Proc. Natl. Acad. Sci. USA
Vol. 78, No. 6, pp. 3483–3486, June 1981
Biochemistry

Control of liver 6-phosphofructokinase by fructose 2,6-bisphosphate and other effectors

(fructose 1,6-bisphosphate/glycolysis)

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Communicated by Christian de Duve, March 16, 1981

ABSTRACT **Rat liver 6-phosphofructokinase (ATP-D-fructose-6-phosphate 1-phosphotransferase, EC 2.7.1.11) was partially purified free of interfering enzymes by a rapid procedure. Fructose 2,6-bisphosphate, at micromolar concentrations, greatly stimulated the enzyme by increasing its affinity for fructose 6-phosphate and relieving the inhibition by ATP. Its action was synergistic with that of AMP. As a stimulator of liver phosphofructokinase, fructose 2,6-bisphosphate was approximately 1000- and 2500-fold more efficient than fructose 1,6-bisphosphate and glucose 1,6-bisphosphate, respectively. The concentration at which a half-maximal effect was obtained with the hexose bisphosphates was dependent upon the experimental conditions. It was relatively high at physiological concentrations of substrates, AMP, and P_i, and under these conditions the positive effect of fructose 1,6-bisphosphate was no longer detectable. This was probably due to the negative effect of fructose 1,6-bisphosphate as a reaction product inhibitor. It is concluded that fructose 2,6-bisphosphate rather than fructose 1,6-bisphosphate controls, in association with other effectors, the activity of phosphofructokinase in the liver.**

There is a general agreement in the literature that 6-phosphofructokinase (ATP:D-fructose-6-phosphate 1-phosphotransferase, EC 2.7.1.11) plays a major role in the control of glycolysis in nearly all types of cells (1–6). The activity of this enzyme is controlled by several metabolites, most notably its two substrates, fructose 6-phosphate and ATP. The rate of the reaction is a sigmoidal function of fructose 6-phosphate concentration, and the saturation curve is shifted to the left in the presence of positive effectors, among which fructose 1,6-bisphosphate (7, 8), the product of the reaction, and AMP are currently believed to play a major role. Negative effectors such as ATP and citrate have the opposite effect. The rate of the reaction plotted as a function of ATP concentration exhibits a typical inhibition by excess substrate. This inhibition is relieved by the positive effectors and by large concentrations of fructose 6-phosphate, whereas it is intensified by the negative effectors.

Fructose 2,6-bisphosphate is a newly recognized, extremely effective, positive effector of liver and muscle phosphofructokinase (9). Its concentration in the liver is greatly increased under conditions in which glycolysis is active and is decreased by glucagon (10). It has also been found to inhibit, at micromolar concentrations, liver and muscle fructose-1,6-bisphosphatase (11). It therefore appears to be a major regulator of both glycolysis and gluconeogenesis in the liver. The purpose of the present work was to investigate the action of fructose 2,6-bisphosphate on the kinetics of liver phosphofructokinase and to compare it to that of its isomers fructose 1,6-bisphosphate and glucose 1,6-bisphosphate and that of AMP.

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MATERIALS AND METHODS

Materials. Phospho*enol*pyruvate, glucose 1,6-bisphosphate, and auxiliary enzymes were purchased from Boehringer Mannheim. The source of other reagents and chemicals, including fructose 2,6-bisphosphate, was as described (11).

Assay of Enzymes. Unless otherwise stated, phosphofructokinase was assayed spectrophotometrically by the aldolase/triose phosphate isomerase/glycerol phosphate dehydrogenase assay (Fru-1,6-P_2-coupled assay). During purification of the enzyme, the assay was performed at pH 8 under the optimal conditions of Reinhart and Lardy (12). For kinetic measurement, the assay was performed at pH 7.1 and 22°C (13). The incubation mixture contained 100 mM KCl, 1 mM NH_4Cl, 0.15 mM NADH, 50 mM Hepes buffer, aldolase at 50 μg/ml, triose phosphate isomerase at 1 μg/ml, and glycerol-3-phosphate dehydrogenase at 10 μg/ml, as well as substrates and effectors at the concentrations indicated in the figures. $MgCl_2$ was also present at a concentration 5 mM greater than that of ATP. Optimal activity at pH 7.1 was measured in the presence of 5 mM fructose 6-phosphate, 1.5 mM ATP, 1 μM fructose 2,6-bisphosphate, 0.1 mM AMP, and 5 mM P_i and amounted to 0.5 μmol of substrate converted per min per mg protein, which corresponds to 70% of the activity measured at pH 8.0.

