REGULATION OF PHOSPHOFRUCTOKINASE BY PHOSPHOCREATINE AND PHOSPHORYLATED GLYCOLYTIC INTERMEDIATES

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Summary: P-fructokinase from brain and muscle is inhibited in vitro by P-creatine, 3-P-glycerate, P-enolpyruvate, 2-P-glycerate and 2,3-P_2-glycerate. This inhibition is most pronounced at neutral pH, in the presence of inhibitory levels of ATP and of essentially non-activating levels of fructose-6-P. Evidence found in the literature suggests that inhibition in vivo of P-fructokinase by these compounds plays a role in regulation of glycolysis.

The activity of P-fructokinase is evidently regulated by a large variety of physiological substances (1-9). It has been shown that ATP and citrate are potent inhibitors (1-4) and that ADP, 5'-AMP, 3',5'AMP, P_i, fructose-6-P, fructose-1,6-P_2 and NH_3 are activators and/or deinhibitors of the enzyme (5-9). In a recent report concerning the kinetic properties of the muscle enzyme, glucose-1,6-P_2 has been added to the group of activators and/or deinhibitors (9). However, conflicting results have been reported (2,7) regarding a possible inhibitory action of P-creatine and P-enolpyruvate. Since we were unaware of any study that followed up these experiments, the in vitro influence of the latter two compounds and of several other metabolites on the activity of P-fructokinase from brain, muscle and yeast was reinvestigated. It was found that at pH 7.1 P-creatine, 3-P-glycerate, 2-P-glycerate, 2,3-P_2-glycerate and P-enolpyruvate substantially decreased the activity of both brain and muscle P-fructokinase. However, this inhibition was only observed when inhibitory levels of ATP and essentially non-activating concen-

trations of fructose-6-P were employed. P-fructokinase from yeast, under comparable assay conditions, was not affected by these inhibitors. Muscle and brain enzymes inhibited by the compounds above were deinhibited by fructose-6-P and glucose-1, 6-P_2. When studied at pH 8.1 with conditions otherwise unchanged, these inhibitors and activators alike were ineffective. If, however, ATP was raised 15-fold to levels which were inhibitory at this higher pH, the enzyme from brain became susceptible to inhibition by physiological levels of 3-P-glycerate. This susceptibility to inhibition did not extend to the other inhibitors. It is not unreasonable to suppose that these *in vitro* studies are of significance for the understanding of regulation of glycolysis.

METHODS

P-fructokinase was assayed fluorometrically according to the method of Lowry *et al.* (6), except that the pH was altered and that no extra sulfate was added to the reagent (See legend to Fig. 1). On the day of use P-fructokinase was diluted in 10 mM potassium phosphate, pH 7.5, containing 100 μM 5-'AMP, 100 μM EDTA and 0.02 % bovine serum albumin. The enzymes from muscle and yeast were obtained from Boehringer and Sons. They were freed of most of the $(NH_4)_2SO_4$ by centrifugation and dissolved in the above buffer. The brain enzyme was prepared from sheep brain according to Ling *et al.* (10). The enzyme was purified 140-fold resulting in an activity of 62 μmoles/mg protein/hour. The brain enzyme was stored at -80° in 0.1 M potassium phosphate, pH 7.4, which contained 2 mM EDTA. The auxillary enzymes, essentially free of sulfate, were kept at 4°. All other biochemicals were purchased from Sigma or Boehringer and were standardized by familiar enzymatic fluorometric or spec-

