LIVER PEPTIDE STABILIZING FACTOR PROTECTS PHOSPHOFRUCTOKINASE AGAINST INACTIVATION BY FRUCTOSE-1,6-BISPHOSPHATASE*

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Received September 5,1975

SUMMARY. Rabbit liver fructose-1,6-bisphosphatase (FDPase) can reversibly inactivate both rabbit muscle and rat liver phosphofructokinases (PFK) under appropriate conditions. The peptide factor which stabilizes rat liver PFK-L_2 against thermal inactivation has now been found to protect both PFKs from inactivation by FDPase. Assay at high ATP (ca. 3 mM) is necessary to demonstrate these reversible changes. In addition, the activation of FDPase by liver cytosol, by oleate plus cytosol, or by oleate plus muscle PFK is lowered about 50% in the presence of peptide factor. These observations suggest an active participation of the peptide factor in regulation of liver glycolysis and gluconeogenesis.

Changes in the relative magnitudes of glycolysis vs. gluconeogenesis in liver are presumed to be controlled by the activities and concentrations of key regulatory enzymes for these two metabolic pathways (1). One important site is the "futile" cycle between fructose-6-P and fructose-1,6-P_2, controlled by the relative activities of phosphofructokinase (PFK) and fructose-1,6-bisphosphatase (FDPase), respectively. Conditions which can serve as regulatory mechanisms for these two enzymes include allosteric modulation by metabolites (2-6), rates of enzyme turnover under varying metabolic states (7), and

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(p. 1) * This work was supported by Grants CA-12080 and AM-08873 from the National Institutes of Health.

[+] Recipient of National Institutes of Health Postdoctoral Fellowship CA-05331.

direct, reversible interactions (8, 9).

Dunaway and Segal (10) have described a peptide factor in liver which stabilizes rat liver PFK-L_2 against thermal inactivation. Furthermore, changes in the level of this factor paralleled those of PFK-L_2 in liver, decreasing during starvation and diabetes and increasing following refeeding or insulin administration.

In this communication, we show that the peptide factor can prevent the reversible inactivation of both rabbit muscle and rat liver PFKs by rabbit liver FDPase. In addition, the factor reduces the activation of liver FDPase in the presence of liver cytosol, oleate plus cytosol, or oleate plus muscle PFK. These results provide further evidence that the peptide factor plays an important in vivo role in the regulation of hepatic carbohydrate metabolism.

MATERIALS AND METHODS

Rabbit muscle PFK (Sigma, Type III, 175 units/mg protein) was diluted to 4-6 mg/ml in 10 mM K-phosphate (pH 8)-0.1 mM DTT and dialyzed overnight against the same buffer; protein concentration was determined by the $A_{280/260}$ method (11). Rat liver PFK-L_2 was purified as described (12) (50 units/mg protein). Homogeneous "neutral" FDPase was prepared as previously described (13, 14) (26 units/mg protein). Enzyme was diluted with 50 mM Tris-HCl (pH 7.2)-0.5 M KCl from concentrated stocks in 5 mM Na-malonate (pH 6.0). Peptide stabilizing factor was prepared by the procedure of Dunaway and Segal[1] and stored in 50 mM Tris-0.25 M glucose (pH 8.3). Pyruvate kinase (rabbit muscle, Sigma, Type II, 645 units/mg) and lactic dehydrogenase (rabbit muscle, Sigma, Type II, 715 units/mg) were diluted to 600 units/ml and 900 units/ml, respectively, with 10 mM Tris-HCl (7.5) and dialyzed overnight at 4° against two changes of the same buffer.

Reversibility of changes in PFK activity was checked by the addition of 25 µl of a mixture of PFK activators (0.25 M $(NH_4)_2SO_4$, 0.125 M K_2HPO_4, and 10 mM AMP) to assays after linear rates were observed.

All enzyme assays were at 30°. Other assay conditions are described in the legends of Figures and Tables.

RESULTS

PFK Activity. As shown in Fig. 1, rabbit muscle PFK which has been incubated with FDPase at pH 7.5 and 30° in the absence of Mg^{2+} shows very low

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[1] manuscript submitted for publication.

activity when assayed in the presence of 3 mM ATP (9). This inactivation was completely prevented by including the liver stabilizing factor for PFK in the incubation mixture. Factor alone had no effect on PFK activity under these conditions. The complete reversibility of the change in PFK activity is shown by the increased rate after the addition of a mixture of PFK activators (P_i, $(NH_4)_2SO_4$, and AMP). Rates after addition of these activators were identical in all cases. In a similar experiment where complete inactivation by FDPase was found after 3 min, 85% of the initial PFK activity remained after 13 min of incubation at 30° in the presence of stabilizing factor and FDPase.

