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THE ROLE OF FRUCTOSE 2,6-BISPHOSPHATE IN THE LONG-TERM CONTROL OF PHOSPHOFRUCTOKINASE IN RAT LIVER

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SUMMARY : The phosphofructokinase stabilizing factor, believed to be a peptide of molecular weight 3,800 (Dunaway G.A. and Segal H.L., 1976, J. Biol. Chem. 251, 2323-2329), shares many chemical and biological properties with fructose 2,6-bisphosphate. It co-migrated with it upon gel filtration in the molecular weight range 300-400 or 3,000-4,000 depending upon the ionic strength of the solution. Fructose 2,6-bisphosphate is the most potent phosphofructokinase stabilizing agent present in the liver of a fed rat. Its disappearance during fasting and diabetes could account for the faster rate of degradation of phosphofructokinase reported to occur under these conditions. The effect of starvation to decrease by 60% the phosphofructokinase content of the liver is, however, for its greatest part, related to a non-specific decrease in liver mass.

Dunaway and his co-workers (1-4) have concluded that the reduction in liver PFK-L_2 (the major isoenzyme of PFK in the liver) in fasting and diabetes is the result of an increased degradation rate with little or no change in synthetic rate. They also reported that the livers of fed rats contain a PFK-stabilizing factor, believed to be a polypeptide for which a molecular weight of 3,800 was determined by gel filtration. This factor protected PFK-L_2 against thermal or enzymic inactivation and was also a stimulator of the enzyme, since it increased its affinity for fructose 6-phosphate. It was unstable at slightly acid pH and it was also an inhibitor of fructose-1,6-bisphosphatase (3). Its concentration in the liver was inversely proportional to the rate of PFK degradation. It was therefore proposed that insulin mediates the rate of degradation of PFK-L_2 by controlling the level of the stabilizing factor (4).

Abbreviations : PFK, 6-phosphofructo 1-kinase; LDH, lactate dehydrogenase; GSH, reduced glutathione; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid

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Fructose 2,6-bisphosphate was discovered as a potent, acid-labile stimulator of liver PFK (5,6; reviewed in 7). It also has a remarkable ability to protect PFK against thermal or enzymatic inactivation (8-10) and is an inhibitor of fructose-1,6-bisphosphatase (11, 12). Furthermore, it appears to have an abnormal behaviour upon gel filtration when performed at low ionic strength (the procedure used for the determination of the molecular weight of the stabilizing peptide), being then eluted in the molecular weight range of 3,000-4,000 (13).

Because of its property to stabilize PFK and to disappear in fasting and in diabetes, fructose 2,6-bisphosphate can play the role attributed by Dunaway et al. (4) to the stabilizing peptide in the long-term control of liver PFK. Furthermore, because of the many similarities that exist between fructose 2,6-bisphosphate and the stabilizing peptide, the mere existence of the latter needs to be reconsidered. These problems are the subject of the present paper.

MATERIALS AND METHODS

Sephadex G-50 (medium) was from Pharmacia (Uppsala, Sweden), DEAE from Whatman (Springfield Mills, England), chemicals from Merck (Darmstadt, Germany) or Fluka (Buchs, Switzerland) and biochemicals and enzymes from Boehringer (Mannheim, Germany) or Sigma (St Louis, MO.). Male Wistar rats were used. Fructose 2,6-bisphosphate was prepared as in (14).

PFK was measured at 20°C in the presence of 5 mM fructose 6-phosphate, 17.5 mM glucose 6-phosphate, 1 mM ATP, 1 mM AMP, 6 mM $MgCl_2$, 20 mM $(NH_4)_2SO_4$, 100 mM KCl and 50 mM Hepes pH 7.1, as well as 50 µg/ml aldolase, 1 µg/ml triosephosphate isomerase and 10 µg/ml glycerol 3-phosphate dehydrogenase. Rat liver PFK was purified as described by Dunaway and Weber (15) up to and including the DEAE cellulose step. The active fractions were combined and mixed with 0.6 g $(NH_4)_2SO_4$ per ml, left overnight at 4°C and centrifuged during 15 min at 10,000 x g. The resulting pellet was dissolved in a solution containing 1mM ATP, 20 mM NaF, 2 mM dithiothreitol and 25 mM Tris pH 8. This solution contained approx. 20 U of PFK and 7 mg of protein per ml; it was stored at 4°C.

