An enzyme activity that catalyzes the hydrolysis of phosphate from the C-2 position of fructose 2,6-bisphosphate has been detected in rat liver cytoplasm. The \( K_{m} \) for fructose 2,6-bisphosphate was about 15 \( \mu \)M and the enzyme was inhibited by fructose 6-phosphate \( (K_{i} = 40 \mu M) \) and activated by P\(_i\) \( (K_{a} = 1 \text{ mM}) \). Fructose 2,6-bisphosphatase activity was purified to homogeneity by specific elution from phosphocellulose with fructose 6-phosphate and had an apparent molecular weight of about 100,000. 6-phosphofructo 2-kinase activity copurified with fructose 2,6-bisphosphatase activity at each step of the purification scheme. Incubation of the purified protein with \( \gamma^{32} \text{P}ATP \) and the catalytic subunit of ATPase resulted in an inhibition of 6-phosphofructo 2-kinase measured in cell extracts, suggesting that the hormone regulates the level of fructose 2,6-bisphosphate by affecting both synthesis and degradation of the compound. These findings suggest that this enzyme has both phosphorylase and phosphotransferase activities i.e. that it is bifunctional, and that both activities can be regulated by cAMP-dependent phosphorylation.

Fructose 2,6-bisphosphate plays an important role in the regulation of hepatic carbohydrate metabolism (1-5). Glucagon addition to isolated hepatocytes results in a decrease in the level of the compound (6-12) and to inactivation of the enzyme responsible for its synthesis, 6-phosphofructo 2-kinase (9-15). These results suggest that the effect of glucagon to lower fructose 2,6-P\(_2\) levels is due at least in part to a glucagon-induced phosphorylation and inactivation of 6-phosphofructo 2-kinase (10, 11). However, maximal concentrations of glucagon lower the level of fructose 2,6-P\(_2\) by greater than 90% within minutes (11) suggesting that the rate of degradation of the compound may also be affected. The enzyme responsible for the degradation of fructose 2,6-P\(_2\) has not yet been identified. The purpose of this study was to purify and characterize the enzyme which degrades fructose 2,6-P\(_2\) and to investigate its regulation by glucagon.

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**Preparation and Incubation of Isolated Hepatocytes—**Isolated hepatocytes were prepared from fed rats (male, Sprague-Dawley, 175-225 g) as previously described (16). After a 10-min incubation the cells (approximately 0.5-0.5 g of liver/flask) were homogenized for 90 s (30 s, three times) with an ultraturrax homogenizer in 10 ml of cold homogenizing buffer that contained 50 mM N-\{tris[hydroxymethyl]methyl-2-amino\}ethanesulfonic acid, pH 7.5, 50 mM KCl, 2 mM EDTA, 1 mM dithiothreitol, 0.2 mM phenylmethanesulfonyl fluoride and 0.5 \( \mu \)g/ml of leupeptin, and the homogenate was centrifuged at 30,000 \( \times \) g for 30 min. Solid (NH\(_4\))\(_2\)SO\(_4\) was added to the supernatant fraction to achieve 30% saturation and the precipitate was discarded. The supernatant fraction was then made 70% saturated with (NH\(_4\))\(_2\)SO\(_4\) and the precipitate redissolved in 1 ml of buffer A which contained 20 \( \mu \)M TES, pH 7.5, 50 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol, 0.5 \( \mu \)g/ml of leupeptin and 0.2 mM PMSF and the (NH\(_4\))\(_2\)SO\(_4\) was removed by dialysis against the same buffer. The (NH\(_4\))\(_2\)SO\(_4\)-treated hepatocyte extracts were then assayed for 6-phosphofructo 2-kinase activity, pyruvate kinase, and fructose 2,6-P\(_2\) degrading activity.

