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GLUCAGON STIMULATION OF FRUCTOSE 1,6-BISPHOSPHATASE PHOSPHORYLATION IN RAT HEPATOCYTES

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SUMMARY

Hepatocytes from fed rats were incubated with [^{32}P]PO_4 in the absence and presence of glucagon. Fructose 1,6-bisphosphatase was isolated from extracts of the hepatocytes by the addition of antiserum specific for the enzyme, and the amount of ^{32}P incorporated into the enzyme was determined after sodium dodecyl sulfate/gel electrophoresis. Glucagon (10 nM) stimulated ^{32}P incorporation by 60 percent. Half-maximal stimulation of ^{32}P-incorporation was observed with 1.5 nM glucagon. The enzyme had an apparent M_r of 41,000 after sodium dodecyl sulfate/slab gel electrophoresis. The relationship of phosphorylation to activity of the enzyme is discussed.

INTRODUCTION

It has been proposed that glucagon stimulates gluconeogenesis in part by affecting the cyclic AMP-dependent phosphorylation of several enzymes in the pathway (1). The phosphorylation of pyruvate kinase, phosphofructokinase, and fructose 1,6-bisphosphatase from rat liver have been shown to be catalyzed *in vitro* by the catalytic subunit of the cyclic AMP-dependent protein kinase (2-6). All three enzymes are also phosphorylated *in vivo* (5,7,8). Glucagon stimulates the phosphorylation of pyruvate kinase (9-11) and phosphofructokinase (11) in isolated rat hepatocytes, but no reports have appeared on the stimulation of fructose 1,6-bisphosphatase phosphorylation by glucagon. This report demonstrates that glucagon stimulates the phosphorylation of this enzyme as well.

METHODS

Preparation and Incubation of Hepatocytes. Isolated hepatocytes were prepared from fed rats (Male Sprague-Dawley, 200-300 g) as described (11). The cells were suspended to a final concentration of 50 mg of liver/ml in low phosphate (0.1 mM) Krebs-Henseleit buffer that contained 0.5 percent bacitracin, and 1 mCi of [^{32}P]PO_4 per g of liver cells 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

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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 (50 mM potassium phosphate, pH 7.5/100 mM NaF/1 mM EDTA/20 mM β-mercaptoethanol/0.1 mM phenylmethylsulfonyl fluoride) as described (11).

Immunoprecipitation of Phosphofructokinase and Fructose 1,6-Bisphosphatase. Phosphofructokinase was precipitated from heated extracts by the addition of saturated $(NH_4)_2SO_4$ to a final concentration of 35 percent (11). Fructose 1,6-bisphosphatase was precipitated from the 35 percent $(NH_4)_2SO_4$ supernatant fraction by increasing the $(NH_4)_2SO_4$ concentration to 55 percent. Each pellet was resuspended in 1 ml of homogenizing buffer, assayed for total activity, and treated with enough rat liver phosphofructokinase or fructose 1,6-bisphosphatase antiserum to ensure a 50 percent excess. The activity of each enzyme was determined as described previously (5,11). Usually 2-3 units of phosphofructokinase and 1 unit of fructose 1,6-bisphosphatase were precipitated. After standing overnight at 4°, the immunoprecipitates were collected by centrifugation, and then washed as described previously (11). They were then dissolved in $NaDodSO_4$ and dithiothreitol and subjected to $NaDodSO_4$/polyacrylamide gel electrophoresis. The amount of ^{32}P incorporated was calculated by adding the radioactivity of the fractions corresponding to the enzyme band (3 or 4-2 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 $[\gamma\text{-}^{32}P]ATP$, 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 fructose 1,6-bisphosphatase were assumed to be 40 units per mg and 164,000.

Antiserum specific for phosphofructokinase was prepared as described (11) and had a titer of 27 units per ml. Fructose 1,6-bisphosphatase antiserum was a gift from Dr. B.L. Horecker and had a titer of 40 units per ml.

Determination of Specific Radioactivity of $[\gamma\text{-}^{32}P]ATP$. The specific radioactivity of $[\gamma\text{-}^{32}P]ATP$ was determined by the method of England and Walsh (12) as described previously (11). The specific radioactivity of the ATP was 19 ± 4 cpm//pmol (n=4) and was unaffected by glucagon.

