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Some Properties of Phosphofructokinase from Kidney Cortex and their Relation to Glucose Metabolism

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1. Phosphofructokinase from rat kidney cortex has been partially purified by using a combination of isoelectric and ammonium sulphate precipitation. This preparation was free of enzymes which interfered with the measurement of either product of phosphofructokinase. 2. At concentrations greater than the optimum, ATP caused inhibition which was decreased by raising the fructose 6-phosphate concentration. This suggested that ATP reduced the affinity of phosphofructokinase for the other substrate. Citrate potentiated the ATP inhibition. 3. AMP and fructose 1,6-diphosphate relieved the inhibition by ATP or citrate by increasing the affinity of the enzyme for fructose 6-phosphate. 4. K^+ is shown to stimulate and Ca^{2+} to inhibit phosphofructokinase. 5. The similarity between the complex properties of phosphofructokinase from kidney cortex and other tissues (e.g. cardiac and skeletal muscle, brain and liver) suggests that the enzyme in kidney cortex tissue is normally subject to metabolic control, similar to that in other tissues.

Both liver and kidney cortex are capable of carrying out glucose degradation or glucose synthesis. The two enzymes PFK‡ (ATP–D-fructose 6-phosphate 1-phosphotransferase, EC 2.7.1.11) and FDPase (D-fructose 1,6-diphosphate 1-phosphohydrolase, EC 3.1.3.11) provide a point of control for both pathways (see Wu, 1963; Krebs, 1964; Newsholme & Underwood, 1966). The properties of liver PFK and FDPase have been investigated and, on the basis of these properties, a common control mechanism for both enzyme activities was proposed (Underwood & Newsholme, 1965*a*,*b*). The properties of FDPase from kidney were very similar to those of the liver enzyme (Mendicino & Vasarhely, 1963). The properties of PFK from kidney cortex were therefore investigated to ascertain whether the postulated common control mechanism of PFK and FDPase in liver was also applicable to kidney cortex.

As with liver, the presence in kidney cortex of a very active FDPase, which has a very low K_m for fructose diphosphate, could interfere in the assay for PFK activity. This paper describes a partial purification procedure for kidney cortex PFK that results in very low activities of a number of enzymes that could interfere in the PFK assay. With this preparation it has been found that some properties of kidney cortex PFK are very similar to those of the enzyme isolated from liver. A preliminary account of the purification procedure and the properties of the enzyme has been published (Underwood & Newsholme, 1965*c*).

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‡ Abbreviations: PFK, phosphofructokinase; FDPase, fructose 1,6-diphosphatase.

METHODS

Materials. Animals, enzymes and chemicals were obtained from sources given by Underwood & Newsholme (1965*b*).

Preparation of crude extracts. The kidney cortex from a freshly killed rat was cooled on ice, weighed, cut into small pieces and homogenized in 3 vol. of a solution containing 20 mM-tris, 5 mM-$MgSO_4$ and 0·1 mM-EDTA, pH 8·0, in a manually operated glass Potter–Elvehjem homogenizer. This extract was used to determine total PFK activity in kidney cortex.

Partial purification of kidney cortex phosphofructokinase. The capsule surrounding the kidneys of a freshly killed male Wistar rat was removed and the organs were bisected longitudinally. Scissors were used to cut away the medulla and chop the cortex into pieces, which were then homogenized in 3 vol. of ice-cold 20 mM-potassium phosphate, pH 8·0, in a manually operated Potter–Elvehjem glass homogenizer. The homogenate was centrifuged at 38000*g* for 45 min. at 2°. All subsequent steps took place at 0°. The pH of the clear supernatant solution was adjusted to 5·9 with 0·05 N-acetic acid. After 15 min., the resulting precipitate was centri-

fuged at 9000*g* for 10 min. and washed once by resuspending in the original volume of 10 mM-potassium phosphate, pH 6·0, and centrifuging again. The supernatant solution was discarded and the precipitate dissolved in 100 mM-potassium phosphate, pH 8·0. To this solution was added sufficient saturated $(NH_4)_2SO_4$ solution to bring it to 35% saturation. After mixing for a further 20 min. the precipitate was centrifuged at 9000*g* for 10 min. and discarded. The concentration of $(NH_4)_2SO_4$ in the supernatant was brought to 55% saturation with saturated $(NH_4)_2SO_4$ solution and after a further 20 min. was again centrifuged at 9000*g* for 10 min. This precipitate was washed as before but with a solution containing 55%-saturated $(NH_4)_2SO_4$ and 48% 100 mM-potassium phosphate, pH 8·0, and then dissolved in 20 mM-tris–5 mM-$MgSO_4$–0·1 mM-EDTA, pH 8·0, to avoid complications which might arise with phosphate buffer, which is an activator and possibly a de-inhibitor of the enzyme. Ammonium sulphate was removed from this preparation by passage through a column of Sephadex G-25. Although not unstable enough to necessitate correction of results for activity loss, the Sephadex-treated enzyme lost 20–30% of its activity in 24 hr. at 0°. This preparation contained very little FDPase and aldolase and virtually no phosphoglucose isomerase, glycerophosphate dehydrogenase or adenylate kinase.

