The Purification and Properties of Pig Spleen Phosphofructokinase

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Pig spleen phosphofructokinase has been purified 800-fold with a yield of 17%. Two isoenzymes that appear to be kinetically identical can be separated by DEAE-cellulose column chromatography. In common with the enzyme from other mammalian sources, the spleen enzyme has a pH optimum of 8.2. At pH 7.0 it displays sigmoidal kinetics with respect to fructose 6-phosphate concentration but its co-operative behaviour is very dependent on pH, protein concentration and the concentration of MgATP. MgGTP and MgITP can replace MgATP as phosphate donors but, unlike MgATP, these nucleotides do not cause significant inhibition. Mn^{2+} and Co^{2+} (as the metal ion-ATP complexes) act as cofactors and in the free form are far more inhibitory than free Mg^{2+} . The spleen enzyme responds to a wide variety of potential effector molecules: ADP, AMP, cyclic AMP, aspartate, NH_4^+ , fructose 6-phosphate, fructose 1,6-diphosphate and P_i all act as either activators or protectors, whereas Mg-ATP, Mg^{2+} , citrate, phosphoenolpyruvate and the phosphoglycerates are inhibitors.

Phosphofructokinase (ATP-D-fructose 6-phosphate 1-phosphotransferase; EC 2.7.1.11) has been positively identified as a major regulatory enzyme in a number of mammalian tissues (Bloxham & Lardy, 1973). It has been purified from a number of sources and its properties have been exhaustively examined. Hickman & Weidemann (1974) found that phosphofructokinase is also an important regulatory enzyme in rat spleen and thymus. Although the enzyme from rat thymus has been highly purified (Yamada & Ohyama, 1972), no detailed investigation of the kinetic properties of the lymphoid-tissue enzyme has been reported. In the present paper, we report the partial purification and some of the kinetic properties of pig spleen phosphofructokinase.

Materials and Methods

Materials

All nucleotides and fine chemicals were purchased from Boehringer Mannheim G.m.b.H., Mannheim, West Germany. They were either purchased as the sodium salts, or were converted into this form with Dowex (Na^+ form) before use. All other chemicals used were of analytical grade.

The coupling enzymes used, namely aldolase, triose phosphate isomerase, α -glycerophosphate dehydrogenase, pyruvate kinase and lactate dehydrogenase were purchased from either Boehringer Mannheim, or the Sigma Chemical Co., St. Louis, Mo., U.S.A.

Hepes [2-(*N*-2-hydroxyethylpiperazin-*N'*-yl)-ethanesulphonic acid] was purchased from Sigma,

and DEAE-cellulose DE52 from Whatman Biochemicals Ltd., Maidstone, Kent, U.K.

Pig spleens were obtained from the Canberra Abattoirs. They were placed on ice within 5 min of death, and transported to the laboratory in a large Dewar flask. Fat was trimmed off samples, which were then cut into pieces weighing approx. 10 g before being frozen and stored for up to 7 days at $-20^\circ C$.

Methods

Enzyme assays. Measurements of phosphofructokinase activity were made at two distinct pH regions: (i) pH 8.0–8.2, where the activity is maximum; or (ii) pH 6.8–7.2, which is near physiological, and where the regulatory properties of the enzyme can be clearly demonstrated.

Hepes buffer has a pK_a intermediate between these pH regions (7.55) and, in addition, does not bind metal ions (Good *et al.*, 1966). It was used in all studies reported in the present paper.

For examination of its regulatory properties, phosphofructokinase was assayed at $23^\circ C$ by following NADH oxidation in the presence of 50 mM-Hepes-NaOH, pH 7.0, ATP, fructose 6-phosphate and effectors as indicated. Because NADH at high concentration inhibits the coupling enzymes used in this assay (Newsholme *et al.*, 1970), the initial amount of NADH added was kept constant and low (0.1 mM). Unless specifically stated, the Mg^{2+} concentration was always 3 mM in excess of the ATP concentrations. Phosphofructokinase activity was measured by following the decrease in absorption at 340 nm with a Zeiss PMQII spectrophotometer,

with aldolase (0.7 unit), triose phosphate isomerase (3.0 units) and α -glycerophosphate dehydrogenase (1.7 units) added as coupling enzymes. Coupling enzymes were dialysed for 24 h against three 1-litre volumes of 50 mM-Tris-HCl, pH 8.0, containing 0.05 mM-EDTA, to remove $(\text{NH}_4)_2\text{SO}_4$ and the amount added to the assay cuvette exceeded by approx. tenfold the maximum activity of phosphofructokinase. The final concentration of Tris added to the assay cuvette with the coupling enzymes never exceeded 1.5 mM and no NH_4^+ could be detected in the dialysed coupling-enzyme mixture by enzymic assay. Immediately before assay, stock solutions of phosphofructokinase were diluted to about 1–2 mg of protein/ml with potassium phosphate buffer (80 mM, pH 8.0) containing EDTA (1 mM), and the reaction was initiated with either 10 or 20 μl of this solution, depending on which volume gave a suitable absorption change. Under these conditions the reaction velocity obtained was linear with time from zero time onwards, and gave the same rate as did direct measurement of fructose 1,6-diphosphate production (Fig. 7), which indicates that sufficient coupling-enzyme activity was present at all times (McClure, 1969).