Polyacrylamide Gel Electrophoresis. Polyacrylamide gel electrophoresis in the presence of $NaDodSO_4$ was performed on 5 percent disc gels as described previously (5,11) or on 10 cm x 14 cm x 0.75 mm slabs as described (13). The slab gels were 9 percent (wt/vol) in polyacrylamide and were electrophoresed at 40 mA per slab. After completion of electrophoresis, gels were fixed in 10 percent methanol/10 percent acetic acid, stained overnight in 0.05 percent Coomassie brillant blue R/10 percent methanol/10 percent acetic acid, and destained in 10 percent methanol/10 percent acetic acid. The gels were scanned in an Ortec Model No. 4310 densitometer, and the migration of the proteins measured relative to the bromophenol blue dye front.

RESULTS

We sought to determine whether the addition of glucagon to isolated hepatocytes would stimulate the phosphorylation of fructose 1,6-bisphosphatase. Figure 1 shows the $NaDodSO_4$/gel electrophoresis pattern obtained when

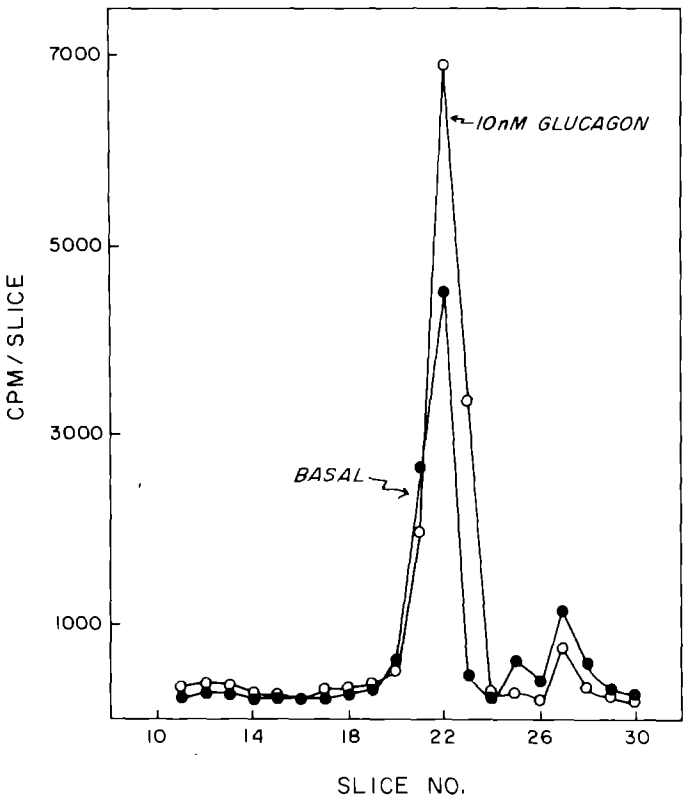

Fig. 1. NaDodSO$_4$/disc gel electrophoresis of immunoprecipitates of ^{32}P-labeled fructose 1,6-bisphosphatase isolated from basal or glucagon-treated hepatocytes. Radioactivity profiles were obtained by assaying 2 mm sections. The ^{32}P-labeled enzyme comigrated with the purified rat liver enzyme; both had an R_F value of 0.56 corresponding to a M_r of 36,000.

fructose 1,6-bisphosphatase was isolated by specific immunoprecipitation from extracts of hepatocytes that had been equilibrated with [^{32}P]PO_4. It shows a single peak of ^{32}P radioactivity. A similar pattern was seen when phosphofructokinase was specifically immunoprecipitated from cell extracts (11). In both cases the peak of radioactivity was coincident with the peak of enzyme protein (data not shown). The addition of 10 nM glucagon to the hepatocytes for 10 min increased the amount of ^{32}P incorporated into fructose 1,6-bisphophatase by about 60 percent. No radioactivity was found in the subunit band of either fructose 1,6-bisphosphatase or phosphofructokinase when hepatocytes from control or glucagon-treated hepatocytes were homogenized in buffer that

