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Fructose 2,6-bisphosphate, the probable structure of the glucose- and glucagon-sensitive stimulator of phosphofructokinase

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The low-molecular-weight stimulator of phosphofructokinase [Van Schaftingen, Hue & Hers (1980) *Biochem. J.* **192**, 887–895] has been purified from rat liver. It was completely destroyed upon incubation with 0.01 M-HCl for 10 min at 20°C and fructose 6-phosphate and a reducing power equivalent in amount to the acid-labile organic phosphate were formed. It was therefore tentatively identified as fructose 2,6-bisphosphate.

Several groups of workers (Castaño *et al.*, 1979; Pilkis *et al.*, 1979; Kagimoto & Uyeda, 1979) have reported that incubation of isolated hepatocytes in the presence of glucagon caused a decrease in the activity of phosphofructokinase measured at subsaturating concentrations of fructose 6-phosphate. This change was reported to be stable upon gel filtration as well as upon partial purification. We have recently observed (Van Schaftingen *et al.*, 1980) that a similar change could be obtained by gel filtration of a liver extract and could be reversed by the addition of the low-molecular-weight fraction. We have also reported that the substance responsible for this effect is acid labile even at 0°C but could be separated from proteins by ultrafiltration of a liver homogenate or by heating at 80°C followed by centrifugation. The stimulator has a molecular weight similar to that of fructose 1,6-bisphosphate and was destroyed by incubation in the presence of alkaline phosphatase; it appeared therefore to be an acid-labile phosphoric ester.

In the present paper we report other properties of the stimulator, which indicate that it is probably fructose 2,6-bisphosphate.

Materials and methods

Chemicals and enzymes

Inorganic pyrophosphatase and rabbit muscle phosphofructokinase were obtained from Boehringer (Mannheim, Germany) and rabbit muscle fructose bisphosphatase from Sigma (St. Louis, MO, U.S.A.). [U-^{14}C]Fructose 1,6-bisphosphate and [8-^{14}C]adenosine 5′-triphosphate were purchased from The Radiochemical Centre (Amersham, Bucks., U.K.), Chelex 100 (100–200 mesh, Na^+ form) and AG1 (X8; 200–400 mesh, Cl^- form) were from Bio-Rad Laboratories (Richmond, CA, U.S.A.).

Abbreviation used: Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

Analytical procedures

Reducing sugars were determined as described by Park & Johnson (1949). Phosphate was assayed by the sensitized method developed by Itaya & Ui (1966); the absorbance was read 15 min after the addition of the colour-developing reagent, which contained no detergent. Pyrophosphate was determined as phosphate after incubation for 15 min with pyrophosphatase (2 units/ml) in the presence of 1.2 mM-$MgCl_2$ and 50 mM-Tris/HCl adjusted to pH 7.0. Esterified phosphate was determined after hydrolysis by alkaline phosphatase; samples were incubated at 30°C for 30 min with 2 units of calf intestine alkaline phosphatase/ml in the presence of 0.25 mM-$ZnCl_2$, 2.5 mM-$MgCl_2$ and 0.1 M-Tris/HCl buffer, pH 8.0. To test the action of fructose bisphosphatase on the stimulator, samples of the stimulator purified by chromatography (see below) were incubated for 15 min with 1 mM-$MgCl_2$, 0.1 mM-EDTA and 20 mM-Hepes, pH 7.1, in the presence or in the absence of 1 unit of fructose bisphosphatase/ml which had been previously desalted by filtration on Sephadex G-25 equilibrated with 10 mM-Hepes, pH 7.1. At the end of the incubation the samples were heated at 80°C for 5 min to destroy fructose bisphosphatase. It has been verified that this procedure leads to the destruction of fructose 1,6-bisphosphate. Other experimental procedures, including the assay of the stimulator as well as the

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source of other chemicals and reagents were as described elsewhere (Van Schaftingen *et al.*, 1980).

Results

Purification of the stimulator of phosphofructokinase from rat liver

When designing the following purification procedure, we took into consideration the fact (Van Schaftingen *et al.*, 1980) that perfusion of a liver at a high concentration of glucose considerably increased its content of stimulator. We also knew that the stimulator does not resist acid extraction or boiling, but that it can be extracted at 80°C. Since the stimulator was expected to be a phosphoric ester, we applied the classical methodology used to isolate this type of compound (Cardini & Leloir, 1957).

Extraction from the liver. Well-fed rats were anaesthetized and their livers were perfused for 20min with Krebs–Henseleit (1932) bicarbonate buffer enriched with 50mM-glucose. The livers were then cut into pieces and homogenized with an Ultra-Turrax (Janke and Kunkel, Staufen im Breisgau, Germany) with 5vol. of 10mM-Hepes adjusted to pH7.1 at 20°C with KOH and heated at 80°C. The homogenate was maintained at 80°C for 5min with constant stirring in a water bath. It was then cooled in ice and centrifuged at 15000*g* for 10min and the sediment was discarded.