For assays of maximum activity during enzyme purification, phosphofructokinase was assayed in 50 mM-Hepes-NaOH, pH 8.0, containing 0.4 mM-ATP, 2.0 mM-fructose 6-phosphate, 3.0 mM- Mg^{2+} and 0.1 mM-NADH. Coupling enzymes were added in approx. tenfold excess, but without prior dialysis.

Reported methods of initiating phosphofructokinase assays vary. For example, Kemp (1971) and Mansour & Ahlfors (1968) preincubated the enzyme in buffer and ATP before initiating the reaction with fructose 6-phosphate. Other workers (e.g. Ling *et al.*, 1965; Tarui *et al.*, 1972; Underwood & Newsholme, 1965) initiated the reaction with enzyme. In preliminary studies we found that spleen phosphofructokinase was rapidly inactivated by preincubation with ATP (Hickman, 1974). When considered in terms of the association-dissociation model proposed by Mansour (1972) for regulation of phosphofructokinase, it is apparent that preincubation of the enzyme with individual substrates or effectors may promote the formation of a wide variety of aggregated or disaggregated states, with more or less activity, before initiation of the reaction. On the other hand, if the reaction is initiated with the enzyme, it is exposed to all the substrates and effectors simultaneously and the appropriate equilibrium position between active and inactive forms should be reached more rapidly. For this reason, in all of the kinetic experiments reported below reactions were initiated by addition of enzyme to the complete reaction mixture.

Enzyme purification. Pig spleen phosphofructokinase was purified as far as the 'wash' stage of

Massey & Deal (1973), then dialysed overnight against three 1-litre volumes of 80 mM-potassium phosphate buffer, pH 8.0, containing 1 mM-EDTA. With the exception of one step in the Massey & Deal (1973) purification schedule, which involved incubating the enzyme for 45 min at 41°C, all other operations were carried out at 4°C. Much of the kinetic work reported here used the enzyme purified only to this stage.

A number of additional kinetic experiments were conducted with a more highly purified form of the enzyme prepared as follows. The dialysed enzyme was applied to a column (15 cm $\times$ 1.5 cm) containing DEAE-cellulose equilibrated with the dialysis buffer, and washed on to the column with 50 ml of the same buffer. A continuous gradient was then introduced consisting of the dialysis buffer plus 0–500 mM- $(\text{NH}_4)_2\text{SO}_4$. Samples (5.0 ml) were collected and assayed for phosphofructokinase activity. Fractions containing most of the activity in each peak were pooled, and the enzyme was precipitated by the addition of solid $(\text{NH}_4)_2\text{SO}_4$ to 65% saturation. After stirring overnight in the cold, precipitates were collected by centrifugation (30 000 g for 15 min), dissolved in a minimum amount of potassium phosphate buffer and dialysed extensively against the same buffer. The resultant solution contained the most highly purified phosphofructokinase obtained.

Results

Purification and storage

A typical purification schedule for pig spleen phosphofructokinase is shown in Table 1.

Both 'wash' and 'column' phosphofructokinases had similar stabilities in the potassium phosphate-EDTA buffer, losing 10–15% of the initial activity in 1 month and retaining approx. 40% of their activities after 3 months. Of several additional stabilizing compounds examined (ATP, $(\text{NH}_4)_2\text{SO}_4$, fructose 1,6-diphosphate and fructose 6-phosphate) fructose 1,6-diphosphate was by far the most effective. However, stabilizing molecules of this type have profound effects on the kinetic behaviour of phosphofructokinase at low concentrations. Potassium phosphate, on the other hand, although less effective as a stabilizer, has very little activating effect on phosphofructokinase kinetics at the low P_i concentrations added to the assay cuvette with the enzyme (<0.5 mM). Because sensitivity to a wide variety of molecules rather than stability is of primary importance in this study, the enzyme was stored in unsupplemented potassium phosphate buffer at optimum pH (pH 8.0). The enzyme is active enough for only about 2–3 months of routine work under these conditions, whereas up to 70% of its original activity is retained after 4 months of storage

Table 1. *Purification schedule for pig spleen phosphofructokinase*

Experimental details are given in the Materials and Methods section. One unit of activity is defined as 1 μ mol of fructose 6-phosphate converted into fructose 1,6-diphosphate/min at 23°C.

