Proc. Natl. Acad. Sci. USA
Vol. 79, pp. 325–329, January 1982
Biochemistry

Regulation of fructose-6-phosphate 2-kinase by phosphorylation and dephosphorylation: Possible mechanism for coordinated control of glycolysis and glycogenolysis

(phosphofructokinase)

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Communicated by Jesse C. Rabinowitz, September 28, 1981

ABSTRACT **The kinetic properties and the control mechanism of fructose-6-phosphate 2-kinase (ATP: D-fructose-6-phosphate 2-phosphotransferase) were investigated. The molecular weight of the enzyme is ≈100,000 as determined by gel filtration. The plot of initial velocity versus ATP concentration is hyperbolic with a K_m of 1.2 mM. However, the plot of enzyme activity as a function of fructose 6-phosphate is sigmoidal. The apparent $K_{0.5}$ for fructose 6-phosphate is 20 μM. Fructose-6-phosphate 2-kinase is inactivated by the catalytic subunit of cyclic AMP-dependent protein kinase, and the inactivation is closely correlated with phosphorylation. The enzyme is also inactivated by phosphorylase kinase in the presence of Ca^{2+} and calmodulin. The phosphorylated fructose-6-phosphate 2-kinase, which is inactive, is activated by phosphorylase phosphatase and alkaline phosphatase. The possible physiological significance of these observations in the coordinated control of glycogen metabolism and glycolysis is discussed.**

Phosphofructokinase is a key regulatory enzyme of glycolysis whose activity is controlled by various metabolites (1). Our investigation into the biochemical mechanism of hormonal control of phosphofructokinase in liver has revealed the existence of an "activation factor" that effectively releases ATP inhibition of the enzyme (2, 3). This factor appears to play an important role in the control of phosphofructokinase in hepatocytes: (*i*) its concentration is altered rapidly by glucagon or glucose (4), (*ii*) the enzyme has an extremely high affinity for the factor (5), and (*iii*) this affinity varies depending on the phosphorylation state of phosphofructokinase (6). Moreover, the discovery of the activation factor has provided a plausible explanation for a puzzling phenomenon. Glycolysis in the liver proceeds at a rate that requires at least 30% of the total hepatic phosphofructokinase to be active (2). However, a calculation based on the *in vitro* determination of hepatic phosphofructokinase activity in the presence of *in vivo* concentrations of ATP, fructose 6-phosphate, and other known effectors shows that the enzyme would be totally inactive. It now appears that the activation factor may relieve this inhibition of phosphofructokinase under *in vivo* conditions and thus allow hepatic glycolysis to proceed. A similar factor has also been isolated by van Schaftingen and Hers (7), who tentatively characterized it as fructose 2,6-bisphosphate (8). Subsequently, its structure was determined as β-D-fructose-2,6-bisphosphate (9, 10).

We have recently found that the enzyme fructose-6-phosphate 2-kinase (ATP:D-fructose-6-phosphate 2-phosphotransferase) catalyzes the synthesis of fructose 2,6-bisphosphate in the presence of fructose 6-phosphate and ATP (11) (Eq. 1).

Fructose 6-phosphate + ATP
→ Fructose 2,6-bisphosphate + ADP. [1]

We have shown that the administration of extremely low concentrations of glucagon (0.1 fM) or high concentrations of epinephrine (10 μM) to hepatocytes results in inactivation of fructose-6-phosphate 2-kinase and concomitant decrease in the fructose 2,6-bisphosphate level (12). These results, as well as more recent data using Ca^{2+} and the Ca^{2+} ionophore A23187 (unpublished results) suggest that the regulation of fructose-6-phosphate 2-kinase is complex and may involve both cyclic AMP (cAMP)-dependent and Ca^{2+}-mediated mechanisms (12). In this communication, we present evidence that fructose-6-phosphate 2-kinase is inactivated by phosphorylation and activated by dephosphorylation *in vitro*.