Separation from nucleotides and from glycogen. To each 100ml fraction of extract, 5g of charcoal (Darco) was added. The mixture was shaken from time to time and after 10min was centrifuged at 15000*g* for 10min. It has been checked that a tracer amount of [^{14}C]ATP added to the mixture was completely removed by this treatment. The supernatant was then mixed with an equal volume of 95% (v/v) ethanol and this mixture was left for 30min at 0°C. It was then centrifuged at 15000*g* for 10min and the pellet of glycogen was discarded.

Barium precipitation and anion-exchange chromatography. The ethanolic supernatant (1vol.) was mixed at 0–4°C with 1vol. of water and 0.2vol. of 1M-barium acetate and a few drops of a 1% ethanolic solution of phenolphthalein. A fresh solution of 1M-NaOH was then added dropwise until a pink coloration was obtained. The mixture was left for 1h with the occasional addition of NaOH to maintain the pink coloration. It was then centrifuged at 1000*g* for 10min and the supernatant was discarded. The sediment was washed twice with 95% (v/v) ethanol (one-tenth of the volume of initial ethanolic supernatant), once with diethyl ether and was immediately resuspended in water. Through this procedure, about 10mg of barium precipitate per g of liver was obtained.

The amount of barium precipitate obtained from 250ml of the original (heat-treated) extract was suspended in 25ml of water at 0–4°C and shaken in the presence of 5g of Chelex. After 15min, the mixture was centrifuged and the sediment was extracted again with another 25ml of water and centrifuged. The supernatants were combined and passed through a column (1.4cm × 3cm) of Chelex and, immediately afterwards, through a column (0.9cm × 20cm) of Dowex AG 1 (X8; Cl^- form) and a gradient of NaCl at 0–4°C (0–500mM in 300ml) was applied. The stimulator was eluted after glucose 6-phosphate and inorganic phosphate at a concentration of NaCl of approx. 220mM. The active fractions were combined, diluted with 2vol. of water and chromatographed again on Dowex with an NaCl gradient (100–400mM in 200ml). Except for the volume of liquid contained in the charcoal and glycogen pellets, the recovery of the stimulator through this purification procedure was nearly complete.

Acid hydrolysis and treatment with fructose bisphosphatase

The extreme acid-lability of the stimulator of phosphofructokinase (Van Schaftingen *et al.*, 1980) is illustrated in Fig. 1. In the presence of 0.01M-HCl, half of the stimulator was destroyed within about 15min at 0°C. This time was decreased to less than 3min at 20°C. Since phosphate determination is done in the presence of a strong acid, it was not possible to show with the technique used a progressive liberation of inorganic phosphate in the course of acid hydrolysis. However, the reaction was characterized by the formation of fructose 6-phosphate in an amount equimolar to the phosphate measured in the sample. Since inorganic phosphate had been removed by the column chromatography, this phosphate may be considered as acid-labile esterified phosphate. A reducing power appeared simultaneously with the fructose 6-phosphate and was entirely accounted for by it. There was little reducing power before acid hydrolysis, indicating that the reducing group of fructose 6-phosphate was not free before acid treatment. No pyrophosphate or glucose 6-phosphate could be detected among the products of acid hydrolysis.

Treatment of the active fractions with purified fructose bisphosphatase did not destroy the stimulator.

Purity and tissue concentration

The presence of an acid-labile fructose 6-phosphate moiety in the stimulator allows one to assay it on a molar basis. We show in Fig. 2 that, upon anion exchange chromatography, the stimulatory property was eluted in parallel with acid-labile phosphate, and with the acid-liberated fructose 6-phosphate and reducing power in nearly equimolar concentrations.