When the effect of fructose 1,6-bisphosphate was examined, pyruvate kinase (20 μg/ml) and lactate dehydrogenase (25 μg/ml) were used as coupling enzymes in the presence of 1 mM phospho*enol*pyruvate (ADP-coupled assay). The concentration of other reagents was the same in both assay systems and the reaction was always initiated by the addition of ATP. In both methods, the reaction rate decreased during the first 5 min and then remained almost constant for another 5 min; this latter rate was taken for the measurement of activity. Lactate dehydrogenase was assayed according to Bergmeyer and Bernt (14). One unit of phosphofructokinase or lactate dehydrogenase is the amount of enzyme that converts 1 μmol of substrate per min under the conditions of the assay. Protein concentrations were determined by the method of Lowry *et al.* (15) after precipitation in 10% trichloroacetic acid.

Purification of Liver Phosphofructokinase. The object of the purification was to render phosphofructokinase essentially free of enzymes that might interfere with kinetic studies. The procedure developed is simple and can be carried out in a single day. It takes advantage of the relatively uncommon propensities of liver phosphofructokinase to precipitate at low concentrations of ammonium sulfate and to form aggregates that can be separated from most interfering enzymes by gel filtration. The initial steps (homogenization, fractionation by high-speed centrifugation, and ammonium sulfate precipitation) are the same as

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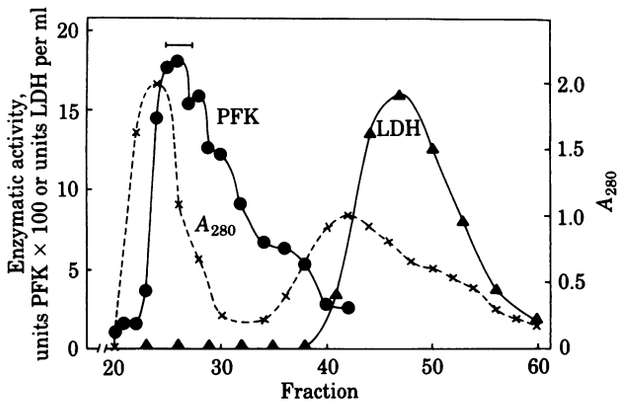

FIG. 1. Purification of liver phosphofructokinase (PFK) by filtration on Sepharose CL-6B. Fractions 24–26 (⊢——⊣) were pooled and used for kinetic investigations. The elution profile of lactate dehydrogenase (LDH) is also shown.

described by Reinhart and Lardy (12), with the main exception that the heat-treatment step was omitted. After the ammonium sulfate precipitation, the clarified solution (4 ml, corresponding to 8 g of liver) was layered onto a column (2.6 × 40 cm) of Sepharose CL-6B (molecular weight fractionation range, 10,000–4,000,000) equilibrated with a buffer containing 20 mM KP_i, 50 mM NaF, 1 mM fructose 6-phosphate, 20 μM EDTA, and 5 mM dithiothreitol (pH 7.6). The elution was performed with the same buffer at a rate of 0.6 ml/min, and 3-ml fractions were collected. Phosphofructokinase was eluted soon after the first peak of proteins corresponding to the void volume, and well before lactate dehydrogenase (M_r 140,000) (Fig. 1). The peak fractions were pooled and dialyzed overnight against 200 vol of a buffer containing 20 mM KP_i, 2 mM $MgSO_4$, 1 mM fructose 6-phosphate, 100 μM EDTA, 2 mM dithiothreitol, and 25% (wt/vol) glycerol. The purified enzyme (0.8 unit/mg) contained only trace amounts of NADH oxidoreductase, lactate dehydrogenase, fructose-1,6-bisphosphatase, aldolase, and myokinase.