**Partition of Rat Liver Fructose 2,6-Bisphosphatase—**This enzyme was purified from rat liver by a modification of the method of El-Maghrabi et al. (11). This method was also used to purify 6-phosphofructo 2-kinase. The conditions of homogenization, polyethylene glycol fractionation and chromatography on DEAE sephadex A 50 were as previously described (11). The pooled enzyme fractions from the DEAE-Septadex step were then subjected to (NH\(_4\))\(_2\)SO\(_4\) fractionation and the 30 to 60 percent (NH\(_4\))\(_2\)SO\(_4\) pellet dissolved in buffer A (50 mM KCl and subjected to gel filtration on Sephacyr S-200 superfine (11). The pooled enzyme fractions (616 mg of protein and 700 milliunits of 6-phosphofructo 2-kinase) were then equilibrated with 20 mM TES, pH 7.5, 50 mM KCl, 1 mM dithiothreitol, 0.1 mM EDTA, 5% glycerol, 0.5 \( \mu \)g/ml of leupeptin, and 0.2 mM PMSF (buffer B) by ultrafiltration and dialysis and applied to a phosphocellulose column (2.5 X 8 cm). The column was washed with buffer until the absorbance at 280 nm fell below 0.02. 6-Phosphofructo 2-kinase and fructose 2,6-bisphosphatase activities co-eluted with 5 mM fructose 2,6-phosphate in buffer B. The protein appeared homogeneous by the criterion of SDS-disc gel electrophoresis and the specific activities of the purified enzyme after specific elution from phosphocelullose were 50 milliunits of 6-phosphofructo 2-kinase/mg of protein and 20 milliunits of fructose 2,6-bisphosphatase/mg of protein (measured at submaximal fructose 2,6-bisphosphate concentrations). The enzyme pool (3 mg) was dialyzed extensively against buffer A containing 20% glycerol in order to remove fructose 6-phosphate and stored at -20°C.

**Assay of 6-Phosphofructo 2-Kinase Activity—**This assay was assayed by measuring the production of \( ^{32} \text{P} \) from fructose 2,6-[\( ^{32} \text{P} \)P]2. Various concentrations were assayed by incubating various aliquots of liver extract or purified enzyme with 10 \( \mu \)M fructose 2,6-P\(_2\) and 5 \( \mu \)M MgCl\(_2\) in Buffer A. Aliquots were removed at 0, 5, 10, and 30 min and NaOH added to a final concentration of 0.25 M. The samples were then heated for 30 min at 90°C, neutralized, and assayed for fructose 2,6-P\(_2\) with the 6-phosphofructo 1-kinase activation assay. This assay is most sensitive when low concentrations of fructose 2,6-P\(_2\) (5-20 \( \mu \)M) are used. Consequently, fructose 2,6-bisphosphatase measurements by this assay were carried out at the submaximal concentration of 10 \( \mu \)M fructose 2,6-P\(_2\).

**Assay of Fructose 2,6-P\(_2\) in Hepatocytes—**Hepatocyte fructose 2,6-P\(_2\) levels were measured. The method described (12, 17).

**Assay of Fructose 2,6-Bisphosphatase Activity—**Fructose 2,6-bisphosphatase activity was assayed by incubating various aliquots of liver extract or purified enzyme with 10 \( \mu \)M fructose 2,6-P\(_2\) and 5 \( \mu \)M MgCl\(_2\) in Buffer A. Aliquots were removed at 0, 5, 10, and 30 min and NaOH added to a final concentration of 0.25 M. The samples were then heated for 30 min at 90°C, neutralized, and assayed for fructose 2,6-P\(_2\) with the 6-phosphofructo 1-kinase activation assay. This assay is most sensitive when low concentrations of fructose 2,6-P\(_2\) (5-20 \( \mu \)M) are used. Consequently, fructose 2,6-bisphosphatase measurements by this assay were carried out at the submaximal concentration of 10 \( \mu \)M fructose 2,6-P\(_2\).

