue was homogenized in 5 vol. of 50 mM-tris–HCl buffer, pH 7·4, containing EDTA (1 mM). This and all subsequent procedures including centrifugation were carried out at 3–5°. The crude homogenates were centrifuged at 160000$g_{av.}$ for 30 min.

The supernatant solution from each tissue homogenate was brought to 45% saturation with $(NH_4)_2SO_4$ and kept at 0° for 30 min. The mixture was centrifuged at 160000$g_{av.}$ for 10 min. The second supernatant solution was collected and brought to 55% saturation with $(NH_4)_2SO_4$ and kept at 0° for 30 min. The mixture was centrifuged at 160000$g_{av.}$ for 10 min., the supernatant solution discarded and the precipitated protein dissolved in 0·5 ml. of 50 mM-tris–HCl buffer, pH 7·4, containing EDTA (1 mM).

For each tissue the 45–55%-saturation-$(NH_4)_2SO_4$ fraction contained 50% of the total PyK activity of the tissue. The specific activities of the fat-body and flight-muscle enzymes were 4 and 20 μmoles/min./mg. of protein respectively.

Assay of PyK activity. PyK activity was assayed by the method of Bücher & Pfleiderer (1955). The reaction was coupled with lactate dehydrogenase to give oxidation of NADH, which was followed continuously by measuring the change in E_{340} with a Beckman DB spectrophotometer and Sargent SR chart recorder. The reaction was carried out at 25°. The final volume of the reaction mixture was always 1·5 ml., and except where it was necessary to vary conditions for the purpose of a particular experiment the final concentrations were as follows: triethanolamine–HCl buffer, pH 7·5, 40 mM; KCl, 70 mM; NADH, 0·15 mM; ADP, 1·0 mM; $MgSO_4$, 8·0 mM; lactate dehydrogenase (rabbit muscle), 10 units. The concentrations of PEP and FDP are given in the legends to the Tables and Figures. Unless otherwise stated the reactions were started by the simultaneous addition of PEP and enzyme. In preincubation experiments the reaction mixture, including enzyme, was preincubated at 25° for the time stated and the reaction started by the addition of PEP.

RESULTS

Effect of FDP on fat-body PyK activity. The stimulation of the fat-body enzyme by FDP depends not only on the FDP concentration but also on the PEP concentration and on pH. The interrelationship between FDP and PEP is illustrated by showing the influence of FDP on the PEP concentration–activity curve (Fig. 1). In the absence of FDP the curve for the initial velocity plotted against PEP concentration is sigmoidal and half-maximal activity is attained at 0·2 mM-PEP. In the presence of high concentrations of FDP (0·5 mM) the response to PEP concentrations is transformed to give a normal Michaelis–Menten curve, the half-maximal concentration of PEP being 0·05 mM, i.e. one-quarter of that in the absence of FDP.

The low concentration of FDP capable of stimulating the enzyme activity is demonstrated in Fig. 2, which shows the activities of the enzyme in the presence of 0·1 mM-PEP and various amounts of FDP. The activation values are calculated assuming that 0·5 mM-FDP gives 100% activation. Fig. 2 shows that half-maximal stimulation occurs at 1 μM-FDP.

Effects of compounds other than FDP on fat-body PyK activity. Table 1 shows the effect of various metabolites, two of which [AMP and 3′,5′-(cyclic)-AMP] are known to stimulate PyK from other sources, on the PyK activity of the locust fat body. None of the metabolites, tested at 1 mM, had any effect on the enzyme activity; similar results were obtained with the metabolites at 0·1 mM.

