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Inhibition of fructose-1,6-bisphosphatase by fructose 2,6-bisphosphate

(AMP/gluconeogenesis)

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ABSTRACT **Fructose 2,6-bisphosphate, a known powerful stimulator of phosphofructokinase [Van Schaftingen, E., Hue, L. & Hers, H.-G. (1980) *Biochem. J.* 192, 897–901] was found to inhibit, at micromolar concentrations, liver and muscle fructose-1,6-bisphosphatase (D-fructose-1,6-bisphosphate 1-phosphohydrolase, EC 3.1.3.11). The main characteristics of this inhibition are that (*i*) it is much stronger at low than at high substrate concentrations, (*ii*) it changes the substrate saturation curve from almost hyperbolic to sigmoidal, and (*iii*) it is synergistic with the inhibition by AMP. This inhibition may play an important role in the stimulation of gluconeogenesis by glucagon, because this hormone is known to decrease the concentration of fructose 2,6-bisphosphate in the liver [Van Schaftingen, E., Hue, L. & Hers, H.-G. (1980) *Biochem. J.* 192, 887–895].**

We have recently reported that the activity of liver phosphofructokinase, when measured at low concentrations of fructose 6-phosphate, can be greatly stimulated by an extremely acid-labile phosphoric ester; the concentration of this stimulator in isolated hepatocytes was greatly increased in the presence of high concentrations of glucose and decreased by glucagon (1). This ester has been identified as fructose 2,6-bisphosphate (2) and synthesized chemically (3). It is a potent positive effector of liver phosphofructokinase and its stimulatory effect on that enzyme is synergistically increased by AMP (unpublished results). Because the activity of liver phosphofructokinase is antagonized by that of fructose-1,6-bisphosphatase (D-fructose-1,6-bisphosphate 1-phosphohydrolase, EC 3.1.3.11), it was of interest to investigate the potential effect of fructose 2,6-bisphosphate on fructose-1,6-bisphosphatase. This investigation has been made possible because fructose 2,6-bisphosphate has recently been prepared in millimolar amounts and in an almost pure state.

MATERIALS AND METHODS

Chemicals and Enzymes. Fructose 1,6-bisphosphate, Hepes [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid], NADP, glucose-6-phosphate dehydrogenase, and phosphoglucose isomerase were obtained from Boehringer (Mannheim, Germany). Ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA) was purchased from Sigma; Sephadex G-25, from Pharmacia; and carboxymethylcellulose (CM 52 microgranular, preswollen), from Whatman Biochemicals (Springfield Mill, England). Other chemicals were from Merck (Darmstadt, Germany).

Fructose 2,6-bisphosphate was prepared from fructose 1,6-bisphosphate by an adaptation (4) of the method developed by Pontis and Fischer (5) for the synthesis of fructose 2-phosphate. The treatment of fructose-1,6-bisphosphate with dicyclohexylcarbodiimide resulted in the formation of its 1,2-cyclic phosphodiester derivative which, upon alkaline hydrolysis, was converted into a mixture of fructose 1,6-bisphosphate (85%) and fructose 2,6-bisphosphate (15%). Because the two fructose bisphosphates are difficult to separate, fructose 1,6-bisphosphate was converted to fructose 6-phosphate and P_i by specific enzymic hydrolysis and fructose 2,6-bisphosphate was then easily separated from fructose 6-phosphate by anion-exchange chromatography. The preparation was at least 90% pure as indicated by the amount of fructose 6-phosphate and P_i formed upon mild acid hydrolysis (2) related to the amount of total phosphate. NMR analysis of the product confirmed its structure and revealed that only the β anomer was present (6). Natural fructose 2,6-bisphosphate was prepared as described in ref. 2.

Purification and Assay of Fructose 1,6-Bisphosphatase. Fructose 1,6-bisphosphatase was purified from rat liver according to Tejwani *et al.* (7) except that the final ammonium sulfate precipitation was omitted. One milligram of protein (measured by the absorbance at 280 nm) hydrolyzed 9 μmol of fructose 1,6-bisphosphate per min at 22°C. This preparation was found to be devoid of aldolase activity.

Crude preparations of rat liver and rabbit leg muscle were obtained as follows. The tissues were homogenized with 2 vol of a solution containing 0.25 M sucrose, 1 mM EDTA, and 10 mM 2-mercaptoethanol. This homogenate was centrifuged for 30 min at 125,000 × *g* and the supernatant was used for the assay, either as such (liver) or after filtration through Sephadex G-25 equilibrated with 20 mM Hepes, pH 7.2/1 mM EDTA/10 mM mercaptoethanol (muscle).

