Mechanism of Activation of Glycogen Phosphorylase by Fructose in the Liver

STIMULATION OF PHOSPHORYLASE KINASE RELATED TO THE CONSUMPTION OF ADENOSINE TRIPHOSPHATE

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(Received 23 May 1978)

1. A dose-dependent activation of phosphorylase and consumption of ATP was observed in isolated hepatocytes incubated in the presence of fructose; histone kinase and phosphorylase kinase activities were unchanged at doses of this sugar that were fully effective on phosphorylase. The activation of phosphorylase by fructose was also observed in cells incubated in a Ca^{2+} -free medium as well as in the livers of rats *in vivo*. 2. In a liver high-speed supernatant, fructose, tagatose and sorbose stimulated the activity of phosphorylase kinase; this effect was dependent on the presence of K^+ ions, which are required for the activity of fructokinase; it was accompanied by the transformation of ATP into ADP. In the presence of hexokinase, glucose also stimulated phosphorylase kinase, both in an Na^+ or a K^+ medium. 3. The activities of partially purified muscle or liver phosphorylase kinase were unchanged in the presence of fructose. 4. Some properties of liver phosphorylase kinase are described, including a high molecular weight and an inhibition at ATP/Mg ratios above 0.5, as well as an effect of ATP concentration on the hysteretic behaviour of this enzyme. 5. The effect of fructose on the activation of phosphorylase is discussed in relation to the consumption of ATP.

An activation of phosphorylase by fructose in isolated perfused rat liver has been reported by Walli *et al.* (1975) and by Jakob (1976) and was related to the decrease in the ATP/AMP ratio that was observed under these conditions. More recently, Miller (1978) has observed a similar effect of high concentrations of fructose in isolated liver hepatocytes and has attributed this effect to an increase in cyclic AMP concentration. The purpose of the present paper was to reinvestigate the mechanism of this property of fructose to activate phosphorylase. We have now observed this effect not only in isolated hepatocytes but also in rats *in vivo* and in a cell-free system.

Materials and Methods

Chemicals

Fructose, glucose, sorbose and glycerol were purchased from E. Merck A. G., Darmstadt, Germany. Mannose was from Pfanstiehl Laboratories, Waukegan, IL, U.S.A. Tagatose, glucose 6-phosphate, ATP and AMP were from Sigma Chemical Co., St. Louis, MO, U.S.A. Fructose 1-phosphate, hexokinase and ADP were purchased from Boehringer, Mannheim, Germany. Glucose 1-phosphate was from Fluka A.G., Buchs, Switzerland. [γ - ^{32}P]ATP was purchased from The Radiochemical

Centre, Amersham, Bucks., U.K. Sepharose 6B was from Pharmacia A.B., Uppsala, Sweden. Adenosine 3':5'-cyclic monophosphate was obtained from Calbiochem, San Diego, CA, U.S.A. and was further purified by paper chromatography (van de Werve *et al.*, 1974). ^{125}I -labelled thyroglobulin (mol.wt. 660000) was a generous gift of Dr. M. F. van den Hove-Vandenbroucke.

Biological material

Experiments with anaesthetized rats as well as the isolation and incubation of hepatocytes were carried out by the procedure previously described (van de Werve *et al.*, 1977a). The viability of the hepatocytes was checked by staining with Trypan Blue and/or determination of ATP concentration; the amount of cells excluding the stain was $>87\%$ and the ATP concentration was $2.96 \pm 0.22 \mu\text{mol/g}$ of liver (mean $\pm$ S.E.M. for four cell preparations) when the cells were routinely preincubated for 30 min at 37°C in the presence of 10 mM-glucose and bacitracin (1 mg/ml).

A liver high-speed supernatant, obtained by centrifugation for 30 min at 100000g of a liver extract, was filtered through glass wool. The extract was obtained by homogenization of the liver of overnight-starved rats in a Potter-Elvehjem tube with 2 vol. of ice-cold 10 mM-glycylglycine, pH 7.4. The high-speed supernatant was fractionated by gel filtra-

tion by layering 0.1ml of the preparation on a column (8cm×0.6cm) filled with 1.7 ml of Sepharose 6B gel and equilibrated with a solution containing 5mM-dithiothreitol and 10mM-glycylglycine at pH 7.4. The column was washed with the same buffer and fractions of 0.2ml were collected: phosphorylase kinase was eluted in the first fractions as a single peak preceding thyroglobulin.