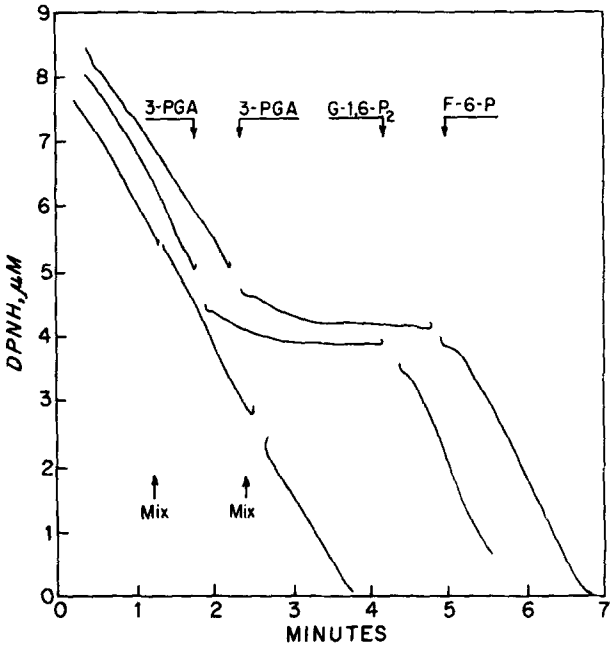

Fig. 1. Inhibition of brain P-fructokinase by 3-P-glycerate and deinhibition by glucose-1, 6-P_2 and fructose-6-P.

The reaction was conducted at 25° in 1 ml of imidazole-HCl buffer, pH 7.1, with 150 mM potassium acetate, 5 mM $MgCl_2$, 260 μM ATP, 75 μM fructose-6-P, 10 μM DPNH, 5 μg/ml of glycero-P-DH, 30 μM/ml of aldolase and 10 μg/ml of triose-P-isomerase. The reaction was started with 1.5 μg/ml P-fructokinase. Additions were made as indicated: 78 μM 3-P-glycerate (3-PGA), 43 μM glucose-1,6-P_2 (G-1,6-P_2) and an additional 181 μM (total 256 μM) fructose-6-P (F-6-P).

trophotometric methods (11). DPNH was freed of 5'-AMP by treatment with alkaline phosphatase, and was then removed from the enzyme by dialysis.

RESULTS AND DISCUSSION

The effects of 3-P-glycerate and of the two deinhibitors glucose-1,6-P_2 and fructose-6-P added to brain P-fructokinase are shown (Fig. 1) to illustrate the assay procedure. The complete inhibition caused by 3-P-glycerate was entirely removed by the sugar phosphates. All data reported in this paper were obtained by the use of similar experiments.

A comparison of the curves in Fig. 2 shows that 3-P-glycerate is the most potent and P-creatine is the least potent inhibitor of the group of compounds tested. The three other phosphorylated tricarbon compounds hold an intermediate position. Concentrations of inhibitors resulting in 50 % inhibition were of the order of magnitude previously shown to occur in a variety of

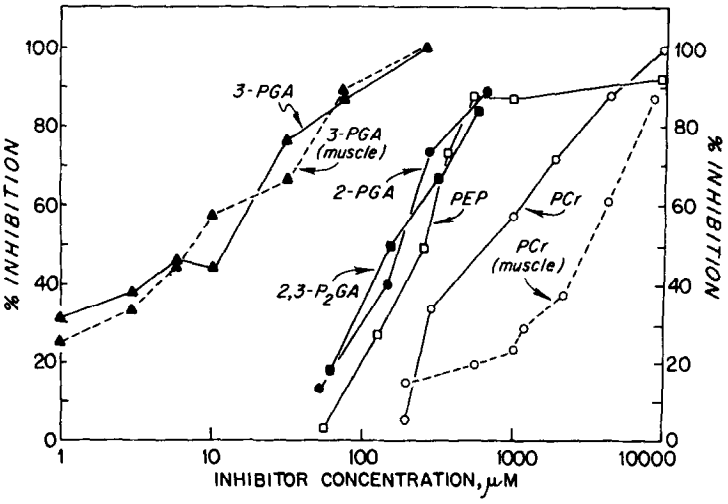

Fig. 2. Inhibition of P-fructokinase from brain and muscle by five phosphate compounds.

