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In vivo and *in vitro* phosphorylation of rat liver fructose-1,6-bisphosphatase

(hexosediphosphatase/protein kinase/gluconeogenic enzyme/cyclic AMP)

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ABSTRACT Incorporation of ^{32}P from [γ-^{32}P]ATP into a homogeneous preparation of rat hepatic fructose-1,6-bisphosphatase (D-fructose-1,6-bisphosphatase 1-phosphohydrolase, EC 3.1.3.11) was catalyzed by a homogeneous preparation of the catalytic subunit of cyclic AMP-dependent protein kinase from bovine liver. Approximately 4 mol of phosphate were incorporated per mol of the tetrameric enzyme. This phosphorylation was associated with an increase in enzyme activity. In addition, *in vivo* phosphorylation of the enzyme was observed after injection of radioactive inorganic phosphate into rats and subsequent isolation of the enzyme by conventional purification methods and by immunoprecipitation. All of the labeled phosphate incorporation into the enzyme, both *in vitro* and *in vivo*, was precipitated by antibody specific for the enzyme. Furthermore, the $^{32}P_i$ counts were coincident with the enzyme subunit band when the immunoprecipitates were examined by sodium dodecyl sulfate/disc gel electrophoresis. Acid hydrolysis of the immunoprecipitated enzyme that was phosphorylated *in vitro* revealed that only seryl residues were labeled. On the basis of the concentration of protein kinase (0.2–1.0 μM) necessary to phosphorylate physiological amounts of fructose-1,6-bisphosphatase (1.0–4.0 μM), it is suggested that cyclic AMP-dependent protein kinase may catalyze the phosphorylation of fructose-1,6-bisphosphatase *in vivo*.

Neither the mechanism nor the site(s) of action of glucagon on gluconeogenesis has been clearly defined. An attractive hypothesis is that the hormone acts by affecting phosphorylation of an enzyme(s) in the pathway. Recently, it was reported that the activity of and the flux through L-pyruvate kinase, phosphofructokinase, and fructose-1,6-bisphosphatase (Fru-P_2ase; D-fructose-1,6-bisphosphate 1-phosphohydrolase, EC 3.1.3.11) are affected by glucagon (1–8). Both pyruvate kinase and phosphofructokinase are phosphorylated *in vitro* by protein kinases (9, 10). However, neither enzyme has been shown to be phosphorylated *in vivo*. The present work provides evidence that rat hepatic Fru-P_2ase is phosphorylated *in vivo* and that phosphorylation of the enzyme *in vitro* can be catalyzed by the catalytic subunit of a cyclic AMP (cAMP)-dependent protein kinase, with an associated increase in enzyme activity.

MATERIALS AND METHODS

Purification and Assay of Hepatic Fru-P_2ase. Minced rat livers (80 g) were added to 2 volumes (wt/vol) of 0.25 M sucrose/20 mM Na, K phosphate/0.1 mM dithiothreitol/1 mM EDTA, pH 7.4, and homogenized in a Waring blender for 2 min. The homogenate was centrifuged at 30,000 × *g* for 45 min. The supernatant was then rapidly heated to 65° for 5 min and immediately cooled to 4° by the method of Carlson *et al.* (11). After centrifugation the enzyme activity was recovered from the supernatant in a 30–50% ammonium sulfate pellet and then dissolved and equilibrated in 20 mM Na, K phosphate/0.1 mM dithiothreitol, pH 7.4, by gel filtration. The enzyme was then applied to a 2.5 × 35 cm DEAE-cellulose column and eluted with a 2-liter gradient of 20–160 mM Na, K phosphate/0.1 mM dithiothreitol, pH 7.4. The peak fractions (specific activity, 6 units/mg), eluting between 70 and 90 mM phosphate, were pooled and concentrated by ultrafiltration (Amicon PM 10). The enzyme was then equilibrated with 5 mM malonate/0.1 mM dithiothreitol, pH 5.8, by gel filtration and applied to a 0.6 × 15 cm CM-cellulose column. Fru-P_2ase was specifically eluted with 2 mM fructose bisphosphate (12) in 5 mM malonate buffer, pH 6.0. The enzyme solution was concentrated to 5 mg/ml and stored at −70° in 5 mM malonate buffer (pH 6.0) containing 1 mM dithiothreitol.

