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

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1. Phosphofructokinase was isolated, and partially purified by ammonium sulphate fractionation, from the fat body and flight muscle of the desert locust. 2. Ammonium sulphate appears to stabilize the enzymes, but does not activate them. 3. Both flight-muscle and fat-body enzymes give sigmoidal hexose monophosphate concentration-activity curves, which are characteristic of regulatory enzymes. 4. At low ATP concentrations both the enzyme activities increase rapidly with increasing ATP concentrations, but above an optimum concentration ATP becomes inhibitory. This optimum concentration is 0.2 mM for the fat-body enzyme and 0.1 mM for the flight-muscle enzyme. 5. AMP activates both the enzymes; half-maximal activation occurs at 10 μ M in each case, the effect being independent of substrate concentration. 6. 3',5'-(cyclic)-AMP (0.5 mM) and P₁ (1 mM) activate the flight-muscle enzyme, but have no effect on the fat-body enzyme. 7. FDP (1 mM) inhibits both enzymes, and with the flight-muscle enzyme this inhibition is increased by increasing the ATP concentration. 8. Citrate, phosphoenolpyruvate and α -glycerophosphate have no effect on either enzyme under the assay conditions used. 9. The properties of phosphofructokinases from the locust are compared with those of phosphofructokinases from other sources.

It is now considered that PFK* plays an important regulatory role in carbohydrate metabolism in bacterial, yeast, plant and mammalian systems. PFK isolated from these sources is allosterically activated by one of its substrates, FDP, and usually inhibited by the other substrate, ATP. The enzyme is also affected by its products and some other metabolites. The effects of these compounds on the PFK enzymes already studied are summarized in Table 1.

Despite the large number of reports of the properties of PFK from various sources, those of an insect PFK have not hitherto been described. It was therefore decided to isolate PFK from two major tissues of the locust, and compare their properties with those of the other PFK enzymes that have been studied.

EXPERIMENTAL

Materials. ATP, ADP, AMP, 3',5'-(cyclic)-AMP, glucose 6-phosphate, NADH, FDP (all as sodium salts), PEP (tricyclohexylammonium salt), phosphoglucose isomerase, pyruvate kinase, α -glycerophosphate dehydrogenase, triose

phosphate isomerase, lactate dehydrogenase and aldolase were obtained from Boehringer Corp. (London) Ltd. (London, W. 5). Sephadex G-25 was obtained from Pharmacia (Uppsala, Sweden). All other chemicals were obtained from British Drug Houses Ltd. (Poole, Dorset).

Insects. Treatment of insects was as described by Bailey & Walker (1969).

Preparation of PFK from flight muscle and fat body. Dissection of flight-muscle and fat-body tissues and preparation of homogenates were as described by Bailey & Walker (1969).

The supernatant from each homogenate was collected and brought to 43% saturation with (NH₄)₂SO₄ (26 g./100 ml. of solution) and kept at 0° for 20 min. The mixture was then centrifuged for 10 min. at 160 000 *g*_{av.}. The second supernatant was collected, brought to 50% saturation with (NH₄)₂SO₄ (4 g./100 ml. of solution) and again kept at 0° for 20 min. After centrifugation for 10 min. as before, the supernatant was discarded and the precipitated protein dissolved in 0.5 ml. of 50 mM-tris-HCl buffer, pH 7.4, containing EDTA (1 mM).

This fraction gave specific activities of 0.4 and 3 μ moles/min./mg. of protein for the fat-body and flight-muscle enzymes respectively. The (NH₄)₂SO₄ concentration in these fractions was 150 mM as determined by the method of Seligson & Hirahara (1957).

The flight-muscle preparation was free from adenylate kinase, adenosine triphosphatase and NADH oxidase. Fructose diphosphatase activity was less than 2% of the PFK activity and similar results were obtained with either

* Abbreviations: PFK, phosphofructokinase (ATP-D-fructose 6-phosphate 1-phosphotransferase, EC 2.7.1.11); FDP, fructose 1,6-diphosphate; PEP, phosphoenolpyruvate.