Phosphorylase *b* was purified from rabbit muscle by the method of Fischer & Krebs (1962). Phosphorylase kinase was prepared from rabbit muscle as described by Krebs (1966). The heat-stable protein inhibitor of cyclic AMP-dependent protein kinase was purified from rabbit muscle, up to the step of trichloroacetic acid precipitation (Walsh *et al.*, 1971).

Measurement of enzyme activities

In biopsies and isolated hepatocytes, phosphorylase *a* activity was measured as described by Hue *et al.* (1975) and the activity ratio of histone kinase as described by van de Werve *et al.* (1977b), phosphorylase kinase was measured as described before (van de Werve *et al.*, 1977a) except that the temperature was 30°C and that 3.3mM-EGTA was equilibrated with calcium to obtain a free Ca^{2+} concentration of 0.3 μM (Portzehl *et al.*, 1964); when the enzyme was assayed in a high-speed supernatant, the inhibitor of cyclic AMP-dependent protein kinase was omitted. One unit of phosphorylase kinase is the amount of enzyme that catalyses the transformation of 1 unit of phosphorylase *b* into phosphorylase *a* per min under

the conditions of the assay, when the reaction rate has reached a constant value. One unit of phosphorylase *a* is the amount of enzyme that converts 1 μmol of substrate/min in the conditions of the assay. A similar method was used to measure the activity of purified muscle phosphorylase kinase except that fluoride and KCl were omitted in the incubation mixture.

Determination of metabolites

Fructose was determined by the method of Roe *et al.* (1949), after deproteinization with equimolecular amounts of ZnSO_4 and Ba(OH)_2 by the method of Weichselbaum & Somogyi (1941). Adenine nucleotides were measured in neutralized perchloric acid extracts by the methods of Lamprecht & Trautshold (1963) for ATP and of Adam (1963) for ADP and AMP.

Results

The data shown are illustrative of a series of experiments that gave consistent results.

Activation of phosphorylase in isolated hepatocytes incubated in the presence of fructose

Fig. 1 shows that the addition of fructose to the incubation medium caused within 2min a dose-dependent activation of phosphorylase and a decrease in the concentration of ATP, but no change in

Fig. 1. Effect of various concentrations of fructose on the concentration of ATP ($\blacktriangle$) and on the activity of cyclic AMP-dependent histone kinase ($\blacksquare$) and phosphorylase *a* ($\bullet$) in isolated hepatocytes. Samples were taken 2min after the addition of fructose.

the activity of histone kinase. In another similar experiment, it was observed that 7–28 mM-fructose did not induce a stable change in the activity of phosphorylase kinase, although the activity of phosphorylase was greatly increased (results not shown). It is only at higher fructose concentrations (about 30 mM) that an activation of histone kinase (ratio up to 0.4) and phosphorylase kinase (up to 20% activation) was irregularly observed. The effect of fructose in causing a decrease in ATP concentration and an activation of phosphorylase, although not of histone kinase, was shared by 28 mM-tagatose and to a smaller extent by 15 mM-glycerol (results not shown).

The activation of phosphorylase by fructose was also observed in cells incubated in a Ca^{2+} -free medium (results not shown).

In all these experimental conditions, we have measured a concentration of AMP that was unchanged and averaged $0.19 \pm 0.09 \mu\text{mol/g}$ of liver (mean $\pm$ S.E.M. for four cell preparations).

Effect of fructose on the activation of phosphorylase in a liver high-speed supernatant

Figs. 2 and 3(b) show the effect of 2.5 mM-fructose on the activation of muscle phosphorylase by a liver high-speed supernatant incubated in the presence of 2 mM-ATP, 1.25 mM-Mg and 0.14 M- K^+ . The rate of reaction increased with time and became linear in these experiments only after 20–30 min; the rate of reaction was severalfold faster in the presence of fructose, which progressively disappeared from the

medium and also caused a conversion of ATP into ADP.