Fru-P_2ase was assayed essentially by the spectrophotometric method of Pontremoli *et al.* (13). The assay mixture (1 ml) contained, unless otherwise indicated, 100 mM Tris·HCl (pH 7.5), 2 mM $MgCl_2$, 2 mM $(NH_4)_2SO_4$, 2.5 mM 2-mercaptoethanol, 0.05 mM EDTA, 0.2 mM $NADP^+$, 2 μg of glucose-6-phosphate dehydrogenase, and 2 μg of phosphoglucose isomerase. The enzyme was preincubated for 5 min at 30° in this solution and the reaction was started by the addition of fructose bisphosphate (70 μM). One unit of enzyme is defined as the amount that catalyzes the hydrolysis of 1 μmol of fructose bisphosphate per min at 30°.

Protein was estimated by the method of Lowry *et al.* (14) with bovine serum albumin as a standard. Rat hepatic Fru-P_2ase has been reported to contain no tryptophan (15). The protein content of homogeneous Fru-P_2ase was calculated from the $E^{1\%}_{1\,cm}$ of 6.3 at 280 nm for pure enzyme as described by Tejwani *et al.* (15).

Purification of the Catalytic Subunit of cAMP-Dependent Protein Kinase from Bovine Liver. Homogeneous catalytic subunit of bovine liver cAMP-dependent protein kinase (type II) was prepared by the method of Sugden *et al.* (16). The specific activity was 3×10^6 units/mg (1 unit is the amount of enzyme that catalyzes the transfer of 1 pmol of ^{32}P from [γ-^{32}P]ATP to histone per min at 30°). Homogeneous catalytic subunit of bovine heart was prepared by the same method (16). It had similar specific activity and properties (17) as the liver enzyme. The catalytic subunit of either preparation was free of Fru-P_2ase and phosphofructokinase activities.

Phosphorylation of Fru-P_2ase. Homogeneous rat liver Fru-P_2ase (30–60 μg) was incubated at 30° in a final volume of 0.09 ml that contained 20 mM Tris·HCl (pH 7.4), 35 mM K

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Abbreviations: Fru-P_2ase, fructose-1,6-bisphosphatase (D-fructose-1,6-bisphosphate 1-phosphohydrolase, EC 3.1.3.11); cAMP, cyclic AMP; $NaDodSO_4$, sodium dodecyl sulfate; $S_{0.5}$, substrate concentration giving half-maximal velocity.

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Table 1. Purification of rat hepatic Fru-P_2ase

Stage	Fru-P_2ase, units	Protein, mg	Specific activity, units/mg protein	Yield, %
Homogenate	1235	14,700	0.08	—
Heat step	974	2,877	0.34	79
$(NH_4)_2SO_4$ step (30–50%)	750	582	1.30	61
Sephadex G-25	744	501	1.50	60
DEAE-cellulose	412	70	5.90	33
CM-cellulose	312	6.8	46.00	30

Livers (80 g wet weight) from eight Sprague–Dawley rats fed Purina Laboratory Chow *ad libitum* were used. Except for the second step, all procedures were performed at 4°. The enzyme was specifically eluted from the CM-cellulose with 2 mM fructose 1,6-bisphosphate.

phosphate, 10 mM 2-mercaptoethanol, 6 mM $MgCl_2$, 0.3 mM [γ-^{32}P]ATP (50–200 cpm/pmol), and catalytic subunit of cAMP-dependent protein kinase (1000–10,000 units). Incorporation [^{32}P]P into Fru-P_2ase was estimated by the method of Corbin and Reimann (18). The number of moles of ^{32}P incorporated per mol of enzyme was calculated on the basis of a molecular weight of 144,000 for Fru-P_2ase (15).

