Mechanism of action of glucagon on hepatocyte phosphofructokinase activity

(ADP-fructose-6-phosphate 1-phosphotransferase/phosphorylation/fructose bisphosphate/enzyme regulation/pyruvate kinase)

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ABSTRACT Addition of glucagon to isolated hepatocytes reduced the activity of 6-phosphofructokinase (ADP-fructose-6-phosphate 1-phosphotransferase, EC 2.7.1.11) and pyruvate kinase (ATP:pyruvate 2-O-phosphotransferase, EC 2.7.1.40). Phosphorylation contributed to the inhibition of pyruvate kinase, but several lines of evidence indicated that this reaction was not responsible for the inhibition of phosphofructokinase. First, the increase in phosphorylation in intact cells induced by increasing the concentration of glucagon did not correlate well with the decrease in enzyme activity. Second, phosphorylation of phosphofructokinase induced by addition of cyclic AMP and MgATP or by addition of MgATP and the catalytic subunit of the cyclic AMP-dependent protein kinase to hepatocyte extracts had no effect on enzyme activity. Third, ammonium sulfate precipitation of the enzyme from extracts of cells incubated with glucagon abolished the hormone effect. The effect could be restored, however, by the addition of a phosphofructokinase-free extract from glucagon-treated cells to the ammonium sulfate-treated enzyme from either untreated or glucagon-treated cells. These results suggest that the inhibition of phosphofructokinase by glucagon is due to changes in the level of an allosteric effector(s).

The addition of glucagon to isolated rat hepatocytes inhibits the activity of both phosphofructokinase (ADP-fructose-6-phosphate 1-phosphotransferase, EC 2.7.1.11) (1–3) and pyruvate kinase (ATP:pyruvate 2-O-phosphotransferase, EC 2.7.1.40) (4–8). These effects account in part for the stimulation of gluconeogenesis by the hormone. The inhibition of both enzymes is characterized by increases in the substrate concentration needed for half-maximal activity (S0.5) (1, 2, 4–8) and in sensitivity to inhibition by ATP (3, 5, 7). Glucagon also stimulates the in vivo phosphorylation of both enzymes (3, 9), and this has led to the hypothesis that enzyme inhibition by phosphorylation is the mechanism by which the hormone stimulates gluconeogenesis (10). The results we report here support this hypothesis in the case of pyruvate kinase, but not in the case of phosphofructokinase.

MATERIALS AND METHODS

Preparation and Incubation of Hepatocytes. Isolated hepatocytes were prepared from fed rats (male Sprague-Dawley, 200–300 g) as described (8). The cells were suspended to a final concentration of 50 mg of liver/ml in Krebs-Henseleit buffer that contained 0.5% bactracin. In experiments in which only phosphofructokinase activity was measured, heated extracts from 5-ml aliquots of cell suspension were prepared as described (1), except that the cells were homogenized in 1.5 ml of buffer (50 mM potassium phosphate, pH 7.5/100 mM NaF/1 mM EDTA/20 mM 3-mercaptoethanol/0.1 mM phenylmethylsulfonyl fluoride). In experiments in which 32P incor-

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poration was also determined, hepatocytes were suspended in low-phosphate (0.1 mM) Krebs-Henseleit buffer that contained 0.5% bactracin, and 1 mCi of $[^32P]PO_4$ per g of liver cells (1 Ci = $3.7 \times 10^{10}$ becquerels) was added to the cell suspension. Aliquots (30–45 ml) were transferred to 500-ml plastic erlenmeyer flasks and incubated for 45 min, with continuous gassing, to allow the intracellular ATP to reach a constant specific activity (11). The appropriate additions were then made to each flask, and the incubation was continued for 10 min. The cell suspension was transferred to 50-ml plastic round-bottom centrifuge tubes, 3-ml samples were withdrawn for ATP specific activity determination, and the remainder of the cells was centrifuged rapidly. The supernatant fluid was decanted, and the cells were homogenized in 8 ml of buffer as described above.

Assay for Phosphofructokinase and Pyruvate Kinase. Phosphofructokinase activity was assayed in heated extracts as described (1), except that the pH was 7.4. Pyruvate kinase activity was measured in the 27,000 × g supernatant fraction as described (8), except that the assay was initiated by using substrate instead of enzyme.