When K^+ was replaced by Na^+ (Fig. 3a) in the incubation medium, fructose was barely phosphorylated and had little or no effect on the activation of phosphorylase. Since a high concentration in K^+ ions is required for a good activity of fructokinase in a crude liver extract (Hers, 1952), the stimulation of phosphorylase kinase by fructose appears to be related to the activity of fructokinase. The latter enzyme phosphorylates other oxo-hexoses, including tagatose and sorbose (Leuthardt & Testa, 1950). These ketoses had an effect on phosphorylase kinase similar to that of fructose, whereas glucose, mannose, sucrose and also fructose 1-phosphate (up to 2.5 mM) were without action (results not shown). Glucose, however, had an effect similar to that of fructose if purified hexokinase was added to the incubation medium. Under these conditions, the stimulatory effect of glucose was obtained in the presence of both K^+ (Fig. 4) and Na^+ ions.

Absence of fructose effect on purified phosphorylase kinase

We have checked that fructose had no effect on the activity of purified rabbit muscle phosphorylase kinase. We have also obtained similar negative results when using as a source of phosphorylase kinase the high-molecular-weight liver protein fraction obtained by gel filtration of a high-speed supernatant.

Fig. 2. Effect of fructose on the concentrations of ATP (a, i), ADP (a, ii) and AMP (a, iii) and on the formation of phosphorylase a (b) in a liver high-speed supernatant incubated in the presence of ATP

A high-speed supernatant from rat liver was incubated with 6 units of muscle phosphorylase b, 2 mM-ATP, 1.25 mM- MgSO_4 , 100 mM-KCl, 40 mM-KF, 20 mM-glycylglycine, pH 7.4, and 3.3 mM-EGTA, equilibrated with calcium to obtain a free Ca^{2+} concentration of $0.3 \mu\text{M}$ (Portzehl *et al.*, 1964), in the presence (●) or absence (○) of 2.5 mM-fructose.

Fig. 3. Effect of Na^+ and K^+ ions on the utilization of fructose and on the stimulation by this sugar of the activity of phosphorylase kinase in a liver high-speed supernatant

Experimental conditions were as described in the legend to Fig. 2 except that in (a) K^+ ions were replaced by Na^+ ions. Phosphorylase activation was measured in the presence (●) or absence (○) of 2.5 mM-fructose. ▲, Fructose concentration (a) 0.14M- Na^+ ; (b) 0.14M- K^+ .

Fig. 4. Effect of hexokinase on the utilization of glucose and on the activity of phosphorylase kinase at two ATP concentrations in a liver high-speed supernatant

A rat liver high-speed supernatant was incubated as described in the legend of Fig. 2, but with 0.5 mM-glucose and 2 mM-ATP (a) or 0.2 mM-ATP, in the absence (○, △) or presence (●, ▲) of 0.5 μg of purified yeast hexokinase. ○ and ●, Phosphorylase a activity; △ and ▲, glucose concentration.

Effect of ATP concentration on the activity of liver phosphorylase kinase

Fig. 5 shows the effect of varying the concentration of ATP at constant concentrations of MgSO_4 . It appears that there is an optimum in the concentration

of ATP that is dependent on magnesium concentration and that approximately equals one-half that of that concentration. An increase in the ATP/Mg ratio above 0.5 is inhibitory.

This inhibition of the rate of reaction by a high ATP/Mg ratio suggests that the fructose effect

could be explained by the drop in ATP concentration relative to Mg; a similar explanation would apply to the effect of glucose with hexokinase. A further

Fig. 5. Phosphorylase kinase activity at various concentrations of ATP and at constant concentrations of MgSO_4 . Phosphorylase kinase activity was measured in a liver high-speed supernatant as described in the Materials and Methods section, except that MgSO_4 concentration was kept constant at 1.25 mM (solid line) or 2.5 mM (broken line) and that ATP concentration was varied from 0 to 6 mM.

complexity in the fructose effect is, however, introduced by the hysteretic behaviour of phosphorylase kinase (Kim & Graves, 1973). Fig. 6 shows that indeed, when the concentration of ATP exceeded that of magnesium, fructose did stimulate the rate of reaction not only if added at the beginning of the reaction, but also when a constant rate was reached. In contrast, when the concentration of ATP was below that of Mg, a stimulation by fructose was only observed when the hexose was added at time zero. The latter effect was also observed with glucose and hexokinase, indicating that it is indeed attributable to a relative change in ATP concentration towards Mg (Fig. 4b).