Estimation of ^{32}P Radioactivity in Fru-P_2ase by Immunoprecipitation and Gel Electrophoresis. Fru-P_2ase was isolated from the phosphorylation mixture by immunoprecipitation using a double antibody technique. Twice the equivalence point of rabbit antiserum specific for rat liver Fru-P_2ase was added to a known amount of Fru-P_2ase. After 2–3 hr of incubation at 0°, goat antirabbit serum was added and the mixture was incubated overnight at 0–4°. Controls were run with normal rabbit serum instead of antiserum to Fru-P_2ase. The immunoprecipitates were washed extensively, dissolved in a buffer containing (final concentrations) 1% sodium dodecyl sulfate ($NaDodSO_4$), 40 mM dithiothreitol, 1 mM EDTA, pyronin Y (10 μg/ml), and 10 mM Tris·HCl (pH 8.0), and incubated for 30 min at 70°. The samples were then subjected to $NaDodSO_4$/polyacrylamide electrophoresis (19).

Isolation of Phosphoserine from Phosphorylated Fru-P_2ase. After phosphorylation of the enzyme by the catalytic subunit of protein kinase from bovine liver, ^{32}P-labeled Fru-P_2ase was separated from [γ-^{32}P]ATP and catalytic subunit by immunoprecipitation as described above. Phosphoseryl residues were isolated from phosphorylated Fru-P_2ase essentially by the method of Schaffer (20).

Materials. Preswollen DEAE-cellulose (DE 52) and CM-cellulose (CM 52) were obtained from Whatman Ltd., Sephadex G-25 came from Pharmacia Fine Chemicals. Glucose-6-phosphate dehydrogenase and phosphoglucose isomerase were from Boehringer-Mannheim. Muscle Fru-P_2ase and *o*-phosphoserine came from Sigma. [^{32}P]Phosphoric acid in water was obtained from Amersham/Searle. Antibody to hepatic Fru-P_2ase was the kind gift of B. L. Horecker. Glucagon was a gift from Eli Lilly and Co. All other reagents were of the highest purity commercially available.

RESULTS

Characteristics of Rat Hepatic Fru-P_2ase. Table 1 summarizes the purification of rat hepatic Fru-P_2ase. The entire procedure could be completed in 2 days, with an overall recovery of 20–40% and a specific activity varying from 34 to 46 units/mg of protein. The ratio of activity at pH 7.5 to activity at pH 9.2 was 3.4, indicating that little or no proteolysis occurred during the purification (21). The enzyme appeared to be homogeneous on $NaDodSO_4$/disc gels (e.g., see Fig. 3) and the molecular weight of the enzyme subunit was 36,000. It gave a single precipitin line against antiserum to rat liver Fru-P_2ase in an Ouchterlony double-diffusion test (data not shown). The purified enzyme showed half-maximal velocity with fructose 1,6-bisphosphate at 5 μM $S_{0.5}$, and concentrations greater than 90 μM inhibited enzyme activity. The apparent K_i for AMP was 23 μM. This preparation, which contained no detectable pyruvate kinase, histone kinase, or phosphofructokinase activity, was used in the subsequent *in vitro* phosphorylation experiments.

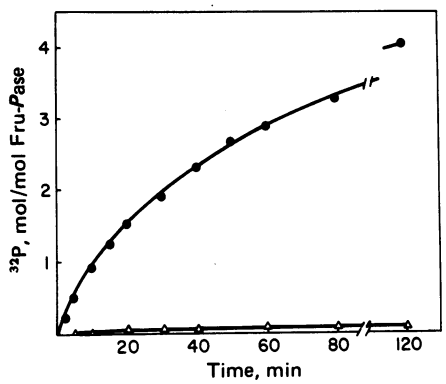

FIG. 1. Time course of phosphorylation of Fru-P_2ase by the catalytic subunit of cAMP-dependent protein kinase from bovine liver: 2.5 units (60 μg or 4 μM) of rat liver Fru-P_2ase (●) or 2.5 units of rabbit muscle Fru-P_2ase (△) incubated with 5000 units of catalytic subunit (0.46 μM). At the indicated times, 5-μl aliquots were withdrawn and the ^{32}P incorporation into Fru-P_2ase was determined.