Fructose 1,6-bisphosphatase activity was assayed spectrophotometrically (8). The incubation mixture contained 100 mM KCl, 5 mM EGTA, 1 mM $MgSO_4$, 1 mM NH_4Cl, 0.2 mM NADP, 50 mM Hepes at pH 7.2, 5 μg of glucose-6-phosphate dehydrogenase per ml, and 10 μg of phosphoglucose isomerase per ml, as well as fructose 1,6-bisphosphate, AMP, and fructose 2,6-bisphosphate at the concentrations indicated in the figures. Auxiliary enzymes were desalted on Sephadex G-25. All the constituents of the assay except fructose 1,6-bisphosphate were incubated at the indicated temperature for 3 min. The assay was started by the addition of fructose 1,6-bisphosphate. Except for an initial lag period of less than 1 min, the reaction rates were constant over the time period studied (1–2 min). One unit of fructose-1,6-bisphosphatase activity corresponds to the hydrolysis of 1 μmol of substrate per min in the conditions of the assay.

RESULTS

Inhibitory Effect of Fructose 2,6-Bisphosphate at Various Concentrations of Fructose 1,6-Bisphosphate. The saturation curve of fructose-1,6-bisphosphatase for its substrate, measured

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Abbreviation: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid.

at pH 7.2 and 37°C, is shown in Fig. 1A. The curve was almost hyperbolic, with an apparent K_m of approximately 5 μM and a slight inhibition by excess substrate. A concentration-dependent inhibition by fructose 2,6-bisphosphate was observed. This inhibition was much stronger at low than at high concentrations of substrate, without, however, being completely released at the highest concentrations used. Another characteristic of this inhibition is that it changed the saturation curve from hyperbolic to sigmoidal. This effect was clearly apparent at 25 μM fructose 2,6-bisphosphate.

It has been checked that an identical inhibition was obtained with the natural fructose 2,6-bisphosphate used at the same concentration. Furthermore, no inhibition was obtained with a preparation of inhibitor that had been preincubated for 10 min in 0.01 M HCl at 20°C. This treatment is known to hydrolyze fructose 2,6-bisphosphate into fructose 6-phosphate and P_i (2). In the conditions of the enzymatic assay, there was no detectable production of fructose 6-phosphate from fructose 2,6-bisphosphate.

Effect of AMP, Temperature, and pH. An experiment similar to the one described above was repeated in the presence of 25 μM AMP, a well-known inhibitor of fructose-1,6-bisphosphatase (8). From the data shown in Fig. 1*B*, it appears that the inhibitory actions of fructose 2,6-bisphosphate and of AMP were synergistic. Indeed, at 10 μM fructose 1,6-bisphosphate, the addition of AMP alone caused a 35% inhibition but it increased the inhibition by 1 μM fructose 2,6-bisphosphate from 20% to 80%. Similar effects were noted at other concentrations of the substrate and of the two inhibitors. In the presence of 25 μM AMP, the sigmoidal shape of the saturation curve for the substrate became clearly apparent at 2.5 and 5 μM fructose 2,6-bisphosphate.

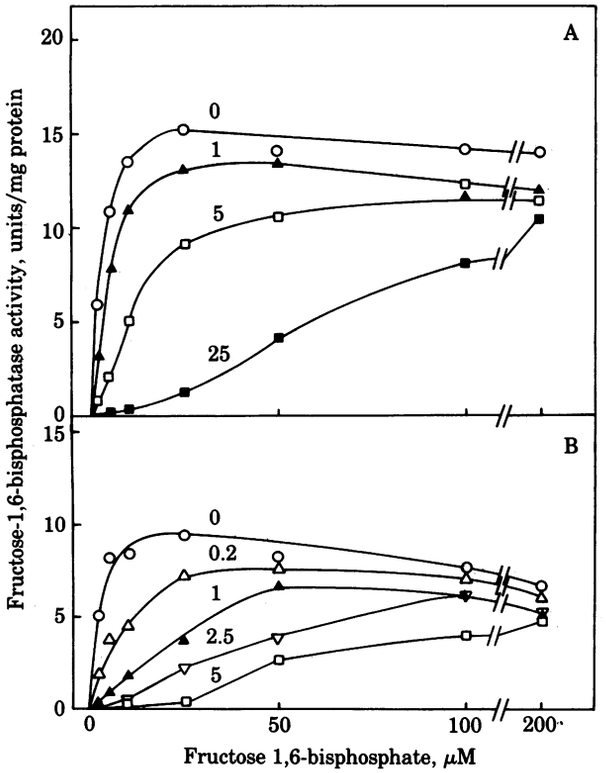

FIG. 1. Inhibition of fructose-1,6-bisphosphatase by fructose 2,6-bisphosphate at various concentrations of substrate in the absence (*A*) or in the presence (*B*) of 25 μM AMP. Concentrations of fructose 2,6-bisphosphate, in μM, are indicated above the curves. The activity was measured at 37°C.

