Regulation of 6-phosphofructo-2-kinase activity by cyclic AMP-dependent phosphorylation
(AMP:D-fructose-6-phosphate 2-phosphotransferase/fructose 2,6-bisphosphate/enzyme regulation)

M. Raafat El-Maghrabi, Thomas H. Claus, Jo Pilkis, and Simon J. Pilkis

Department of Physiology, Vanderbilt University School of Medicine, Nashville, Tennessee 37232

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ABSTRACT
Addition of glucagon to isolated rat hepatocytes resulted in inhibition of 6-phosphofructo-2-kinase (AMP:D-fructose-6-phosphate 2-phosphotransferase) activity in extracts of the cells and in a decrease in the intracellular level of fructose 2,6-bisphosphate. The effect on 6-phosphofructo-2-kinase was characterized by a decrease in the affinity of the enzyme for fructose 6-phosphate. To investigate the mechanism of action of glucagon, 6-phosphofructo-2-kinase from rat liver was partially purified by polyethylene glycol precipitation, DEAE-cellulose chromatography, (NH₄)₂SO₄ fractionation, Sephacryl S-200 gel filtration, DEAE-Sephadex chromatography, and Sephadex G-100 gel filtration. Incubation of the purified enzyme with the catalytic subunit of the cyclic AMP-dependent protein kinase from rat liver and [γ-³²P]ATP resulted in ³²P incorporation into a protein with a subunit M₉ of 49,000 as determined by NaDodSO₄ disc gel electrophoresis. Associated with this phosphorylation was an inhibition of 6-phosphofructo-2-kinase activity that was also characterized by a decrease in the affinity of the enzyme for fructose 6-phosphate. Both the phosphorylation and the inhibition of the purified 6-phosphofructo-2-kinase were blocked by addition of the heat-stable protein kinase inhibitor. It is concluded that the glucagon-induced decrease in fructose 2,6-bisphosphate levels observed in isolated hepatocytes is due, at least in part, to cyclic AMP-dependent phosphorylation and inhibition of 6-phosphofructo-2-kinase.

A number of groups have presented evidence for the existence in liver of a novel effector of 6-phosphofructo-1-kinase (1–7). The structure of this compound was first put forth as fructose 2,6-bisphosphate by Van Schaftingen and Hers (1) and subsequently confirmed by us spectroscopically and by ¹³C NMR (5). Fructose 2,6-bisphosphate is an allosteric activator of 6-phosphofructo-1-kinase (1, 2, 4–7) and an inhibitor of fructose 1,6-bisphosphatase (8–10). The level of fructose 2,6-bisphosphate has been shown to vary widely depending on the hormonal and dietary state (2, 11–13). For example, glucagon addition to isolated hepatocytes results in a 90% decrease in its level within minutes (2, 4, 11, 12). These results suggest that the enzyme responsible for the synthesis or degradation (or both) of fructose 2,6-bisphosphate is regulated by a cyclic AMP (cAMP)-dependent phosphorylation mechanism (11). Recently, the enzyme responsible for fructose 2,6-bisphosphate synthesis was isolated and shown to catalyze the transfer of the γ phosphate of ATP to the C-2 position of fructose 6-phosphate (14–16). The goal of the present study was to determine whether rat liver 6-phosphofructo-2-kinase is regulated by cAMP-dependent protein kinase-catalyzed phosphorylation and to characterize this regulation.

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MATERIALS AND METHODS
Preparation and Incubation of Isolated Hepatocytes. Isolated rat hepatocytes were prepared from fed rats (male, Sprague–Dawley, 175–225 g) as described (3). The cells were suspended (final concentration, 50 mg of liver/ml) in Krebs–Henseleit bicarbonate buffer/0.5% bacitracin and incubated without any additions for 20 min at 37°C. Cells = 2 g of liver per flask) were incubated with and without 10 nM glucagon for 10 min and then rapidly centrifuged. The cells were homogenized for 90 sec (30 sec, three times) with an Ultraturrax homogenizer in 10 ml of cold 50 mM Tris·HCl, pH 7.5/50 mM KH₂PO₄/2 mM EDTA/0.5 mM dithiothreitol, and the homogenate was centrifuged at 27,000 × g for 30 min. Enough solid (NH₄)₂SO₄ was added to the supernatant to make it 30% saturated and the precipitate was centrifuged and discarded. The supernatant was then made 65% saturated with (NH₄)₂SO₄, and the precipitate was dissolved in 2 ml of 20 mM Tris·HCl, pH 7.5/50 mM KCl/10 mM MgCl₂/5 mM EDTA/0.5 mM dithiothreitol, and the (NH₄)₂SO₄ was removed by dialysis against the same buffer. The (NH₄)₂SO₄-treated hepatocyte extracts were then assayed for 6-phosphofructo-2-kinase activity.