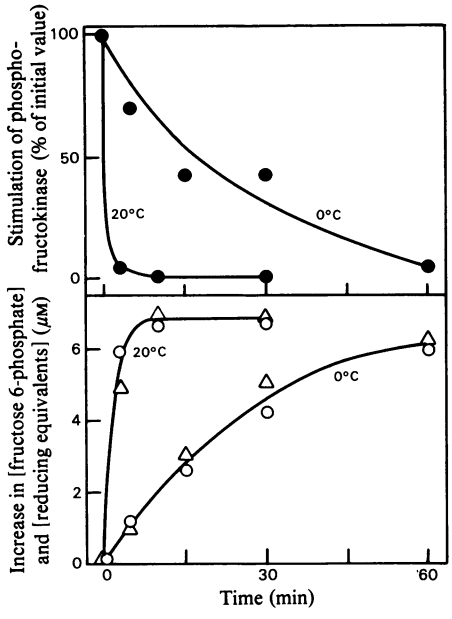

Fig. 1. *Acid hydrolysis of the stimulator*
Fractions 32–36, obtained by elution of the Dowex column (see Fig. 2) were pooled. A sample of this solution was mixed with 0.01 vol. of 1 M-HCl and incubated either at 0°C or at 20°C. At the time indicated, the reaction was stopped by addition of an equal volume of 200 mM-Hepes, pH 7.1, for the estimation of the stimulatory power (●) and of the concentration of fructose 6-phosphate (○). For the determination of the reducing power (△), 0.1 vol. of 0.1 M-NaOH was added. One reducing equivalent corresponds to the reducing power of one mol of fructose 6-phosphate. At time zero, fructose 6-phosphate was not detectable and the reducing equivalents were 3.5 μM.

The active fractions appear therefore to contain little, if any, other acid-labile phosphoric ester.

When the same fractions were treated with alkaline phosphatase, phosphate was liberated in an about 20-fold excess over acid-labile phosphate. This indicates that other phosphoric esters, presumably, according to their position of elution, diphosphoric esters, contaminate the active fractions. A small proportion of these esters appear responsible for the low reducing power that was detected in the active fractions before acid treatment. Heating the fractions for 5 min at 100°C in 1 M-HCl did not liberate glucose 6-phosphate, indicating that glucose 1,6-bisphosphate was not among the contaminants. The specific assay for fructose 1,6-bisphosphate did not indicate the presence of any significant amount of this compound in the active fractions. Incubation with pyrophosphatase did not liberate inorganic phosphate. It therefore appears that neither fructose

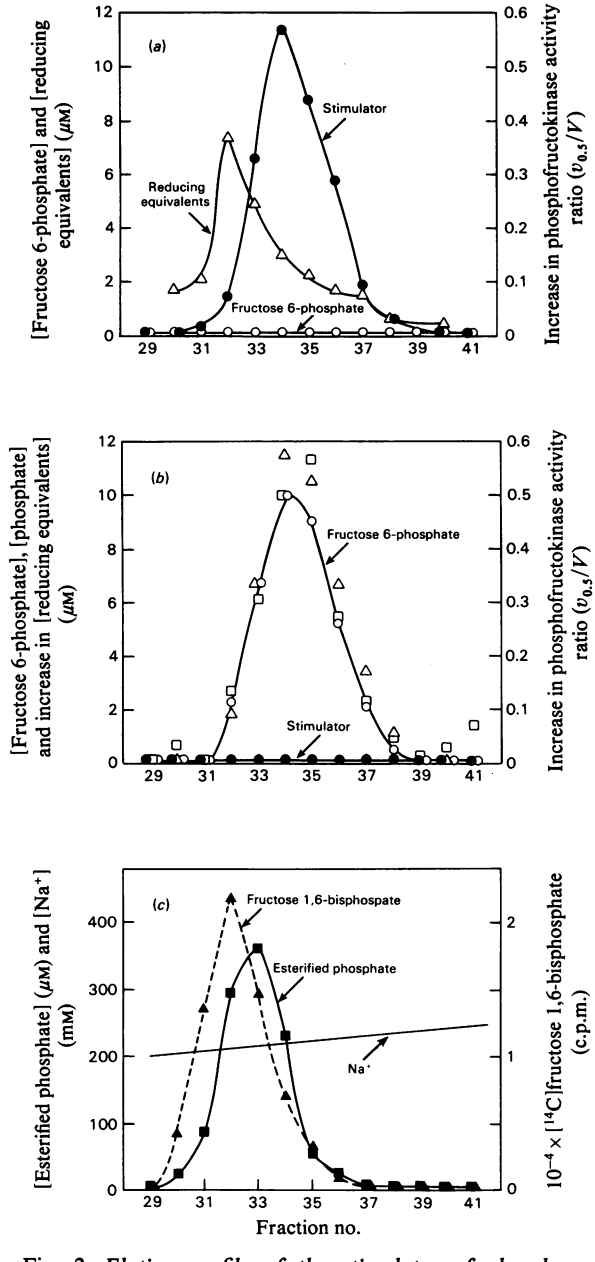

Fig. 2. *Elution profile of the stimulator of phosphofructokinase from Dowex AG1*
The Figures show the elution of the stimulator and of other phosphoric compounds in the course of the second chromatography (see the text). Fractions (3 ml) were collected. The stimulator (●), fructose 6-phosphate (○) and reducing equivalents (△) were measured before (*a*) and after (*b*) incubation for 30 min in 0.01 M-HCl at 20°C. Phosphate (□), esterified phosphate (■), and [^{14}C]fructose 1,6-bisphosphate (▲) (*c*) were also measured. In (*b*), the value of reducing equivalents shown in (*a*) has been subtracted. Samples (10 μl) were used for the assay of the stimulator.