The thermal denaturation of PFK was measured by diluting 1 µl of the enzyme preparation in 1 ml of a solution containing 100 mM KCl and 20 mM Hepes, pH 7.1. When this mixture was incubated at 37°C, PFK activity decayed rapidly during the first 20 min and then more slowly but the semi-log plot was not linear. Since we could not express this decay in terms of half-life, we represented it by the fractional residual activity after 40 min of incubation. At that time PFK activity was 5-10% of its initial value, but maintained a higher value in the presence of a stabilizing agent.

Fructose 2,6-bisphosphate (16), reduced glutathione (17), protein (18), lactate dehydrogenase (19) and aldolase (20) were measured as indicated. One unit of enzyme is the amount that converts 1 µmol of substrate per min at 20°C.

RESULTS

PFK-stabilizing effect of fructose 2,6-bisphosphate and of other low-molecular weight agents. Fructose 2,6-bisphosphate can protect

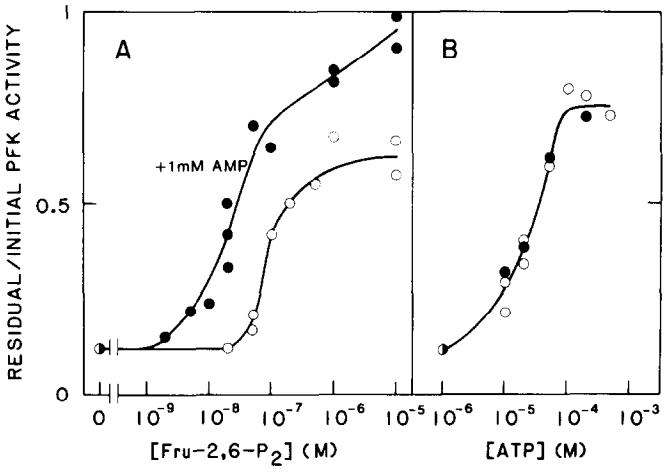

Fig. 1. Stabilization of PFK by fructose 2,6-bisphosphate and by ATP, with and without AMP.

PFK-L_2 against heat denaturation at about the same concentrations at which it is known to stimulate the enzyme activity (Fig. 1). AMP, which had no stabilizing effect by itself, increased the sensitivity of the system to fructose 2,6-bisphosphate by about 1 order of magnitude and also increased the amplitude of the effect. ATP also stabilized the enzyme but at much higher concentrations and its action was not affected by the presence of AMP. Citrate (0.2 mM), fructose 6-phosphate (0.5 mM), sulfate and P_i (5 mM) could also stabilize the enzyme, showing half maximal effects at the concentrations indicated.

Gel filtration of a liver extract. Liver extracts (1.5 ml) obtained from fed rats were filtered through 41 ml of Sephadex G-50 either under the conditions of Dunaway and Segal or in the presence of 200 mM KCl. In the first condition (Fig. 2), fructose 2,6-bisphosphate was eluted together with the stabilizing factor soon after the protein peak, in the range of molecular weight 3,000-4,000. In the presence of 200 mM KCl (Fig. 3), fructose 2,6-bisphosphate was eluted much later together with the major part of the stabilizing factor and directly before reduced glutathione. In both experiments, a minor part of the stabilizing factor was also found with the protein peak. Furthermore, when comparing the data from Figs. 2 and 3 with those from Fig. 1, it appears that fructose 2,6-bisphosphate can account for all the stabilizing effect of the corresponding peak in Fig. 3, although not in Fig. 2. This discrepancy could be explained by the fact that the protein peak as well as fraction 18 to 30 contained an unknown substance that increased the stabilizing effect of fructose 2,6-bisphosphate. This unknown substance was also present in the livers of rats that had been fasted for 2 days and therefore cannot correspond to the PFK-stabilizing peptide.