6-Phosphofructo-2-Kinase Assay. 6-Phosphofructo-2-kinase activity was measured by following the production of fructose 2,6-bisphosphate as described (13, 15). The maximal activity of the enzyme was measured with 5 mM fructose 6-phosphate and 2 mM ATP. The reaction was terminated by the addition of 0.25 M NaOH followed by heating at 90°C for 30 min. The pH was adjusted to 7 to 8 with 1 M HClO₄, and the amount of fructose 2,6-bisphosphate formed was determined by the 6-phosphofructo-1-kinase activation assay. Under our assay conditions, the rate of the reaction was a linear function of enzyme concentration and time up to 30 min. One unit of enzyme is defined as the amount that catalyzes the synthesis of 1 μmol of fructose 2,6-bisphosphate per min.

6-Phosphofructo-1-Kinase Activation Assay for Fructose 2,6-Bisphosphate. Fructose 2,6-bisphosphate was assayed by comparing the amount of activation of 6-phosphofructo-1-kinase obtained with base-treated samples with the activation obtained with known concentrations of fructose 2,6-bisphosphate. The activation of homogeneous rat hepatic 6-phosphofructo-1-kinase by fructose 2,6-bisphosphate was roughly linear over the range 3–10 mM when the enzyme was assayed with 0.2 mM fructose 6-phosphate and 1 mM ATP as substrate (Fig. 1). Various aliquots of the base-treated samples were tested for their ability to activate the enzyme and those volumes that activated the enzyme 10–45% of the maximum activity were used to determine the amount of fructose 2,6-bisphosphate. An aliquot of

Abbreviation: cAMP, cyclic AMP.
Preparation of 6-Phosphofructo-1-Kinase and the Catalytic Subunit of cAMP-Dependent Protein Kinase. Rat liver 6-phosphofructo-1-kinase was purified to homogeneity by a modification (unpublished results) of the method of Kemp (18). The enzyme had a specific activity of 90 units/mg. The catalytic subunit of cAMP-dependent protein kinase from rat liver was purified as described (15).

Preparation of Rat Hepatic 6-Phosphofructo-2-Kinase. The enzyme was purified by a modification of the method of El-Maghrabi et al. (15). Livers from 25 fed male Sprague-Dawley rats (250–300 g) were homogenized in a Waring Blender at high speed (two times, 30 sec each) in 3 vol of buffer A (20 mM Tris-HCl, pH 7.5/50 mM KCl/5 mM MgCl₂/2 mM EDTA/1 mM dithiothreitol/0.1 mM phenylmethylsulfonyl fluoride). The homogenate was centrifuged at 30,000 × g for 30 min and then the supernatant was centrifuged at 100,000 × g for 60 min. Solid polyethylene glycol 6000 was added to the clear supernatant to give 6% saturation. After 15 min, the precipitate was collected by centrifugation at 30,000 × g for 20 min and discarded. Enough solid polyethylene glycol 6000 was added to this supernatant to give 12% saturation. The 6–12% polyethylene glycol precipitate was collected by centrifugation at 30,000 × g for 20 min and dissolved in 100 ml of buffer A. Undissolved precipitate was collected by centrifugation and extracted with 20 ml of buffer A, and the clear supernatants were combined and applied to a DEAE-cellulose column (1.0 × 20 cm) equilibrated with buffer A. The column was washed with 100 ml of buffer A, and then the 6-phosphofructo-2-kinase activity was eluted with 300 ml of a linear 50–400 mM KCl gradient in buffer A. The enzyme eluted at ~170 mM KCl. The fractions that contained 6-phosphofructo-2-kinase activity were pooled and enough solid (NH₄)₂SO₄ was added to give 45% saturation. It was found that less (NH₄)₂SO₄ was needed to precipitate the enzyme in the presence of ~200 mM KCl than was required to precipitate in the presence of 50 mM KCl (45–65%) (15). The precipitate was collected by centrifugation, dissolved in a minimum volume of buffer A, and applied to a Sephacryl S-200 superfine (1.8 × 115 cm) column equilibrated with the same buffer. The enzyme activity eluted as a single symmetrical peak with an apparent M₉ of ~90,000 (15).