Fructose 2,6-bisphosphatase was also assayed by measuring the production of \( ^{32} \text{P} \) from fructose 2,6-[\( ^{32} \text{P} \)P]2. Various concentrations...
of fructose 2,6-[2-32P]P2 (100–200 cpm/pmol) were incubated at 30 °C with enzyme in buffer A + 5 mM MgCl₂ for varying periods of time. The reaction was stopped by adding NaOH to a final concentration of 0.25 M and heating for 30 min at 90 °C. The mixture was then neutralized, diluted 10-fold with 20 mM triethylamine HCO₃ (TEA-HCO₃), pH 8.2, and applied to a DEAE-Sephadex column (0.7 × 5 cm). The column was then washed with 2 column volumes of 200 mM triethylamine HCO₃, pH 8.2, and the eluate collected directly into scintillation vials and counted for 32P radioactivity. Fructose 2,6-bisphosphatase activity was also assayed by measuring the production of fructose 6-phosphate spectrophotometrically as previously described for fructose 1,6-bisphosphatase (4). One unit of enzyme activity is the amount of fructose 2,6-bisphosphatase that catalyzes the hydrolysis of 1 μmol of fructose 2,6-P₂/min.

Preparation of Fructose 2,6-[2-32P]P₂—Fructose 2,6-P₂, labeled with 32P at the C-2 position was prepared by incubating 0.2 milliunits of 6-phosphofructo 2-kinase with 5 mM fructose 6-phosphate and 0.5 mM [γ-32P]ATP-Mg (2000–4000 cpm/pmol) in a total volume of 200 μl of buffer A for 2 h. Fructose 2,6-[2-32P]P₂, was purified by chromatography on DEAE-Sephadex as previously described (2).

Assay of Hepatocyte L-type Pyruvate Kinase Activity—Pyruvate kinase was assayed by previously described methods (16). Protein was assayed by the method of Lowry (18) with bovine serum albumin as a standard.

Materials—Rabbit muscle aldolase, triose phosphate isomerase, and a glycerol phosphate dehydrogenase were obtained from Boehringer Mannheim. Trisodium fructose 1,6-bisphosphate, fructose 6-phosphate and ATP were obtained from Sigma. [γ-32P]ATP was prepared by the method of Walseth and Johnson (19). Fructose 2,6-bisphosphate was prepared and measured as described by Fiklis et al. (2).

RESULTS

Identification and Isolation of Fructose 2,6-Bisphosphatase—Initial attempts to detect an activity which degraded fructose 2,6-P₂ in crude rat liver extracts were unsuccessful because of the presence of large amounts of endogenous fructose 2,6-P₂. An extract free of all low molecular weight effectors was prepared by (NH₄)₂SO₄ (0–70%) fractionation of a 100,000 × g supernatant fraction. When aliquots of this (NH₄)₂SO₄ treated extract were incubated with 10 μM fructose 2,6-P₂ and 5 mM MgCl₂ it was possible to detect the disappearance of fructose 2,6-P₂ with time by using the 6-phosphofructo 1-kinase activation assay (Fig. 1). No fructose 2,6-P₂ was degraded in the absence of extract. In preliminary attempts to purify this degrading activity, it was found to copurify with 6-phosphofructo 2-kinase activity. We then decided to use the purification scheme, previously described by El-Maghrabi et al. (11) for the kinase, to purify the degrading activity. The pooled enzyme fractions from the Sephacryl S-200 stage (700 milliunits of 6-phosphofructo 2-kinase) was applied to a phosphocellulose column and 6-phosphofructo 2-kinase and fructose 2,6-P₂ degrading activities eluted with 5 mM fructose 6-phosphate as described under "Experimental Procedures" (A). An aliquot of the enzyme pool from the phosphocellulose stage (20 milliunits of 6-phosphofructo 2-kinase) was concentrated by ultrafiltration to 1 ml and applied to a Sephadex G-100 superfine column (1.5 × 90 cm) equilibrated with buffer A + 50 mM KCl (B). Aliquots of the fractions were assayed for 6-phosphofructo 2-kinase activity with 5 mM fructose 6-phosphate (F₆P) and 2 mM ATP (C) and for fructose 2,6-bisphosphate degrading activity (F₂,6-P₂ase) with 10 μM fructose 2,6-P₂ (C) in a total volume of 100 μl as described under "Experimental Procedures."
Rat Liver Fructose 2,6-Bisphosphatase

