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Comparison of purified bovine heart and rat liver 6-phosphofructo-2-kinase

Evidence for distinct isoenzymes

Mark H. RIDER, Dominique FORET and Louis HUE
Hormone and Metabolic Research Unit, University of Louvain, and International Institute of Cellular and Molecular Pathology, UCL 7529, Avenue Hippocrate 75, B-1200 Brussels, Belgium

Rat liver and bovine heart 6-phosphofructo-2-kinase were purified by the same procedure. Compared with the liver enzyme, the heart enzyme had a smaller apparent M_r, different kinetic properties, was not inactivated by cyclic AMP-dependent protein kinase, and contained less fructose-2,6-bisphosphatase activity. These differences suggest that heart and liver 6-phosphofructo-2-kinase are distinct isoenzymes. Likewise, 6-phosphofructo-2-kinase from rat heart and skeletal muscle was not inactivated on treatment with cyclic AMP-dependent protein kinase.

INTRODUCTION

Fru-2,6-P_2 is an important regulator of glycolysis in various mammalian tissues. It is involved in the glucose-induced stimulation of glycolysis not only in liver (Van Schaftingen *et al.*, 1980; Hue *et al.*, 1984), but also in epididymal adipocytes (Rider & Hue, 1985), pancreatic islets (Sener *et al.*, 1984), enterocytes (Louis *et al.*, 1985) and rat hepatoma (HTC) cells (Loiseau *et al.*, 1985). In the liver, Fru-2,6-P_2 is involved in the control of gluconeogenesis by glucagon (Hers & Hue, 1983). An increase in Fru-2,6-P_2 concentration has been observed after insulin stimulation in skeletal muscle and heart, but not in white adipose tissue (Hue *et al.*, 1982; Rider & Hue, 1984, 1985).

In rat liver, 6-phosphofructo-2-kinase (PFK-2) and fructose-2,6-bisphosphatase (FBPase-2), which are responsible for the synthesis and degradation of Fru-2,6-P_2, co-purify (Van Schaftingen *et al.*, 1982; El-Maghrabi *et al.*, 1982), and have been shown to be dual catalytic functions of a single protein (El-Maghrabi *et al.*, 1982). Treatment of the protein with the cyclic AMP-dependent protein kinase causes reciprocal changes in the activity of PFK-2 (inactivation) and FBPase-2 (activation) (Van Schaftingen *et al.*, 1982; El-Maghrabi *et al.*, 1982; reviewed by Hers & Hue, 1983; Claus *et al.*, 1984). However, the enzymology of the Fru-2,6-P_2-synthesizing and -degrading systems of other mammalian tissues has received little attention. PFK-2 activity has been detected in brain, skeletal muscle, kidney (Kuwajima & Uyeda, 1982) and partially purified from adipose tissue (Rider & Hue, 1985). In pancreatic islets, PFK-2 activity was not inactivated by glucagon (Malaisse *et al.*, 1982). In rat heart, insulin was found to double PFK-2 activity (Rider & Hue, 1984). We have compared the rat liver and bovine heart PFK-2 and show that their properties are different.

MATERIALS AND METHODS

Chemicals

Chemicals were obtained from sources described by Rider & Hue (1984, 1985). In addition, histone IIA was from Sigma Chemical Co., and [γ-^{32}P]ATP was from Amersham International. Gel-filtration protein standards, hydroxyapatite and Agarose A-0.5 m were from Bio-Rad Chemical Division. DEAE-Sephacel and electrophoresis standards were from Pharmacia and DEAE-Trisacryl was from LKB. Ultrafilters with a nominal M_r limit of 50000 were from Filtron Corp. (Clinton, MA, U.S.A.).