The fractions that contained activity were then applied to a DEAE-Sephadex column (0.9 × 5 cm) equilibrated with buffer A, and the column was washed with 20 ml of 100 mM KCl in buffer A and eluted with a 100–300 mM linear KCl gradient (total volume, 70 ml) in buffer A. Two peaks of enzyme activity were eluted from the DEAE-Sephadex column, one eluting at ~210 mM KCl comprising 30% of the total eluted enzyme activity and the other eluting at ~240 mM KCl comprising 70% of the enzyme activity. The fractions of each peak that contained the enzyme activity were pooled and concentrated by ultrafiltration and applied separately to Sephadex G-100 superfine columns (1.5 × 90 cm) equilibrated with buffer A. The fractions that contained 6-phosphofructo-2-kinase activity were pooled and concentrated by ultrafiltration. The specific activity of the enzyme from peak II was 15.2 milliunits/mg of protein, representing 500-fold purification over the polyethylene glycol step.

Protein was assayed by the method of Lowry et al. (19) with bovine serum albumin as a standard.

Materials. Rabbit muscle aldolase, triose phosphate isomerase, and α-glycerol phosphate dehydrogenase were obtained from Boehringer Mannheim. Trisodium fructose 1,6-bisphosphate, fructose 6-phosphate, and allophanate monohydrate were obtained from Sigma. The heat-stable protein kinase inhibitor was a gift from Thomas Soderling (Department of Physiology, Vanderbilt University).
RESULTS

Effect of Glucagon on the Activity of Hepatocyte 6-Phosphofructo-2-Kinase. The effect of 10 nM glucagon on the activity of hepatocyte 6-phosphofructo-2-kinase is shown in Fig. 2. In the absence of hormone, enzyme activity measured with 1 mM ATP was dependent on the concentration of fructose 6-phosphate with an apparent $K_m$ for fructose 6-phosphate of 0.2 mM. The addition of hormone had little effect on the activity of the enzyme measured with saturating concentrations of fructose 6-phosphate but increased the $K_m$ for fructose 6-phosphate to 2 mM. This change was probably due to covalent modification of the enzyme, since activity was measured in (NH$_4$)$_2$SO$_4$-treated extracts and after dialysis, by which low molecular weight effectors should be removed or greatly diluted. The effect of glucagon on 6-phosphofructo-2-kinase activity was apparent within 2 min (data not shown). Glucagon also caused a large decrease in the level of fructose 2,6-bisphosphate in hepatocytes; in three separate experiments, the level in hepatocytes incubated with no additions was $10.1 \pm 1.5$ nmol/g of liver and 10 min after hormone addition the level was $1.0 \pm 0.5$ nmol/g of liver.

Characterization of Partially Purified Rat Liver 6-Phosphofructo-2-Kinase. The results shown in Fig. 2 suggest that glucagon inhibits the activity of 6-phosphofructo-2-kinase by covalent modification of the enzyme. To test this possibility, we undertook to purify the enzyme from rat liver and to study the effect of phosphorylation on its activity. The purification procedure is summarized in Table 1. This procedure is an improvement of the method of El-Maghralbi et al. (15) that takes advantage of the observation of Furuuya and Uyeda (14) that the enzyme can be precipitated with polyethylene glycol. The purification factor of the enzyme was calculated from the polyethylene glycol step because the homogenate contains a high background level of fructose 2,6-bisphosphate, as well as some MgATP and fructose 6-phosphate, which make it difficult to estimate the activity of the enzyme accurately at this stage.

The elution of 6-phosphofructo-2-kinase from the DEAE-Sephadex column showed two peaks of enzyme activity, which are designated peak I (210 mM KCl) and peak II (240 mM) (Fig. 3). Although two peaks were obtained by anion exchange chromatography, the enzyme always eluted as a single symmetrical peak on gel filtration columns (15), which suggests that the two forms have similar molecular weights. Since peak II was separated from the bulk of protein on the DEAE-Sephadex column, it was carried through the final Sephadex G-100 gel filtration step, which gave a final specific activity of 15.2 million units/mg with a recovery of 11%. This is the highest specific activity so far reported. Although five distinct peptide bands were detected by NaDodSO$_4$ disc gel electrophoresis, there was an enrichment during the purification of a major peptide band that migrated with a relative mobility of 0.45 and had a subunit $M_r$ of $\approx 49,000$ (Fig. 4).

The apparent $K_m$ values of the enzyme for fructose 6-phosphate and ATP were 0.05–0.15 mM and 0.2–0.4 mM, respectively, for both peak I and peak II. The enzyme preparation contained no 6-phosphofructo-1-kinase, fructose 1,6-bisphosphate, pyruvate kinase, aldolase, lactic dehydrogenase, or cAMP-dependent protein kinase activity (15).