MATERIALS AND METHODS

Fructose 2,6-bisphosphate was prepared as described (10). [γ-^{32}P]ATP (3000 Ci/mmol; 1 Ci = 3.7 × 10^{10} becquerels) was from Amersham. A catalytic subunit of porcine cAMP-dependent protein kinase (type II; specific activity, 20 μmol of ^{32}P incorporated into histone·min^{-1}·mg^{-1}) was a gift from James T. Stull (University of Texas Health Science Center at Dallas). Phosphorylase phosphatase was provided by D. L. Brautigan and E. H. Fischer (University of Washington, Seattle, WA). Rabbit muscle phosphofructokinase was purified as described (13). Rabbit skeletal muscle phosphorylase kinase was purified according to the method of Cohen (14) through the Sepharose 4B step. Phosphorylase kinase/protein kinase inhibitor was from Sigma. All other enzymes were products of Boehringer Mannheim. A high-pressure liquid chromatography (HPLC) exclusion gel column, Micro Pak TSK 3000 SW, was from Varian.

Assay for Fructose 2,6-bisphosphate. Fructose 2,6-bisphosphate was assayed by using rabbit muscle phosphofructokinase as described (5).

Assay for Fructose-6-phosphate 2-kinase. Fructose-6-phosphate 2-kinase was assayed in 0.05 M Tris·HCl, pH 7.4/5 mM ATP/0.2 mM fructose 6-phosphate/7.5 mM $MgCl_2$ (final vol, 0.1 ml). The mixture was incubated at 30°C and, at various time intervals, aliquots (10 μl) were removed and transferred to 90

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Abbreviations: cAMP, cyclic AMP; HPLC, high-pressure liquid chromatography; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid.

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μl of 0.1 M NaOH to stop the reaction. Aliquots of the alkali-treated reaction mixture were then assayed for fructose 2,6-bisphosphate as described (5). One unit of activity is defined as the amount of enzyme that catalyzes the formation of 1 μmol of D-fructose 2,6-bisphosphate per min under these conditions (11).

Preparation of Fructose-6-phosphate 2-kinase. Fructose-6-phosphate 2-kinase was purified as described (11) with the following modification. Fed rats (usually three) were killed with injection of ≈0.3 ml of Nembutal. The livers were quickly removed and homogenized in a Polytron homogenizer in 3 vol of 50 mM Tris phosphate, pH 8/5 mM ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA) at 4°C. The subsequent centrifugation, polyethylene glycol precipitation, and DEAE-cellulose chromatography were carried out as described (5). The enzyme was eluted from the DEAE-cellulose column with 50 mM Na phosphate/0.2 mM ATP/0.20 M NaCl. The enzyme solution was concentrated to 2 ml with an Amicon concentrator using a PM30 membrane, and the concentrated enzyme was placed on a Sephadex G-200 column (1.5 cm × 65 cm) that had been equilibrated with 50 mM Tris phosphate, pH 8.0/0.2 mM ATP/0.2 mM EGTA/1 mM dithiothreitol/0.1 M NaCl. The enzyme was eluted with the same solution. The fractions containing the enzyme (usually 1.5 times the void volume) were pooled, concentrated with an Amicon concentrator as above, and stored at −75°C. The specific activity of the enzyme was ≈20 milliunits/mg.

HPLC. High speed gel filtration of fructose-6-phosphate 2-kinase was carried out with a TSK 3000 SW column using a HPLC instrument model 204 (Waters). The column was equilibrated with 50 mM Na phosphate, pH 7.4/0.1 mM ATP/0.2 mM dithiothreitol. The enzyme was eluted with the same buffer at 25°C at a flow rate of 1 ml/min and 0.5-ml aliquots were collected.

RESULTS

Properties of Fructose-6-phosphate 2-kinase. The M_r of fructose-6-phosphate 2-kinase, as determined by gel filtration on a Sephadex G-200 column, is ≈100,000 (Fig. 1). When the phosphorylated enzyme was subjected to $NaDodSO_4$/acrylamide gel electrophoresis as discussed below, the dissociated enzyme showed a M_r of ≈55,000. These results suggest that the native enzyme consists of two subunits.