Fractionation step	Volume (ml)	Total activity (units)	Total protein (mg)	Specific activity (units/mg of protein)	Yield (%)	Purification (fold)
1. Initial supernatant	1040	1139	51376	0.023	100	1
2. Heat (1°C)-ethanol supernatant	912	753	7852	0.096	67	4
3. Mg ²⁺ precipitation and wash	7.5	235	31	7.45	21	324
4. DEAE-cellulose+(NH ₄) ₂ SO ₄						
Peak (1)	3	108	} 24	5.6	} 17	—
Peak (2)	2	88		18.6		
						815

Fig. 1. *Sigmoidal response of pig spleen phosphofructokinase to fructose 6-phosphate*

The assay was performed at pH7.0, as described in the text, at two MgATP concentrations: ■, 0.8mM-MgATP; △, 1.5mM-MgATP.

in the same buffer containing 0.2mM-ATP, 0.1mM-fructose 6-phosphate, 2mM-(NH₄)₂SO₄ and 0.2mM-dithiothreitol. Some stabilizers are obviously essential, as the enzyme loses all of its activity within 3 days if dialysed against Tris-HCl, pH8.0.

Of the two isoenzymes separated on the column, neither displayed very strong affinity for the DEAE-cellulose. The first peak came through in the buffer wash, and the second was eluted with a low concentration of (NH₄)₂SO₄ (<50mM). Initial attempts to purify spleen phosphofructokinase by using the batch DEAE-cellulose method of Tarui *et al.* (1972) were rejected when it was found that less than 50% of the initial activity was absorbed.

The two isoenzymes from the DEAE-cellulose column were compared with respect to the following effectors: NH₄⁺, AMP and ATP. Kemp (1971) has shown that the two principal forms of phosphofructokinase, namely 'muscle-type' and 'liver-type', can be differentiated on the basis of their relative sensitivities to AMP and ATP. In this case the two isoenzymes from spleen had almost identical kinetic

Table 2. *Michaelis constants for the substrates of pig spleen phosphofructokinase*

For each *K_m* determination the second substrate was fixed at 2.0mM. Other experimental conditions and details of the assay method used are given in the text.

	<i>K_m</i> (μM)	
	Fructose 6-phosphate	MgATP
pH7.0	350	40
pH8.0	32	60

properties and could not be distinguished from each other. Because their stabilities were also very similar, all further experiments were conducted with either a 1:1 mixture of the two types, or with the 'wash' enzyme.

General properties

Fig. 1 shows the sigmoidal response of phosphofructokinase to increasing fructose 6-phosphate concentration, when assayed at pH7.0 in the presence of fixed concentrations of MgATP. The intensity of the sigmoidal response observed is a function of the MgATP concentration. For example, when 0.8mM-MgATP was present, the Hill coefficient was 1.41 and it increased to 1.79 at 1.5mM-MgATP. It would appear from Fig. 1 that the maximum velocity observed is also a function of the MgATP concentration. The situation is more complex, however, as when the protein concentration is doubled, the maximum velocities at both concentrations of MgATP are increased, and the disparity between them (at high fructose 6-phosphate concentrations) becomes less pronounced. Accompanying the increase in protein concentration, the Hill coefficients fall.

Both Table 2 and Fig. 2 demonstrate the sensitivity of phosphofructokinase to variation in pH. In Table 2, the concentrations of substrates required to give half-maximum velocity (*K_m*) at both pH7.0 and 8.0 are shown. At each pH,

Fig. 2. Effect of pH on pig spleen phosphofructokinase activity

Assays were performed at the indicated pH, as described in the text, under the following conditions: 0.2mm-fructose 6-phosphate, 2.0mm-MgATP (Δ); 0.2mm-fructose 6-phosphate, 0.4mm-MgATP ($\bullet$); and 1.0mm-fructose 6-phosphate, 0.2mm-MgATP ($\square$). For each curve the velocity is expressed as a percentage of the V_{max} value found at pH 8.2 (optimum pH).

one substrate was fixed at a constant saturating value (2.0mm) and the activity was monitored with respect to the second substrate. Of greatest interest is the large increase in K_m value for fructose 6-phosphate as the pH decreases from 8.0 to 7.0. When the normal amounts of fructose 6-phosphate *in vivo* in spleen are considered (0.045 ± 0.002 mm; ten determinations; Hickman, 1974) it is apparent that, in the presence of physiological concentrations of ATP (4.35 ± 0.18 mm; ten determinations), phosphofructokinase will be strongly inhibited at the expected intracellular pH range (7.3–7.4). Although no values are shown here, the apparent K_m for fructose 6-phosphate at pH 7.0 also increases as the MgATP concentration is increased.