Effect of preincubation and FDP on fat-body and flight-muscle PyK activity. Since Bailey *et al.* (1968) demonstrated that preincubation at 25° profoundly effects the activity of rat liver 'L'-type PyK, the effect of this treatment on the enzyme preparations

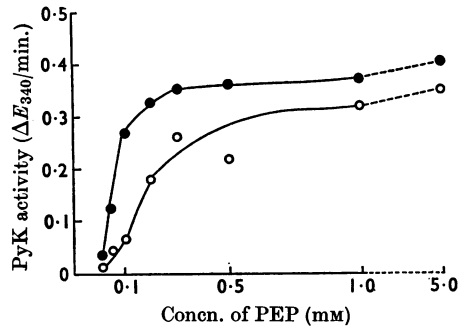

Fig. 1. Influence of FDP on the relationship between locust fat-body PyK activity and PEP concentration. Assay conditions were as described in the Experimental section, with 25 μg. of protein. ○, No FDP; ●, 0·5 mM-FDP.

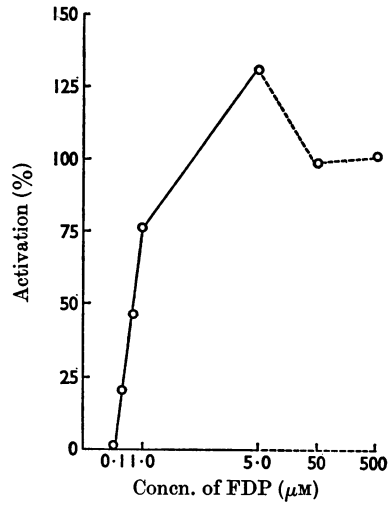

Fig. 2. Activation of locust fat-body PyK by FDP. Assay conditions were as described in the Experimental section, with 0·1 mM-PEP and 25 μg. of protein. The percentage activation of the enzyme was calculated assuming that 0·5 mM-FDP gives 100% activation.

Table 1. *Effect of various compounds on locust fat-body PyK activity*

The enzyme used was a 45–55%-saturation-$(NH_4)_2SO_4$ fraction (see the text). Assay conditions were as described in the Experimental section, with 0·1 mM-PEP and 25 μg. of protein. After measurement of the initial rate the compounds indicated (final concn. 1 mM) were added.

Addition	Final rate (% of initial rate)
DL-Glycerol 3-phosphate	110
D-2-Phosphoglycerate	122
Glucose 6-phosphate	104
Glucose 1-phosphate	90
6-Phosphogluconate	105
DL-2,3-Diphosphoglycerate	83
D-3-Phosphoglycerate	116
Glyceraldehyde 3-phosphate	87
AMP	96
3′,5′-(cyclic)-AMP	91

Table 2. *Effect of preincubation and FDP on locust fat-body and flight-muscle PyK activity*

The enzyme used was a 45–55%-saturation-$(NH_4)_2SO_4$ fraction (see the text). Preincubations were carried out at 25° for the times indicated. Assay conditions were as described in the Experimental section, 0·1 mM-PEP (final concn.) and 25 μg. of protein being used for the fat-body enzyme and 1 mM-PEP and 5 μg. of protein for the flight-muscle enzyme. —, Not assayed.

Duration of preincubation (min.)	PyK activity (ΔE_{340}/min.) Flight-muscle PyK −FDP	Flight-muscle PyK +FDP (0·5 mM)	Fat-body PyK −FDP	Fat-body PyK +FDP (0·5 mM)
0	0·225	0·255	0·065	0·270
5	0·352	0·371	—	—
10	0·360	0·375	0·080	0·260
30	0·285	0·332	0·090	0·224
60	0·325	0·332	0·084	0·224

used here was tested. The results are shown in Table 2. Whereas preincubation of the fat-body enzyme for as long as 60 min. had no effect on its activity, as little as 5 min. preincubation of the flight-muscle enzyme caused a considerable stimulation of activity. FDP, however, although stimulating the fat-body enzyme, had no effect on the flight-muscle enzyme at the PEP concentration (1 mM) shown, or indeed at 0·1 mM-PEP.