***In Vitro* Phosphorylation of Hepatic Fru-P_2ase.** The time course of phosphorylation of Fru-P_2ase by the catalytic subunit of cAMP-dependent protein kinase from bovine liver is shown in Fig. 1. The incorporation of 1 mol of phosphate per mol of Fru-P_2ase occurred in about 10 min. A maximum of 4 mol of phosphate per mol of enzyme was reached in about 2 hr. The curvilinearity could be accounted for in part by the lability of the bovine liver catalytic subunit under the conditions used. Because Fru-P_2ase is a tetramer (15), there is apparently 1 mol of phosphate incorporated per mol of enzyme subunit. No incorporation was observed when the Fru-P_2ase was incubated without the catalytic subunit. Partially purified rabbit skeletal muscle Fru-P_2ase (11 units/mg of protein) was not phosphorylated under the same conditions. No phosphorylation of the muscle enzyme was observed even after AMP and other low molecular weight effectors were removed by gel filtration. The muscle Fru-P_2ase contained no protein kinase inhibitor activity.

The phosphorylation of hepatic Fru-P_2ase was dependent on the concentrations of both catalytic subunit and and Fru-P_2ase. When 0.1, 0.2, 0.4, and 0.8 μM concentrations of catalytic subunit were incubated for 20 min with 4 μM Fru-P_2ase, 0.15, 0.40, 1.0, and 2.2 mol of phosphate were incorporated per mol of Fru-P_2ase, respectively. This range of concentrations is about the same as the calculated concentration of protein kinase (0.2–1 μM) in the intact cell (16). Increasing the concentration of Fru-P_2ase also increased the rate of its phosphorylation but even at a low concentration of catalytic subunit (0.1 μM), maximal rates of phosphorylation were not obtained (Fig. 2). Addition of AMP (1–100 μM) or fructose 1,6-bisphosphate (1–100 μM) or both had no effect on the rate of phosphorylation. The phosphorylation was completely blocked by the addition of either the protein inhibitor of protein kinase or the regulatory subunit of type I bovine heart protein kinase. The latter effect

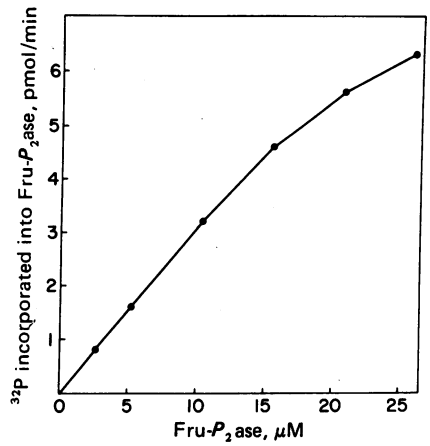

FIG. 2. Effect of Fru-P_2ase concentration on its phosphorylation by the bovine liver catalytic subunit of cAMP-dependent protein kinase. Varying amounts of hepatic Fru-P_2ase were incubated with 380 units of catalytic subunit, 240 nmol of $MgCl_2$, and 12 nmol of [γ-^{32}P]ATP in a total volume of 40 μl. The amount of ^{32}P incorporation into Fru-P_2ase was determined by the method of Corbin and Reimann (18).