In Vitro Phosphorylation of Purified 6-Phosphofructo-2-Kinase. Incubation of partially purified 6-phosphofructo-2-kinase (peak II) with [$\gamma$-$^32$P]ATP and the catalytic subunit of cAMP-dependent protein kinase resulted in time-dependent incor-

![Figure 2](image2.png)

**Fig. 2.** Substrate dependence of the glucagon effect on hepatocyte 6-phosphofructo-2-kinase activity. Forty milliliters of hepatocytes (120 $\mu$g of DNA/ml) from fed rats was incubated for 10 min in the presence (○) or absence (●) of 10 nM glucagon. Enzyme activity was determined with various fructose 6-phosphate concentrations. The experiment was repeated five times with identical results. Fru-2,6-P$_2$, fructose 2,6-bisphosphate.

![Table 1](image1.png)

**Table 1.** Purification of rat hepatic 6-phosphofructo-2-kinase

<table>
<thead>
<tr>
<th>Stage</th>
<th>Enzyme activity, milliunits</th>
<th>Protein, mg</th>
<th>Specific activity, milliunits/mg of protein</th>
<th>Yield, %</th>
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</thead>
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<tr>
<td>Homogenate</td>
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<td>40</td>
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<tr>
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<td>21</td>
<td>3.4</td>
<td>36</td>
</tr>
<tr>
<td>Sephacryl S-200</td>
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<td>3.9</td>
<td>2.7</td>
<td>5</td>
</tr>
<tr>
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<td>15</td>
</tr>
<tr>
<td>DEAE-Sephadex (peak II)</td>
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<td>1.44</td>
<td>15.2</td>
<td>11</td>
</tr>
<tr>
<td>Sephadex G-100 (peak I)</td>
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<td>1.44</td>
<td>15.2</td>
<td>11</td>
</tr>
<tr>
<td>Sephadex G-100 (peak II)</td>
<td>22</td>
<td>1.44</td>
<td>15.2</td>
<td>11</td>
</tr>
</tbody>
</table>

Livers (303 g wet weight) from 25 fed Sprague–Dawley male rats were used. All steps were performed at 4°C.

The apparent $K_m$ values of the enzyme for fructose 6-phosphate and ATP were 0.05–0.15 mM and 0.2–0.4 mM, respectively, for both peak I and peak II. The enzyme preparation contained no 6-phosphofructo-1-kinase, fructose 1,6-bisphosphate, pyruvate kinase, aldolase, lactic dehydrogenase, or cAMP-dependent protein kinase activity (15).

In Vitro Phosphorylation of Purified 6-Phosphofructo-2-Kinase. Incubation of partially purified 6-phosphofructo-2-kinase (peak II) with [$\gamma$-$^32$P]ATP and the catalytic subunit of cAMP-dependent protein kinase resulted in time-dependent incor-

![Figure 3](image3.png)

**Fig. 3.** DEAE-Sephadex chromatography of rat liver 6-phosphofructo-2-kinase. Seventy-one milliunits of 6-phosphofructo-2-kinase from the Sephacryl S-200 gel filtration step was applied to a DEAE-Sephadex column and the enzyme was eluted with a linear KCl gradient (-----). $A_{280}$, 6-phosphofructo-2-kinase activity.
Fig. 4. *In vitro* phosphorylation of rat liver 6-phosphofructo-2-kinase catalyzed by the catalytic subunit of cAMP-dependent protein kinase. Purified 6-phosphofructo-2-kinase (18 μg) was incubated with [γ-32P]ATP and 40 units of catalytic subunit. (Inset) Time course of phosphorylation of 6-phosphofructo-2-kinase in the absence (○) and presence (□) of heat-stable protein kinase inhibitor (250 units). At the indicated times, 5-μl aliquots were withdrawn and 32P incorporation into protein was determined by the method of Corbin and Reimann (17). After incubation for 60 min, the entire incubation mixture was subjected to NaDodSO4 disc gel electrophoresis. The gel was then stained with Coomassie blue and, after destaining, scanned at 580 nm (-----). The radioactivity profile (○) of the gel was obtained by assaying 3-mm sections. The relative mobility of the major 32P-containing peptide band and of the major Coomassie blue-stained band was 0.45, corresponding to a subunit Mr of 49,000. This subunit molecular weight was determined by comparing the relative mobility with those of proteins of known molecular weight as described (20). The phosphorylation of this protein (data not shown). Since the enzyme was not homogeneous, the labeled products were subjected to NaDodSO4 disc gel electrophoresis (Fig. 4). Only one peak of 32P radioactivity was detected. This peak of radioactivity migrated with a relative mobility of 0.45 and had an apparent subunit Mr of 49,000. The catalytic subunit of cAMP-dependent protein kinase migrated with a subunit Mr of 42,000, but no 32P radioactivity was incorporated into it under the conditions used. Both peak I and peak II of 6-phosphofructo-2-kinase could be phosphorylated by cAMP-dependent protein kinase (data not shown).