Table 1. *Activators and inhibitors of PFK enzymes from different sources*

+, Enzyme activator; -, enzyme inhibitor; ±, enzyme activator at low concentrations and inhibitor at higher concentrations; 0, no effect on enzyme.								
Source of enzyme	AMP	ADP	P _i	FDP	3',5'-(cyclic)-AMP	Citrate	PEP	Reference
Sheep brain	+	+	+	+	+	-	-	Lowry & Passonneau (1966)
Rabbit skeletal muscle	+	+	+	+	+	-	-	Passonneau & Lowry (1962); Uyeda & Racker (1965); Parmeggiani, Luft, Love & Krebs (1966)
Guinea-pig heart muscle	+	+	+	+	+	+	+	Mansour (1963)
Rat heart muscle	+	+	+	+	+	-	-	Garland, Randle & Newsholme (1963)
Ox heart muscle	+	+	+	+	+	+	+	Frenkel (1968)
Kidney cortex	+	+	+	+	+	-	0	Underwood & Newsholme (1967)
Adipose tissue	+	+	+	+	+	-	0	Denton & Randle (1966)
Liver	+	+	+	+	+	-	0	Underwood & Newsholme (1965)
Liver fluke (<i>Fasciola hepatica</i>)	0	0	+	+	+	+	+	Mansour & Mansour (1962)
Plant enzymes	-	-	+	+	0	-	-	Dennis & Coulter (1966, 1967); Kelly & Turner (1968)
Yeast (<i>Saccharomyces cerevisiae</i>)	+	0	0	±	0	0	0	Betz & Moore (1967); Lindell & Stellwagen (1968)
<i>Escherichia coli</i>	0	±	0	0	0	0	-	Griffin, Houek & Brand (1967); Blangy, Buc & Monod (1968)

the ADP-linked assay or the FDP-linked assay. The fat-body preparation was also free from adenylate kinase, but fructose diphosphatase activity was 25% of the PFK activity. The ADP-linked assay was therefore used throughout. A low endogenous rate due to NADH oxidase and adenosine triphosphatase was present and the results were corrected for this. This rate was not sufficient to produce an appreciable change in reaction concentrations during the assay.

Assay of PFK activity. PFK activity was assayed at 25° by the ADP-linked and FDP-linked assays described by Underwood & Newsholme (1965). The following reactants were used in the FDP-linked assay (final concns.): KCl (0.2M), imidazole-HCl buffer, pH 7.0 (20mM), MgCl₂ (5mM) and NADH (0.1mM). The following amounts of enzymes were added: aldolase (50μg.), triose phosphate isomerase (5μg.), α-glycerophosphate dehydrogenase (5μg.) and phosphoglucose isomerase (5μg.). In the ADP-linked assay the final concentrations and amounts of enzymes were: KCl (67mM), imidazole-HCl buffer, pH 7.0 (20mM), MgCl₂ (5mM), NADH (0.1mM), PEP (7.5mM), lactate dehydrogenase (5μg.), pyruvate kinase (5μg.) and phosphoglucose isomerase (5μg.). ATP, glucose 6-phosphate and AMP were added as required. In both assay systems the final volume was 3ml. and the reaction was started with 5μl. of the PFK preparation, after incubation of the reactants for 5min. at 25°. The change in *E*₃₄₀ was followed with a Beckman DB spectrophotometer and a Sargent SR chart recorder. Initial rates were determined over a 3-5min. period before addition of effectors. Progress curves were linear throughout the assay. All reactions were compared with controls from which glucose 6-phosphate was omitted. To study the effects of (NH₄)₂SO₄ on PFK Sephadex G-25 was used to remove the (NH₄)₂SO₄ from the commercial enzymes; without such treatment the final concentration of (NH₄)₂SO₄ was 4.6mM.

RESULTS

Effect of ammonium sulphate on PFK activity and sensitivity towards effectors. Ammonium sulphate has been shown to activate PFK from rat liver (Underwood & Newsholme, 1965) and rabbit skeletal muscle (Parmeggiani *et al.* 1966). However, 4.6mM-ammonium sulphate had no significant effect on locust flight-muscle and fat-body PFK activity and did not effect AMP activation of either enzyme. Ammonium sulphate was removed for the study of other effectors.