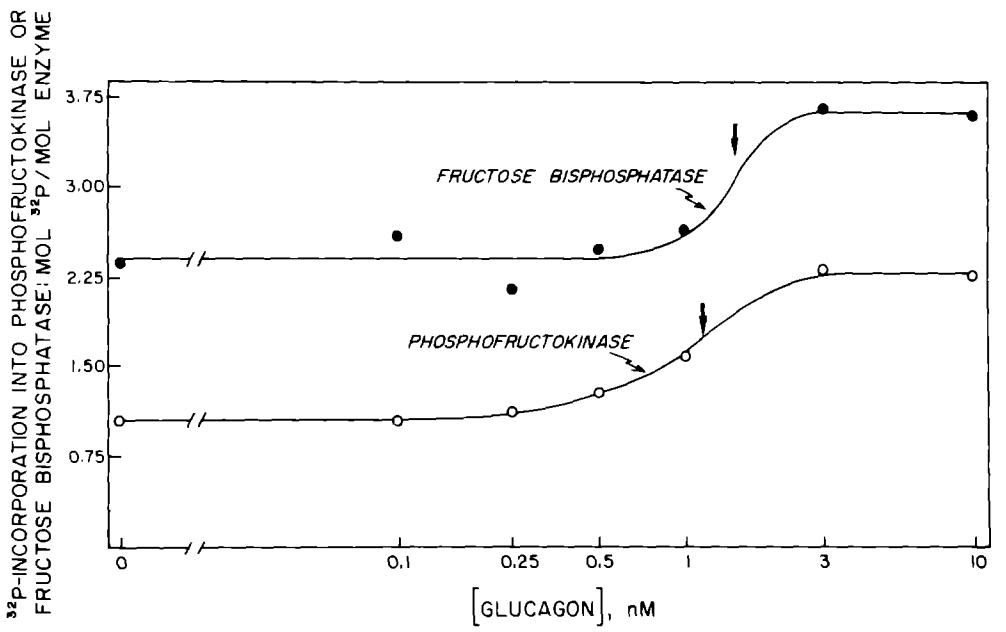

Fig. 2. Effect of glucagon concentration on ^{32}P incorporation into fructose 1,6-bisphosphatase and phosphofructokinase. ^{32}P-incorporation was determined after immunoprecipitation of the enzymes. The arrows represent the glucagon concentrations needed for half-maximal stimulation of ^{32}P-incorporation. The values were 1.1 nM and 1.5 nM for phosphofructokinase and fructose 1,6-bisphosphatase, respectively.

contained [γ-^{32}P]ATP (data not shown). Thus, neither enzyme was phosphorylated during the isolation procedure.

We next investigated the effects of increasing glucagon concentration of ^{32}P incorporation into fructose 1,6-bisphosphatase and into phosphofructokinase (Fig. 2). The ^{32}P incorporation into fructose 1,6-bisphosphatase increased from 2.3 to 3.6 mol/mol of enzyme in the presence of 10 nM glucagon while the ^{32}P incorporation into phosphofructokinase increased from 1.0 to 2.1 mol/mol of enzyme. Half-maximal incorporation into both enzymes occurred with 1.1-to-1.5 nM glucagon.

There is some controversy about the subunit molecular weight of rat liver fructose 1,6-bisphosphatase (14). We found a M_r of 36,000 after $NaDodSO_4$/disc gel electrophoresis (Fig. 1 and ref. 5). However, when the relative mobility of the rat liver enzyme was compared to that of other

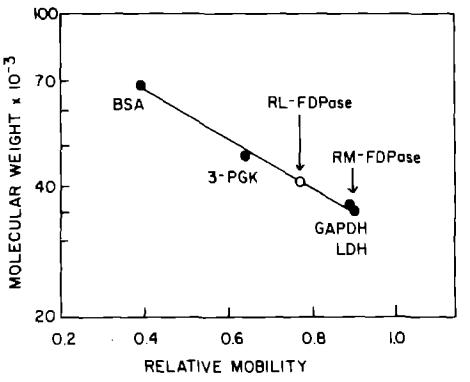

Fig. 3. The subunit size of rat liver fructose 1,6-bisphosphatase (RL-FDPase) as determined by $NaDodSO_4$/slab gel electrophoresis. Migration was measured relative to the dye front. The standard proteins run were: bovine serum albumin (BSA); 3-phosphoglycerate kinase (3-PFK) from yeast; glyceraldehyde-3-phosphate dehydrogenase (GAPDH) frm rabbit muscle; and lactate dehydrogenase (LDH) from rabbit muscle. The subunit size of rabbit muscle fructose 1,6-bisphosphatase (RM-FDPase) is also indicated. RL-FDPase was purified as described (5).

proteins after $NaDodSO_4$/slab gel electrophoresis a higher M_r was found (Fig. 3). Under these conditions, the purified enzyme migrated as a single sharp band with a mobility corresponding to an apparent M_r of 41,000. The reason for the difference between the two systems is not clear. A commericial preparation of rabbit muscle fructose 1,6-bisphosphatase had a mobility corresponding to an apparent M_r of 36,000 on the slab gels (Fig. 3) and 32,000 on the disc gels (data not shown). ^{32}P-Labeled fructose 1,6-bisphosphatase, immunoprecipitated from hepatocyte extracts, had the same M_r as the purified rat liver enzyme in both systems (data not shown).