Fig. 5. Effect of phosphorylation on substrate dependence of 6-phosphofructo-2-kinase activity. Purified 6-phosphofructo-2-kinase (30 μg) was incubated in 20 mM Tris-HCl, pH 7.5/1 mM ATP/6 mM MgCl2/50 mM KCl/2 mM EDTA/0.5 mM diithiothreitol with and without catalytic subunit (40 units) from rat liver for 30 min at 20°C. The enzyme was then assayed for 6-phosphofructo-2-kinase activity as a function of fructose 6-phosphate concentration with 2 mM ATP. ○, incubation with MgATP; ●, incubation with MgATP/catalytic subunit; ■, incubation with MgATP/catalytic subunit/heat-stable protein kinase inhibitor (250 units). Fru-2,6-P2, fructose 2,6-bisphosphate.

DISCUSSION

We have shown that partially purified 6-phosphofructo-2-kinase is subject to *in vitro* phosphorylation by the catalytic subunit of cAMP-dependent protein kinase. 32P radioactivity is incorporated into a 49,000-dalton peptide band. Since the native enzyme has an estimated apparent Mr of ~90,000 as determined by gel filtration (15), it is possible that the enzyme is a dimer of two 49,000-dalton subunits. Although elucidation of the definitive subunit structure of the enzyme must await further
studies, it is clear that it differs from 6-phosphofructo-1-kinase, which is a tetrameric enzyme with a $M_r$ of 320,000.

Two isozymes of 6-phosphofructo-1-kinase have been detected in rat liver (21), and there may be two forms of 6-phosphofructo-2-kinase as well (Fig. 4). Van Schaftingen and Hers (16) have also detected two peaks of 6-phosphofructo-2-kinase activity after anion exchange chromatography. Our results suggest that the two peaks have a number of similarities. They have the same subunit molecular weights and the same affinity for fructose 6-phosphate. Phosphorylation of both enzymes is catalyzed by cAMP-dependent protein kinase, and phosphorylation inhibits both forms to the same extent.

We have also shown that the glucagon-induced alteration in 6-phosphofructo-2-kinase activity in hepatocytes and the cAMP-dependent phosphorylation-induced changes in the purified enzyme in vitro are similar. In both cases, the apparent $K_m$ for fructose 6-phosphate was increased while the maximum activity of the enzyme was essentially unaffected. That the effect of glucagon is due to covalent modification is further supported by the fact that the hormone effect was observed after $(NH_4)_2SO_4$ fractionation, where all low molecular weight effectors should have been removed. From these results, it seems reasonable to postulate that the ability of glucagon to cause a rapid and large decrease in the level of fructose 2,6-bisphosphate is a result, at least in part, of cAMP-dependent phosphorylation-induced inhibition of 6-phosphofructo-2-kinase. However, it is not possible to rule out an effect of glucagon to cause a cAMP-independent alteration of 6-phosphofructo-2-kinase activity (14) or to accelerate the degradation of fructose 2,6-bisphosphate by activating a fructose 2,6-bisphosphatase. In fact, the rapid and large drop in fructose 2,6-bisphosphate levels is difficult to explain solely by a decrease in kinase activity unless the turnover of the compound is very rapid. It may be that glucagon also activates a phosphatase.

Regulation of the level of fructose 2,6-bisphosphate by hormones and substrate is a possible mechanism for modulating the rate of hepatic glycolysis and gluconeogenesis (12). We have recently shown that the level of fructose 2,6-bisphosphate is decreased in livers from diabetic and starved rats and that the activity of 6-phosphofructo-2-kinase was depressed in the diabetic state (13). It seems reasonable to postulate that the regulation of the level of fructose 2,6-bisphosphate by alterations in 6-phosphofructo-2-kinase activity is a complex function of changes in its phosphorylation state, the amount of enzyme protein, and various, as yet unidentified, effectors of the enzyme.