Because glucagon does not affect the $V_{max}$ of phosphofructokinase (1–3) or pyruvate kinase (4–8), the change in rate at submaximal concentrations of substrate is the best way to measure the interconversion of the inactive and active forms of the enzymes. Therefore, the rate at submaximal concentrations ($r$) was expressed as the fraction ($r/V_{max}$) of the maximum rate obtained with 4 mM fructose 6-phosphate plus 1 mM AMP for phosphofructokinase and with 4 mM phosphoenolpyruvate for pyruvate kinase. The maximum activities of phosphofructokinase and pyruvate kinase in hepatocytes from fed rats were 2.4 and 43 units per g of liver, respectively, where 1 unit is the amount of enzyme that converts 1 μmol of substrate per min under the conditions of the assay.

Immunoprecipitation of Phosphofructokinase and Pyruvate Kinase. Phosphofructokinase was precipitated from the heated extract by using 35% (NH₄)₂SO₄. The pellet was resuspended in 1 ml of homogenizing buffer, assayed for total activity, and treated with enough rat liver phosphofructokinase antiserum to ensure a 50% excess. Usually, $\approx$2–3 units of phosphofructokinase was precipitated. Pyruvate kinase was precipitated by adding the same excess of rat liver pyruvate kinase antiserum to the 27,000 × g supernatant fraction. After standing overnight at 4°C, the immunoprecipitates were collected by centrifugation, resuspended in 0.2 ml of homogenizing buffer, and centrifuged at 1800 × $g_{max}$ for 25 min through a two-step gradient (12) made of 0.6 ml of 1 M sucrose and 0.3 ml of 0.5 M sucrose in 10 mM sodium phosphate (pH 7.5) and 0.2% Tween-100. The precipitates were resuspended in 0.5 ml of the 0.5 M sucrose solution and redsedimented. Fi-

Abbreviations: cAMP, cyclic AMP; $S_{0.5}$, substrate concentration giving half-maximal velocity.
nally, the immunoprecipitates were dissolved in NaDodSO4 and dithiothreitol and subjected to NaDodSO4/polyacrylamide gel electrophoresis (13) as described (9, 14). The amount of $^{32}$P incorporated was calculated by adding the radioactivity of the fractions corresponding to the enzyme band (three or four 2-mm slices) and subtracting a background estimated from the radioactivity of the slices immediately preceding and following. The moles of $^{32}$P incorporated per mole of enzyme were calculated from the radioactivity in the enzyme, the specific radioactivity of the $^{[\gamma -32]P}$ATP, and the specific activity and molecular weight of the pure enzyme. The specific activity and molecular weight of phosphofructokinase were assumed to be 100 units per mg and 320,000, respectively, and those of pyruvate kinase were assumed to be 400 units per mg and 228,000.

Preparation of Antisera Specific for Rat Liver Phosphofructokinase and Pyruvate Kinase. Antisera specific for phosphofructokinase and pyruvate kinase were raised by subcutaneous injection of purified preparations of the enzyme into rabbits, essentially as described (15, 16). Phosphofructokinase and pyruvate kinase were purified as described by Pilkis et al. (17) and Riou et al. (14), respectively. Both antisera gave single lines in the Ouchterlony double-diffusion test against either the purified enzyme or a crude rat hepatocyte extract. The phosphofructokinase antiserum had a titer of 27 units per ml, while that of pyruvate kinase was 60 units per ml.

Determination of Specific Radioactivity of $[\gamma -32]P$ATP. Aliquots of cells (3 ml) that had been incubated with $[32]$P]PO4 were rapidly separated from the suspending medium by the method of Cornell (18). The cell suspension was layered over 1.9 ml of bromododecane and 0.5 ml of 2.5% HClO4 in 10% (wt/vol) sucrose and rapidly centrifuged. After removal of the medium and most of the oil layer, 0.4 ml of the HClO4 extract was removed and neutralized with 1 M K2CO3. The specific radioactivity of the $[\gamma -32]$ATP was determined on 150-μl aliquots of the neutralized extract by the method of England and Walsh (19). The specific radioactivity of the ATP was 26 ± 6 cpm/pmol (n = 7) and was unaffected by the addition of glucagon.

Preparation of (NH4)2SO4-Treated Extracts and of Heated Extracts Free of Phosphofructokinase Activity. Phosphofructokinase was precipitated from portions of the heated extracts by the addition of saturated (NH4)2SO4 (35%). The precipitate was resuspended to 10% of the original volume in homogenizing buffer and dialyzed overnight against the same buffer (2 × 1 liter). The remaining portion of each extract was incubated overnight with enough phosphofructokinase antiserum (=0.1 ml/ml of extract) to completely precipitate the activity. The precipitate was removed by centrifugation, leaving an activity-free extract. The extracts were diluted about 14-fold from their original intracellular concentrations.

Materials. All enzymes were from Boehringer Mannheim, Sigma, or Worthington. Glucagon was a gift from Eli Lilly. All other reagents were of the highest purity commercially available.