It was verified that P_i (0–5 mM) and ADP up to 0.5 mM did not change phosphorylase kinase activity in a high-speed supernatant.

Activation of liver phosphorylase by fructose *in vivo*

We checked that the activation of phosphorylase by fructose, observed previously only in isolated liver preparations, also occurs in the liver *in vivo*. Fig. 7 shows that this is the case, since the intravenous injection of fructose to anaesthetized rats caused a very significant, rapid and dose-dependent activation of phosphorylase in the liver. Again, there was no statistically significant change in either the activity of protein kinase or phosphorylase kinase. The decrease

Fig. 6. Effect of ATP concentration on the stimulation of phosphorylase kinase by fructose added at various times in a liver high-speed supernatant

A high-speed supernatant from rat liver was incubated as described in the legend of Fig. 2, but with 2 mM-ATP (a) or 0.2 mM-ATP (b), in the presence (●) or absence (○) of 2.5 mM-fructose added either at time zero or at 40 min (arrows).

Fig. 7. Effect of the administration of fructose on the activities of cyclic AMP-dependent histone kinase (a), phosphorylase kinase (b) and phosphorylase a (c) in the liver of anaesthetized rats

At zero time, fructose [0.2 (●) or 1 mg/g body wt. (▲)] or 0.9% (w/v) NaCl (○) were injected intravenously into anaesthetized rats. The vertical bars are means for three determinations $\pm$ S.E.M.

in ATP concentration under similar experimental conditions is well documented (Mäenpää *et al.*, 1968; Raivio *et al.*, 1969).

Discussion

Properties of liver phosphorylase kinase

Comparison with muscle phosphorylase kinase. The properties of liver phosphorylase kinase that are described in the present paper are similar to those previously reported for purified rabbit muscle phosphorylase kinase (Walsh & Krebs, 1973). The liver enzyme has indeed a high molecular weight and a value of about 1 300 000 has been reported for purified muscle phosphorylase kinase (Hayakawa *et al.*, 1973; Cohen, 1973).

Hysteresis is another property common to both liver and muscle phosphorylase kinases. In the case of the muscle enzyme, this property has been attributed to autophosphorylation (Wang *et al.*, 1976; Carlson & Graves, 1976). The inhibition by ATP in excess of Mg is also common to both enzymes (Krebs *et al.*, 1964; Shimazu & Amakawa, 1968).

The liver and the muscle enzymes seem, however, not to be identical as indicated by DEAE-cellulose chromatography and immunological investigation (Proux *et al.*, 1974); furthermore, it appears from the work of Vandenheede *et al.* (1977a) that the influence of pH on the activity of the less active form as well as the effect of the interconversion of the two forms on the K_m for phosphorylase *b* are different in the case of the liver and muscle phosphorylase kinase.

Control of activity of phosphorylase kinase by the ATP/Mg ratio. The fact that both the liver and the muscle phosphorylase kinases are optimally active at an ATP/Mg ratio between 0.3 (Krebs *et al.*, 1964) and 0.5 (the present paper) may have important physiological implications. Indeed, it indicates that the rate of reaction is influenced by the concentration of both ATP-Mg and free Mg^{2+} . The inhibition by an excess of ATP over Mg could be due to competition by free ATP for ATP-Mg or to a decrease in the concentration of Mg^{2+} . Arguments against the first interpretation have been presented by Clerch & Huijing (1972) in the case of muscle phosphorylase kinase. Knowing that ATP is present in the liver mostly as an ATP-Mg (1:1) complex with only a slight excess of magnesium (Veloso *et al.*, 1973), phosphorylase kinase activity may not be optimal and would be augmented by an increase in free Mg^{2+} as is expected to occur when the concentration of ATP is decreased. This is a simple and potentially important mechanism by which ATP consumption would increase glycogenolysis. A similar mechanism was previously proposed by Villar-Palasi & Wei (1970) for the control of glycogenolysis during muscle contraction.