RESULTS

As seen in Fig. 2, fructose 2,6-bisphosphate displayed the typical properties of a positive effector of phosphofructokinase. It greatly increased the affinity of the enzyme for fructose 6-phosphate (Fig. 2*A*) and decreased the inhibitory action of ATP (Fig. 2*B*). These effects were obtained at concentrations of the effector below 1 μM.

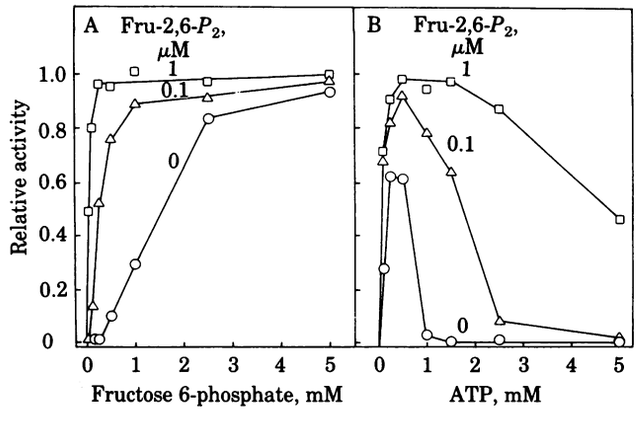

FIG. 2. Effect of fructose 2,6-bisphosphate on the affinity of phosphofructokinase for fructose 6-phosphate (*A*) and the inhibition of the enzyme by ATP (*B*). All assays were performed in the presence of 5 mM P_i and 0.1 mM AMP. In *A*, ATP was 1.5 mM; in *B*, fructose 6-phosphate was 0.25 mM. The activity is related to the optimal activity as defined in *Materials and Methods*.

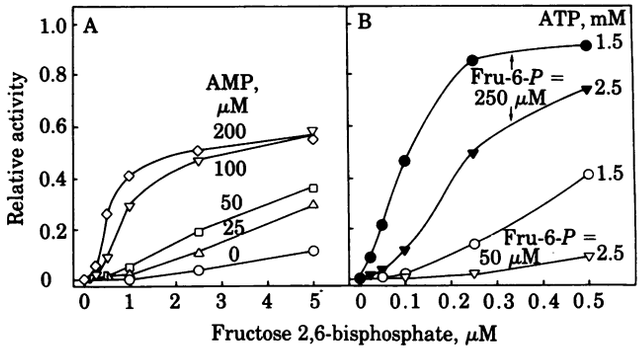

FIG. 3. Effect of fructose 2,6-bisphosphate on the activity of phosphofructokinase measured at various concentrations of AMP (*A*) or fructose 6-phosphate and ATP (*B*). All assays were performed in the presence of 5 mM P_i. In *A*, fructose 6-phosphate was 50 μM and ATP was 2.5 mM; in *B*, AMP was 100 μM.

In order to study the concentration dependence of the stimulatory effect, the enzyme was assayed under conditions in which no activity could be detected in the absence of fructose 2,6-bisphosphate. It is apparent from Fig. 3 that the response was greatly dependent on the concentration of AMP (Fig. 3*A*) and the two substrates, fructose 6-phosphate and ATP (Fig. 3*B*). A greater sensitivity was observed in the presence of increasing concentrations of fructose 6-phosphate or AMP and decreasing concentrations of ATP. Under all conditions a clear cooperativity for fructose 2,6-bisphosphate was observed.

The synergism with AMP is further illustrated by the data shown in Fig. 4. In this experiment concentrations of ATP (2.5 mM) and fructose 6-phosphate (50 μM) were close to physiological and almost no activity was observed in the absence of the positive effectors, AMP and fructose 2,6-bisphosphate. Neither AMP (2.5 mM) nor fructose 2,6-bisphosphate (2.5 μM) alone was able to stimulate phosphofructokinase activity. When the two effectors were combined, however, the enzyme was stimulated to various degrees. The plateau reached in the presence of 2.5 μM fructose 2,6-bisphosphate and 0.25 mM AMP cor-