Assay of phosphofructokinase activity. PFK activity in crude and partially purified extracts was assayed as described by Underwood & Newsholme (1965*b*). However, as the partially purified extract of kidney cortex PFK contained very little FDPase activity it could also be assayed by following fructose 1,6-diphosphate formation by a coupled enzyme system in a Gilford recording spectrophotometer at 25°. The assay medium comprised 20 mM-imidazole, 5 mM-$MgCl_2$, 0·1 mM-$NADH_2$, 0·1 mM-EDTA, 50 μg. of aldolase, 5 μg. of α-glycerophosphate dehydrogenase and triose phosphate isomerase in 2 ml., and varying concentrations of fructose 6-phosphate and ATP, at pH 7·0. The coupling enzymes were passed down a column of Sephadex G-25 before use to remove $(NH_4)_2SO_4$ (see Underwood & Newsholme, 1965*b*). The reaction was initiated by addition of 25 μl. of extract to the cuvette, and, after an equilibration period of 2–3 min., the reaction was linear for 5–10 min. This linear rate was used to calculate the activity of PFK. Control measurements were carried out in the absence of either fructose 6-phosphate or ATP. Unlike the liver enzyme, the concentration of kidney cortex PFK did not affect its specific activity under any condition.

RESULTS

Properties of kidney cortex phosphofructokinase. The partially purified preparation of kidney cortex PFK contained very little FDPase and so could be assayed by following the formation of fructose 1,6-diphosphate. As with the enzyme from liver (Underwood & Newsholme, 1965*b*), the formation of ADP could also be followed. This latter assay required the addition of K^+ and phosphoenolpyruvate, which are cofactor and substrate respectively of the auxiliary enzyme pyruvate kinase. The activity of PFK was not inhibited by phosphoenolpyruvate, but was increased markedly by K^+. Under conditions of identical substrate, Mg^{2+} and K^+ concentrations, both assays gave the same activity.

Effect of K^+. As it was not possible to separate hepatic PFK from FDPase, Underwood & Newsholme (1965*b*) assayed PFK by following ADP formation, and K^+ is a necessary cofactor for this assay; the effect of K^+ on PFK activity could not therefore be investigated. Since kidney cortex PFK is separated from FDPase, it can be assayed by following fructose diphosphate formation, and therefore the effects of K^+ can be studied. Table 1 shows the stimulation of activity caused by increasing concentrations of K^+. A maximal stimulation of about 15-fold is given by 50 mM-K^+.

Effect of ATP and fructose 6-phosphate. As in liver, ATP inhibits kidney cortex PFK by reducing its affinity for fructose 6-phosphate. Fig. 1 shows the effect of increasing fructose 6-phosphate concentration at two different ATP concentrations in the presence of 100 mM-K^+. Omission of K^+ gave essentially the same results but with lower activities. Both curves are sigmoid, although this is less pronounced at the lower ATP concentration. At an ATP concentration of 0·01 mM the $V_{max.}$ was lower than at an ATP concentration of 0·1 mM,

Table 1. *Effect of* K^+, *AMP, citrate, inorganic phosphate, ADP and* Ca^{2+} *on partially purified kidney cortex phosphofructokinase*

PFK was assayed by following the formation of fructose diphosphate in the presence of 100 mM-KCl, except when K^+ was investigated, and its activity is expressed in μmoles of product formed/g. wet wt. of tissue/hr. Citrate, AMP and ADP were used as the sodium salts and inorganic phosphate as the potassium salt. All solutions were at pH 7·0.