Effect of hexose monophosphate concentration on PFK activity. Figs. 1 and 2 show the effect of hexose monophosphate concentration on the velocity of the enzyme reaction. Both flight-muscle and fat-body enzymes show very similar patterns. In the presence of 1mM-ATP the curves are sigmoidal and half-maximal velocity is attained at approx. 3mM-hexose monophosphate with the flight-muscle enzyme and 2mM-hexose monophosphate with the fat-body enzyme. With 2mM-ATP the curves are displaced to the right, showing that

Fig. 1. Effect of hexose monophosphate on locust flight-muscle PFK activity. $(\text{NH}_4)_2\text{SO}_4$ (4.6 mM) was present in all the assays. Other conditions were as described in the Experimental section. $\blacktriangle$, 2 mM-ATP; $\bullet$, 1 mM-ATP; $\circ$, 1 mM-ATP, plus 2 mM-AMP (added to the reaction mixture after the initial rate had been measured).

Fig. 2. Effect of hexose monophosphate on locust fat-body PFK activity. $(\text{NH}_4)_2\text{SO}_4$ (4.6 mM) was present in all the assays. Other conditions were as described in the Experimental section. $\blacktriangle$, 2 mM-ATP; $\bullet$, 1 mM-ATP; $\circ$, 1 mM-ATP, plus 2 mM-AMP (added to the reaction mixture after the initial rate had been measured).

ATP inhibits the enzyme by increasing the K_m with respect to hexose monophosphate.

Effect of ATP concentration on PFK activity. The effects of ATP concentration on the activity of the flight-muscle and the fat-body enzymes are shown in Figs. 3(a) and 3(b) respectively. The curves show that activity increases rapidly at very low ATP concentrations, whereas higher concentrations inhibit. Inhibition by ATP is less at high hexose monophosphate concentration. The curves are very similar for both enzymes at 2 mM- and 5 mM-hexose monophosphate, and AMP relieves ATP inhibition to the same extent with both enzymes.

Effect of AMP concentration on PFK activity. Figs. 1 and 2 show the effect of 2 mM-AMP on the hexose monophosphate concentration-activity curves of the enzymes in the presence of 1 mM-ATP. AMP converts the sigmoidal curves into hyperbolic curves, lowering the concentration for half-maximal velocity to 1.5 mM- and 1 mM-hexose monophosphate for the flight-muscle and fat-body enzymes

Fig. 3. Inhibition of locust flight-muscle (a) and fat-body (b) PFK activity by ATP. $(\text{NH}_4)_2\text{SO}_4$ (4.6 mM) was present in all the assays. Other conditions were as described in the Experimental section. $\blacktriangle$, 2 mM-Hexose monophosphate; $\bullet$, 5 mM-hexose monophosphate; $\circ$, 5 mM-hexose monophosphate, plus 2 mM-AMP (added to the reaction mixture after the initial rate had been measured).

respectively. Figs. 3(a) and 3(b) show that 2 mM-AMP relieves inhibition of ATP and also activates the enzymes at non-inhibitory ATP concentrations. Thus AMP appears to act by relieving inhibition of PFK by ATP and by lowering the K_m with respect to hexose monophosphate at both inhibitory and non-inhibitory concentrations of ATP. Fig. 4 gives the AMP activation curves for both fat-body and flight-muscle PFK in the presence of 1 mM-ATP and 2 mM-hexose monophosphate. Both activation patterns are very similar and 50% stimulation is given by $10 \mu\text{M}$ -AMP in both cases. AMP at 2 mM is slightly inhibitory to both enzymes.

Effect of FDP and P_i on PFK activity. The effects of these compounds on flight-muscle and fat-body PFK are shown in Tables 2 and 3 respectively. P_i at 1 mM activates the flight-muscle enzyme in the presence of 1 mM-ATP, but has no effect on the fat-body enzyme. FDP at 1 mM inhibits both the fat-body enzyme and the flight-muscle enzyme. This effect was studied in more detail on the flight-muscle PFK and the results are shown in Fig. 5. Fig. 5(a) shows that the inhibition increases with increased FDP concentration and

Fig. 4. Stimulation of locust flight-muscle (O) and fat-body (▲) PFK activity by AMP. $(\text{NH}_4)_2\text{SO}_4$ (4.6 mM) was present in all the assays. The hexose monophosphate concentration was 2 mM and the ATP concentration 1 mM throughout. Other conditions were as described in the Experimental section. Percentage activation is calculated assuming that 200 μM -AMP gives 100% activation.