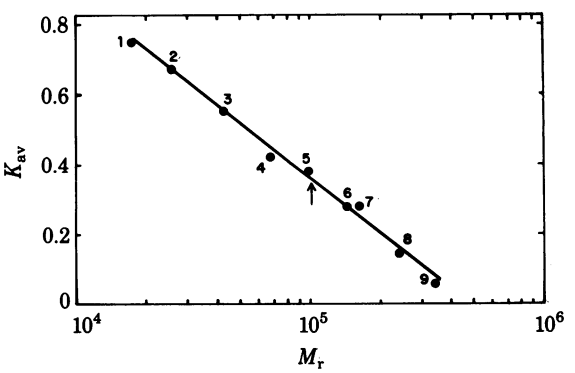


FIG. 1. Determination of M_r of fructose-6-phosphate 2-kinase by gel filtration on Sephadex G-200. Fructose-6-phosphate 2-kinase and marker enzymes (total vol, 0.8 ml) were chromatographed on a Sephadex G-200 column. Arrow, elution volume of fructose-6-phosphate 2-kinase. Marker proteins: 1, myoglobin; 2, chymotrypsin; 3, ovalbumin; 4, bovine serum albumin; 5, citrate synthase; 6, lactate dehydrogenase; 7, aldolase; 8, pyruvate kinase; 9, glutamate dehydrogenase. K_{av} is calculated as $(V_e - V_0)/(V_t - V_0)$, where V_0, V_t, and V_e are void volume, total column volume, and the peak elution volume, respectively.

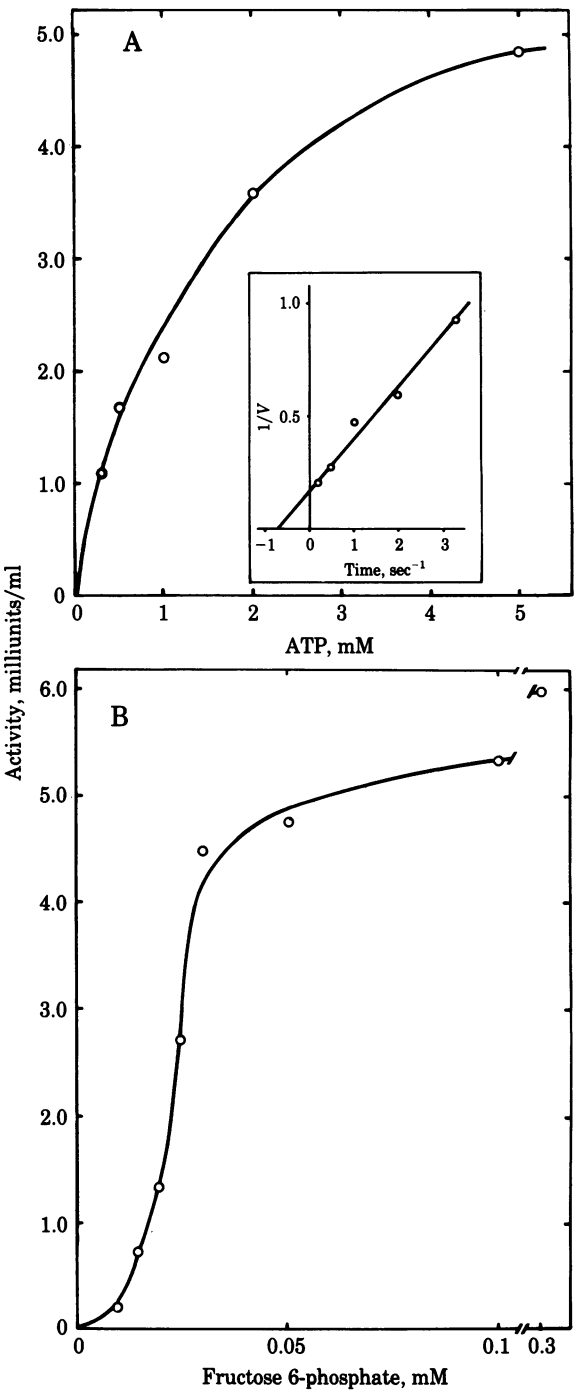


FIG. 2. Fructose-6-phosphate 2-kinase activity as a function of ATP or fructose 6-phosphate concentration. (*A*) Fructose 6-phosphate concentration was 3 mM. (*B*) ATP concentration was 5 mM and $MgCl_2$ concentration was 7.5 mM. The reaction was initiated by addition of 6 milliunits of fructose-6-phosphate 2-kinase.