RESULTS

Effect of Glucagon on Hepatocyte Phosphofructokinase Activity. When 10 nM glucagon was added to isolated hepatocytes from fed rats, the phosphofructokinase activity measured in heated extracts of the cells was inhibited (Fig. 1; ref. 1). Glucagon caused a shift in the fructose 6-phosphate concentration curve to the right, with the result that the $S_{0.5}$ increased from 0.7 mM to 1.4 mM. A similar 2-fold increase in the $S_{0.5}$ was observed when phosphofructokinase activity was measured in unheated extracts (data not shown; ref. 2).

![Fig. 1](image1.png)

**Fig. 1.** Effect of glucagon on hepatocyte phosphofructokinase activity. Isolated hepatocytes from fed rats were incubated in the presence (+) and absence (×) of 10 nM glucagon from 10 min, and activity was assayed in heated extracts. Arrows denote $S_{0.5}$ values—0.7 mM in the absence and 1.4 mM in the presence of glucagon.

Effect of Glucagon on $^{32}$P Incorporation into Phosphofructokinase and Pyruvate Kinase. We next sought to determine whether the inhibition by glucagon might be explained by phosphorylation of the phosphofructokinase. The NaDodSO4/gel electrophoresis pattern obtained when phosphofructokinase was isolated by specific immunoprecipitation from extracts of hepatocytes that had been equilibrated with $[32]$P]PO4 showed a single peak of $^{32}$P radioactivity that comigrated with the homogeneous phosphofructokinase subunit band (Fig. 2). The $R_F$ values for $^{32}$P radioactivity and the homogeneous subunit were both 0.28, and both migrated with an apparent $M_r$ of 82,000. The addition of 10 nM glucagon to the hepatocytes for 10 min more than doubled the amount of $^{32}$P incorporation. No radioactivity was found in the subunit band when hepatocytes from control or glucagon-treated hepatocytes were

![Fig. 2](image2.png)

**Fig. 2.** NaDodSO4/disc gel electrophoresis of immunoprecipitates of $^{32}$P-labeled phosphofructokinase. Radioactivity profiles from untreated (●) and glucagon-treated (○) cells were obtained by assaying 2-mm sections. Arrow denotes the migration of the homogeneous phosphofructokinase subunit having an $R_F$ value of 0.28, corresponding to a molecular weight of 82,000.
homogenized in buffer that contained [γ-32P]ATP (data not shown). Thus, phosphofructokinase was not phosphorylated during the isolation procedure.

We next investigated the effects of increasing glucagon concentration on [32P] incorporation into phosphofructokinase and on the activity of the enzyme (Fig. 3). The [32P] incorporation increased from 1.0 to 2.1 mol/mol of enzyme in the presence of 10 nM glucagon. Half-maximal incorporation occurred at about 1 nM glucagon. However, half-maximal inhibition of activity occurred at 0.3 nM glucagon, a concentration where there was no perceptible increase in [32P] incorporation into the enzyme. In contrast to these results, the concentration of glucagon needed for both half-maximal stimulation of [32P] incorporation into pyruvate kinase and half-maximal inhibition of pyruvate kinase activity was 0.3 nM. The lack of correlation between [32P] incorporation into and activity of phosphofructokinase suggests that the increase in [32P] incorporation may not be responsible for the change in enzyme activity.

Effects of Addition of cAMP to Hepatocyte Extracts on [32P] Incorporation into and Activity of Phosphofructokinase and Pyruvate Kinase. Phosphofructokinase activity was not inhibited by the addition of cAMP and Mg2+-ATP to an hepatocyte extract, even though the addition increased [32P] incorporation into the enzyme from 0.2 to 0.7 mol/mol of enzyme (Table 1). The increase in activity must have been due to the cyclic nucleotide acting as an effector; no increase or decrease in activity was observed when the enzyme was assayed after the reaction had been stopped with 50% (NH4)2SO4. The addition of large amounts of a homogeneous preparation of the catalytic subunit of the cAMP-dependent protein kinase to the extract also did not inactivate the enzyme, even though [32P] incorporation increased to 4.1 mol/mol of enzyme. On the other hand, the addition of cAMP or the catalytic subunit and Mg2+-ATP increased the [32P] incorporation into pyruvate kinase 9- to 10-fold and inhibited the enzyme by 80%. These results also suggest that phosphorylation of phosphofructokinase by the cAMP-dependent protein kinase is not associated with a change in activity of the enzyme.

