

SPECIFIC ACTIVATION BY FRUCTOSE 2,6-BISPHOSPHATE
AND INHIBITION BY P-ENOLPYRUVATE
OF ASCITES TUMOR PHOSPHOFRUCTOKINASE

Lisardo Boscá, Juan J. Aragón and Alberto Sols

Instituto de Enzimología y Patología Molecular del CSIC
and Facultad de Medicina, Universidad Autónoma de Madrid, Spain

Received April 15, 1982

Phosphofructokinase of Ehrlich ascites tumor is very sensitive to activation by fructose 2,6-bisphosphate while it is unaffected by fructose 1,6-bisphosphate. This finding shows that in this enzyme a specific site for fructose 2,6-bisphosphate exists and suggests that the isozymes sensitive to both bisphosphates are likely to have two different regulatory sites for these compounds. The tumor isozyme has also been found to be allosterically inhibited by P-enolpyruvate in contrast with the muscle enzyme.

INTRODUCTION

Fructose-2,6-P₂ has been recently discovered as a potent activator of the phosphofructokinases (EC 2.7.1.11) from liver and muscle (1-3). Since these enzymes are activated by fructose-1,6-P₂, it was an open question whether both esters acted at a common regulatory site or at different specific sites (4). The fact that the isozyme of phosphofructokinase that occurs in ascites tumor was found to be insensitive to fructose-1,6-P₂ (4) offered a possibility to approach this question. We have found that the tumor isozyme is strongly activated by fructose-2,6-P₂, which indicates a regulatory site for this effector unrelated to fructose-1,6-P₂. In the course of this work it was also found that P-enolpyruvate is an allosteric inhibitor of the tumor isozyme.

MATERIALS AND METHODS

ATP, fructose-6-P, fructose-1,6-P₂, glucose-6-P, P-enolpyruvate, 2-P-glycerate, NADH, Agarose-ATP, rabbit muscle phosphofructokinase and auxiliary enzymes were purchased from Sigma (St. Louis, MO). Fructose-2,6-P₂ was kindly donated by Professor H.-G. Hers, Laboratoire de Chimie Physiologique, Université Catholique de Louvain, Belgium. Other chemicals were from Calbiochem (San Diego, CA) and Rieder (Mannheim, FRG).

Biological material. A hypertriploid strain of Ehrlich-Lettré carcinoma cells was grown in the abdominal cavity of young adult male Swiss mice, and

harvested about the seventh day after inoculation. The cells were withdrawn from the sacrificed animal, washed in an isotonic saline solution (115 mM NaCl, 5 mM potassium phosphate, pH 7.4) and centrifuged at $1000 \times g$ for 5 min at $2-4^{\circ}\text{C}$. When red cell contamination was apparent, the packed cells were resuspended in 1:4 diluted saline solution for 60 seconds, in order to lyse the erythrocytes; the cells were then returned to isotonicity, centrifuged two or three times and finally suspended in 1 vol. of the saline solution.

Purification of ascites tumor phosphofructokinase. Washed cells were frozen and thawed twice in liquid nitrogen. The resulting homogenate was centrifuged at $31,000 \times g$ for 30 min and the pellet discarded. Phosphofructokinase was partially purified by ammonium sulfate fractionation of the supernatant between 30-43% saturation. The precipitated material was collected, dissolved in 2 vol. of buffer A (10 mM Hepes, pH 7.4 and 20% (v/v) glycerol) and filtered through a column of coarse Sephadex G-25 (0.5 x 22 cm) equilibrated with buffer A and eluted with the same buffer. Fractions containing protein were pooled and applied to a 2-ml column of Agarose-ATP equilibrated with buffer A. After washing, the enzyme was eluted with equilibration buffer plus 0.1 mM fructose-6-P and 1 mM ATP. The preparation of this partially purified phosphofructokinase had less than 1 % of the following enzymatic activities: fructose-1,6-bisphosphatase, aldolase, adenylate kinase and adenosine triphosphatase. It was filtered through Sephadex G-25 before use.