DISCUSSION

Glucagon has been shown to increase carbon flux through fructose 1,6-bisphosphatase in the intact rat hepatocyte (15), and to increase activity of the enzyme in hepatocyte extracts (16,17). The glucagon-induced increase in phosphorylation of the enzyme (Figs. 1 and 2) would seem to provide a mechanism for these effects. Consistent with these results is the observation that phosphorylation of the enzyme *in vitro* by the catalytic subunit of the cyclic AMP-dependent protein kinase is associated with a small increase in enzyme activity

(5). However, we have been unable to observe any glucagon-induced change in activity of the enzyme (data not shown). This may be due to the fact that the enzyme contains a relatively large amount of phosphate (2 mol/mol of enzyme) even in the absence of glucagon (Fig. 2).

While glucagon stimulates the phosphorylation of fructose 1,6-bisphosphatase, the concentration of hormone required for half-maximal stimulation of phosphorylation (> 1 nM; Fig. 2) was more than three times that needed for half-maximal stimulation of gluconeogenesis (0.3 nM)(18). The same high concentration of glucagon also was needed for half-maximal stimulation of phosphofructokinase phosphorylation (Fig. 2)(11). In this case, it has been shown that the glucagon-induced increase in phosphorylation of phosphofructokinase is not responsible for the inhibition of enzyme activity observed in crude hepatocyte extracts (11). Instead, glucagon causes inhibition of the enzyme by decreasing the level of fructose 2,6-bisphosphate, a potent activator of phosphofructokinase (19-22). This same effector also has been shown recently to be a potent inhibitor of fructose 1,6-bisphosphatase (23). Thus, it is possible that glucagon also may regulate the activity of fructose 1,6-bisphosphatease by virtue of its ability to lower the level of fructose 2,6-bisphosphate. The role of phosphorylation of fructose 1,6-bisphosphatase may be to offer a fine-tuning of the enzyme by altering the affinity of the enzyme for fructose 2,6-bisphosphate as has been proposed recently for phosphofructokinase (24). Studies on this question are in progress in our laboratory.

Different subunit molecular weights have been reported for fructose 1,6-bisphosphatase (see ref. 14). Part of these differences may be due to the different methods used (Fig. 1 vs Fig. 3). The differences may also be due to proteolysis. It is well known that fructose 1,6-bisphosphatase from several species is highly susceptible to attack by intracellular proteases (25). Early preparations of the enzyme were characterized by an alkaline pH optimum, and they were subsequently shown to be proteolytically modified at the NH_2-terminus of the molecule (25). Recent experiments suggest that proteolytic modi-

fication may also occur at the COOH-terminal region and that this may be important in regard to phosphorylation of the enzyme. Pilkis et al. (26) and Humble et al. (27) have shown that proteolytic digestion of the purified rat liver enzyme removes a peptide from the COOH-terminus that contains the site phosphorylated by cAMP-dependent protein kinase. Hosey et al. (14) confirmed this finding. They also found that fructose 1,6-bisphosphatase from pig kidney, and mouse and rabbit liver had a lower M_r than the rat liver enzyme. Likewise, a commerical preparation of rabbit muscle fructose 1,6-bisphosphatase had a lower M_r than the rat liver enzyme (Fig. 3). Just as we found for the rabbit muscle enzyme (5), Hosey et al. (14) found that the other fructose 1,6-bisphosphatases were not substrates for the cyclic AMP-dependent protein kinase. A possible explanation for these negative results is that a COOH-terminal peptide containing the phosphorylation site had been removed from the enzyme during purification. Cleavage of the COOH-terminal peptide apparently does not occur when the rat liver enzyme is purified by the method of Riou et al. (5) or when it is rapidly isolated by immunological methods (Fig. 1).

If the molecular weight of the fructose 1,6-bisphosphatase subunit is 41,000 as shown in Fig. 3 and suggested by Hosey et al. (14), it is possible that an increase in the phosphorylation of this enzyme by glucagon was also detected by Garrison and Borland (28). They observed that glucagon caused a 35 percent increase in the phosphorylation of a M_r 41,000 peptide isolated from the cytosol of rat hepatocytes by $NaDodSO_4$/slab gel electrophoresis but they did not identify this band.

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