Effect of PEP concentration and preincubation on flight-muscle PyK activity. The PEP concentration–activity curve for the flight-muscle enzyme (Fig. 3) has a Michaelis–Menten form. Preincubation for 10 min. at 25° gave considerable stimulation of

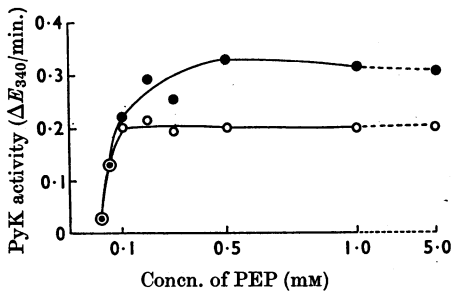

Fig. 3. Influence of preincubation on the relationship between locust flight-muscle PyK activity and PEP concentration. Assay conditions were as described in the Experimental section, with 5 μg. of protein. ○, No preincubation; ●, 10 min. preincubation at 25°.

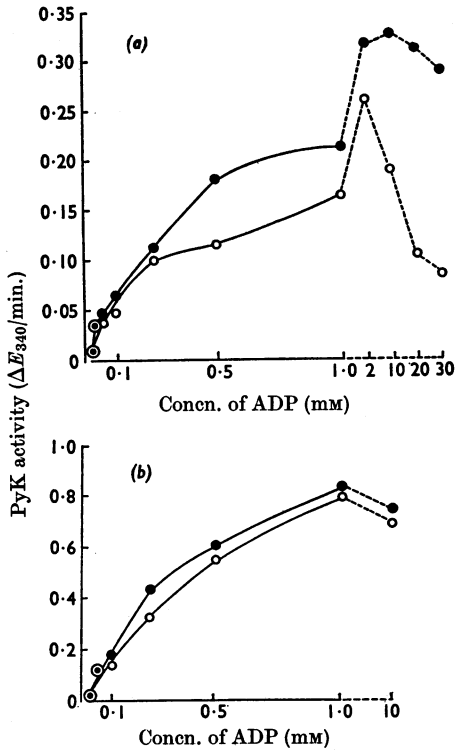

Fig. 4. Effect of FDP on the relationship between locust fat-body (*a*) and flight-muscle (*b*) PyK activity and ADP concentration. The enzyme preparations were preincubated at 25° for 10 min. Assay conditions were as described in the Experimental section, with 1 mM-PEP and 25 μg. of protein being used for the fat-body enzyme and 1 mM-PEP and 10 μg. of protein for the flight-muscle enzyme. ○, No FDP; ●, 0·5 mM-FDP.

activity at PEP concentrations above 0·1 mM. The curve for the preincubated enzyme closely resembles the curve (Fig. 1) obtained for fat-body PyK in the

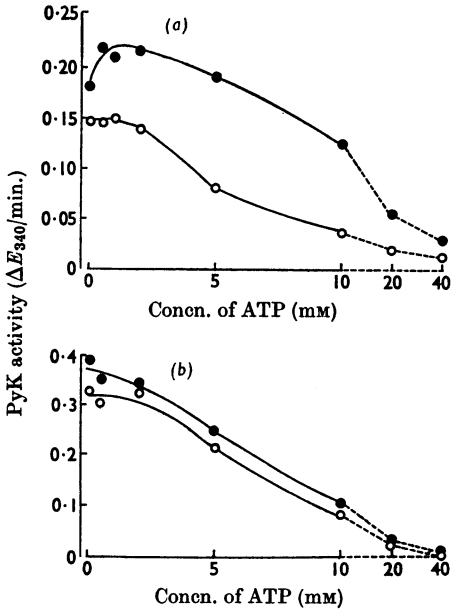

Fig. 5. Effect of FDP on the relationship between locust fat-body (*a*) and flight-muscle (*b*) PyK activity and ATP concentration. The enzyme preparations were preincubated at 25° for 10min. Assay conditions were as described in the Experimental section, with 1mM-PEP and 25μg. of protein being used for the fat-body enzyme and 1mM-PEP and 5μg. of protein for the flight-muscle enzyme. ○, No FDP; ●, 0·5mM-FDP.