K_m for Fructose 6-phosphate and ATP. The plot of fructose-6-phosphate 2-kinase activity versus ATP concentration (Fig. 2*A*) shows hyperbolic kinetics. The Lineweaver–Burk plot (Fig. 2*A Inset*) is linear and the K_m for MgATP is estimated as 1.2 mM. On the other hand, the plot of enzyme activity as a function of fructose 6-phosphate concentration (Fig. 2*B*) is sigmoidal, suggesting positive cooperativity and the apparent $K_{0.5}$ for fructose 6-phosphate is approximately 20 μM.

Inactivation of Fructose-6-phosphate 2-kinase by Protein Kinase and Activation by Phosphatases. When fructose-6-phos-

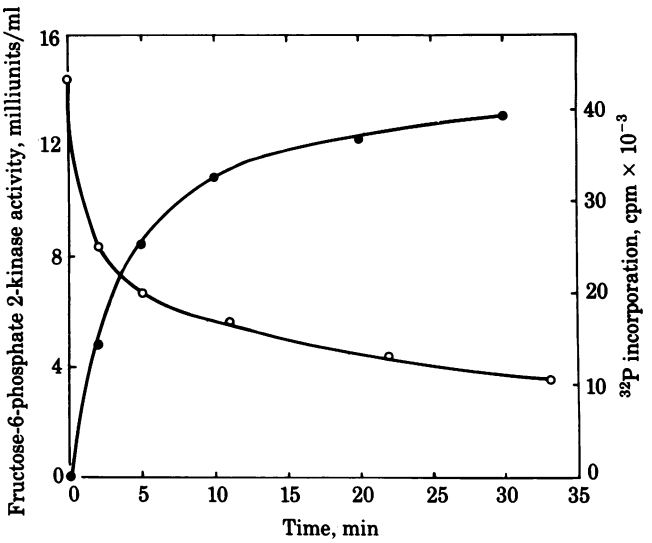


FIG. 3. Time course of inactivation (○) and phosphorylation (●) of fructose-6-phosphate 2-kinase by protein kinase. The reaction mixture was 0.17 μg of fructose-6-phosphate 2-kinase in 0.12 ml of 50 mM Na phosphate, pH 6.8/2 μM [γ-^{32}P]ATP (19.8 μCi)/0.3 mM $MgCl_2$/10 mM dithiothreitol. Reaction was initiated by the addition of 3.2 milliunits (0.16 μg) of the catalytic subunit of cAMP-dependent protein kinase and incubation was at 30°C. At the indicated times, 5-μl aliquots were removed for assay of fructose-6-phosphate 2-kinase activity and 10-μl aliquots were removed and transferred into 0.1 ml of bovine serum solution (10 mg/ml) at 0°C. To this solution, 1 ml of 10% trichloroacetic acid was immediately added at 0°C. The acidified solution was allowed to stand 10 min and then centrifuged. The precipitate was dissolved in 1 ml of 0.1 M NaOH and this solution was treated with 1 ml of 10% trichloroacetic acid. The solubilization and precipitation steps were repeated twice. Finally, the precipitate was dissolved in 0.2 ml of 0.1 M NaOH and 100-μl aliquots were removed for ^{32}P determination.

phate 2-kinase was incubated with MgATP and the catalytic subunit of cAMP-dependent protein kinase, the enzyme was rapidly inactivated (Fig. 3). To avoid dilution of [^{32}P]ATP, a limited amount (2 μM) of ATP was used in this experiment. The enzyme was, however, inactivated completely when additional ATP (8 mM) was added to the reaction mixture after 35 min. The rate of ^{32}P incorporation into the enzyme was determined in the same reaction mixture by removing aliquots at given time intervals, and ^{32}P in the trichloroacetic acid-precipitated protein was measured. As shown, the rates of ^{32}P incorporation and the inactivation were similar. The ^{32}P in the trichloroacetic acid-precipitated protein was completely hydrolyzed in 0.3 M NaOH when incubated for 20 hr at 37°C, suggesting that the phosphate is linked to a serine (or threonine) residue in the protein.

