The Regulation of Rabbit Liver Fructose 1,6-Diphosphatase Activity by Phospholipids *in vitro*

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The loss of activity in the neutral pH region that occurs during the purification of fructose 1,6-diphosphatase (D-fructose 1,6-diphosphate 1-phosphohydrolase; EC 3.1.3.11) has been a source of speculation and controversy since the discovery of the enzyme by Gomori (1943).

The loss of activity has been attributed to two factors, limited proteolysis (Nakashima & Horecker, 1971) and the loss of a natural activator (Pogell *et al.*, 1968). Both factors are probably contributory. Recent purification procedures for the enzyme from rabbit liver claim to yield undegraded 'natural' enzyme (Traniello *et al.*, 1971, 1972), yet the enzyme still requires an activator for maximal activity at neutral pH values. Pogell *et al.* (1968) describe a natural protein activator with properties similar to those of phosphofructokinase, whereas the Pontremoli-Horecker school use the unnatural activator EDTA (Traniello *et al.*, 1971, 1972). Baxter *et al.* (1972) have reported that under certain conditions oleic acid considerably activates fructose 1,6-diphosphatase from rabbit liver, and they propose that oleic acid is the natural activator of the enzyme. We now present evidence that certain phospholipids are activators of this enzyme, and that it is the elimination of these phospholipids during purification that results in a loss of activity at neutral pH values. Other phospholipids are inhibitors of the enzyme.

Experimental and results

Evidence for the presence of both an activator and an inhibitor of fructose 1,6-diphosphatase in crude extracts containing the enzyme comes from a study of the effect of dilution on the specific activity. For these experiments rabbit liver (20g) was homogenized in 1 vol. of 0.154M-KCl and centrifuged at 100000g for 1 h at 4°C. A portion of the supernatant (0.5 ml) was freed from small molecules by gel filtration through a column (8 cm × 1 cm) of Sephadex G-25. As shown in Fig. 1, in the absence of any added activator the specific activity of fructose 1,6-diphosphatase falls as the protein concentration in the assay mixture decreases. This can be interpreted in a number of ways. (1) The enzyme dissociates into inactive subunits; this possibility has been eliminated by showing that the molecular weight of the enzyme, as estimated by both gel filtration through Sephadex

G-100 and sucrose-density-gradient centrifugation, remains constant as the extract is diluted (M. B. Allen & J. McD. Blair, unpublished work). (2) The enzyme plates out on to surfaces in dilute solution; this is not a significant factor, because, if the crude extract diluted at pH 7.4 is transferred to another vessel and assayed at pH 9.3, the specific activity at pH 9.3 is not lowered. (3) There may be an inhibitor in the assay system. This appears to be supported by the fact that low concentrations of EDTA restore the activity; however, pre-treatment of all solutions with a chelating resin (Chelex-100; Bio-Rad Laboratories, Richmond, Calif., U.S.A.) is not effective in restoring activity. It is thought that EDTA exerts its effect on fructose 1,6-diphosphatase by some means other than chelation of inhibitory metal ions (Pogell *et al.*, 1968; Van Tol *et al.*, 1972). (4) Dilution results in the dissociation of an activator not removed by gel filtration; further evidence supports this interpretation.

In the presence of saturating concentrations of EDTA (0.1 mM), the specific activity of fructose 1,6-diphosphatase in the crude extract falls as the protein concentration increases (see Fig. 1). This effect is not due to competition for the substrate as the protein concentration increases, because increasing the substrate concentration does not overcome the effect. Neither is the effect due to competition for the products or limitation of the assay system, as the same result is found either by using the coupled assay system or by assaying for phosphate release. We believe that the effect is due to an inhibitor in the crude extract that is not removed by gel filtration through Sephadex G-25.

The effects of dilution on the specific activity of fructose 1,6-diphosphatase both in the presence and in the absence of EDTA have been demonstrated repeatedly, but the form and precise location of the two curves are variable.

Direct evidence for the presence of both an activator and an inhibitor of fructose 1,6-diphosphatase in crude extracts of the enzyme comes from DEAE-cellulose chromatography (43 cm × 2.5 cm column of Whatman DE 52 DEAE-cellulose run at pH 8.5 in 20 mM-triethanolamine-HCl buffer with a concave gradient of 0–1 M-KCl). Separate peaks, of enzyme with low activity at neutral pH values, of an activator that restores neutral activity to the enzyme, and of an inhibitor of the enzyme, are obtained. T.l.c. of

Fig. 1. Effect of dilution in the presence and in the absence of EDTA on the specific activity of fructose 1,6-diphosphatase in a crude extract of rabbit liver

Assay mixtures contained 0.154M-KCl, 20 mM-triethanolamine hydrochloride and 5 mM-MgCl₂, adjusted to pH 7.4 with KOH, 0.2 mM-NADP⁺, and 17.5 EC units of both phosphoglucose isomerase and glucose 6-phosphate dehydrogenase [yeast enzymes from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany, freed from (NH₄)₂SO₄ by gel filtration]. High-speed supernatant, with small molecules removed by gel filtration (40 mg of protein/ml, assuming that 1 mg of protein/ml has $E_{280} = 1.0$), was added to give the protein concentrations indicated. Reactions were initiated without preincubation by the addition of fructose 1,6-diphosphate to give 80 μM in the cuvette. The total volume was 1 ml, and the temperature 37°C. Rates were measured after an initial slight lag phase. At no time did the coupling system become rate-limiting. ○, No EDTA; △, EDTA (0.1 mM).