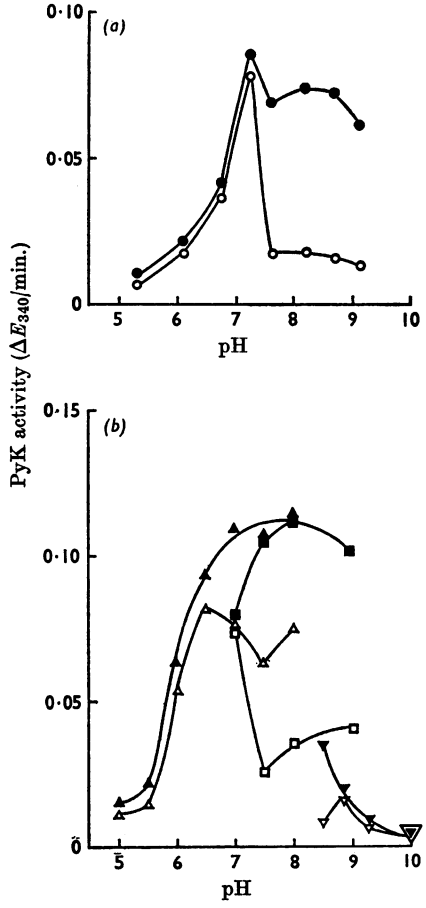

Fig. 6. Effect of FDP on the relationship between locust fat-body PyK activity and pH. Assay conditions were as described in the Experimental section, with the buffers indicated and with 0·1mM-PEP and 10μg. of protein. (*a*) Sodium diethylbarbiturate (veronal)–HCl buffer was used at a final concentration of 120mM. ○, No FDP; ●, 0·5mM-FDP. (*b*) Various buffers were used at a final concentration of 60mM: △ and ▲, potassium phosphate buffer; □ and ■, triethanolamine–HCl buffer; ▽ and ▼, glycine–NaOH buffer. △, □ and ▽, No FDP; ▲, ■ and ▼, 0·5mM-FDP.

presence of FDP, giving half-maximal activity at 0·05mM-PEP.

Effect of ADP concentration on fat-body and flight-muscle PyK activity. The effect of ADP concentration on the activity of the two enzymes in the presence and absence of FDP (0·5mM) is shown in Fig. 4. Both enzymes require 1mM-ADP or higher for maximal activity and are inhibited by high concentrations of ADP. With the fat-body enzyme, however, the inhibition by high concentrations of ADP is prevented by FDP.

Inhibition of PyK activity by ATP and the effect of FDP. The inhibition of both the fat-body and flight-muscle enzymes by ATP is shown in Fig. 5. In both cases half-maximal inhibition is caused by about 5mM-ATP. FDP has little effect on the inhibited flight-muscle enzyme, but addition of FDP to the inhibited fat-body enzyme appears to cause significant reversal of the ATP inhibition.

Effect of pH *and FDP on fat-body and flight-muscle PyK activity.* Fig. 6(*a*) shows the effect of pH on the activity of the fat-body enzyme with veronal buffer in the reaction mixture. The pH–activity curve in the absence of FDP shows a sharp peak at about pH 7·2 and a rapid decline as the pH of the reaction mixture is raised further. In the presence of FDP, however, the activity of the enzyme is reasonably constant over the range pH 7·5–9·0. Fig. 6(*b*) shows results obtained with phosphate, triethanolamine and glycine buffers for different parts of the curve. A similar overall pattern is found to that in Fig. 6(*a*), but it is noteworthy that at pH 7·5 (the pH usually used for assays) the activity in phosphate buffer, in the absence of FDP, is considerably greater than that in triethanolamine buffer. However, the FDP-stimulated activities are similar.