Assay conditions for brain enzyme are as given in Fig. 1. The reagent used for muscle enzyme had a higher level of ATP (0.77 mM). The rate observed prior to addition of the inhibitor served as control for calculating inhibition (see also Fig. 1.). Abbreviations not defined above are: 2-PGA (2-P-glyceric acid), 2,3-P_2-GA (2, 3-P_2-glyceric acid), PEP (P-enolpyruvic acid) and PCr (P-creatine).

organs in vivo (11-14). It is of interest that for a similar degree of inhibition of muscle P-fructokinase higher levels of all inhibitors were required (only the results for 3-P-glycerate and P-creatine are shown). The difference in sensitivity of the latter enzyme extends to ATP which had to be tripled in order to elicit inhibition of the same magnitude as that observed with brain enzyme. The yeast enzyme, which was sensitive to inhibition by ATP and citrate, was not affected by 3-P-glycerate, P-enolpyruvate, or P-creatine under conditions where the mammalian enzymes were inhibited.

The efficacy of all inhibitors depended on the levels of ATP and fructose-6-P present in the reagent (Figures 3 and 4). Inhibition became more apparent with increasing concentrations of ATP, but became less pronounced when fructose-6-P levels were elevated. Similar data were obtained for muscle P-fructokinase, except that higher levels of ATP were needed in order to evoke changes of the same extent (not shown).

The pH of the reagent proved to be crucial for the kinetic properties of the enzyme (Table 1), a point evident from the

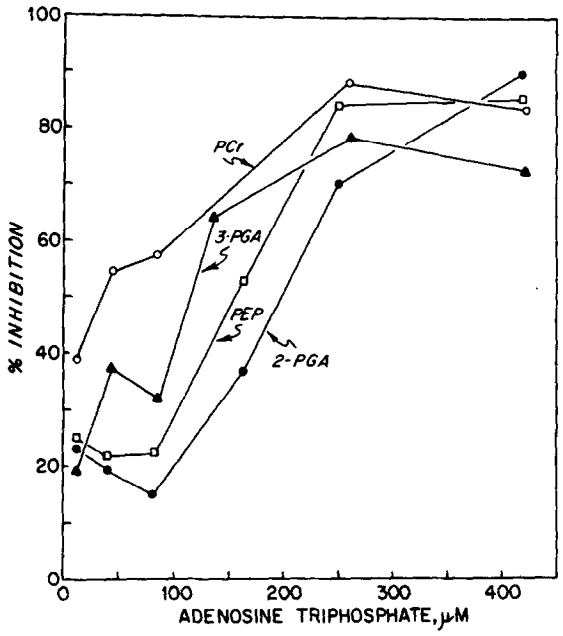

Fig. 3. Enhancement by ATP of the action of 4 inhibitors of brain P-fructokinase.

Inhibition is expressed as % of rate with the same ATP level in absence of inhibitor. Inhibitors were: 32 μM 3-PGA, 262 μM 2-PGA, 384 μM PEP and 4.41 mM PCr. Fructose-6-P was 75 μM throughout, the pH was 7.1.

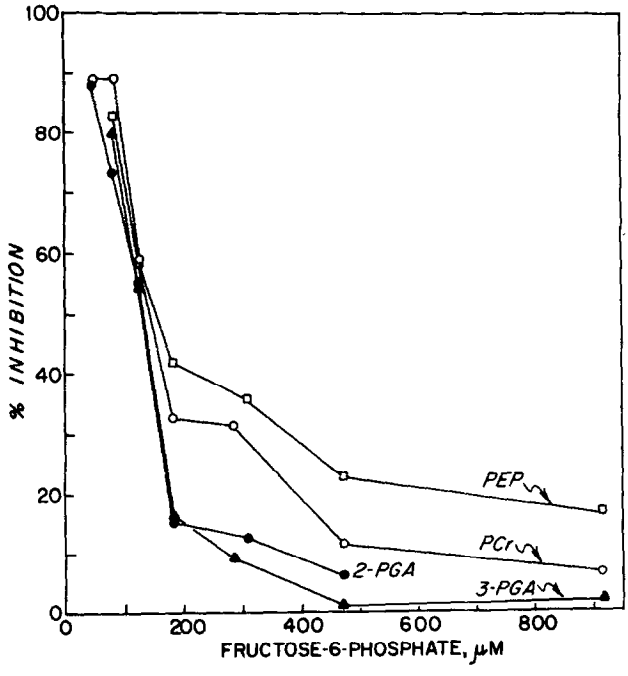

Fig. 4. Fructose-6-P as an antagonist of 4 inhibitors of brain P-fructokinase.