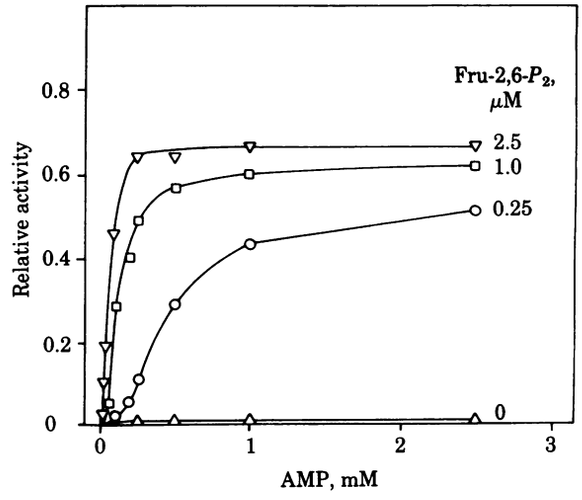

FIG. 4. Effect of AMP on the activity of the phosphofructokinase measured at various concentrations of fructose 2,6-bisphosphate. All assays were performed in the presence of 5 mM P_i, 2.5 mM ATP, and 50 μM fructose 6-phosphate.

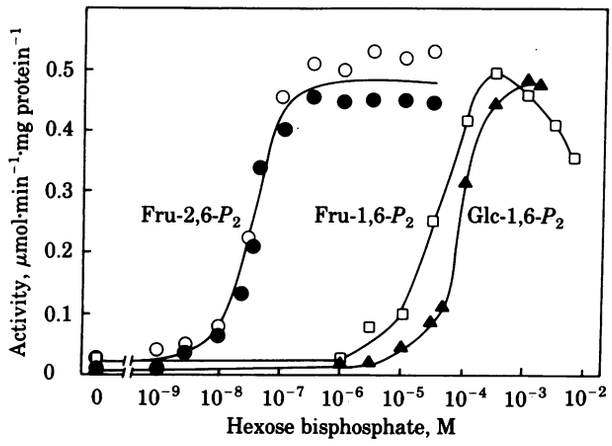

FIG. 5. Stimulation of phosphofructokinase by hexose bisphosphates. The assays were performed in the presence of 3 mM ATP, 5 mM $MgCl_2$, 1 mM AMP, 2 mM fructose 6-phosphate, and 0.2 mM P_i. Open symbols, ADP-coupled assay; closed symbols, Fru-1-6-P_2-coupled assay.

responds to 65% of the maximal value that could be obtained at higher fructose 6-phosphate concentrations.

Because the action of fructose 2,6-bisphosphate and other positive effectors is greatly dependent upon the conditions of the assay, it was important to compare the effectiveness of fructose 2,6-bisphosphate with that of its isomers fructose 1,6-bisphosphate and glucose 1,6-bisphosphate under identical conditions. In doing this type of experiment, we initially encountered great difficulties in demonstrating the stimulatory action of fructose 1,6-bisphosphate under near-physiological conditions analogous to those of Figs. 3*A* and 4. It then became apparent that this effect could be demonstrated only under certain conditions that sensitized the enzyme to hexose bisphosphates. Fig. 5 shows that in the presence of 2 mM fructose 6-phosphate and 1 mM AMP, a half-maximal effect was obtained at approximately 0.04 μM fructose 2,6-bisphosphate, 40 μM fructose 1,6-bisphosphate, and 100 μM glucose 1,6-bisphosphate. An inhibition by millimolar concentrations of fructose 1,6-bisphosphate was also apparent. At more physiological concentrations of substrate and effectors, all curves were shifted to the right and the stimulatory effect of fructose 1,6-bisphosphate was barely apparent (not shown). No effect was discernible at 50 μM fructose 6-phosphate, 0.1 mM AMP, 2.5 mM ATP, and 5 mM P_i (physiological concentrations).

DISCUSSION

In a recent investigation of the kinetics of rat liver phosphofructokinase, Reinhart and Lardy (12) reached the conclusion that, in the presence of 3 mM ATP, physiological concentrations of positive effectors (fructose 1,6-bisphosphate, AMP, and P_i) failed to activate phosphofructokinase sufficiently to account for its function at fructose 6-phosphate levels found in the cell. Our data (Fig. 4) confirm this observation and indicate that fructose 2,6-bisphosphate is most likely the missing stimulator, which was unknown by Reinhart and Lardy (12) and which allows glycolysis to operate in the liver.