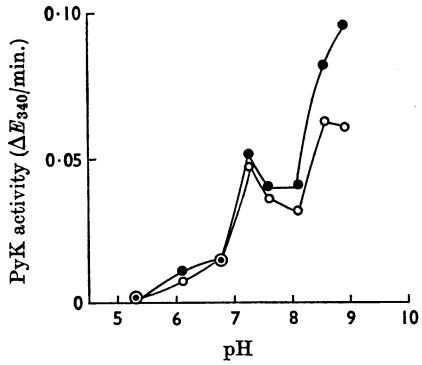

Fig. 7. Effect of FDP on the relationship between locust flight-muscle PyK activity and pH. The enzyme preparation was preincubated at 25° for 10min. Assay conditions were as described in the Experimental section, with 120mM-sodium diethylbarbiturate (veronal)–HCl buffer at the pH indicated, 1mM-PEP and 0·5μg. of protein. ○, No FDP; ●, 0·5mM-FDP.

Fig. 7 shows the dependence of flight-muscle PyK activity on pH. There appear to be two activity maxima, one at about pH 7·2 as with the fat-body enzyme and the other at pH 8·5–9·0. Stimulation of the enzyme activity by FDP, not observed at neutral pH, is evident above pH 8·0.

DISCUSSION

It has been shown that FDP can exert a positive-feedforward effect on PyK of rat liver and adipose tissue but not on the skeletal-muscle enzyme (Taylor & Bailey, 1967; Tanaka *et al.* 1967*a*; Pogson, 1968). It is therefore not surprising to find that FDP stimulates the activity of the PyK of locust fat body (which in many ways functions as a combined liver and adipose tissue) but not that of the flight-muscle enzyme. The fat-body enzyme resembles the rat liver enzyme further in that only FDP appears to have a stimulating effect whereas bacterial (Maeba & Sanwal, 1968) and fish (Milman & Yurowitzki, 1967) PyK enzymes are activated by AMP and 3′,5′-(cyclic)-AMP respectively. The concentration (1μM) of FDP required for half-maximal stimulation of the fat-body enzyme is very similar to that for the rat liver enzyme (Taylor & Bailey, 1967) and is about one-hundredth of that required to activate the yeast enzyme (Hess *et al.* 1966).

Although the locust enzymes studied are similar in a number of ways to their mammalian counterparts, they have several distinct characteristics. In contrast with the situation in rat liver (Tanaka *et al.* 1967*a*) no evidence could be found in the fat body for the presence of a PyK with properties similar to those of the muscle enzyme. The locust and rat enzymes differ profoundly in their response to preincubation. Bailey *et al.* (1968) showed that, under the conditions of the normal assay, preincubation causes a loss of activity in both rat liver and muscle enzymes and that, with the liver enzyme only, the activity is restored by the addition of FDP. In contrast preincubation is without effect on the fat-body enzyme but causes a marked stimulation of the flight-muscle enzyme.

As with the rat liver enzyme (Tanaka *et al.* 1967*a*) ADP at high concentrations (2mM and above) inhibits the enzyme activity. In the presence of FDP, however, inhibition of the fat-body enzyme is prevented. The significance of this effect of FDP is not clear, since the concentrations of ADP necessary for inhibition are very high and probably non-physiological.

ATP inhibits both the locust enzymes, with half-maximal inhibition occurring at about 5mM in each case. The concentration of ATP required is much higher than that required for half-maximal inhibition of the rat liver enzyme (0·16mM) but similar to that required for half-maximal inhibition of rat muscle enzyme (3mM) (Tanaka *et al.* 1967*b*). However, although higher concentrations of ATP are necessary for inhibition of the fat-body enzyme than of the rat liver enzyme, the two enzymes are similar in that FDP can reverse the ATP inhibition (Tanaka *et al.* 1967*b*).

The two locust enzymes studied have different pH–activity curves, with the fat-body enzyme having only one pH optimum but the flight-muscle enzyme having two. With the fat-body enzyme the stimulating effect of FDP is best demonstrated at high pH, when the unstimulated activity is very low. The results with different buffers (Fig. 6) clearly indicate that the stimulated/unstimulated activity ratio depends not only on the pH but also on the nature of the buffer. It would be interesting to know whether the stimulation by FDP of the muscle enzyme at high pH is a property of the locust system alone or is a property of all muscle PyK enzymes.

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