Substrate concn. 0·63 mM-fructose 6-phosphate and 0·1 mM-ATP			Substrate concn. 2·5 mM-fructose 6-phosphate and 0·5 mM-ATP		
Concn. (mM)		PFK activity	Concn. (mM)		PFK activity
KCl	0	0·5	Citrate	0	3·0
	5	3·4		0·25	1·9
	10	4·7		0·50	1·3
	25	6·7		0·75	1·1
	50	7·1		1·00	1·0
	100	6·1	AMP	0	7·6
Inorganic phosphate	0	0·4		0·08	12·4
	2·5	2·0		0·15	14·9
	5·0	2·5		0·3	16·0
	10·0	2·7		0·45	16·5
ADP	0	0·4		0·75	16·2
	0·1	1·3	$CaCl_2$	0	3·0
	0·4	1·9		2·6	2·4
	1·0	2·3		5·5	1·8
				11·0	1·2

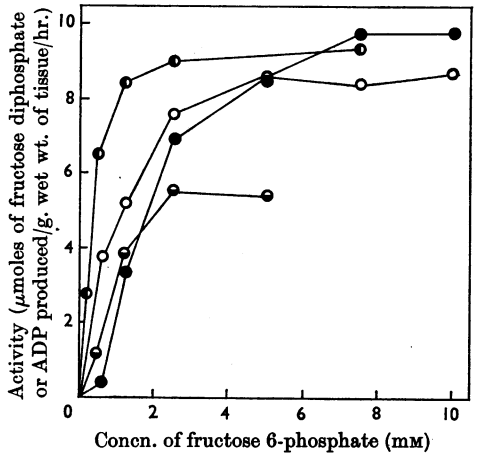

Fig. 1. Effect of fructose 6-phosphate on the activity of partially purified kidney cortex PFK. Conditions of assay are described in the text. ◒, 0·01 mM-ATP; ●, 0·1 mM-ATP; ◐, 0·1 mM-ATP and 0·25 mM-AMP; ○, 0·1 mM-ATP and 1·25 mM-fructose diphosphate.

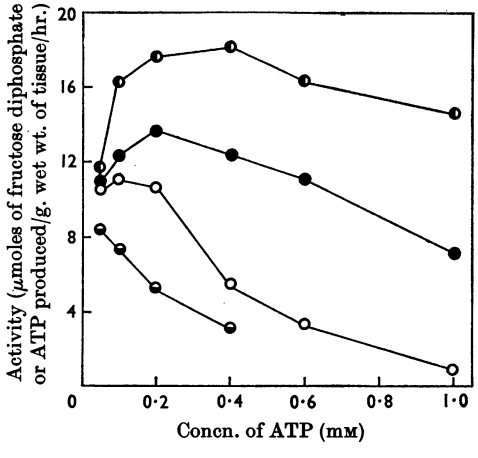

Fig. 2. Effect of ATP on the activity of partially purified kidney cortex PFK. Conditions of assay are described in the text, and 100 mM-KCl was always present. ◒, 1·25 mM-Fructose 6-phosphate; ○, 2·5 mM-fructose 6-phosphate; ●, 2·5 mM-fructose 6-phosphate and 1·25 mM-fructose diphosphate; ◐, 2·5 mM-fructose 6-phosphate and 0·25 mM-AMP.

probably because ATP was limiting for its substrate site. Fig. 2 shows the effect of increasing ATP at two concentrations of fructose 6-phosphate: ATP inhibition is relieved by increasing the concentration of fructose 6-phosphate.

Effect of AMP and fructose diphosphate. AMP and fructose disphosphate both relieve the inhibition by ATP. They reduce the concentration of fructose 6-phosphate necessary to achieve $V_{max.}$, which is the same whether or not these activators are present. In their presence the curve of increasing fructose 6-phosphate against activity is not sigmoid (Fig. 1). The effect of both activators became greater as the ATP concentration increased (Fig. 2). The range of AMP concentrations which stimulate PFK activity is shown in Table 1. Very low concentrations of fructose diphosphate were effective in relieving ATP inhibition (10 μM with 2·5 mM-fructose 6-phosphate and 0·5 mM-ATP).

Effect of citrate. Low concentrations of citrate inhibit PFK (Table 1) and the extent of this inhibition depends on the ATP concentration. As the latter increased at a fixed fructose 6-phosphate concentration the percentage inhibition by citrate increased (Table 2). Citrate therefore potentiates ATP inhibition.

Table 2. *Effect of ATP on the citrate inhibition of partially purified kidney cortex phosphofructokinase*

Activity was measured by following the formation of fructose diphosphate, and is expressed as μmoles of product formed/g. wet wt. of tissue/hr. Fructose 6-phosphate concentration was 2·5 mM.

Concn. of ATP (mM)	PFK activity: No addition	PFK activity: With citrate (0·5 mM)	Inhibition by citrate (%)
0·05	2·6	2·2	18
0·1	3·5	2·4	31
0·2	3·6	2·6	28
0·5	2·9	1·6	45
1·0	1·0	0·3	70

Table 3. *Effects of intermediates of tricarboxylic acid cycle and acetoacetate on partially purified kidney cortex phosphofructokinase activity*

Activity was measured by following the formation of fructose diphosphate in the presence of 100 mM-KCl, and is expressed as μmoles of product formed/g. wet wt. of tissue/hr. If necessary compounds were adjusted to pH 7·0 before addition to the cuvette.