Effects of (NH4)2SO4 Treatment on Activity of Phosphofructokinase. If the inactivation of phosphofructokinase by glucagon were due to its phosphorylation, the effect would be expected to persist after (NH4)2SO4 treatment, as it does for pyruvate kinase (5, 6, 8). Comparison of the fructose 6-phosphate concentration curves for phosphofructokinase in (NH4)2SO4-treated and dialyzed extracts of hepatocytes incubated with and without 10 nM glucagon shows that (NH4)2SO4 treatment plus dialysis shifted the substrate curve to the right and increased the S0.5 from 0.7 mM (see Fig. 1) to more than 4 mM (Fig. 4). No effect of glucagon was observed after this treatment, even when the enzyme was partially reactivated by the addition of 2 mM (NH4)2SO4 to the assay mixture [despite the fact that the increase in [32P] incorporation into the enzyme by glucagon was retained after (NH4)2SO4 treatment]. These results suggest that inhibition of phosphofructokinase by glucagon may be due to changes in the level of an allosteric effector(s) or to a phosphorylation-induced change in the sensitivity of the enzyme to an allosteric effector(s).

**Table 1. Activity of and [32P] Incorporation into Phosphofructokinase and Pyruvate Kinase in Hepatocyte Extracts**

<table>
<thead>
<tr>
<th>Addition</th>
<th>Phosphofructokinase</th>
<th>Pyruvate kinase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>v0.5/Vmax mol/mol</td>
<td>v0.5/Vmax mol/mol</td>
</tr>
<tr>
<td>None (control)</td>
<td>0.35 (0.33)</td>
<td>0.43 (0.3)</td>
</tr>
<tr>
<td>cAMP</td>
<td>0.51 (0.33)</td>
<td>0.7 (0.10)</td>
</tr>
<tr>
<td>Catalytic subunit</td>
<td>(0.34)</td>
<td>4.1 (0.08)</td>
</tr>
</tbody>
</table>

Aliquots (5 ml) of the isolated hepatocyte cell suspension were centrifuged, and the cell pellets were homogenized in 1.5 ml of buffer (50 mM Tris, pH 7.5/50 mM NaF/1 mM EDTA/1 mM dithiothreitol) for 90 sec (3 × 30 sec) in an ultraturrax homogenizer. The homogenate was centrifuged at 27,000 × g max for 45 min. Aliquots (0.5 ml) of the supernatant fraction were incubated at 30°C for 30 min in 1 mM theophylline/10 mM MgCl2/0.3 mM [γ-32P]ATP (30 cpm/pmole) with or without 50 μM of cAMP or 70,000 units of a homogenous preparation of the catalytic subunit of the cAMP-dependent protein kinase from rat liver. For phosphofructokinase, the reaction was stopped either by heating at 58°C for 3 min or by addition of 50% (NH4)2SO4 (results shown in parentheses). For pyruvate kinase, the reaction was stopped by addition of 60% (NH4)2SO4. The (NH4)2SO4 pellets were redissolved in buffer. Phosphofructokinase activity was assayed with 0.2 and 4 mM fructose 6-phosphate. Pyruvate kinase activity was assayed with 0.4 and 4 mM phosphoenolpyruvate. [32P] incorporated into each enzyme (mol/mol) was estimated as described.

![Fig. 3. Effect of glucagon concentration on [32P] incorporation into and activity of phosphofructokinase and pyruvate kinase (PK) in isolated hepatocytes. Activity of phosphofructokinase (•) was measured in heated extracts with 0.1 and 4 mM fructose 6-phosphate. Pyruvate kinase activity (●) was measured in the 27,000 × g supernatant fraction with 0.4 and 4 mM phosphoenolpyruvate. [32P] Incorporation into phosphofructokinase (○) and pyruvate kinase (△) was determined after immunoprecipitation of the enzymes. The glucagon concentrations needed for half-maximal stimulation of [32P] incorporation into phosphofructokinase (1) and into pyruvate kinase (1) were 1 and 0.3 nM, respectively. Half-maximal inhibition of both occurred at 0.3 nM glucagon.](image)

![Fig. 4. Phosphofructokinase activity in (NH4)2SO4-treated extracts of hepatocytes incubated with and without 10 nM glucagon. Activity in (NH4)2SO4-treated and dialyzed extracts from control (●, •) and glucagon-treated (○, △) hepatocytes was assayed in the absence or presence of 2 mM (NH4)2SO4.](image)
involvcd in the glucagon-induced changes in enzyme activity. Activity was measured after recombination of (NH₄)₂SO₄-treated and dialyzed extracts from untreated (basal) hepatocytes and phosphofructokinase-free extracts of untreated (●) or glucagon-treated cells (▲) or after recombination of (NH₄)₂SO₄-treated and dialyzed extracts from glucagon-treated cells and phosphofructokinase-free extracts of untreated (●) or glucagon-treated cells (▲).