Table 2. Action of effectors and products on locust flight-muscle PFK activity

The hexose monophosphate concentration was 2 mM. $(\text{NH}_4)_2\text{SO}_4$ was removed by Sephadex G-25 treatment. Other conditions were as described in the text.

Concn. of ATP (mM)	Addition	Rate (% of control)
1.0	2 mM-AMP	180
1.0	0.5 mM-3',5'-(cyclic)-AMP	140
1.0	1 mM- P_i	142
1.0	1 mM-FDP*	50
0.1	0.5 mM-3',5'-(cyclic)-AMP	100

* ADP-linked assay used (see the text).

Table 3. Action of effectors and products on locust fat-body PFK activity

Conditions were as described in the text.

Concn. of ATP (mM)	Addition	Rate (% of control)
1.0	2 mM-AMP	230
1.0	0.5 mM-3',5'-(cyclic)-AMP	100
1.0	1 mM- P_i	100
0.1	1 mM-FDP	22

that this inhibition is increased by increasing the ATP concentration. Fig. 5(b) shows the effect of 1 mM-FDP on the ATP inhibition curve in the presence of 2 mM-hexose monophosphate.

Effect of 3',5'-(cyclic)-AMP on PFK activity. Table 2 shows that 0.5 mM-3',5'-(cyclic)-AMP

Fig. 5. Inhibition of locust flight-muscle PFK activity by FDP. $(\text{NH}_4)_2\text{SO}_4$ was removed by Sephadex G-25 treatment. The ADP-linked assay was used and the other conditions were as described in the Experimental section. The hexose monophosphate concentration was 2 mM. (a) ▲, 0.1 mM-ATP; ○, 1 mM-ATP. (b) ○, No FDP; ●, 1 mM-FDP.

activates the flight-muscle PFK at 1.0 mM-ATP, but has no effect at 0.1 mM-ATP, suggesting that 3',5'-(cyclic)-AMP activates by relieving ATP inhibition. 3',5'-(cyclic)-AMP has no effect on the fat-body PFK at 1.0 mM-ATP (Table 3).

Effect of citrate, PEP and DL-glycerol 3-phosphate on PFK activity. Citrate has been shown to inhibit PFK from mammalian and plant sources, and PEP has been shown to inhibit *E. coli*, plant and skeletal-muscle PFK (for references see Table 1). PEP at 1 mM and 1 mM-citrate have no effect on either flight-muscle or fat-body PFK when assayed under the conditions described in Table 2; 1 mM-DL-glycerol 3-phosphate also has no effect.

DISCUSSION

A detailed comparison of the properties of PFK enzymes from the various systems that have been studied is difficult because the work has been carried out on preparations of different degrees of purity and various buffers and pH values have been used in the assay systems. The Mg^{2+} concentration and the presence or otherwise of ammonium sulphate are also critical. Some general observations can, however, be made. All the enzymes studied show a sigmoidal fructose 6-phosphate activation

curve typical of regulatory enzymes. The concentration of fructose 6-phosphate for half-maximal activity varies from 0.028 mM in guinea-pig heart (Mansour, 1963) to 5 mM in the liver fluke (Mansour & Mansour, 1962). The values of 1 mM for locust flight muscle and 0.7 mM for fat body fall within this range. All the enzymes show a similar ATP activity pattern, which is dependent on fructose 6-phosphate and Mg^{2+} concentrations. The optimum ATP concentration varies from about 0.02 mM in ox heart (Frenkel, 1968) and rat adipose tissue (Denton & Randle, 1966) to more than 1 mM in *E. coli* (Atkinson & Walton, 1965) and liver fluke (Mansour & Mansour, 1962); the corresponding values for locust flight-muscle and fat-body PFK are about 0.1 mM and 0.2 mM respectively. Above this optimum concentration ATP becomes inhibitory to all the enzymes except that of *E. coli* (Blangy *et al.* 1968).