Addition	Concn. (mM)	PFK activity	Inhibition (%)
Control	—	4·0	—
Citrate	0·5	1·9	53
α-Oxoglutarate	1·25	3·7	7
Malate	1·25	2·7	23
Fumarate	1·25	3·7	7
Succinate	1·25	3·0	25
Acetoacetate	5·0	3·7	7
Acetyl-CoA	0·5	3·5	12

Effect of intermediates of the tricarboxylic acid cycle. Various other intermediates of the tricarboxylic acid cycle were much less inhibitory than citrate (Table 3).

Effect of Ca^{2+}, *inorganic phosphate and ADP.* The presence of Ca^{2+}, inorganic phosphate or ADP altered the activity of PFK; inorganic phosphate and ADP stimulated, whereas Ca^{2+} inhibited, activity (Table 1). The concentration ranges of ADP, Ca^{2+} and inorganic phosphate, which inhibit PFK activity, are similar to those found in the intact tissue, and therefore may be described as physiological. However, the concentrations of these compounds in the immediate environment of PFK in the kidney cortex cell is unknown, so that the suggestion of physiological significance must remain speculative.

DISCUSSION

The present results show that some properties of kidney cortex PFK are very similar to those of the enzyme that is present in skeletal muscle, brain and cardiac muscle (Passonneau & Lowry, 1962, 1963; Mansour, 1963). ATP inhibited the activity of the enzyme by decreasing the affinity for the other substrate, fructose 6-phosphate. The inhibition can therefore be removed by increasing the concentration of fructose 6-phosphate. The inhibition was also reversed by low concentrations of AMP or fructose diphosphate, which appear to compete with the inhibitory function of ATP, but not with its action as a substrate for PFK. The enzyme activity was also inhibited by citrate, which became more inhibitory as the ATP concentration was increased; this suggests that citrate potentiates the inhibition by ATP. The decrease of citrate inhibition by AMP or fructose diphosphate, or conversely the ability of citrate to decrease the effects of AMP or fructose diphosphate on the inhibition by ATP, support the above suggestion. Other intermediates of the tricarboxylic acid cycle also inhibited PFK activity, but they were much less effective than citrate. There is no indication that the inhibitions of PFK by malate or succinate are of physiological significance. The response of kidney cortex PFK to physiological concentrations of these nucleotides and intermediates, and the similarity with PFK from other tissues, suggest that these properties play an important role in the regulation of PFK activity in kidney cortex. In particular, the similarity between the properties of kidney cortex PFK and FDPase to these enzymes from liver suggests that a similar system for the control of glycolysis and gluconeogenesis may operate at this level in both tissues. Newsholme & Underwood (1966) have reported evidence that these properties of PFK, particularly the citrate inhibition, may play some role in the regulation of glucose formation as well as glucose degradation in kidney cortex.

The curves shown in Figs. 1 and 2 are important because they show the sigmoid rate curves for PFK when the fructose 6-phosphate concentration is varied, and a maximum activity when the ATP concentration is varied. These characteristic properties could be explained by reference to the allosteric model of Monod, Wyman & Changeux (1965), or to a kinetic interpretation based on a preferred pathway for formation of the enzyme ternary complex (see Ferdinand, 1966).

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REFERENCES

Ferdinand, W. (1966). *Biochem. J.* **98**, 278.
Krebs, H. A. (1964). *Proc. Roy. Soc.* B, **159**, 545.
Mansour, T. E. (1963). *J. biol. Chem.* **238**, 2285.
Mendicino, J. & Vasarhely, F. (1963). *J. biol. Chem.* **238**, 3528.
Monod, J., Wyman, J. & Changeux, J.-P. (1965). *J. molec. Biol.* **12**, 88.
Newsholme, E. A. & Underwood, A. H. (1966). *Biochem. J.* **99**, 24c.
Passonneau, S. V. & Lowry, O. H. (1962). *Biochem. biophys. Res. Commun.* **7**, 10.
Passonneau, J. V. & Lowry, O. H. (1963). *Biochem. biophys. Res. Commun.* **13**, 372.
Underwood, A. H. & Newsholme, E. A. (1965*a*). *Biochem. J.* **95**, 767.
Underwood, A. H. & Newsholme, E. A. (1965*b*). *Biochem. J.* **95**, 868.
Underwood, A. H. & Newsholme, E. A. (1965*c*). *Biochem. J.* **97**, 6P.
Wu, R. (1963). *Biochem. biophys. Res. Commun.* **14**, 79.