Effect of cAMP-dependent phosphorylation on the activity of purified rat liver fructose 2,6-bisphosphatase and 6-phosphofructo 2-kinase

Twenty microliters of the enzyme preparation used in Table I were incubated with ATP (0.2 mM for the bisphosphatase experiment or 2 mM for the kinase experiment) and 20 units of the catalytic subunit of cAMP-dependent protein kinase (+ catalytic subunit) or an equivalent volume of protein kinase buffer (−catalytic subunit) for 15 min at 30°C. The enzymes were then assayed for their respective activities at the indicated substrate concentrations as described under "Experimental Procedures." Values represent averages of at least three experiments.

**Table I**

| Fructose 2,6-P2 concentration | Fructose 2,6-bisphosphatase activity |
|------------------------------|-------------------------------------|
| 10 μM                        | 1.7                                  |
| 100 μM                       | 22.5                                 |

| Fructose 6-phosphate concentration | 6-Phosphofructo 2-kinase activity |
|-----------------------------------|-----------------------------------|
| 100 μM                            | 2.5                               |
| 5 mM                              | 4.3                               |

**Table II**

| Fructose 2,6-P2 concentration | Phosphorylation of Fructose 2,6-bisphosphatase/6-Phosphofructo 2-Kinase | Fructose 2,6-bisphosphatase activity |
|-------------------------------|------------------------------------------------------------------------|-------------------------------------|
| 5 mM                          | +F6P* (20 μM)                                                          | 1.4                                 |
| 10                            | +F6P (40 μM)                                                           | 1.7                                 |
|                               | +F6P (100 μM)                                                          | 0.9                                 |
|                               | +P1 (1 μM)                                                             | 0.01                                |
|                               | +P1 (20 μM)                                                            | 1.8                                 |
|                               | +P1 (10 mM)                                                            | 3.6                                 |
|                               | +F6P (200 μM)                                                          | 4.9                                 |
| 20                            | +F6P (200 μM)                                                          | 13.1                                |
| 100                           | +F6P (200 μM)                                                          | 22.5                                |

* F6P, fructose 6-phosphate.
activity of pyruvate kinase, an enzyme known to be regulated by cyclic AMP-dependent phosphorylation. The concentration of glucagon that caused a 50% reduction in fructose 2,6-P₂ levels was 3 × 10⁻¹¹ M while that necessary for half-maximal inhibition of 6-phosphofructo 2-kinase activity was 7 × 10⁻¹¹ M. These concentrations of hormone are much lower than that necessary to produce half-maximal inhibition of pyruvate kinase (3 × 10⁻⁶ M) (Fig. 4) or half-maximal elevation of cAMP levels (3 × 10⁻⁶ M) (21). Fig. 4 also shows that glucagon enhanced the activity of fructose 2,6-bisphosphatase by about 5-fold and that the glucagon concentration giving half-maximal stimulation of this activity was the same as that necessary for half-maximal reduction of fructose 2,6-P₂ levels. Since fructose 2,6-bisphosphatase activity was measured in (NH₄)₂SO₄-treated extracts, it is likely that the glucagon effect involves covalent modification of the enzyme.