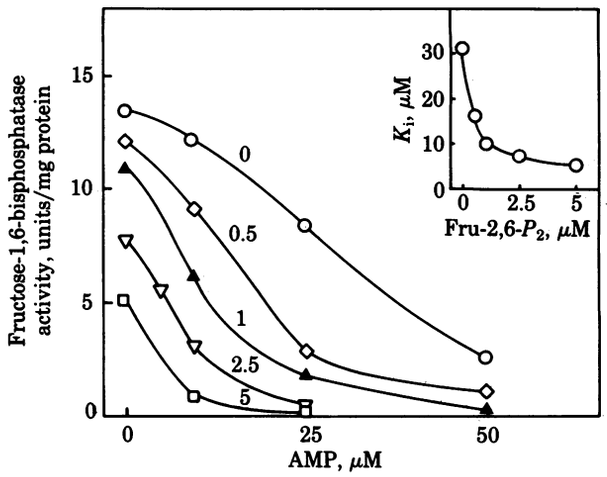

FIG. 2. Effect of fructose 2,6-bisphosphate on the inhibition of fructose-1,6-bisphosphatase by AMP. Concentrations of fructose 2,6-bisphosphate, in μM, are indicated above the curves. The activity was measured at 37°C in the presence of 10 μM fructose 1,6-bisphosphate. (*Inset*) Effect of fructose 2,6-bisphosphate on K_i, defined as the concentration of AMP that decreased the activity to 50% of that measured in the absence of the nucleotide.

The synergism between the inhibitory action of fructose 2,6-bisphosphate and of AMP is further illustrated in Figs. 2 and 3. Fig. 2 is a plot of the enzyme activity as a function of AMP concentration in the presence of various concentrations of fructose 2,6-bisphosphate. A 50% inhibition was reached at much lower concentrations of AMP in the presence than in the absence of fructose 2,6-bisphosphate (see *Inset*). Fig. 3 shows that the concentration of fructose 2,6-bisphosphate that caused a 50% inhibition was decreased by the addition of AMP. A Hill plot of the inhibition by fructose 2,6-bisphosphate gave coefficients (H) ranging between 1.2 and 1.4 (not shown).

It has also been observed that the inhibition by fructose 2,6-bisphosphate was less at pH 8 than at pH 7.2 and less at 37°C

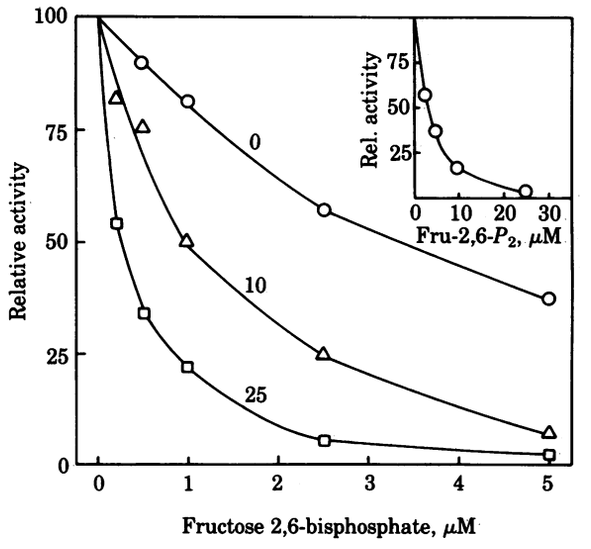

FIG. 3. Effect of AMP on the inhibition of fructose-1,6-bisphosphatase by fructose 2,6-bisphosphate at 37°C. The activity was measured in the presence of 10 μM fructose 1,6-bisphosphate. In the absence of fructose 2,6-bisphosphate the activities were 13.5, 12.2, and 8.4 units/mg of protein in the presence of 0 (○), 10 (△), and 25 (□) μM AMP, respectively. (*Inset*) Graph with compressed abscissa showing higher concentrations of fructose 2,6-bisphosphate.

than at 22°C. A similar effect of pH and temperature on the inhibition by AMP has been previously reported (8).