In Fig. 2, phosphofructokinase was assayed at different pH values with substrates present: (i) at near-optimum concentration of substrates for maximal activity (1.0mm-fructose 6-phosphate and 0.2mm-ATP); (ii) at concentrations of substrates that are mildly inhibitory (0.2mm-fructose 6-phosphate and 0.4mm-ATP); and (iii) at highly inhibitory substrate concentrations (0.2mm-fructose 6-phosphate and 2.0mm-ATP). The three curves are drawn on the same scale to emphasize the degree of inhibition observed at each pH value. At the optimum pH (8.2), the velocity at optimum substrate concentrations was only 20% higher than that observed when the substrate concentrations were highly inhibitory. Evidently the different velocities observed at different pH values represent

Table 3. Ability of various nucleoside triphosphates to act as substrates and inhibitors of pig spleen phosphofructokinase

Assays were conducted at pH 7.0 as described in the text, at a fixed concentration of fructose 6-phosphate (0.2mm). Mg^{2+} was always added so that it exceeded the nucleoside triphosphate concentration by 3mm. All values are expressed as a percentage of the activity measured at 0.3mm-ATP, which is given as 100, although 0.3mm-ATP itself is already mildly inhibitory.

Nucleotide concn. ...	Activity		Ratio of velocities (V) $V_{low \text{ nucleoside triphosphate}} / V_{high \text{ nucleoside triphosphate}}$
	0.3mm	3.0mm	
ATP	100	22	4.55
dATP	15	1	15.0
dATP+0.3mm-ITP	44	0	—
ITP	89	83	1.07
GTP	103	67	1.54
UTP	54	42	1.29
CTP	47	34	1.38

primarily differing sensitivities to MgATP inhibition.

Although phosphofructokinase uses ATP as phosphate donor *in vivo* the enzyme displays little specificity for this nucleotide, and various nucleoside triphosphates can be substituted for ATP. Table 3 shows the ability of several nucleoside triphosphates to act as phosphate donors and also demonstrates their ability to inhibit the reaction at higher concentrations. The ratio ($V_{low \text{ nucleoside triphosphate}} / V_{high \text{ nucleoside triphosphate}}$) indicates the extent of this inhibition. In this context, when a nucleoside triphosphate is referred to as a substrate or inhibitor, it is known to be acting in this manner only when bound as a Mg^{2+} -nucleotide complex. In Table 3, the nucleoside triphosphates were tested both at a low concentration, where they should maximally activate the enzyme, and at a concentration ten times higher, where any inhibitory effects should be apparent. ATP, GTP and ITP appear to be good substrates, whereas UTP and CTP are significantly less effective, and dATP is a very poor phosphate donor. Both ATP and dATP are strongly inhibitory at the higher concentrations, whereas GTP, CTP and UTP are only slightly inhibitory. The strong inhibition by dATP is also observed in the presence of ITP, which, on the basis of insignificant inhibition at 3.0mm, apparently serves only as a substrate.

MgATP is the natural substrate for phosphofructokinase. Muntz (1953) reported that the brain enzyme could also use MnATP and CoATP as substrate. In Table 4 the ability of several bivalent metal ions to substitute for Mg^{2+} is demonstrated. To ensure that any inhibition due to the presence of free metal ions was minimized, only 1.5mm-metal ion was added in the presence of 1.0mm-ATP. As with the brain enzyme, Mg^{2+} , Mn^{2+} and Co^{2+} (as the

Table 4. Ability of some bivalent metal ions to replace Mg^{2+} as substrate for the pig spleen phosphofructokinase reaction

Assays were conducted at pH 7.0, as described in the text, with 0.2mm-fructose 6-phosphate and 1.0mm-ATP present. All metal ions were added to give a final concentration of 1.5mm.

Addition	Relative activity
$MgCl_2$	100
$CaCl_2$	0
$MnCl_2$	63
$CoCl_2$	39
$SrCl_2$	1
$CdCl_2$	0
$BaCl_2$	0

Fig. 3. Effect of bivalent metal-ion concentration on pig spleen phosphofructokinase activity

Assays were performed at pH 7.0, as described in the text, with 0.2mm-fructose 6-phosphate, 1.0mm-ATP and metal-ion concentrations as indicated: Mg^{2+} (■), Mn^{2+} (▲) and Co^{2+} (○).