In studies with rat liver PFK-L_2, it was necessary to replace Tris with 50 mM imidazole plus 10 mM $(NH_4)_2SO_4$ in the assays in order to obtain linear rates under conditions suitable to study reversible inactivation by FDPase (pH 7.5, 3 mM ATP). Experiment 1 in Table I shows that rat liver PFK-L_2 was also reversibly inactivated after incubation with FDPase. When the ATP con-

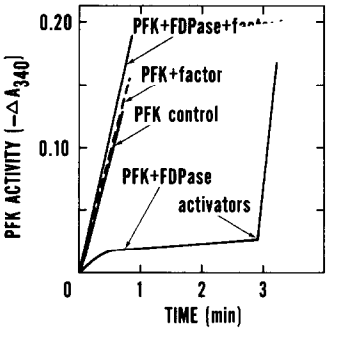

Fig. 1. Protection of rabbit muscle PFK from FDPase inactivation by peptide stabilizing factor. Each incubation mixture contained PFK (0.18 mg/ml) in 50 mM Tris-HCl (pH 7.5)-2 mM K-phosphate (pH 7.5)-0.2 mM DTT, plus FDPase (0.34 mg/ml) and liver stabilizing factor (0.08 mg/ml) where indicated. After incubating each mixture at 30° for 1 min, a 5 µl aliquot was removed and added to the assay mixture to measure the rate of ADP formation. The PFK assay mixture contained 50 mM Tris (pH 7.5), 5 mM $MgCl_2$, 0.1 M KCl, 3 mM ATP, 0.1 mM fructose-6-P, 0.5 mM P-enolpyruvate, 0.15 mM NADH, 3 units of pyruvate kinase, 4.5 units of lactic dehydrogenase, and PFK in a final volume of 0.5 ml.

TABLE I

FDPase Inactivation of Rat Liver PFK: Restoration of Activity at Low ATP Levels in Assay and Protection by Peptide Stabilizing Factor

In Exp. 1, 0.18 mg/ml of liver PFK-L_2 was incubated at 30°, alone or with 0.42 mg/ml of FDPase in 50 mM Tris-HCl (pH 7.5), 2 mM K-phosphate (pH 7.5), and 0.2 mM DTT. After incubation, a 10 µl aliquot was added to an assay mixture containing 50 mM imidazole (pH 7.5), 10 mM $(NH_4)_2SO_4$, either 1 mM or 3 mM ATP, and the other components listed in the legend to Fig. 1 in a final volume of 0.5 ml. In Exp. 2, 1.8 µg PFK was added to the above assay mixture containing 0.4 µg FDPase and 4 µg peptide factor where indicated.

	Rate ($-\Delta A_{340}$/min)			
	1 mM ATP in assay		3 mM ATP in assay	
	No activators	Plus activators	No activators	Plus activators
Exp. 1[a]				
PFK control	0.121	0.286	0.020	0.370
PFK + FDPase	0.100	0.392	0.004	0.353
Exp. 2				
PFK control			0.0241	0.275
PFK + FDPase			0.0008	0.361
PFK + FDPase + peptide stabilizing factor			0.0196	0.315

[a] Samples assayed at 1 and 3 mM ATP were incubated for 6 to 5 min, respectively.

centration in the subsequent assay was reduced to 1 mM, very little inactivation (<20%) was observed. Similar results have been found with muscle PFK (9).

Liver stabilizing factor (80-133 µg/ml) protected liver PFK-L_2 from inac

tivation by FDPase in preincubations similar to those in Fig. 1 and Exp. 1 of Table I. In these experiments, the initial rates of PFK activity after preincubation with stabilizing factor and FDPase were higher than control values and then declined abruptly after about 30 sec. The decline is undoubtedly due to a dilution of the PFK-stabilizing factor complex upon transfer to the assay cuvette and a consequent inactivation by the high FDPase level in the assay (8.5 μg/ml). However, we have no explanation at present for the initial high rates. Presence of additional stabilizing factor in the cuvette during the assay slowed, but did not prevent the observed rate decrease.