the same activity as in the original extract. This addition diluted the phosphofructokinase-free extract less than 10%. When the enzyme from untreated (basal) cells was recombined with the enzyme-free extract from untreated cells, the substrate concentration curve was shifted to the left and the enzyme had the same kinetic properties as the heated extracts of untreated cells. When the enzyme from glucagon-treated cells was recombined with enzyme-free extract from glucagon-treated cells, the hormone effect—i.e., the shift of the substrate concentration curve to the right—was restored. However, when the enzyme from glucagon-treated cells was recombined with enzyme-free extract from untreated cells, the enzyme had the same kinetic properties as the enzyme from heated extracts of untreated cells. Conversely, recombination of the enzyme from untreated cells and the enzyme-free extract from glucagon-treated cells gave an enzyme that had the same kinetic properties as the enzyme from heated extracts of glucagon-treated cells. In other words, the glucagon effect was conferred on the enzyme by the properties of the glucagon-treated extract that was free of activity rather than by the enzyme itself. The same results were obtained when the enzyme-free extracts were added to enzyme that had been precipitated with (NH₄)₂SO₄ from the unheated 27,000 × g supernatant fraction (data not shown).

**DISCUSSION**

Glucagon is believed to affect the phosphorylation of specific enzymes involved in the gluconeogenic-glycolytic pathway. The best support for this hypothesis comes from studies on pyruvate kinase, in which in vitro phosphorylation of the purified enzyme, catalyzed by the cAMP-dependent protein kinase, increased the S₀₅ to the same extent as did glucagon addition to isolated hepatocytes. The correlation between inactivation of pyruvate kinase and incorporation into the enzyme by glucagon (see Fig. 3) also supports this hypothesis. The glucagon-induced changes in rat liver phosphofructokinase activity have been reported to persist after partial purification of the enzyme. The involvement in the inactivation of this enzyme as well. However, we found that the in vitro phosphorylation of phosphofructokinase, catalyzed by the cAMP-dependent protein kinase, did not lead to any change in activity of the purified enzyme or of the enzyme in crude extracts. That the hormone-induced increase in the S₀₅ could be restored by addition of an enzyme-free extract from glucagon-treated cells to enzyme from either untreated or glucagon-treated cells (see Fig. 5) suggests that the inactivation is due to changes in an allosteric effector(s). Consistent with this idea is the observation that (NH₄)₂SO₄ fractionation results in disappearance of the hormone effect (see Fig. 4).

Glucagon inhibits the flux through phosphofructokinase in intact hepatocytes, as well as the phosphofructokinase activity in hepatocyte extracts (see Fig. 1; ref. 24). Because changes in the level of allosteric effectors appear to be the mechanism by which the activity of the enzyme is affected (see Fig. 5), the flux through the enzyme may also be inhibited by changes in allosteric effectors. Phosphofructokinase can be regulated by many effectors. Of the known activators, the only one that changes in such a way as to cause inactivation is fructose bisphosphate. Glucagon decreases the concentration of this metabolite, which is located in the mitochondria. It is possible that the hormone causes the cytosolic concentration of this activator to decrease, but this has not been measured. Glucagon increases the concentration of 2- and 3-phosphoglycerate and phosphoenolpyruvate, weak inhibitors of phosphofructokinase, and may thus contribute to the inhibition. More work is required to determine which of these effectors, or possibly other as-yet unknown ones, is responsible for the inactivation. Also, it will be necessary to determine whether the level of effectors in the extracts, where the glucagon effect on activity is observed, accurately reflects changes in effectors that occur in the cell.

Purified rat liver phosphofructokinase has been reported to contain 3–4 mol of phosphate per mol of enzyme subunit. One mol of phosphate per mol of subunit is present in sites where the phosphorylation is catalyzed by the cAMP-dependent protein kinase. The presence of this phosphate, if it turns over slowly, may explain why glucagon stimulation of incorporation was less for phosphofructokinase than for pyruvate kinase (see Fig. 3). If phosphate is already present in cAMP-dependent sites, the incorporation of another mole by glucagon may not have any further effect on activity of the enzyme. However, removal from purified phosphofructokinase of phosphate in the cAMP-dependent sites did not alter activity of the enzyme (unpublished results). It is possible that removal of phosphate from both cAMP-dependent and cAMP-independent sites may yet show some change in the kinetic properties of the enzyme. However, this study suggests that the ability of glucagon to cause an increase in the S₀₅ does not depend on the amount of phosphate in the enzyme but rather results from a change in the level of an allosteric effector(s).

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