M-ATP complexes) were able to act as substrates for phosphofructokinase.

Fig. 3 shows the effects on enzyme activity when these three metal ions were titrated at a fixed concentration of ATP. Under the conditions of the assay (pH 7.0 and 1.0mm-ATP) only 82% of the maximum velocity was reached at an Mg^{2+} /ATP ratio of 1.0 and maximum activity was not achieved until the total Mg^{2+} concentration exceeded 5.0mm. At 1.0mm-ATP and pH 7.0, the addition of 1.0mm- Mg^{2+} complexes 84% of the total ATP as $MgATP^{2-}$, leaving only 8% in the form of free ATP^{4-} (calculated by the method of Morrison & Heyde, 1972). The simplest explanation for the rise in activity observed between 1 and 5mm- Mg^{2+} is that the enzyme is not fully saturated with substrate ($MgATP^{2-}$) until the Mg^{2+} concentration reaches 5mm, when all of the ATP is effectively present in the $MgATP^{2-}$ form. There are two alternative explanations, namely: (i) that free ATP^{4-} itself may be so highly inhibitory that its removal, even by the addition of a large excess of another inhibitor [free Mg^{2+} is inhibitory (Lowry & Passonneau, 1966; Hickman, 1974)] causes a further rise in activity; and (ii) that low concentrations of free Mg^{2+} may be necessary for enzyme activity (as with several kinases) so that full activity is not achieved until a sufficient concentration of free metal ion is generated in the cuvette. Although it is not possible to distinguish between these alternatives, the close correlation between the percentage of the maximum activity achieved and the percentage of ATP present as $MgATP^{2-}$ throughout the Mg^{2+} concentration range would tend to favour the first explanation. When either Mn^{2+} or Co^{2+} was titrated at the same fixed concentration of ATP (1.0mm), maximum activity was reached at an ATP/metal-ion ratio of approx. 1:1. The maximum activity may be reached at a lower metal-ion concentration in this instance because the stability constants for the

ATP-metal-ion complexes are much higher than that reported for $MgATP$ (O'Sullivan, 1969). The rapid fall in activity when the 1:1 ratio is exceeded indicates either that free Mn^{2+} and Co^{2+} are much more inhibitory than free Mg^{2+} or that an inhibitory 2:1 M_2ATP complex is formed which competes with $MATP^{2-}$ at the substrate-binding site.

Activators and inhibitors

Activators. Regulation of phosphofructokinase activity by adenine nucleotides is well documented (Passonneau & Lowry, 1962; Underwood & Newsholme, 1965). Perhaps the most important single factor governing phosphofructokinase activity *in vivo* is its powerful inhibition by ATP at the very low concentrations of fructose 6-phosphate found in living tissues. Small percentage changes in ATP concentrations may be amplified via the adenylate kinase reaction into much larger percentage changes in the amounts of ADP and AMP (Newsholme, 1972). Fig. 4 shows the ability of ADP, AMP and cyclic AMP to activate phosphofructokinase. The calculated apparent K_A values for these three nucleotides were 0.035, 0.028 and 0.031mm respectively.

Fig. 5 shows the activation of phosphofructokinase by aspartate. The apparent K_A was calculated to be 0.68 ± 0.14 mm (three determinations). As far as is known, there have been no previous reports of activation of other mammalian phosphofructokinases by aspartate.

Both K^+ and NH_4^+ are potent stimulators of phosphofructokinase activity (Abrahams &

Fig. 4. Activation of pig spleen phosphofructokinase by ADP (a), AMP (b) and cyclic AMP (c)

Assay conditions, at pH 7.0, were as described in the text with substrate concentrations fixed at 0.2 mM-fructose 6-phosphate and 1.0 mM-MgATP. Adenine nucleotides were added at the concentrations indicated.

Younathan, 1971). In Fig. 6 the ability of K^+ and NH_4^+ , as well as Rb^+ , to stimulate enzyme activity is shown. The order of stimulatory capacity is: $K^+ > NH_4^+ > Rb^+$. Of greatest interest is the difference in apparent activation constants (K_A values) for the three ions. Double-reciprocal plots of data obtained in the appropriate concentration range, for each ion independently, gave K_A values of the order of 10–12 mM for K^+ and Rb^+ , whereas for NH_4^+ this value was only 0.35 mM. There is some suggestion in Fig. 6 that each cation at a high concentration causes a slight inhibition of enzyme activity.