Assay for phosphofructokinase. Unless otherwise stated, the assay mixture contained 50 mM Pipes, pH 7.0, 100 mM KCl, 5 mM MgCl_2 , 0.15 mM NADH, 1 mM MgATP, 100 μg of aldolase, 70 μg of triosephosphate isomerase, 7 μg of glycerol-3-P dehydrogenase and 50 μl of enzymic preparation in a total volume of 1 ml. After 4 min the reaction was started by adding 4 mM fructose-6-P and the decrease in absorbance at 340 nm was measured at 30°C . Glucose-6-P was always added to fructose-6-P in a relation of concentration to 3:1. When the effect of fructose-1,6- P_2 was examined, pyruvate kinase (9 μg) and lactate dehydrogenase (8 μg) were used as auxiliary enzymes in the presence of 0.5 mM P-enolpyruvate. Auxiliary enzymes, as well as rabbit muscle phosphofructokinase, were desalted by gel filtration on a column (0.5 x 20 cm) of coarse Sephadex G-25. Enzymic activities were eluted with buffer A. Protein was determined by the Bradford method (5).

RESULTS

Effect of fructose-2,6- P_2 and fructose-1,6- P_2 on the activity of ascites tumor phosphofructokinase. As shown in Figure 1, fructose-2,6- P_2 strongly activated phosphofructokinase activity. Half-maximal activation was observed with 1,7 μM fructose-2,6- P_2 . By contrast, fructose-1,6- P_2 at concentrations up to 2 mM had no effect on the enzyme when assayed in similar conditions, or when ATP was increased up to 4 mM and the pH was lowered to 6.8; in these more inhibitory conditions the muscle enzyme exhibited a 20-fold activation by 0.5 mM fructose-1,6- P_2 (results not shown).

Effect of P-enolpyruvate on the activity of ascites tumor phosphofructokinase. P-enolpyruvate markedly inhibited the tumor enzyme, while the muscle isozyme was very little affected by this metabolite (Fig. 2). Half-maximal inhibi-

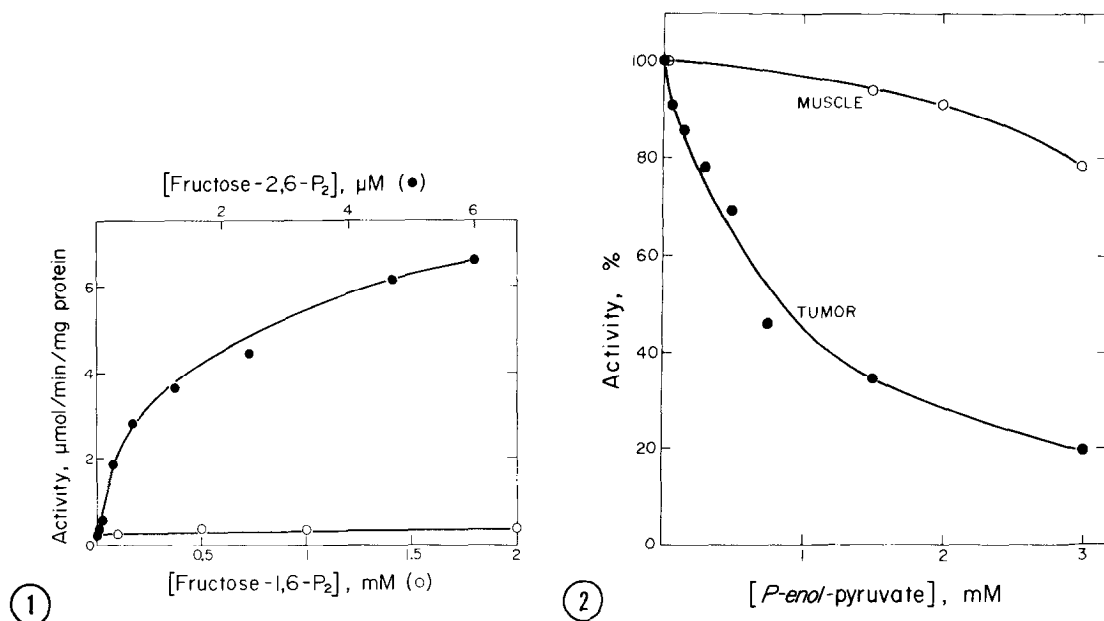


Fig. 1. Effect of fructose-2,6-P₂ and fructose-1,6-P₂ on the activity of ascites tumor phosphofructokinase. Fructose-6-P was 1 mM.