**DISCUSSION**

The present results provide evidence for the existence of a fructose 2,6-P₂ degrading activity in rat liver. This activity can be clearly designated as a fructose 2,6-bisphosphatase since a highly purified preparation of the enzyme was shown to hydrolyze phosphate from the C-2 position of the compound. Others (9, 10, 22) have also reported the existence of a fructose 2,6-P₂ degrading activity but did not show that it specifically removes phosphate from the C-2 position. These authors also claimed that glucagon had no effect on this activity. This activity was not due to fructose 1,6-bisphosphatase since 1) the purified fructose 2,6-bisphosphatase did not contain any measurable fructose 1,6-bisphosphatase activity (data not shown) and 2) fructose 1,6-bisphosphatase has been shown not to hydrolyze fructose 2,6-P₂ (1, 4). The fructose 2,6-bisphosphatase reaction is also dependent on divalent cations and is inhibited by micromolar concentrations of fructose 6-phosphate. Various estimates place the level of fructose 6-phosphate in liver cytosol at 100–200 μM and that of fructose 2,6-P₂ at 8–16 μM (12, 17). From the data in Table II, one would expect that fructose 6-phosphate would be an effective inhibitor of fructose 2,6-bisphosphatase in vivo.

Fig. 4 provides support for the hypothesis that glucagon not only regulates the enzyme responsible for synthesis of fructose 2,6-P₂, 6-phosphofructo 2-kinase, but that the hormone also activates fructose 2,6-bisphosphatase. This may explain the great sensitivity of hepatic fructose 2,6-P₂ levels to glucagon. Since these changes occur at concentrations of hormone which have no measurable effect on cAMP levels (1, 21) or on the inactivation of pyruvate kinase (16), glucagon may act on these enzymes by a cAMP-independent mechanism or by a cAMP-dependent mechanism where only very small, perhaps localized changes in cAMP, are necessary to induce phosphorylation of these enzymes. The results shown in Table II provide additional support for a role for cAMP in the regulation of these enzyme activities. Incubation of purified 6-phosphofructo 2-kinase/fructose 2,6-bisphosphatase with Mg-ATP and catalytic subunit of the cAMP-dependent protein kinase resulted in activation of the hydrolytic activity and inhibition of the kinase. Regardless of the mechanism, the ability of glucagon to affect these activities in a reciprocal manner provides an extremely rapid, efficient, and sensitive means for regulating hepatic glycolytic and gluconeogenic flux.

The results shown in Fig. 2 suggest that either the two enzymes are different proteins but very similar in terms of charge and size or that the two activities are present in the same enzyme (i.e. the enzyme is bifunctional). Support for the latter alternative includes the following: 1) both activities coeluted from a phosphocellulose column by fructose 6-phosphate; 2) the resulting preparation contained a single peptide band following SDS-disc gel electrophoresis; 3) both activities coeluted on a Sephadex G-100 column with an apparent molecular weight of about 100,000 (14); and 4) incubation of the enzyme preparation with catalytic subunit and [γ-³²P]ATP resulted in the phosphorylation of the peptide (Mᵣ = 50,000), concomitant with a reciprocal effect on the enzyme activities. However, the specific activity of the enzyme is low compared to that of other phosphoryl transferring enzymes, suggesting that the enzyme may have a low turnover number. Further studies on the characterization of the two activities and their interrelationship are in progress.

**FIG. 4. Effect of varying glucagon concentrations on hepatocyte 6-phosphofructo 2-kinase, fructose 2,6-bisphosphatase, and pyruvate kinase activities and on fructose 2,6-P₂ levels.** Hepatocytes from fed rats were incubated with increasing concentrations of glucagon. Hepatocytes were homogenized and treated with (NH₄)₂SO₄ as described under "Experimental Procedures." 6-Phosphofructo 2-kinase was measured by following fructose 2,6-P₂ production and is expressed as the ratio of activity at 0.5 mM and 5 mM fructose 6-phosphate. Pyruvate kinase was measured with 0.4 mM and 4 mM phosphoenolpyruvate. Fructose 2,6-bisphosphatase was measured with 10 μM fructose 2,6-P₂. Disappearance of fructose 2,6-P₂ was followed by the 6-phosphofructo 1-kinase activation assay. Hepatic fructose 2,6-P₂ levels were measured as described under "Experimental Procedures."
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