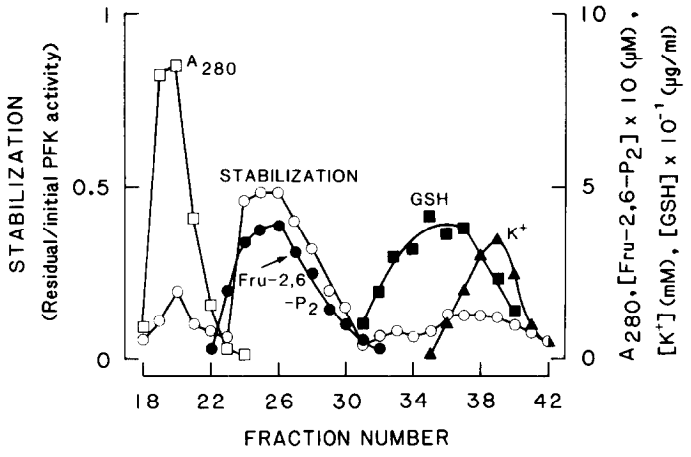

Fig. 2. Chromatography of a liver extract on Sephadex G-50 in the absence of salt. The liver of a fed rat was homogenized in 5 vol of a solution containing 250 mM glucose and 50 mM Tris, pH 8. The homogenate was centrifuged for 30 min at 100,000 x g and 1.5 ml of the resulting supernatant was filtered through a column (27 x 1.4 cm) equilibrated with the homogenization buffer. The flow rate was 10 ml/hr and fractions of 1 ml were collected. Stabilization against thermal denaturation was tested with 200 μl of each fraction.

The effect of prolonged fasting on the concentration of PFK and of other proteins in the liver. Dunaway and Weber (1) reported a 60 to 70 % decrease in PFK in the livers of 6-day fasted rats, expressed as U/cell. This observation needed confirmation because of the recent report by Claus et al. (10) that the recovery of PFK from the livers of fasted rats could be greatly impaired because of the absence of fructose 2,6-bisphosphate which, as seen above, is a potent stabilizer of the enzyme. We found, however, that when the livers were extracted with the medium recommended by Dunaway and Weber (1), the addition of 10 μM fructose 2,6-bisphosphate had little or no effect on the recovery of PFK

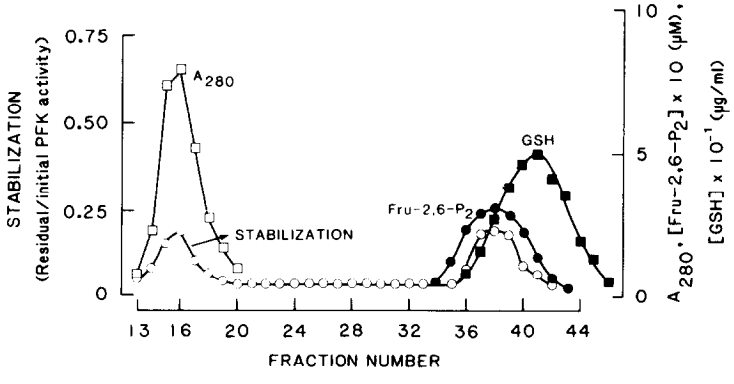

Fig. 3. Chromatography of a liver extract on Sephadex G-50 in the presence of 200 mM KCl. Same general conditions as in Fig. 2, except that 200 mM KCl was included in the filtration buffer.

Table 1. Effect of prolonged food deprivation

Nutritional state	Body weight (g)	Liver weight (g)	[Protein] (mg/g)	PFK (U/g)	LDH (U/g)	Aldolase (U/g)
Fed	193±16	7.8±0.8	121±7	1.13±0.14	278±17	5.7±0.8
Fasted	128±4	3.5±0.2	140±14	0.96±0.13	322±45	6.6±0.8
Significance	$0.001 < p < 0.01$	$p < 0.001$	$0.05 < p < 0.1$	$0.1 < p < 0.2$	$0.1 < p < 0.2$	$0.1 < p < 0.2$