Fig. 2. Effect of P-enolpyruvate on the activity of ascites tumor and muscle phosphofructokinase.

tion of tumor phosphofructokinase was observed with 0.7 mM P-enolpyruvate. Inhibition by P-enolpyruvate was also detected when the phosphoric ester was enzymatically generated from 2-P-glycerate, which is not inhibitory, by using enolase (Table 1). The inhibition effect of P-enolpyruvate was decreased by 2 μM fructose-2,6-P₂ (Table 2). P-enolpyruvate greatly reduced the affinity of phosphofructokinase for ATP but did not alter the ATP inhibition of the enzyme (Fig. 3).

DISCUSSION

There seem to be at least 3 genetically distinct isozymes of phosphofructokinase in animal tissues: the muscle type (M), the liver type (L) and the fibroblast type (F) (6,7). The Ehrlich ascites tumor enzyme probably belongs to the F type and it was fortunate that it is not activated by fructose-1,6-P₂ (4), like that of the Novikoff tumor (8), hence offering the possibility of testing for specific activation by fructose-2,6-P₂. Our finding that tumor phosphofructokinase is strongly activated by fructose-2,6-P₂ indicates that this isozyme has a regulatory site specific for fructose-2,6-P₂, which is independent of

Table 1. Inhibition of ascites tumor phosphofructokinase by enzymatically generated P-enolpyruvate.

| Addition | Activity | |
|--|--------------------------------|-----|
| | $\mu\text{mol/min/mg protein}$ | % |
| None | 2.2 ± 0.2 | 100 |
| 4 mM 2-P-glycerate | 2.2 ± 0.2 | 100 |
| 28 $\mu\text{g/ml}$ enolase | 2.2 ± 0.3 | 100 |
| 4 mM 2-P-glycerate + 28 $\mu\text{g/ml}$ enolase | 0.8 ± 0.2 | 38 |

Additions were incubated with 50 mM Pipes, 100 mM KCl, 5 mM MgCl_2 , pH 7.4, for 60 min at 30 °C as indicated. Reaction mixture was then completed and phosphofructokinase activity was measured as described under "Materials and Methods". Results show the average of three experiments \pm S.D.

fructose-1,6- P_2 . This suggests that there must be an additional regulatory site for activation by fructose-1,6- P_2 in those isozymes (M and L) that are sensitive to both bisphosphate esters. The liver isozyme is sensitive to both bisphosphates, although with very different sensitivities (9). This duality of sites for fructose bisphosphates in animal phosphofructokinases, pending its direct confirmation by binding studies, suggests that each of the bisphospha-

Table 2. Effect of fructose-2,6- P_2 on the inhibition of ascites tumor phosphofructokinase by P-enolpyruvate.

| Addition | Activity | |
|--|--------------------------------|-----|
| | $\mu\text{mol/min/mg protein}$ | % |
| None | 2.1 ± 0.1 | 100 |
| 2 mM P-enolpyruvate | 0.8 ± 0.1 | 38 |
| 2 μM fructose-2,6- P_2 | 3.5 ± 0.2 | 170 |
| 2 μM fructose-2,6- P_2 + 2 mM P-enolpyruvate | 1.7 ± 0.1 | 82 |

Phosphofructokinase activity was measured as described under "Materials and Methods". Results show the average of three experiments \pm S.D.