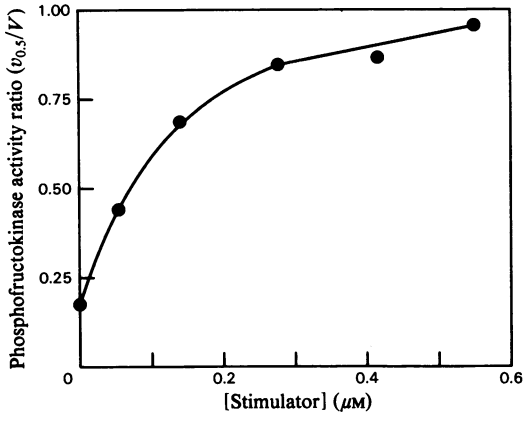

Fig. 3. *Dose–response curve of liver phosphofructokinase to its stimulator*

The concentration of stimulator was estimated by the amount of fructose 6-phosphate liberated by hydrolysis.

1,6-bisphosphate nor pyrophosphate are major contaminants. When a tracer amount of fructose 1,6-bisphosphate was added to the mixture before chromatography, it was eluted slightly before the stimulator (Fig. 2).

As reported elsewhere (–Van Schaftingen *et al.*, 1980) the concentration of the stimulator of phosphofructokinase in isolated hepatocytes, as assayed by its biological properties, varied greatly according to whether glucose or glucagon were present in the incubation medium. The determination of the amount of fructose 6-phosphate liberated in 0.01 M-HCl allows us to calculate that approximately 5 nmol of stimulator were obtained from 1 g of liver through the procedure described above. The concentration of the stimulator is therefore approx. 5×10^{-6} M in the liver perfused with 50 mM-glucose. This concentration is known to be at least 10-fold lower after treatment of the liver with glucagon (Van Schaftingen *et al.*, 1980), although the biological assay used was not sensitive enough to detect very low concentrations.

Effect of stimulator concentration on liver and muscle phosphofructokinase

Fig. 3 shows the saturation of liver phosphofructokinase by the stimulator. The enzymic preparation used was a Sephadex filtrate obtained from the liver of a fed rat. The effect was half-maximal at about 10^{-7} M (expressed as fructose 6-phosphate liberated by hydrolysis). A similar effect with similar doses was obtained with purified muscle phosphofructokinase (results not shown).

Discussion

The stimulator of phosphofructokinase present in livers perfused with glucose at a high concentration was known to be a highly acid-labile phosphoric ester whose molecular weight is close to that of fructose 1,6-bisphosphate (Van Schaftingen *et al.*, 1980). The facts that it is not adsorbed on charcoal, that it is barium-insoluble and that it is eluted from anion exchangers after monophosphoric esters and inorganic phosphate are all in agreement with the hypothesis that the stimulator is a non-nucleotidic diphosphoric ester.

The remarkable parallelism that was observed in the course of limited acid hydrolysis between the loss of the stimulator and the appearance of fructose 6-phosphate together with an equivalent amount of reducing power indicates that fructose 6-phosphate is a constituent of the stimulator, in which its reducing group is hidden. The parallelism between the amount of stimulator and acid-liberated reducing power and fructose 6-phosphate in the elution profile strengthens this view. There was no such a parallelism with the elution of the reducing power measured before acid treatment; furthermore, the amount of this reducing power was too small to account for the hidden fructose 6-phosphate. We therefore conclude that the stimulator is a non-reducing derivative of fructose 6-phosphate.

The parallelism between the elution of the stimulator and that of the acid-labile phosphate, in a concentration equimolar to that of the acid-liberated fructose 6-phosphate, indicates that phosphate is another moiety of the stimulator. To account for the absence of reducing power and for the remarkable lability in acid medium, one has to assume that this phosphate is esterified at position 2 of fructose 6-phosphate. Indeed, furanosylphosphates such as ribose 1-phosphate (Kalckar, 1947) and ribose 1,5-bisphosphate (Klenow, 1953) are known to be acid-labile at low temperature. We therefore propose that the stimulator of phosphofructokinase is fructose 2,6-bisphosphate. The structure proposed may, however, be incomplete and nothing is known about the anomeric form.

The effect of the stimulator is half-maximal at about 10^{-7} M and its concentration in the liver is in the same range. This range of concentration is similar to that of cyclic AMP. The fact that the stimulator acts equally well on crude liver phosphofructokinase and on the purified muscle enzyme indicates that its biological action may extend to tissues other than the liver. It is not known at the present time if it extends to reactions other than that catalysed by phosphofructokinase.

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