Both fructose 1,6-diphosphate and P_i significantly activate phosphofructokinases from several sources (e.g. Lowry & Passonneau, 1966; Tarui *et al.*, 1972; Kühn *et al.*, 1973). Fig. 7 shows an experiment which was designed to compare the coupling-enzyme assay of phosphofructokinase activity with a direct assay in which the products of the reaction were allowed to accumulate until the reaction was stopped by acidification. Cuvette contents were then neutralized, coupling enzymes added, and the total amount of fructose 1,6-diphosphate formed was measured.

Fig. 5. Activation of pig spleen phosphofructokinase by aspartate

Assay conditions were similar to those described for Fig. 4.

Fig. 6. Activation of pig spleen phosphofructokinase by K^+ , NH_4^+ and Rb^+

Assay conditions were similar to those described for Fig. 4 except that univalent-metal-ion concentrations were varied as indicated: K^+ (Δ), NH_4^+ ($\blacksquare$) and Rb^+ ($\bullet$).

No increase in the reaction rate in the presence of the accumulated fructose 1,6-diphosphate was observed, suggesting that this molecule had little direct stimulatory effect on spleen phosphofructokinase. Direct addition of fructose 1,6-diphosphate to the cuvette, using a pyruvate kinase-lactate dehydrogenase coupling assay, confirmed this conclusion, although the activating effect of added K^+ , which is essential for pyruvate kinase activity, may have masked any additional stimulatory effect of fructose 1,6-diphosphate.

Inhibitors. MgATP is a potent inhibitor of pig spleen phosphofructokinase; its ability to inhibit

Fig. 7. Comparison of the coupled and direct enzyme assays for pig spleen phosphofructokinase

Conditions for both enzyme assays are described in the text. The coupled assay (○) was compared, under identical conditions, with a direct assay (▲) in which the reaction was stopped by acidification and fructose 1,6-diphosphate estimated enzymically on the neutralized solution.

Fig. 8. Inhibition of pig spleen phosphofructokinase by MgATP and the ability of fructose 6-phosphate to counter this inhibition

Assays were performed at pH 7.0, as described in the text, at two concentrations of fructose 6-phosphate (▲, 0.5 mM; ○, 0.2 mM) and MgATP as indicated.

Fig. 9. Inhibition of pig spleen phosphofructokinase by citrate and phosphoenolpyruvate

Assay conditions were similar to those described for Fig. 4. Citrate (a) and phosphoenolpyruvate (b) were added at the concentrations indicated.

activity is not reached until 0.3 mM-MgATP is present, whereas at 0.2 mM-fructose 6-phosphate, it is reached at a much lower MgATP concentration (0.1 mM).

In Fig. 9 the inhibitory effects of citrate and phosphoenolpyruvate are shown. Citrate has a differential inhibitory effect on the enzyme from different sources, being a particularly potent inhibitor of muscle phosphofructokinase (Kemp, 1971; Abrahams & Younathan, 1971). In this study the apparent K_i for citrate was found to be of the order of 1.2 mM. Phosphoenolpyruvate had a very similar inhibitory effect on the spleen enzyme, the apparent K_i being approx. 1.5 mM.

The phosphoglycerates are reported to be inhibitors of phosphofructokinase (Krzanowski &

the enzyme at two different concentrations of fructose 6-phosphate is shown in Fig. 8. In the presence of 0.2 mM-fructose 6-phosphate the apparent K_i for MgATP is only 1.3 mM, but it increases to 3.0 mM in the presence of 0.5 mM-fructose 6-phosphate. Also, with 0.5 mM-fructose 6-phosphate, maximum

Fig. 10. Inhibition of pig spleen phosphofructokinase by phosphoglycerates

Assay conditions were similar to those described for Fig. 4. 3-Phosphoglyceric acid (a), 2-phosphoglyceric acid (b) and 2,3-diphosphoglyceric acid (c) were added at the concentrations indicated.

Matschinsky, 1969). Fig. 10 compares the inhibitory capacity of 3-phosphoglyceric acid, 2-phosphoglyceric acid and 2,3-diphosphoglyceric acid. None of these molecules proved to be very effective inhibitors in the physiological concentration range; the apparent K_i for 3-phosphoglyceric acid was approx. 3.0mM, and that for 2,3-diphosphoglyceric acid approx. 2.2mM. Measurement of intracellular amounts of 3-phosphoglyceric acid and 2-phosphoglyceric acid in the freeze-clamped rat spleen gave values of 64 ± 14 and 9.7 ± 0.4 nmol/ml of intracellular water respectively (ten determinations $\pm$ S.E.M.).