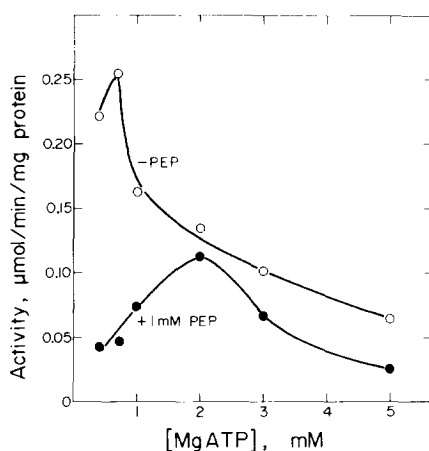


Fig. 3. Effect of MgATP on the inhibition of ascites tumor phosphofructokinase by P-enolpyruvate. PEP is for P-enolpyruvate.

tes has a unique significance in the physiological regulation of phosphofructokinase. Autocatalytic activation of glycolysis by the activation of phosphofructokinase by fructose-1,6- P_2 seems to be potentially important in certain tissues but not in others. In contrast, the universality of the activability by fructose-2,6- P_2 enhances the likely significance of this new ester as a specifically regulatory metabolite in the control of glycolysis.

In contrast with the muscle enzyme, the tumor phosphofructokinase has been found to be specifically inhibited by P-enolpyruvate (Fig. 2 and Table 1). The presence of this metabolite modifies the effect of other modulators of the enzyme, such as fructose-2,6- P_2 and ATP (Table 2 and Fig. 3 respectively). Although we do not yet know the precise regulatory meaning of this effect it opens in certain animal tissues a possibility of feedback regulation of glycolysis of a type well known in bacteria (4) and also present in plants (10). The insensitivity of the muscle enzyme does not confirm an early report of inhibition of this enzyme (11). Additionally this property could be used as a tool to characterize the tumor isozyme, given that the liver enzyme was reported not to be affected by P-enolpyruvate (12).

Phosphofructokinase activity in platelet extracts is specifically activated by fructose-2,6- P_2 without significant activation by fructose-1,6- P_2 and is inhibited by P-enolpyruvate similarly to that of the tumor (unpublished data). The

probable identity of the phosphofructokinase isozyme predominant in platelets and tumors (and embryos?) is under investigation.

Acknowledgements. We thank Drs. C. and J.M. Gancedo for critical reading of the manuscript and Drs. M. Gosálvez and J. Coll for supplying samples of ascites tumor. This work was supported by grants from the Comisión Asesora para la Investigación Científica y Técnica and from the Fondo para Investigaciones Sanitarias.

REFERENCES

- 1.- Van Schaftingen, E., Hué, L. and Hers, H.-G. (1980) *Biochem. J.* 192, 897-901.
- 2.- Pilkis, S.J., Raafat El-Maghrabi, M., Pilkis, J., Claus, T.H., and Cumming, D.A. (1981) *J. Biol. Chem.* 256, 3171-3174.
- 3.- Uyeda, K., Furuya, E., and Luby, L.J. (1981) *J. Biol. Chem.* 256, 8394-8399.
- 4.- Sols, A., Castaño, J.G., Aragón, J.J., Domenech, C., Lazo, P.A., and Nieto, A. (1981) in: *Metabolic interconversion of enzymes 1980* (Holzer, H., ed.), pp.111-123, Springer-Verlag, Berlin.
- 5.- Bradford, M. (1976) *Anal. Biochem.* 72: 248-254.
- 6.- Meienhofer, M.C., Cottreau, D., Dreyfus, J.C., and Kahn, A. (1980) *FEBS Lett.* 110: 219-220.
- 7.- Vora, S. (1981) *Blood* 57:724-732.
- 8.- Wu, R. (1966) *J. Biol. Chem.* 241: 4680-4685.
- 9.- Van Schaftingen, E., Jett, M.-F., Hué, L., and Hers, H.-G. (1981) *Proc. Natl. Acad. Sci. USA* 78: 3483-3486.
- 10.- Sabularse, D.C., and Anderson, R.L. (1981) *Biochem. Biophys. Res. Comm.*, 103:848-855.
- 11.- Uyeda, K., and Racker, E. (1965) *J. Biol. Chem.* 240: 4682-4688.
- 12.- Kemp, R.G. (1971) *J. Biol. Chem.* 246: 245-252.