Further inactivation and protection studies with liver PFK-L_2 were carried out directly in the assay cuvette, since less factor and enzyme were required. Figure 2 shows that liver PFK-L_2 is very sensitive to inactivation by FDPase under assay conditions. In all cases, enzyme activity could be completely recovered by the addition of PFK activators. As shown in Exp. 2 of Table I, the presence of peptide factor in the assay provided greater than 80% protection of liver PFK-L_2 from inactivation by FDPase. Addition of factor to already inactivated enzyme failed to restore any activity. In fact, higher amounts

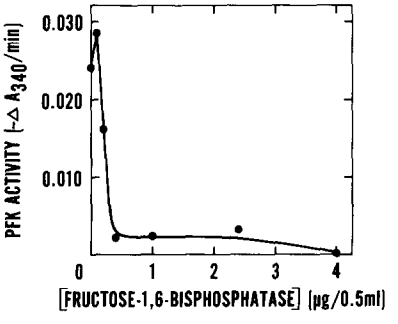

Fig. 2. Inactivation of rat liver PFK-L_2 by FDPase. Each 0.5 ml assay contained the indicated amount of FDPase, 3 mM ATP, and the other assay components listed in Table 1. Assays were started by adding 1.8 μg rat liver PFK-L_2.

of factor (>6 μg/0.5 ml) partially blocked reversal by mixed activators.

FDPase Activity. The kinetic effects of FDPase on PFK described by both Uyeda and coworkers (8) and ourselves (9) can be most reasonably explained

TABLE II

Inhibition of Rabbit Liver FDPase by Rat Liver PFK Stabilizing Factor

The FDPase preincubation and assay using Tris-HCl buffer (pH 7.5) and preparation of dialyzed rabbit liver supernatant freed of FDPase (deFDPased-cytosol) were as described by Carlson et al. (6). Thus, FDPase (0.25 μg) was incubated for 5 min at 30° in the assay cuvette (final volume 0.5 ml) with 10 μl deFDPased-cytosol, or with 30 μM oleate plus 10 μl deFDPased-cytosol, or with 30 μM oleate plus 17 μg muscle PFK (dialyzed overnight against 0.2 M $(NH_4)_2SO_4$ - 2 mM K-phosphate (pH 8.0)), and the indicated amount of peptide stabilizing factor. Assays were initiated by adding a mixture of the auxiliary enzymes, fructose-1,6-P_2, and $NADP^+$. FDPase preincubated without any activators and assayed under these conditions had no measurable activity.

	Relative FDPase activity (per cent)		
μg Factor Added	DeFDPased-cytosol	Oleate + deFDPased-cytosol	Oleate + PFK
0	100*	100*	100*
4	-	53	52
8	56	45	48
12	-	-	39
* Actual rates (ΔA_{340}/min)	0.0101	0.0387	0.0180

by assuming a weak protein-protein interaction between the two enzymes. It therefore was of interest to test directly the effect of the PFK peptide stabilizing factor on FDPase activity. Fatty acids, primarily oleate, bound to cytosol proteins have been shown to activate fully homogeneous enzyme at neutral pH (6, 13) and probably have a role in the physiological control of FDPase activity. Muscle PFK can also act synergistically with oleate to increase FDPase activity (6). The results presented in Table II show that activations of FDPase at pH 7.3 by deFDPased-cytosol, by oleate plus cytosol, and by oleate plus muscle PFK were partially blocked by the peptide factor (about 50% inhibition). However, the factor had no additional effect on either sensitivity to AMP or the sigmoidicity of the AMP inhibition curve (15).

DISCUSSION

Our present observations offer further support for a probable physiological role of the peptide stabilizing factor in regulation of hepatic carbohydrate metabolism. The factor occurs predominantly in liver and kidney, is present in liver at low concentrations during starvation or in diabetes, and rises after refeeding glucose or insulin administration (10). Recent studies by Kaur and Ramaiah[2] indicate the existence of a similar factor in rabbit liver supernatant. These changes in the in vivo concentrations of the peptide factor and its effects in vitro on PFK and FDPase are compatible with a role in the regulation of the relative flux of metabolites through the "futile" cycle between fructose-1,6-P_2 and fructose-6-P. Thus, the high levels of factor which are present in the fed state and after insulin administration would tend to enhance glycolysis by protecting PFK from inactivation, and by lowering the K_m for fructose-6-P (16). At the same time, this high level of factor may reduce gluconeogenesis by

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[2] Ramaiah, A., personal communication.

lowering FDPase activity. During starvation and diabetes, a drop in the level of factor would have opposite effects with lowered PFK and increased FDPase activities, respectively.

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