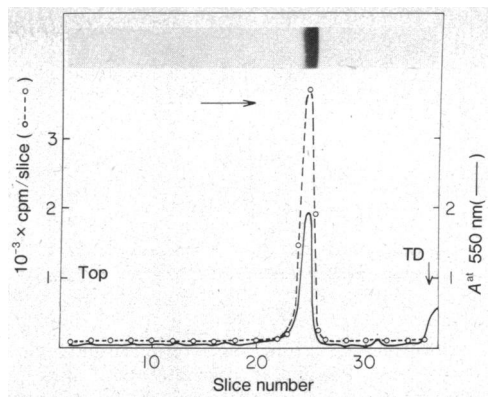

FIG. 3. NaDodSO$_4$/disc electrophoresis of an immunoprecipitate of ^{32}P-labeled Fru-P_2ase. Homogeneous rat hepatic Fru-P_2ase (80 μg) was incubated for 80 min with the liver catalytic subunit. Fru-P_2ase was precipitated with a specific antibody, and the immunoprecipitate was subjected to electrophoresis. The radioactivity profile (O) of the gel was obtained by assaying 2-mm sections. Homogeneous Fru-P_2ase (30 μg), obtained after purification (Table 1), was subjected to electrophoresis at the same time to serve as a marker for the subunits of the enzyme. After staining (17), the gel was photographed and scanned at 550 nm. The molecular weight of the protein (36,000) was determined by comparison of its relative mobility to that of proteins of known molecular weight. TD indicates the position of the tracker dye.

was prevented by cAMP (D. Flockhart, J. P. Riou, S. J. Pilkis, and J. Corbin, unpublished data).

In order to ensure that the ^{32}P incorporation was actually into hepatic Fru-P_2ase and not into the catalytic subunit itself or possible proteolytic degradation products of the Fru-P_2ase, the enzyme was isolated from the phosphorylation mixture by immunoprecipitation. All of the radioactivity incorporated into Fru-P_2ase, as measured by acid precipitation of the enzyme, was recovered in the immunoprecipitate. Analysis of the immunoprecipitate on NaDodSO$_4$/disc gels (Fig. 3) revealed that all the radioactivity coincided with the enzyme subunit band with a molecular weight of 36,000. In separate experiments, no ^{32}P was found in the catalytic subunit under these conditions.

Effect of Phosphorylation on Fru-P_2ase Activity. The changes in Fru-P_2ase activity upon incubation with catalytic subunit, Mg^{2+}, and ATP are shown in Fig. 4. The enzyme activity was increased in a time-dependent manner. The increase in activity was about 40% and was dependent on the presence of both catalytic subunit and ATP. Under the conditions of this experiment (top curve), 4 mol of phosphate per mol of Fru-P_2ase were incorporated in 30 min. No obvious effect of phosphorylation on the $S_{0.5}$ for fructose 1,6-bisphosphate was observed. However, the reaction rates were difficult to measure with substrate concentrations in the range of the $S_{0.5}$ (2–5 μM) and small changes may have been missed. Phosphorylation of the enzyme had no apparent effect on the K_i for AMP.

The Site of Phosphorylation of Fru-P_2ase. In order to study the site of phosphate incorporation into Fru-P_2ase, the ^{32}P-labeled Fru-P_2ase immunoprecipitate was hydrolyzed in 6 M HCl for 20 hr at 100°. Chromatography of the acid hydrolysate on Dowex 50 columns yielded only two radioactive peaks. The initial peak was inorganic phosphate-^{32}P. When the second peak was rechromatographed on Dowex 50, the radioactivity coincided with the peak of *o*-phosphoserine (Fig. 5). No evidence of phosphothreonine was found. Thus, it appears that only seryl residues of Fru-P_2ase are accessible to phosphorylation under the present conditions. The phosphorylated site(s) appears to reside in a 6000–7000 molecular weight peptide that can be isolated after treatment with cyanogen bromide (unpublished data).

***In Vivo* Phosphorylation of Hepatic Fru-P_2ase.** The possibility that hepatic Fru-P_2ase is phosphorylated *in vivo* was investigated. Rats were injected with 1 mCi of phosphate-^{32}P, and Fru-P_2ase was isolated by two different methods. First, Fru-P_2ase was purified to homogeneity by the purification scheme described in Table 1. Fig. 6 shows the elution pattern from the CM-cellulose column. The single peak of enzyme activity (specific activity, 34 units/mg at 30°) coeluted with the peak of protein and the peak of radioactivity. Because only Fru-P_2ase is eluted from this column by fructose 1,6-bisphosphate (see gel photograph, Fig. 3), this experiment clearly demonstrates that Fru-P_2ase is phosphorylated *in vivo*. In order to strengthen this conclusion, Fru-P_2ase was also isolated by immunoprecipitation with a double antibody technique. The Fru-P_2ase was immunoprecipitated with a specific antiserum