To ascertain that ^{32}P is incorporated into fructose-6-phosphate 2-kinase, ^{32}P-labeled and inactivated enzymes were subjected to HPLC molecular sieve filtration using a TSK 3000 SW column (Fig. 4). The elution patterns for fructose-6-phosphate 2-kinase and ATP are shown in Fig. 4*A*. The enzyme eluted at 12–14 min, and ATP eluted after 17 min. When the ^{32}P-labeled fructose-6-phosphate 2-kinase was chromatographed on the same column (Fig. 4*B*), the major ^{32}P-containing protein peak corresponded to the enzyme. The residual activity (5% of the original) of the enzyme is also seen in the same fractions. Furthermore, NaDodSO$_4$/gel electrophoresis of the ^{32}P-containing enzyme peak fraction showed a single radioactive band, suggesting that only fructose-6-phosphate 2-kinase is phosphorylated (data not shown). The ^{32}P-labeled enzyme band (relative mobility, 0.52 where the mobility of bromophenol blue is 1.0) corresponds to a M_r of 55,000 as compared with marker proteins such as citrate synthase and phosphofructokinase.

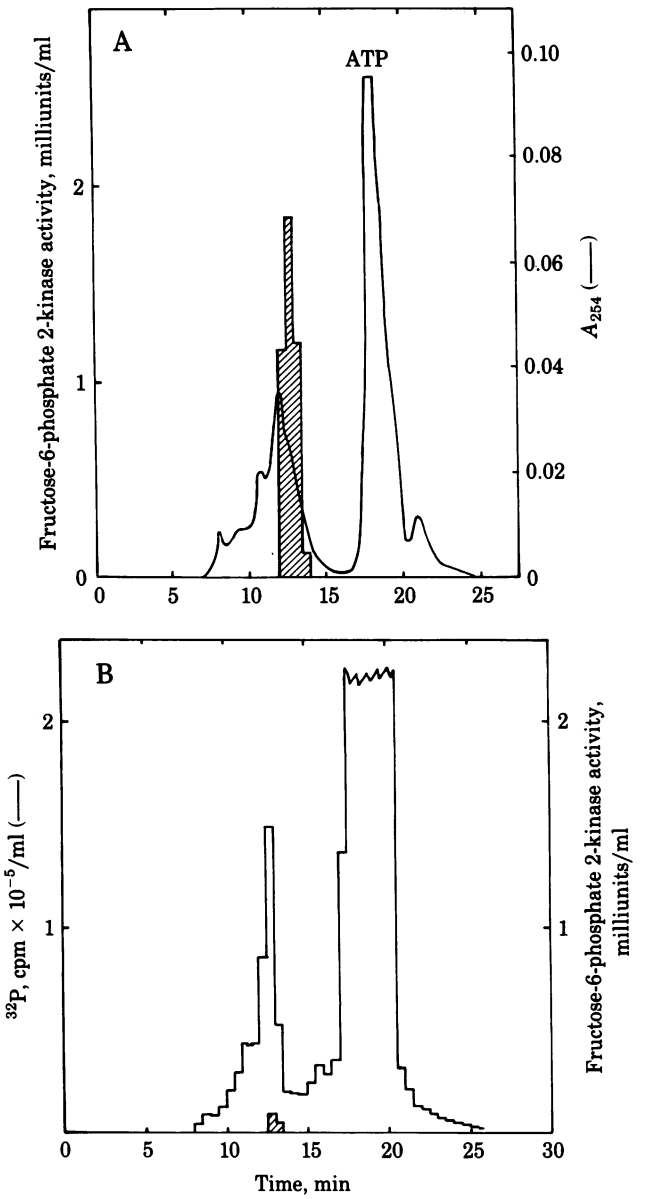


FIG. 4. HPLC gel filtration of ^{32}P-labeled fructose-6-phosphate 2-kinase (□). (*A*) A mixture (0.1 ml) of 2 milliunits of fructose-6-phosphate 2-kinase in 50 mM Na phosphate, pH 7.0/0.1 mM ATP/1 mM dithiothreitol was applied to a TSK 3000 SW column, and the enzyme was eluted. Recovery of enzyme activity was 98–108%. (*B*) Fructose-6-phosphate 2-kinase was phosphorylated in a mixture of 5.1 milliunits of the same enzyme as in *A* in 40 mM Na phosphate, pH 6.8, containing 150 pmol of [^{32}P]ATP (36 μCi), 80 nmol of $MgCl_2$, and 1 μmol of dithiothreitol (final vol, 0.3 ml). Reaction was initiated by the addition of 0.06 unit of protein kinase and incubation was at 30°C for 60 min. An aliquot (80 μl) of the reaction mixture was applied to the column as in *A*.