Discussion

Isoenzyme composition

Phosphofructokinase is an extremely labile enzyme, and considerable difficulty was experienced in developing a satisfactory purification method for the spleen enzyme. The procedure of Massey & Deal

(1973), which was finally adopted, gave two isoenzyme peaks on DEAE-cellulose column chromatography. The low yield obtained raises the possibility that one or more isoenzymes may have been specifically lost during the purification procedure. However, comparison of Massey & Deal's (1973) original observations with that given in Table 1 shows that such an expectation is probably unreasonable. Liver phosphofructokinase is a homogeneous enzyme, and recoveries after successive purification steps were 39, 36 and 17% (to the end of the 'wash' step). Comparable recoveries in the present work were 67%, unknown and 21% (separate determinations were not conducted on the Mg^{2+} -precipitated enzyme). Further evidence that no isoenzyme was specifically lost during purification can be found in the electrophoretic data of Kurata *et al.* (1972), who demonstrated that only two isoenzymes are present in crude extracts from rat spleen. Thus it is assumed, but not proven, that the two isoenzymes separated on the DEAE-cellulose column are identical with those reported by Kurata *et al.* (1972) and that these two forms were conserved during purification. It remains possible, but in our opinion unlikely, that one of the two spleen isoenzymes was completely lost during purification and that the two activities described are an artifact of separation of a single species on the column.

Whereas no distinction can be made on purely kinetic grounds between the two isoenzymes separated on the DEAE-cellulose column, the kinetic properties of the combined activities exclude the possibility that spleen phosphofructokinase is either a purely 'liver' or purely 'muscle' type. The K_A for AMP (0.035mM) is almost identical with that of the muscle enzyme, measured under identical conditions, but the spleen enzyme is far less sensitive to citrate inhibition ($K_i = 1.3$ mM) and far more sensitive to $MgATP$ inhibition ($K_i = 1.5$ mM) than is the muscle enzyme, on the basis of the values reported by Kemp (1971). The strong likelihood that spleen phosphofructokinase is a hybrid form is supported by the electrophoretic data of Kurata *et al.* (1972) which demonstrates that neither isoenzyme band from spleen coincides with the slow (muscle) or the rapidly (liver) migrating species. It remains conjectural whether the erythrocyte enzyme, which has very similar kinetic and antigenic properties to liver phosphofructokinase (Kemp, 1971; Tsai & Kemp, 1973), is a significant component of the pig spleen preparation studied here, although such contamination might be anticipated if pig spleen contains roughly equal numbers of erythrocytes and leucocytes, as has been found with the rodent organ (Suter & Weidemann, 1975). The kinetic similarity of the two isoenzyme peaks obtained on DEAE-cellulose chromatography (cited above) and their obvious dissimilarity to a purely liver type of

activity, does not support the view that one of these activities is uniquely derived from the erythrocyte population, and this conclusion is in harmony with the observation that spleen erythrocytes make an extremely small percentage contribution to the glycolytic activity of spleen slices, in spite of their large numbers in this tissue (Suter & Weidemann, 1975). The separate question of whether one or both isoenzyme components of the spleen enzyme is a unique hybrid type peculiar to leucocytes (or to the lymphocyte subpopulation, which is the major leucocyte type in the spleen) cannot be resolved from the available evidence; clearly, antisera raised to both the liver and muscle enzymes inactivate both isoenzymes from the spleen (Tsai & Kemp, 1973), whereas under the same conditions the enzymes from brain and thymus retain considerable residual activity. It remains an interesting possibility that there is a third 'brain' type of phosphofructokinase that is present in immature thymus-derived lymphocytes but not in mature T-lymphocytes that have lodged in the spleen.

Regulatory properties

It has long been recognized that adenine nucleotides are major potential regulators of phosphofructokinase *in vivo* (Passonneau & Lowry, 1962). Both ATP and MgATP are potent inhibitors of enzyme activity (Lowry & Passonneau, 1966), whereas both ADP and AMP relieve this inhibition (Passonneau & Lowry, 1962). All three adenine nucleotides share a common binding site (Kemp & Krebs, 1967), so it is perhaps most reasonable to consider regulation of phosphofructokinase activity *in vivo* in relation to the physiological concentrations of all three adenine nucleotides (Shen *et al.*, 1968). When this is done at pH 7.0 and with low concentrations of fructose 6-phosphate (0.06 mM), the activity is found to respond steeply to the 'energy-charge' ratio, declining from 95 to 50% of the maximum activity as the energy-charge ratio is increased through the range 0.78–0.96 (Hickman, 1974). An increase in fructose 6-phosphate concentration to 0.2 mM completely overrides the inhibition observed at high energy charge. In contrast, citrate only slightly potentiates the sensitivity of phosphofructokinase to energy-charge inhibition. Although citrate inhibits isolated spleen phosphofructokinase and is undoubtedly a potent physiological inhibitor of phosphofructokinase activity in tissues such as muscle, it appears unlikely that the spleen enzyme, which has a K_i for citrate 5–10 times greater than the estimated concentration in the freeze-clamped organ (0.15–0.25 mM), will be greatly affected by changes in citrate concentration while the MgATP concentration and the energy-charge ratio remain high.