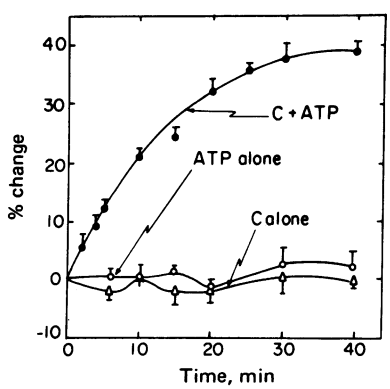

FIG. 4. Time course of the effect of phosphorylation on Fru-P_2ase activity. Fru-P_2ase (20 μg) was incubated with or without the catalytic subunit (C) from bovine heart (8000 units). In addition, 2 mM fructose 1,6-bisphosphate was added to this incubation. At the indicated times, duplicate aliquots (5 μl) were withdrawn and immediately assayed for Fru-P_2ase activity. The assay was started by the addition of the enzyme to the assay mixture containing 70 μM fructose 1,6-bisphosphate and 5 mM 2-mercaptoethanol but no EDTA. Each point represents the mean ± SD of the percentage change in enzyme activity (relative to the zero-time control) from four different experiments. The reproducibility of the assay was ±3–5%. When ATP was present, the concentration of ATP carried over from the phosphorylation incubation mixture to the Fru-P_2ase assay was 1.5 μM.

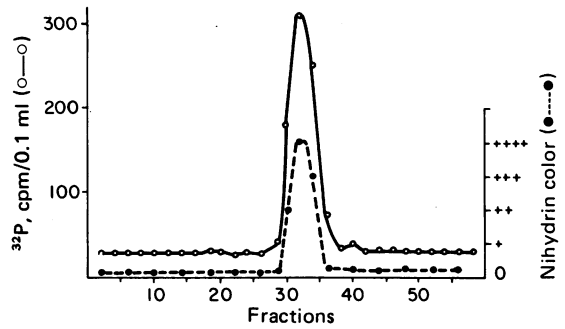

FIG. 5. Dowex 50-X8 chromatography of an acid hydrolysate of ^{32}P-labeled Fru-P_2ase. The immunoprecipitate of 50 μg of ^{32}P-labeled Fru-P_2ase was dissolved in 2 ml of 6 M HCl and hydrolyzed for 20 hr at 100°. After evaporation, the residue was dissolved in 1 ml of 50 mM HCl and the radioactive inorganic phosphate was separated from ^{32}P-labeled amino acid residues by chromatography on Dowex 50-X8 (18). The ^{32}P-labeled amino acid residue was collected, evaporated to dryness, and resuspended in 50 mM HCl. Thirty mg of unlabeled phosphoserine (30 mg) was added, and the sample was applied to another Dowex 50-X8 column (1 × 45 cm) and eluted with 50 mM HCl. Radioactivity in the fractions (o — o) was monitored by liquid scintillation and the elution position of phosphoserine (● — ●) was identified by spotting 50 μl of each fraction onto filter paper and spraying with ninhydrin. The color was estimated subjectively, with ++++ representing the maximal intensity observed.

after the ammonium sulfate step in the purification scheme. When the immunoprecipitate was examined by NaDodSO$_4$/disc gel electrophoresis, only one peak of ^{32}P radioactivity was observed (Fig. 7). It comigrated with the homogeneous Fru-P_2ase subunit band and had an apparent molecular weight of 36,000. When *in vivo* labeled hepatic Fru-P_2ase was isolated from animals injected with saline and compared to that from animals injected with glucagon (125 μg/100 g of body weight), no obvious difference was found in the amount of incorporation of ^{32}P into the enzyme (data not shown).