chloroform-methanol extracts of the activating fraction (on silica gel with no binder, but impregnated with Na₂CO₃) gave no indication of free fatty acids, but did provide evidence from R_F values and reactions with colour-forming reagents for the presence of

Fig. 2. Effects of phosphatidic acid and phosphoinositides on the activity of rabbit liver fructose 1,6-diphosphatase

Assay conditions were the same as for Fig. 1. Fructose 1,6-diphosphatase [purified by the method of Traniello *et al.* (1971), except that EDTA was excluded throughout] (0.001 mg; specific activity 17.4 μmol/min per mg of protein at pH 9.2 and 37°C) was added immediately before assay. Phospholipids were added as a suspension in 20 mM-triethanolamine hydrochloride, pH 7.4. ○, Diphosphoinositide; △, phosphatidic acid (0.003 mg of enzyme); ▲, phosphatidic acid (0.001 mg of enzyme); □, triphosphoinositide. Phospholipid concentrations are plotted on a logarithmic scale.

phospholipids; in particular, one component moved identically with diphosphoinositide in two solvent systems (chloroform-methanol-acetic acid-water, 25:15:4:2, by vol.; propan-1-ol-4M-NH₃, 2:1, v/v).

We therefore screened a selection of phospholipids for their effects on the activity of fructose 1,6-diphosphatase, both in crude extracts and as the pure enzyme prepared by substrate elution from phosphocellulose as described by Traniello *et al.* (1971), except that EDTA was excluded throughout. Phosphatidic acid was obtained from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K., and from Lipid Products, South Nutfield, Surrey, U.K. Triphosphoinositide was a gift from Dr. A. Sheltawy, University of Leeds. The other phospholipids were from Koch-Light. T.l.c. of the phospholipids did not reveal any detectable free fatty acids on testing by charring in the presence of a sulphuric acid-chromic acid mixture.

Of the phospholipids tested, phosphatidic acid gave the greatest activation with purified enzyme, of about 25-fold at approx. 0.1 mM (see Fig. 2). Diphosphoinositide was also an activator, being effective at much lower concentrations than phosphatidic acid (approx. 1–3 μ M). Triphosphoinositide and phosphatidylserine were poor activators, whereas phosphatidylethanolamine and phosphatidylcholine were mild inhibitors (80% inhibition at 0.1 mg/ml). Lyso-phosphatidylcholine and sphingomyelin were without effect on the reaction rate. Pig kidney fructose 1,6-diphosphatase is activated to the same extent as the rabbit liver enzyme by phosphatidic acid (J. McD. Blair & R. Tetlow, unpublished work). As shown in Fig. 2, the enzyme concentration markedly influences both the extent of activation by phosphatidic acid and also the concentration range in which it is effective. As a check on the physical form of the phosphatidic acid in the assay solutions, the amount of scattered light at 300 nm was measured in an Aminco-Bowman spectrofluorimeter in the concentration range 1.7 μ g–0.1 mg/ml of the assay medium. The amount of light-scatter was practically linear with phosphatidic acid concentration over the entire range, and could be extrapolated back to zero at zero concentration, indicating that the phosphatidic acid was micellar throughout this range. Sonication of the phosphatidic acid diluted with assay medium before assay did not measurably influence the reaction rate.

Phosphatidic acid does not affect the substrate inhibition of fructose 1,6-diphosphatase, but decreases the inhibitory effect of AMP. In the presence of 50 μ g of phosphatidic acid/ml, the maximum inhibition of the enzyme obtainable was 60%. In the presence of 0.2 mM-AMP, the addition of 200 μ g of phosphatidic acid/ml increased the activity of fructose 1,6-diphosphatase by a factor of 75.

The phospholipids exerted their effects on the enzyme within seconds, whether added before or after substrate.

With high-speed supernatant from rabbit liver, both phosphatidic acid and diphosphoinositide had an effect very similar to that of EDTA as shown in Fig. 1, in that in their presence the specific activity of fructose 1,6-diphosphatase increased on dilution.

Discussion and conclusions

A potent activator of fructose 1,6-diphosphatase extracted from rabbit liver has been tentatively identified as diphosphoinositide.

In a screening of several phospholipids, those carrying a negative charge activate fructose 1,6-diphosphatase, and those with a positive charge inhibit the enzyme, the effects being roughly in proportion to charge. Triphosphoinositide appears to

be an exception, but as it binds bivalent cations tenaciously (Hendrickson & Ballou, 1964), thus decreasing the net charge on the molecule, it conforms to the pattern. Diphosphoinositide is effective at a much lower concentration than the other phospholipids tested.

The concentrations of phosphatidic acid and diphosphoinositide that have been measured in tissues are in the correct range for them to influence the activity of fructose 1,6-diphosphatase (see, e.g., Koppikar *et al.*, 1969; Tou *et al.*, 1972). Phospholipids *in vivo* are mainly associated with membrane structures, where they must be immobilized to a large extent, so the question of accessibility has to be considered even more carefully than with freely soluble effectors. However, the fact that fructose 1,6-diphosphatase is activated by micelles of phosphatidic acid suggests that *in vivo* it may be attached to membranes and influenced by the state of the membranes.

Both phosphatidic acid (Dawson, 1966) and diphosphoinositide (Tou *et al.*, 1972) are present in low concentrations *in vivo*, but are subject to rapid turnover, which would make their concentrations highly responsive to differential changes in their rates of formation and utilization, and hence make them ideally suited to roles as regulators of metabolism. However, it has not been demonstrated that the concentrations of these phospholipids change with metabolic and hormonal conditions in the manner required for regulation.

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