Among the various effectors that could regulate phosphofructokinase *in vivo*, fructose 1,6-bisphosphate and AMP are usually considered to be the most important (1–8). The role of fructose 1,6-bisphosphate, which has been greatly emphasized (7, 8), deserves special comment. From the work of Mansour and coworkers (16, 17) on heart phosphofructokinase, it appears that this enzyme has two binding sites per aggregate of 100,000 molecular weight. These sites exhibit very different dissociation constants: 0.23 μM and 56 μM. The fructose 1,6-bisphosphate bound to the high-affinity site appears to be essential for the activity because, when it is removed by treatment with fructose 1,6-bisphosphatase or aldolase, phosphofructokinase is rendered inactive. However, this inactivation can be observed only with diluted solutions of phosphofructokinase and there is no indication that it ever occurs *in vivo* (17). It therefore appears likely that the high-affinity site is always saturated under physiological conditions and that the low-affinity site is the only one that is important for the control of the enzymatic activity.

In agreement with this interpretation, a stimulation of phosphofructokinase was perceptible only at concentrations of fructose 1,6-bisphosphate greater than 1 μM (Fig. 5). This observation discounts, therefore, any participation of the high-affinity site in the regulation of phosphofructokinase. A half-maximal effect obtained at 50 μM fructose 1,6-bisphosphate is consistent with binding of this ligand to the low-affinity site. Such results were obtained under conditions in which the enzyme was highly sensitive to the hexose bisphosphates. In contrast, under physiological concentrations of substrates and effectors, no stimulation by fructose 1,6-bisphosphate was observed, presumably because the occupation of the low-affinity site required a concentration of fructose 1,6-bisphosphate at which product inhibition (18) predominated. Because the highest concentration of hexose bisphosphate known to occur in the liver under physiological conditions is approximately 15 μM for fructose 2,6-bisphosphate (unpublished results), 50 μM for fructose 1,6-bisphosphate (13), and 30 μM for glucose 1,6-bisphosphate (19), it seems reasonable to propose that only the first of these phosphoric esters can play a role in the control of glycolysis in the liver.

Fructose 2,6-bisphosphate and AMP appear, therefore, to be the two main positive effectors that, together with the substrate fructose 6-phosphate, control the activity of phosphofructokinase. Cooperativity was observed for fructose 6-phosphate (Fig. 2*A*), fructose 2,6-bisphosphate (Fig. 3), and AMP (Fig. 4). Their effects were also synergistic, indicating that the substrate and the positive effectors cooperate to maintain the enzyme in the same active conformation. It should be noted that fructose 2,6-bisphosphate and AMP act synergistically to inhibit liver and muscle fructose-1,6-bisphosphatase (11). In doing so, they reinforce their stimulatory effect on glycolysis. It may be speculated that an increased concentration of AMP would occur under anaerobic conditions and would be responsible for the Pasteur effect. Fructose 2,6-bisphosphate, on the other hand, would regulate glycolysis and gluconeogenesis under aerobic conditions (10).

The conclusions that we have reached for liver phosphofructokinase apply, at least in part, to the enzyme present in other types of cells. Phosphofructokinases from muscle (9) and from pancreatic islets (20) are also highly sensitive to the action of fructose 2,6-bisphosphate, which increases their affinity for fructose 6-phosphate. Preliminary experiments performed with purified yeast phosphofructokinase (kindly provided by E. Hofmann, Leipzig) have also revealed a large stimulatory effect of fructose 2,6-bisphosphate, but of a more complex type in which there were changes of both K_m and V_{max}.

This work was supported by the Fonds de la Recherche Scientifique Médicale and by the U.S. Public Health Service (Grant AM 9235). E.V.S. is Aspirant and L.H. is Maître de Recherches of the Fonds National de la Recherche Scientifique. M.-F.J. is a recipient of the National Research Service Award (AM 05843) from the National Institute of Arthritis, Metabolism and Digestive Diseases.

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