Purification of enzymes

The catalytic subunit of cyclic AMP-dependent protein kinase was purified from bovine heart by elution from DEAE-cellulose with cyclic AMP, followed by hydroxyapatite chromatography as described by Reimann & Beham (1983). PFK-2 was purified from bovine heart and rat liver by the same procedure. Livers from starved rats which had been re-fed for 12 h on a high-carbohydrate diet (bread and water containing 20 mM-glucose) were homogenized with a Waring Blendor (4 × 30 s) in 2 vol. (v/w) of 20 mM-Hepes/50 mM-KCl/2 mM-EDTA/5 mM-$MgCl_2$/1 mM-dithioerythritol/0.1 mM-fructose 6-phosphate/0.3 mM-glucose 6-phosphate, pH 7.5 at 0 °C (buffer A). Bovine hearts were obtained from a local slaughterhouse. Heart muscle was trimmed away from fat and pericardium, cut into small pieces, and homogenized in 2 vol. (v/w) of buffer A. After centrifugation (10000 *g* for 30 min), the supernatants were filtered through glass wool and were fractionated with poly(ethylene glycol). The 6–15% (w/v) (liver) and 6–20% (w/v) (heart) fractions were resuspended in one-sixth of the homogenate volume in buffer A containing 20% (v/v) glycerol (buffer B). The fractions were then applied to a column (5 cm × 25 cm) of DEAE-Sephacel (heart) or to a column

Abbreviations used: FBPase-2, fructose-2,6-bisphosphatase (EC 3.1.3.46); Fru-2,6-P_2, fructose 2,6-bisphosphate; PFK-1, 6-phosphofructo-1-kinase (EC 2.7.1.11); PFK-2, 6-phosphofructo-2-kinase (EC 2.7.1.105).

(2.5 cm × 20 cm) of DEAE-Trisacryl (liver) equilibrated with buffer B. The column was washed extensively with buffer B until the eluate was free of protein (monitored by the A_{280}). PFK-2 was eluted (at 0.25 M-KCl) with a linear gradient of KCl (buffer B, 50–500 mM-KCl). The fractions containing PFK-2 activity were pooled and applied to a column (2.5 cm × 5 cm) of Blue Sepharose CL-6B equilibrated with buffer B. The column was washed with buffer B, followed by buffer B containing 0.4 M-KCl (buffer C) until the protein concentration in the eluate was negligible. PFK-2 was eluted (at 5–10 mM-ATPMg) with a linear gradient of 0–15 mM-ATPMg in buffer C. The PFK-2-enriched fractions were pooled, concentrated by ultrafiltration, and the concentrated enzyme was stored frozen at −80 °C. Samples (0.3–2 ml) of the frozen stored enzyme were thawed and further purified by gel filtration through a column (1.5 cm × 80 cm) of Agarose A-0.5 m equilibrated with buffer B containing 5 mM-potassium phosphate. The purity of the preparation was determined by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Laemmli, 1970) in 12% polyacrylamide. Proteins were detected by the silver staining method (Merril *et al.*, 1979).

Assay of enzyme activities

The catalytic subunit of cyclic AMP-dependent protein kinase was assayed by the method of Roskoski (1983). One unit is the amount of enzyme that catalyses the transfer of 1 μmol of ^{32}P into histone/min under these conditions.

PFK-2 activity was measured as described by Rider & Hue (1984). Saturation curves describing the activity of PFK-2 as a function of the concentration of each substrate were fitted to hyperbolae by using a computer program described by Barlow (1983). FBPase-2 activity was measured by the production of $[^{32}P]P_i$ from [2-^{32}P]-Fru-2,6-P_2, which was prepared by the method of Van Schaftingen *et al.* (1982). The reaction was performed at 30 °C, in a final volume of 0.2 ml in the presence of 20 mM-Hepes/50 mM-KCl/2 mM-EDTA/10 mM-$MgCl_2$/1 mM-dithioerythritol/5 mM-potassium phosphate/1 mM-GTP/1 mM-*sn*-glycerol 2-phosphate, pH 7.5, and 10 μM-[2-^{32}P]Fru-2,6-P_2 (50000 c.p.m./assay). The product of the reaction, fructose 6-phosphate, which is a potent inhibitor of FBPase-2 (Van Schaftingen *et al.*, 1982), was removed by an enzymic system consisting of 0.1 mM-$NADP^+$, 1 unit of purified yeast phosphoglucose isomerase and 0.5 unit of glucose 6-phosphate dehydrogenase (Boehringer). The reaction was stopped by the addition of 1 vol. of 50 mM-NaOH, and the $[^{32}P]P_i$ was separated from the substrate (Van Schaftingen *et al.*, 1982). One unit of PFK-2 or FBPase-2 is the amount of enzyme that catalyses the formation of 1 μmol of product/min.