The mechanism by which fructose activates liver phosphorylase

The activation of phosphorylase that occurs in rat livers perfused with fructose has been related by Walli *et al.* (1975) and by Jakob (1976) to a conversion of ATP into ADP and AMP. More recently, Miller (1978) has attributed the activation of phosphorylase by fructose in isolated hepatocytes to an increase in cyclic AMP and activation of protein kinase. Our work indicates that the latter mechanism applies only to very high concentrations of fructose (about 30 mM) and even under these conditions is irregularly observed. Fig. 1 indeed indicates that phosphorylase activation is obtained at concentrations of fructose that are about 10 times lower than those that could increase protein kinase and phosphorylase kinase activity. The fact that the withdrawal of Ca^{2+} from the incubation medium of the

hepatocytes did not impair the activation of phosphorylase by fructose suggests that a change in the concentration of this cation is not involved.

The fructose effect on phosphorylase activity has now been observed in a cell-free extract and in this experimental model activation of phosphorylase appears also to be related to the consumption of ATP secondary to the presence of fructose and the activity of fructokinase. Indeed, the fructose effect is only observed in the presence of K^+ ions, which are required for fructokinase activity (Hers, 1952). In a medium where Na^+ is the prevalent cation, there is little utilization of fructose and stimulation of phosphorylase kinase. Furthermore, the fructose effect is shared by tagatose, which is another substrate of fructokinase, but not by glucose unless hexokinase is added. Fructose 1-phosphate has no or little effect on the rate of activation of phosphorylase and glucose 6-phosphate is known to be an inhibitor of the kinase reaction (van de Werve *et al.*, 1974; Vandenheede *et al.*, 1977b). Therefore the common denominator is the consumption of ATP putting in operation the regulatory mechanism suggested in the preceding paragraph. An objection to this mechanism is that this stimulation of phosphorylase kinase by fructose in a high-speed supernatant was also observed at low ATP concentration (0.2mM) with an ATP/Mg ratio below the optimal value. However, the latter effect results only from a cancellation of the hysteretic mechanism whose occurrence in the intact cell has not been elucidated. Indeed, when fructose was added after the latency period, it had no effect at low ATP concentration. The mechanism by which ATP affects the hysteresis of phosphorylase kinase remains to be determined.

The activation of glycogen phosphorylase by fructose in isolated hepatocytes as well as in the liver *in vivo* is in contrast with the general belief that fructose is a good glycogen precursor (see, e.g., Seglen, 1974). In the perfused liver, the activation of phosphorylase appears to be transient (Walli *et al.*, 1975; Jakob, 1976) and to be followed by inactivation of the enzyme, whereas *in vivo* a late inactivation of phosphorylase by fructose in mouse liver has been reported (Van den Berghe *et al.*, 1973; Thurston *et al.*, 1974). A possible explanation for these paradoxical situations may be found in the important decrease of P_i concentration inside the liver cell after a fructose load (Mäenpää *et al.*, 1968). Indeed, it appears that the amount of P_i that remains in the liver under these conditions (about 30% of the initial value) can be accounted for by mitochondrial phosphate (Akerboom *et al.*, 1978) so that the cytosolic concentration could be very low and that the activity of phosphorylase might therefore be greatly impaired. Another factor that can also be taken into consideration is that the drop in ATP concentration is apparently followed by an extrusion of Mg^{2+} and possibly

of other undefined effectors of the liver cell. It has been shown that in patients affected by Hereditary Fructose Intolerance, a fructose load was followed by an increase in Mg concentration in the blood (Levin *et al.*, 1963). A loss of Mg from the liver is expected to decrease the activity of phosphorylase kinase.

This work was supported by the Fonds de la Recherche Médicale and by National Institutes of Health grant AM 9235.

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