Rats, weighing about 170 g, were fed or starved for 6 days. Their livers were homogenized in 10 vol of a solution containing 50 mM NaF, 1mM dithiothreitol, 1 mM ATP and 50 mM Tris pH 8 (14). The homogenates were centrifuged for 30 min at 100,000 x g and protein and enzymes were measured in the resulting supernatant and reported to 1 g of liver. Data shown are the means of 4 observations ± S.D.

in the extract (not shown). The effect of fasting was to decrease by approximately 15 % the activity of PFK (expressed as U/g) and to increase by about 16 % the activity of aldolase and lactic dehydrogenase as well as the content in soluble protein (Table 1). The specific activity of PFK was therefore decreased by about 27 %. Another effect of fasting was to decrease the weight of the livers to about 45 % of the value observed in the fed state. The combination of the two effects accounted for a 62 % decrease in PFK per liver or per cell, as reported by Dunaway and Weber (1). It is, indeed, known that the number of cells per liver remains essentially constant during starvation (21).

DISCUSSION

A comparison of the PFK-stabilizing peptide with fructose 2,6-bisphosphate. As indicated above, the PFK-stabilizing factor and fructose 2,6-bisphosphate share a number of chemical (acid lability), biochemical (stabilization and kinetic effects on liver PFK, inhibition of fructose-1,6-bisphosphatase) and biological (disappearance from the liver in fasting and diabetes) effects. They also co-migrate in gel filtration and therefore have similar apparent molecular weights. Under proper filtration conditions, no stabilizing factor was found in the 3,000-4,000 molecular weight range and nearly all the PFK-stabilizing property of the liver extract could be attributed to fructose 2,6-bisphosphate or to proteins. It is also apparent that, under the conditions of Dunaway and Segal (Fig. 2), fructose 2,6-bisphosphate was eluted directly behind the protein peak and could only be separated from it when the volume of gel was very large relative to that of the liver extract put on the column. This ratio was equal to 27 in Fig. 2 and Sephadex G-50, medium size, was used; it was equal to 5 in the procedure used by Dunaway and Segal (2) for the assay of their factor, performed

with Sephadex G-25 coarse. There is therefore little doubt that this assay measures nearly all the fructose 2,6-bisphosphate present in the extract. A similar explanation applies to the procedure used for the purification of the factor (3). We found that fructose 2,6-bisphosphate is in great part retained by an Amicon ultrafilter UM-2, with nominal molecular weight cut-off of 1,000 (data not shown), as was used to concentrate the factor (3).

Some of the reported properties of the stabilizing factor (3) are not compatible with its identification as fructose 2,6-bisphosphate,including its destruction by pronase, for which we have no explanation, unless pronase was contaminated with traces of a phosphatase. Another is the separation of the factor by high voltage electrophoresis at pH 1.9. The latter property is, however, in contradiction with the reported acid lability of the factor. The effect of actinomycin and cycloheximide to block the resynthesis of the factor after a 6-day fast (3) can be understood, in term of fructose 2,6-bisphosphate synthesis, by an effect on the formation of either glucokinase or phosphofructo 2-kinase or both.

The role of fructose 2,6-bisphosphate in the long-term control of PFK in the liver. One major difference between the livers of fed and fasted animals is in the concentration of fructose 2,6-bisphosphate which decreased 10-20 fold after 2 days of fasting (7). From the above discussion, it appears that the function proposed by Dunaway and co-workers (1-4) for the stabilizing peptide belongs in great part, if not in totality, to fructose 2,6-bisphosphate. This phosphoric ester is, indeed, the only well established PFK-stabilizing agent, whose concentration is greatly decreased in fasting and diabetes. ATP is another potential effective stabilizing factor but its concentration in the liver is not subject to large variations.

The 60-70% decrease in PFK content of hepatocytes after a 6-day fast is due to two factors. One, resulting in a decrease of about 27%, is specific for PFK and, according to the reasoning of Dunaway et al. (4), may be attributed to the disappearance of fructose 2,6-bisphosphate. The other is not specific and affects all liver proteins. The potential role of fructose 2,6-bisphosphate in this phenomenon remains to be substantiated.

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