Treatment of PFK-2 with the catalytic subunit of the cyclic AMP-dependent protein kinase

PFK-2 (0.15 munit) was incubated with 0.2 munit of protein kinase in a final volume of 0.1 ml containing 50 mM-Hepes/1 mM-dithioerythritol, pH 7.4, and 1 mg of albumin/ml. The reaction was started by the addition of 1 mM-ATPMg and continued for 10 min at 30 °C. The reaction was stopped by the addition of 5 mM-EDTA and chilled in ice. Samples (20 μl) were removed, diluted 40-fold, and assayed for PFK-2 activity.

Protein determination

Proteins were measured as described by Bradford (1976), with bovine serum albumin as a standard.

RESULTS AND DISCUSSION

Purification and M_r

Rat liver PFK-2 was about 10–30 times more active than rat heart PFK-2 when compared on a wet-weight basis in crude homogenates. However, the difference was smaller (3-fold) between the rat liver and bovine heart activities. PFK-2 from rat liver and bovine heart were purified by the same procedure (see the Materials and methods section). The major purification was obtained with Blue Sepharose, from which PFK-2 was eluted by a linear gradient of ATPMg. The overall purification was about 2000-fold and the specific activity was about 50 (liver) and 100 (heart) munits/mg of protein. Liver PFK-2 was eluted in two peaks from the DEAE-Trisacryl column. The second peak, eluted at 0.4 M-KCl, represented 20–25% of the activity in the first peak. This second peak was not further purified, and may represent a more negatively charged form of the enzyme, such as a more phosphorylated form. Interestingly, such a second peak was not observed with the heart preparation.

The M_r of the two enzymes was estimated by gel filtration through agarose A-0.5 m with thyroglobulin, γ-globulin, ovalbumin, myoglobulin and cyanocobalamin as markers. The calculated M_r was 112500 for the liver enzyme, a value similar to those previously reported (Pilkis *et al.*, 1983), and 97000 for the heart enzyme (means of two determinations). Filtration on agarose further purified the two enzymes; however, the heart PFK-2 lost about one-third of its activity during this procedure. When analysed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, a major band migrated with a M_r of 58000 (liver) or 52000 (heart). This difference, though small, was consistently observed and confirmed that the M_r of the heart enzyme is slightly less than that of the liver enzyme.

Kinetic properties

Hyperbolic kinetics were obtained with the two enzymes for their substrates. The K_m for ATPMg (0.1 mM) was the same for both enzymes, whereas the K_m for fructose 6-phosphate was 2–3 times smaller for the heart enzyme (0.05 mM) than for the liver enzyme (0.14 mM). This difference might have some physiological implications. Indeed, the concentrations of fructose 6-phosphate usually found in heart, 0.05–0.15 nmol/g (Williamson, 1965; Rider & Hue, 1984), equivalent to 0.15–0.45 mM, assuming a 30% intracellular water space (Williamson, 1965), are above the K_m values, and a change in blood glucose within the physiological range would have little effect on the concentration of Fru-2,6-P_2. Regulation by change in the $V_{max.}$ would be expected; interestingly, such an effect was observed after insulin treatment (Rider & Hue, 1984). By contrast, in liver, the concentration of fructose 6-phosphate (Hue *et al.*, 1984) can be more than 5-fold smaller than the K_m value, and increases in its concentration can be related to an increase in Fru-2,6-P_2 concentration (Hue *et al.*, 1981, 1984).