The locust flight-muscle enzyme resembles the mammalian muscle enzyme in that it is activated by AMP, 3',5'-(cyclic)-AMP and P_i . It is, however, inhibited by FDP, in this respect resembling the yeast enzyme (Betz & Moore, 1967). Sacktor & Hurlbut (1966) have shown that at the onset of flight-muscle activity ADP, P_i and particularly AMP (which increases about 2.5-fold) accumulate. This may cause activation of insect flight-muscle PFK and increase the glycolytic rate. This assumes that there is compartmentation of metabolites in the cell, since the total cellular ATP content, 7 μ moles/g. fresh wt. (Sacktor & Hurlbut, 1966), would inhibit the enzyme at all times. The role of 3',5'-(cyclic)-AMP in insect metabolism is not yet clear. PEP and citrate inhibit mammalian muscle, but have no effect on locust flight-muscle enzyme.

The fat-body enzyme may be expected to resemble the mammalian liver and adipose-tissue enzymes, since the fat body functions in many ways as a combined liver and adipose tissue for insects. Underwood & Newsholme (1965) showed that rat liver PFK is activated by AMP and FDP and inhibited by citrate. Denton & Randle (1966) showed that the adipose-tissue enzyme is activated by 3',5'-(cyclic)-AMP, AMP, P_i and ADP, but is inhibited by citrate. The insect fat-body enzyme is activated by AMP and inhibited by FDP, whereas 3',5'-(cyclic)-AMP and citrate have no effect. This may be advantageous, since the male locust undergoes extensive lipogenesis during development (P. R. Walker, unpublished work) and citrate has been shown to be important in the transfer of carbon from dietary carbohydrate to

acetyl-CoA for fatty acid synthesis (Daikuhara, Tsunemi & Takeda, 1968).

It is noteworthy that the properties of PFK from the two different specialized tissues of the locust resemble each other fairly closely, whereas the properties of another key glycolytic regulatory enzyme, pyruvate kinase, are very different (Bailey & Walker, 1969).

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REFERENCES

- Atkinson, D. E. & Walton, G. M. (1965). *J. biol. Chem.* **240**, 757.
- Bailey, E. & Walker, P. R. (1969). *Biochem. J.* **111**, 359.
- Betz, A. & Moore, C. (1967). *Arch. Biochem. Biophys.* **120**, 268.
- Blangy, D., Buc, H. & Monod, J. (1968). *J. molec. Biol.* **31**, 13.
- Daikuhara, Y., Tsunemi, T. & Takeda, Y. (1968). *Biochim. biophys. Acta*, **158**, 51.
- Dennis, D. T. & Coultate, T. P. (1966). *Biochem. biophys. Res. Commun.* **25**, 187.
- Dennis, D. T. & Coultate, T. P. (1967). *Biochim. biophys. Acta*, **146**, 129.
- Denton, R. M. & Randle, P. J. (1966). *Biochem. J.* **100**, 420.
- Frenkel, R. (1968). *Arch. Biochem. Biophys.* **125**, 166.
- Garland, P. B., Randle, P. J. & Newsholme, E. A. (1963). *Nature, Lond.*, **200**, 169.
- Griffin, C. C., Houck, B. N. & Brand, L. (1967). *Biochem. biophys. Res. Commun.* **27**, 287.
- Kelly, G. J. & Turner, J. F. (1968). *Biochem. biophys. Res. Commun.* **30**, 195.
- Lindell, T. J. & Stellwagen, E. (1968). *J. biol. Chem.* **243**, 907.
- Lowry, O. H. & Passonneau, J. V. (1966). *J. biol. Chem.* **241**, 2268.
- Mansour, T. E. (1963). *J. biol. Chem.* **238**, 2285.
- Mansour, T. E. & Mansour, J. M. (1962). *J. biol. Chem.* **237**, 629.
- Parmeggiani, A., Luft, J. H., Love, D. S. & Krebs, E. G. (1966). *J. biol. Chem.* **241**, 4625.
- Passonneau, J. V. & Lowry, O. H. (1963). *Biochem. biophys. Res. Commun.* **13**, 372.
- Sacktor, B. & Hurlbut, E. C. (1966). *J. biol. Chem.* **241**, 632.
- Seligson, D. & Hirahara, K. (1957). *J. Lab. clin. Med.* **49**, 962.
- Underwood, A. H. & Newsholme, E. A. (1965). *Biochem. J.* **95**, 868.
- Underwood, A. H. & Newsholme, E. A. (1967). *Biochem. J.* **104**, 296.
- Uyeda, K. & Racker, E. (1965). *J. biol. Chem.* **240**, 4682.