Both fructose 6-phosphate and fructose 1,6-diphosphate are potent activators of phosphofructokinases from a number of sources (Lowry & Passonneau, 1966; Underwood & Newsholme, 1965). The strong de-inhibitory role of fructose 6-phosphate is confirmed in Fig. 8. However, as shown in Fig. 7, no stimulatory effect can be found with fructose 1,6-diphosphate. At the longest time-interval used, the fructose 1,6-diphosphate concentration in the cuvette was of the order of 20 μ M, well above the reported K_A value of 1 μ M (Lowry & Passonneau, 1966), yet no significant increase in the rate was observed. On the other hand, fructose 1,6-diphosphate was found to be an excellent stabilizer of the enzyme. The net conclusion reached on the basis of the studies *in vitro* reported here must be that whereas fructose 1,6-diphosphate is an effector of phosphofructokinase, its effect on the spleen enzyme is far less pronounced than that observed with the enzyme isolated from other sources. In terms of mechanism of action, both fructose 1,6-diphosphate and P_i appear to be more effective in blocking inhibition or inactivation of the enzyme by MgATP than in causing direct activation, and may therefore play a 'protective' rather than an activating role (Hickman, 1974).

Studies on the pH-dependence of spleen phosphofructokinase under three separate conditions gave an optimum pH of 8.2, which is essentially similar to that reported for other tissues (Layzer *et al.*, 1969; Mansour & Ahlfors, 1968). As noted earlier, the different velocities observed at different pH values are primarily a function of the relative concentrations of MgATP and fructose 6-phosphate. At physiological pH and in the presence of physiological concentrations of effectors, it is probable that spleen phosphofructokinase exists in a highly inhibited state. Intracellular pH can vary with the metabolic state of the tissue and it is probable, as Trivedi & Danforth (1966) have suggested, that small changes in the intracellular pH may be important in regulating the activity of phosphofructokinase. The intracellular pH of the circulating human lymphocyte is 7.3 (Zieve *et al.*, 1967), although it may decrease significantly during periods of increased metabolic activity, when increased amounts of acidic metabolic end products (such as lactate) accumulate. It is in this pH region that phosphofructokinase is most sensitive to regulation by small changes in the MgATP concentration.

During the preliminary screening of possible effector molecules, aspartate was found to activate the spleen enzyme with an apparent K_A of approx. 0.7 mM. Greenbaum *et al.* (1971) have calculated the cytoplasmic concentration of aspartate in the well-fed rat liver (0.65 mM); this value fell during starvation, and rose during the re-feeding of a high-carbohydrate diet. In addition, the excellent corre-

lation observed between aspartate concentration and phosphofructokinase activity supports the contention that aspartate may be a regulator of phosphofructokinase activity in liver.

The role of aspartate in regulating phosphofructokinase activity in spleen and thymus is less certain. Measurements of the aspartate concentrations in both tissues and of the cytoplasmic concentration by the method of Williamson (1969) gave approximate values of 1.7 and 2.5 mM for spleen and thymus respectively, i.e. both well above the apparent K_A . No conditions were found where the concentrations of aspartate fell to near the K_A concentration. Thus it is considered unlikely that aspartate is an important glycolytic regulator in lymphoid tissue. Changes in cytoplasmic aspartate may be more important in tissues such as liver or muscle, where more extensive 'futile cycling' between fructose 6-phosphate and fructose 1,6-diphosphate occurs which is sensitive to a wide range of effector molecules.

The strong activatory capacity of both K^+ and NH_4^+ has long been known (Muntz & Hurwitz, 1951; Muntz, 1953). Although phosphofructokinase has a greater affinity for NH_4^+ (apparent K_A 0.3 mM) than for K^+ (apparent K_A 10–15 mM), K^+ is present in the cell at concentrations which will saturate phosphofructokinase (Long, 1971). NH_4^+ could still be an important regulatory molecule in the presence of saturating amounts of K^+ if there are separate binding sites on the enzyme for the two ions. Reports have appeared (Abrahams & Younathan, 1971; Hickman & Weidemann, 1973) indicating activation by NH_4^+ in the presence of saturating concentrations of K^+ . However, later results from this laboratory (Hickman, 1974) have served only to emphasize the similarity of the activation by both ions. Final comment on the possible physiological role of NH_4^+ as an activator of phosphofructokinase will have to await competitive-binding experiments with the homogeneous enzyme.

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