Inactivation of fructose-6-phosphate 2-kinase and reactivation by phosphatases was further investigated, and the results are summarized in Table 1. Inactivated fructose-6-phosphate 2-kinase can be reactivated by alkaline phosphatase (*Escherichia coli*) and phosphorylase phosphatase. As shown in Table 1, fructose-6-phosphate 2-kinase, which had been inactivated 95% after phosphorylation with cAMP-dependent protein kinase, regains 68% of the original activity after incubation with alkaline phosphatase. Similarly, the inactivated phosphorylated fructose-6-phosphate 2-kinase is reactivated by a highly purified muscle phosphorylase phosphatase that has been activated by

Table 1. Inactivation of fructose-6-phosphate 2-kinase by protein kinase and reactivation by phosphatases

Step	Addition	Fructose-6-phosphate 2-kinase activity Milliunits/ml	%
	Experiment A		
1	None	5.2	100
2	Protein kinase	0.26	5
3	Alkaline phosphatase	3.5	67
	Experiment B		
1	None	6.0	100
2	Protein kinase	0.0	0
3	Phosphorylase phosphatase	4.3	72
	Experiment C		
1	None	5.8	100
2	Protein kinase	0.6	10
3	Protein kinase inhibitor		
	20 μg	2.0	34
	60 μg	3.9	67

In all experiments, the reaction mixture (step 1) was (final vol, 20 μl) 0.1 M Tris·HCl, pH 8/fructose-6-phosphate 2-kinase (0.13 milliunits, 20 μg)/10 mM dithiothreitol/1 mM ATP/5 mM $MgCl_2$. Then, in step 2, reaction was initiated by the addition of 80 ng of cAMP-dependent protein kinase catalytic subunit and the mixture was incubated at 30°C for 30 min. For reactivation, in experiment A, 10 μg of alkaline phosphatase (step 3) was added to the protein kinase-inactivated fructose-6-phosphate 2-kinase (inactivation at least 95%) and the phosphatase reaction was continued for 60 min at 30°C; in experiment *B*, the reaction mixture after protein kinase treatment was desalted by column centrifugation (15) with a column that had been equilibrated with 20 mM Na phosphate, pH 7.4, and 5.6 μg of phosphorylase phosphatase (step 3) and 10 mM dithiothreitol were then added to the mixture, which was incubated at 30°C for 30 min. In experiment C, the indicated amounts of protein kinase inhibitor were added in step 2.

Mn^{2+}. HPLC gel chromatography of fully activated fructose-6-phosphate 2-kinase following the phosphatase treatment shows that the enzyme contains no ^{32}P, indicating that dephosphorylation has taken place (data not shown).

Inactivation of Fructose-6-phosphate 2-kinase by Phosphorylase Kinase and Reactivation by Phosphatase. As summarized in Table 2 (experiment A), fructose-6-phosphate 2-kinase is also inactivated by purified phosphorylase kinase from skeletal muscle in the presence of Ca^{2+} and calmodulin. The phosphorylase kinase preparations were free of protein kinase; therefore, this inactivation was not due to possible contamination with protein kinase. Moreover, the inactivation by phosphorylase kinase is dependent on both Ca^{2+} and calmodulin. In the absence of Ca^{2+} but the presence of EGTA, inactivation of fructose-6-phosphate 2-kinase does not occur. The requirement for calmodulin is also demonstrated.

The inactivated fructose-6-phosphate 2-kinase after phosphorylation by phosphorylase kinase treatment is also fully reactivated by alkaline phosphatase (Table 2, experiment B).