The conditions were the same as for Fig. 3, except that ATP was held constant at 260 μM.

work of other investigators (6,7,15). Under the conditions chosen, inhibitors, as well as activators, worked at pH 7.1, but were entirely inactive when tested at pH 8.1. When ATP was

Table I

pH Dependency of Kinetics of Brain P-fructokinase

For assay conditions see Fig. 1. In assays made at pH 8.1 the buffer was 0.1 M Tris-HCl. Fructose-6-P was 75 μM except as noted; ATP was 260 μM. Activities were recorded in terms of μmoles of fructose-1,6-P_2 formed per mg protein per hour. See legend of Fig. 2 for abbreviations.

Addition(s)		pH 7.1	pH 8.1
None		46.0	128
PCr	0.48 mM	16.8	
	4.41 mM	1.6	117
3-PGA	7.35 μM	23.7	
	78 μM	1.1	110
2-PGA	138 μM	12.9	
	472 μM	2.4	123
PEP	148 μM	23.6	
	520 μM	3.6	119
3-PGA, 7.35 μM; 2-PGA, 129 μM; PEP, 121 μM; PCr, 875 μM		1.6	122
3-PGA, 7.35 μM; 2-PGA, 129 μM; PEP, 121 μM; PCr, 875 μM; glucose-1,6-P_2, 43 μM		66.8	129
3-PGA, 7.35 μM; 2-PGA, 129 μM; PEP, 121 μM; PCr, 875 μM; fructose-6-P, 256 μM		65.3	156

raised to an inhibitory level of 4.36 mM, however, P-fructokinase again became susceptible to inhibition by 3-P-glycerate (94 % inhibition with 78 μM). In spite of the increased ATP, the other inhibitors remained essentially inactive at the highest concentrations listed in Table 1.

In all cases studied so far, combinations of the various inhibitors were additive or more than additive (Table 1). When the four inhibitors shown were combined at concentrations that individually caused approximately half maximal effects, the activity of P-fructokinase fell to a negligible value. The inhibition was removed by glucose-1,6-P_2 at levels usually present in tissues and also by fructose-6-P at levels which are much higher than commonly found in cells.

The following compounds had little influence on rate when tested in the brain P-fructokinase system at pH 7.1 with 0.26 mM ATP, and 0.075 mM fructose-6-P: 10-100 mM glucose, 2 mM creatine, 200 μM pyruvate, 3 mM lactate, 1 mM α-glycero-phosphate, 60 μM acetyl-S-CoA and 300 μM α-ketoglutarate.

This study adds 3 inhibitors, phosphorylated members of the glycolytic sequence, to the long list of modifiers of P-fructokinase. It also affirms (9) and extends (7) some earlier observations on muscle P-fructokinase regarding the actions of glucose-1,6-P_2 (9), P-enolpyruvate and P-creatine (7), and also clarifies some earlier contradictory results (2,7).

The results of analyses of tissue metabolites under various experimental conditions (11,13) lead one to the conclusion, that the substantial decrease of P-creatine and ATP and the concomitant rise of P_i and AMP, will activate P-fructokinase and cause an acceleration of glycolysis. The rapid and substantial rise of the distal components of the glycolytic pathway seen in such instances (11) could serve as a useful feedback inhibition to curb an overshoot of such a stimulation of glycolysis.

It is also reasonable to speculate that the frequently occurring rise of P-enolpyruvate and 3-P-glycerate together with the accumulation of citrate in liver tissue during enhanced gluconeogenesis (12,14,16) could provide a possible explanation for the P-fructokinase slowdown that appears to be involved in the sequence of events that turn off glycolysis and switch on gluconeogenesis. The validity of the latter hypothesis will obviously depend on the kinetic properties of P-fructokinase from liver.

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