The two enzymes also differ by the extent of inhibition by *sn*-glycerol 3-phosphate when assayed with suboptimal concentrations of substrates (Fig. 1). Moreover, the heart

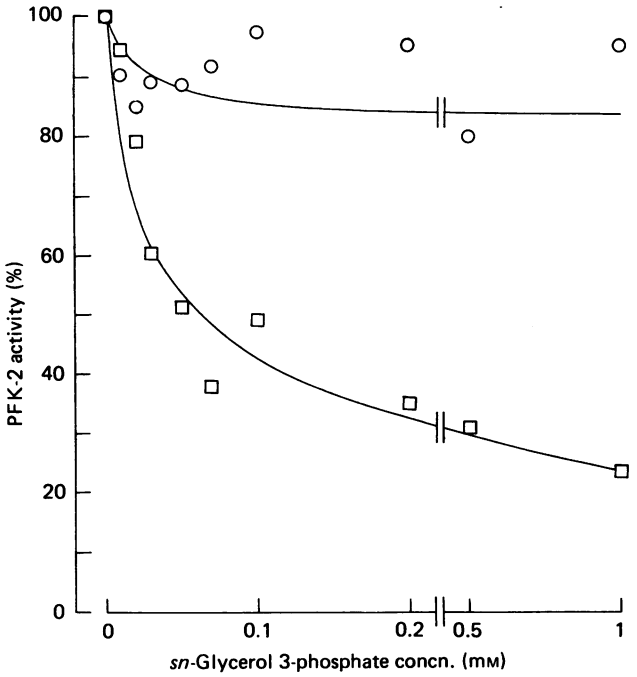

Fig. 1. Effect of *sn*-glycerol 3-phosphate on purified rat liver and bovine heart 6-phosphofructo-2-kinase

Purified rat liver and bovine heart PFK-2 were assayed in 0.2 ml of 50 mM-Hepes/100 mM-KCl/20 mM-KF/1 mM-dithioerythritol, pH 7.1 at 30 °C, with suboptimal concentrations of fructose 6-phosphate (0.1 mM) and ATPMg (0.5 mM) and the indicated concentrations of *sn*-glycerol 3-phosphate. The specific activities of liver (□) and heart (○) PFK-2 corresponding to 100% values were 61.8 and 15.6 munits/mg of protein respectively.

enzyme was more sensitive to inhibition by citrate than was the liver enzyme: for 95% inhibition of activity, 0.5 mM- and 5 mM-citrate respectively were required (results not shown). The optimal activity of liver enzyme was greater at pH 7.1 than at pH 6.5, whereas the converse was true for the heart enzyme (Table 1).

Table 2. Effect of protein kinase catalytic-subunit treatment of poly(ethylene glycol) fractions of rat tissue homogenates on PFK-2 activity

Poly(ethylene glycol) fractions (6–20%) of rat liver, heart and hindlimb muscle homogenates were prepared as described in the Materials and methods section. The fractions (about 1 mg of protein) were incubated with 0.3 munit of protein kinase catalytic subunits in a final volume of 0.1 ml containing 50 mM-Hepes, 1 mM-dithioerythritol and 1 mg of albumin/ml at 30 °C. The reactions were initiated with 1 mM-ATPMg, stopped after 20 min by the addition of 5 mM-EDTA and chilled in ice. Samples (25 μl) of reaction mixture were assayed for PFK-2 under optimal conditions with 2 mM-fructose 6-phosphate, 6 mM-glucose 6-phosphate and 5 mM-ATPMg at pH 7.1 and 30 °C. The values shown are the means ± S.E.M. for four (liver) and three (heart and skeletal muscle) animals: * indicates a statistically significant ($P < 0.05$) effect.