DISCUSSION

Administration of glucagon and cAMP to hepatocytes results in a rapid decrease in the level of fructose 2,6-bisphosphate (4), resulting from inactivation of fructose-6-phosphate 2-kinase (12). Furthermore, epinephrine also decreases the level of fructose 2,6-bisphosphate (12). In additional experiments using Ca^{2+}-depleted hepatocytes and ionophore A23187 to facilitate

Table 2. Inactivation of fructose-6-phosphate 2-kinase by phosphorylase kinase and reactivation by alkaline phosphatases

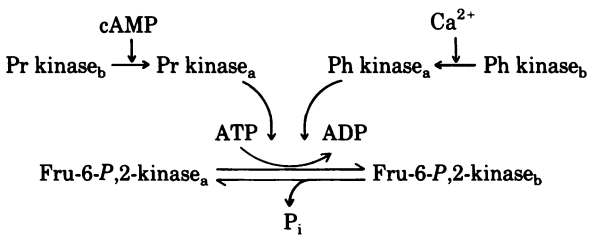
Exp.	Addition	Fructose-6-phosphate 2-kinase activity Milliunits/ml	%
A	None	5.0	100
	Phosphorylase kinase/Ca^{2+}/calmodulin	1.0	20
	Phosphorylase kinase/calmodulin	4.4	88
	Phosphorylase kinase/EGTA/calmodulin	4.2	84
	Phosphorylase kinase/Ca^{2+}	3.9	78
B	None	5.0	100
	Phosphorylase kinase/Ca^{2+}/calmodulin	0.88	18
	Phosphorylase kinase/Ca^{2+}/calmodulin/alkaline phosphatase	4.7	94

In experiment A, the reaction mixture was (final vol, 25 μl) 50 μg of fructose-6-phosphate 2-kinase, 0.25 μmol of dithiothreitol, 50 nmol of ATP, 125 nmol of $MgCl_2$, and 2.5 μmol of Na phosphate, pH 6.8. Additions were 0.4 μg of calmodulin, 2.5 nmol of $CaCl_2$, or 25 nmol of EGTA. Reaction was initiated by the addition of 2.7 μg of phosphorylase kinase and incubation was at 30°C for 15 min. In experiment B, the reaction mixture was (final vol, 50 μl) 0.25 milliunits of fructose-6-phosphate 2-kinase, 5 μmol of Na phosphate (pH 6.8), 0.1 μmol of ATP, and 0.25 μmol of $MgCl_2$. Additions were 5.4 μg of phosphorylase kinase, 1.2 μg of calmodulin, or 5 nmol of $CaCl_2$, and the mixture was incubated for 30 min at 30°C. For the reactivation experiment, the inactivated fructose-6-phosphate 2-kinase was desalted by column centrifugation (15) with a column that had been equilibrated with 50 mM Tris·HCl, pH 8/25 Tris phosphate, pH 8, and 17 μl of the desalted enzyme was incubated with 0.15 μg of alkaline phosphatase in 1 mM $MgCl_2$/10 mM dithiothreitol at 30°C for 30 min.

Ca^{2+} uptake and release, we have shown that the action of epinephrine on fructose 2,6-bisphosphate appears to be Ca^{2+}-mediated (unpublished results). Thus, these results suggest that the control of fructose-6-phosphate 2-kinase involves both cAMP-dependent and Ca^{2+}-mediated mechanisms. The results presented here provide a plausible explanation for both mechanisms, as well as the nature of the enzymes involved in the control of fructose-6-phosphate 2-kinase. We demonstrated, as shown in Scheme I, that partially purified fructose-6-phosphate 2-kinase (Fru-6-*P*,2-kinase$_a$)‡ is inactivated by the phosphorylation reaction catalyzed by either cAMP-dependent protein kinase (Pr kinase) or phosphorylase kinase (Ph kinase), which requires Ca^{2+} and calmodulin. Interestingly, the phosphorylated inactive fructose-6-phosphate 2-kinase (Fru-6-*P*,2-kinase$_b$) is reversibly activated by phosphorylase phosphatase and alkaline phosphatase. Thus, these *in vitro* observations account for the involvement of both cAMP and Ca^{2+} in the control of fructose-6-phosphate 2-kinase.

It is interesting that both protein kinase and phosphorylase kinase phosphorylate fructose-6-phosphate 2-kinase. Phosphorylase kinase and glycogen synthase are also phosphorylated by both kinases (16, 17). Since these kinases show different specificities for the amino acid sequences at the phosphorylation site, one might expect them to phosphorylate different sites on fructose-6-phosphate 2-kinase but this question cannot be resolved until the enzyme is purified further. Moreover, whether both kinases phosphorylate fructose-6-phosphate 2-kinase *in vivo* cannot be certain at present, but these results provide plau-

‡ In accordance with the recommendations of the International Union of Biochemistry Commission on Nomenclature, the active and inactive forms of the enzyme are identified by the subscripts a and b, respectively.