Inhibition of Fructose-1,6-Bisphosphatase in Crude Liver and Muscle Preparations. We found that fructose-1,6-bisphosphatase present in crude liver and muscle preparations is inhibited by fructose 2,6-bisphosphate, that this inhibition is stronger at low than at high concentrations of substrate, and that this effect is synergistic with that of AMP (data not shown).

DISCUSSION

Kinetics of the Fructose 2,6-Bisphosphate Inhibition. The interaction of fructose-1,6-bisphosphatase, which is a tetrameric enzyme (7), with its substrate, fructose 1,6-bisphosphate, and its two inhibitors, AMP and fructose 2,6-bisphosphate, is complex. From the obvious structural similarity between the two fructose bisphosphates, one could hypothesize that the inhibition by fructose 2,6-bisphosphate would be competitive with the substrate. Indeed, this inhibition is the most important at the low concentrations of substrate. However, two striking properties of this inhibition are against this simple interpretation.

First, fructose 2,6-bisphosphate changes the kinetics for fructose 1,6-bisphosphate from almost hyperbolic to sigmoidal, indicating an allosteric type of interaction. In this respect, the effect of fructose 2,6-bisphosphate is therefore in contrast with that of AMP, which does not induce cooperativity for the substrate (8). Its own interaction with the enzyme was only slightly cooperative. The Hill coefficient for fructose 1,6-bisphosphate could not be calculated because of the uncertainty about V_{max}. This difficulty is at least in part related to the well-known inhibition of the enzyme by excess substrate.

A second property of the inhibition by fructose 2,6-bisphosphate is that it is synergistic with the inhibition by AMP. This synergistic effect, associated with the fact that fructose 2,6-bisphosphate changes the substrate saturation curve from hyperbolic to sigmoidal, whereas AMP does not, suggests that the two inhibitors bind to different sites of the enzyme. Increased inhibition at lower temperature and decreased inhibition at higher pH are properties common to both effectors.

The fact that similar results were obtained with crude liver extracts indicates that the inhibition by fructose 2,6-bisphosphate is not due to a modification of the kinetic properties of the enzyme during the purification procedure. Our preliminary investigations of the effect of fructose 2,6-bisphosphate on the muscle enzyme indicate a great analogy with the liver system. However, the presence of aldolase in the crude muscle preparation used has prevented a more precise analysis of these effects.

Physiological Implications. Glucagon and glucose are the two main effectors that, respectively, stimulate and inhibit gluconeogenesis in the liver and have the opposite effect on glycolysis. Fructose 2,6-bisphosphate and AMP have in common the properties of inhibiting fructose-1,6-bisphosphatase and stimulating phosphofructokinase. Changes in the concentration of these effectors could therefore explain the effects of glucagon and of glucose. Thus, the concentration of fructose 2,6-bisphosphate is greatly decreased by glucagon and increased by glucose (1). In contrast, the concentration of AMP in the liver is increased by glucagon and is not affected by glucose (9). Therefore, one reaches the conclusion that fructose 2,6-bisphosphate rather than AMP is a messenger of both glucose and glucagon in their action on gluconeogenesis and glycolysis.

The inhibition of fructose-1,6-bisphosphatase by fructose 2,6-bisphosphate, together with the stimulation of phosphofructokinase by the same effector, explains also the remarkable increase in the concentration of fructose 1,6–bisphosphate in the liver in conditions in which fructose 2,6-bisphosphate is increased (1). Furthermore, the sigmoidal shape of the substrate saturation curve of fructose-1,6-bisphosphatase indicates that there is now a threshold concentration of fructose 1,6-bisphosphate under which there is little conversion to fructose 6-phosphate. The conclusion that the activity of fructose-1,6-bisphosphatase is not simply regulated by the concentration of its substrate had been reached previously (9). It has also been known for many years (9–12) that the concentration of fructose 1,6-bisphosphate in the liver is decreased by the administration of glucagon. Our results indicated that this glucagon effect is mediated by the disappearance of fructose 2,6-bisphosphate.

The increased rate of glycolysis that occurs at high glucose concentrations implies not only that phosphofructokinase is stimulated but also that the activity of pyruvate kinase is increased in parallel. The latter enzyme is greatly stimulated by fructose 1,6-bisphosphate. The increased concentration of fructose 1,6-bisphosphate that is the result of the inhibition of the phosphatase may be the signal that activates pyruvate kinase and allows a rapid rate of metabolite conversion through the glycolytic pathway.

Note Added in Proof. We have recently observed that fructose 2,6-bisphosphate is also a potent inhibitor of fructose-1,6-bisphosphatase purified from yeast (Sigma) or extracted from spinach leaves.

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