Tissue poly(ethylene glycol) fraction	Specific activity of PFK-2 (μunits/mg of protein)	
	No catalytic subunit	Plus catalytic subunit
Liver	137±41	18±4*
Heart	10.0±0.7	9.0±0.9
Skeletal muscle	3.1±0.4	3.1±0.2

Inactivation by the cyclic AMP-dependent protein kinase

Whereas the activity of the liver enzyme was decreased about 3–4-fold by the catalytic subunit of the cyclic AMP-dependent protein kinase, there was no decrease, but even a slight increase, in the activity of the heart enzyme, assayed with optimal or suboptimal concentrations of substrates at pH 7.1 or 6.5 (Table 1). Similarly, PFK-2 was not inactivated when partially purified fractions from either rat heart or skeletal muscle were treated with the cyclic AMP-dependent protein kinase. Liver PFK-2 was, however, inactivated under the same conditions (Table 2). This absence of inactivation of heart PFK-2 confirms a previous observation that perfusion of rat heart with adrenaline, which acts via cyclic AMP, did

Table 1. Effect of treatment with protein kinase catalytic subunit on the activities of purified rat liver and bovine heart 6-phosphofructo-2-kinase

After incubation with the catalytic subunit of cyclic AMP-dependent protein kinase in the presence of 1 mM-$MgCl_2$ or 1 mM-ATPMg, PFK-2 was assayed with 2 mM-fructose-6-phosphate, 5 mM-ATPMg (optimal conditions) or with 0.1 mM-fructose-6-phosphate, 0.5 mM-ATPMg (suboptimal conditions), at both pH 6.5 and 7.1. The values shown are the means of two separate experiments.

Addition to incubation	Specific activity of PFK-2 (munits/mg of protein)							
	Heart				Liver			
	pH 7.1		pH 6.5		pH 7.1		pH 6.5	
Conditions...	Optimal	Suboptimal	Optimal	Suboptimal	Optimal	Suboptimal	Optimal	Suboptimal
Catalytic subunit, $MgCl_2$ (1 mM)	23.3	13.5	31.3	13.1	90.2	34.6	60.8	11.0
Catalytic subunit, ATPMg (1 mM)	26.6	16.3	36.3	17.3	23.9	4.9	19.4	6.1

not decrease the concentration of Fru-2,6-P_2 (Hue *et al.*, 1982).

One could argue that the absence of inactivation of heart PFK-2 could result from the fact that the enzyme was already in a fully phosphorylated form. However, incubation of heart extracts with Mg^{2+} to stimulate phosphatase activity failed to increase PFK-2 activity.

Fructose-2,6-bisphosphatase

The specific activity of FBPase-2 in liver PFK-2 purified by Blue Sepharose was 12 ± 6 munits/mg of protein (mean $\pm$ S.E.M. for three different preparations), i.e. about four times less than the specific activity of PFK-2 in the same purified fraction and than the values previously reported (El-Maghrabi *et al.*, 1982; Sakakibara *et al.*, 1984; Van Schaftingen *et al.*, 1982). The discrepancy between our values of FBPase-2 and the published data might result from differences in assay conditions.

The purified heart PFK-2 contained at least 10 times less FBPase-2 activity than did liver PFK-2, and the specific activity of the heart FBPase-2 (maximal value obtained in two preparations equal to 0.3 munit/mg of protein) was at least two orders of magnitude lower than the specific activity of liver PFK-2 in the same fraction. Thus the liver and heart enzyme differ in their content of FBPase-2. However, since some phosphatase activity is present in the heart enzyme, we cannot draw any conclusion about the bifunctionality, or otherwise, of the heart PFK-2.

General conclusions

Our results clearly show that heart and liver PFK-2 are distinct isoenzymes. The evidence for this interpretation can be summarized as follows: compared with the liver PFK-2, the heart enzyme (i) has a smaller M_r, (ii) has more affinity for fructose 6-phosphate, (iii) is less inhibited by *sn*-glycerol 3-phosphate, but is more sensitive to inhibition by citrate, (iv) is not inactivated by the cyclic AMP-dependent protein kinase, and (v) contains less FBPase-2 activity. These differences in properties confirm differences in the regulation by insulin, namely that, in the liver, insulin changes the K_m for fructose 6-phosphate, by antagonizing the effect of glucagon, whereas in heart insulin increases $V_{max.}$ without change in K_m for fructose 6-phosphate (Rider & Hue, 1984). These differences also confirm the previous observation that treatment of perfused rat hearts or skeletal muscle with adrenaline did not elicit a fall in fructose 2,6-bisphosphate concentration (Hue *et al.*, 1982).

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