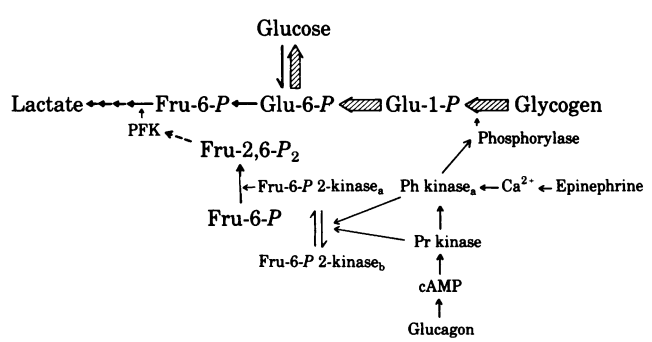


Scheme I

sible mechanisms for cAMP-dependent and -independent inactivation of fructose-6-phosphate 2-kinase.

In addition to the control of fructose-6-phosphate 2-kinase by phosphorylation and dephosphorylation, this enzyme shows sigmoidal saturation kinetics with respect to fructose 6-phosphate (Fig. 1*B*), suggesting that the enzyme activity may also be regulated by the fructose 6-phosphate concentration. This is reminiscent of phosphofructokinase but, unlike phosphofructokinase, this enzyme is not inhibited by ATP.

One of the primary functions of liver is to maintain blood glucose level. When glycogen breakdown is stimulated by fasting or hormonal stimulation, glucose utilization by liver must be slowed to put out more glucose. As shown in Scheme II, when glycogenolysis is stimulated after glucagon release, the concentrations of both glucose 6-phosphate (Glu-6-*P*) and fructose 6-phosphate (Fru-6-*P*) increase severalfold. This increase in fructose 6-phosphate, a positive effector, can cause activation of phosphofructokinase and thus activate glycolysis. However, glycolysis must be stopped to convert glucose 6-phosphate into glucose (heavy arrow). Therefore, activation of glycogen breakdown (heavy arrows) and inhibition of glycolysis must be coordinately controlled. If our *in vitro* observations can be applied to liver, the coordinated control of these pathways can be accomplished as follows. As the cAMP or Ca^{2+} level increases, glycogenolysis is stimulated by the well-known cascade mechanism (18) involving activation of cAMP-dependent protein kinase (Pr kinase), phosphorylase kinase (Ph kinase$_a$), and phosphorylase. At the same time, these protein kinases will inactivate fructose-6-phosphate 2-kinase (Fru-6-*P* 2-kinase$_a$), resulting in a decreased level of fructose 2,6-bisphosphate, and thus inhibition of phosphofructokinase as well as glycolysis occurs. Similarly, the same inhibitory mechanism of fructose-6-phosphate 2-kinase could occur when gluconeogenesis is stimulated in liver.



Scheme II

As the concentration of blood glucose increases, glycogen synthesis and glycolysis may be stimulated by the reversal of the above reactions. The inactive phosphorylated fructose-6-phosphate 2-kinase (Fru-6-*P* 2-kinase$_b$) can be activated by the dephosphorylation reaction catalyzed by a phosphatase such as phosphorylase phosphatase, which results in an increased concentration of fructose 2,6-bisphosphate, and thus activation of phosphofructokinase (PFK) (⇢) and glycolysis. It is especially interesting that phosphorylase phosphatase, which converts phosphorylase a to phosphorylase b, also acts on the phosphorylated fructose-6-phosphate 2-kinase, suggesting that the control of glycogen synthesis is also coordinated with glycolysis. Thus, the activation and the inactivation of fructose-6-phosphate 2-kinase by phosphorylation and dephosphorylation, respectively, may play a key role in homeostasis of glucose in liver.

We thank Drs. David L. Brautigan and Edmond H. Fischer for phosphorylase phosphatase and Dr. James T. Stull for protein kinase. This work was supported by a grant from the Veterans Administration Research Service and by National Institutes of Health Grant 5 RO1 AM16194.

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