It might be argued that Fru-P_2ase was phosphorylated during the isolation procedure and not *in vivo*. To test this possibility, sufficient tracer [γ-^{32}P]ATP was added to a liver homogenate to give a specific activity of ATP that was the same as that found when [^{32}P]P_i was injected *in vivo*. Fru-P_2ase was

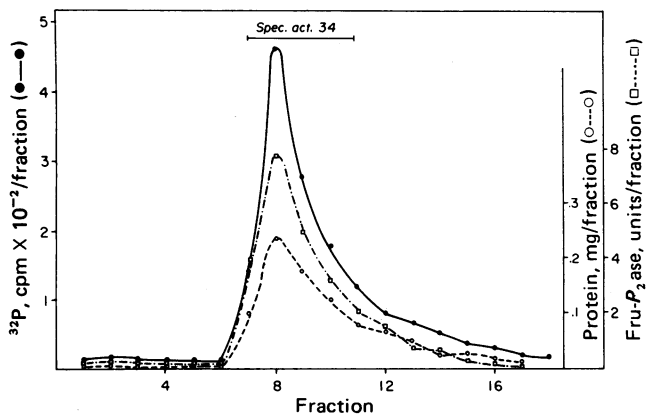

FIG. 6. Chromatography of rat hepatic Fru-P_2ase, labeled *in vivo*, on CM-cellulose. A 200-g rat was fasted overnight and then given 0.45 mCi of carrier-free phosphate-^{32}P intraperitoneally 2.5 hr before sacrifice. An equal dose of ^{32}P was given 30 min before sacrifice and sodium pentobarbital (15 mg) was given 5 min before sacrifice. Liver Fru-P_2ase was purified as described in Table 1. All solutions contained 50 mM NaF until the DEAE-cellulose stage. The enzyme was eluted from the CM-cellulose column (0.4 × 5 cm) with 2 mM fructose 1,6-bisphosphate starting at fraction 1. The volume of each fraction was 0.25 ml.

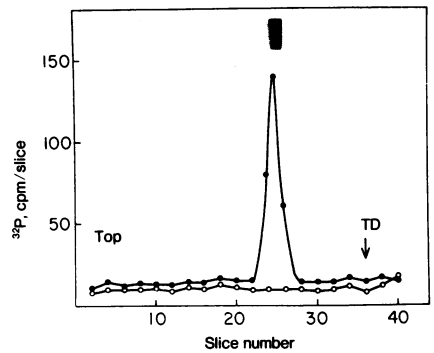

FIG. 7. NaDodSO$_4$/gel electrophoresis of an immunoprecipitate of rat hepatic ^{32}P-labeled Fru-P_2ase labeled *in vivo* as described in Fig. 6. Hepatic Fru-P_2ase was purified as described in Table 1 but with 50 mM NaF present in all solutions. The ammonium sulfate pellet was equilibrated in 20 mM Na, K phosphate/0.1 mM dithiothreitol, pH 7.4, by gel filtration on Sephadex G-25. The Fru-P_2ase (2 units) was then treated with either specific antibody (● — ●) or normal rabbit serum (o — o). The immunoprecipitates were treated as described in Fig. 3. Homogeneous Fru-P_2ase (30 μg), obtained by the purification scheme described in Table 1 and treated as described in Fig. 3, served as a marker for the enzyme subunits (photograph).

then purified, immunoprecipitated, and subjected to NaDodSO$_4$/disc gel electrophoresis as described in Fig. 6. No radioactivity was found in the Fru-P_2ase subunit band (data not shown). Thus, Fru-P_2ase is not phosphorylated during the isolation procedure.

DISCUSSION

This report presents clear evidence that the catalytic subunit of the cAMP-dependent protein kinase catalyzes the phosphorylation of rat hepatic Fru-P_2ase *in vitro* (Fig. 1). The results of NaDodSO$_4$/disc gel electrophoresis of the phosphorylated enzyme rule out the possibility that the radioactivity associated with the enzyme was due to phosphorus-containing compounds that were bound noncovalently. The complete inhibition of this phosphorylation by protein kinase inhibitor or by the addition of homogeneous regulatory subunit from bovine heart cAMP-dependent (type I) protein kinase provides definitive evidence that it is cAMP-dependent. The *in vitro* phosphorylation of Fru-P_2ase is also dependent upon the concentration of protein kinase. If the activity of the protein kinase is expressed in terms of its ability to catalyze phosphorylation of histone, 1000–10,000 units/assay was routinely used. It should be emphasized that these protein kinase concentrations are in the physiological range (16). The need to use these levels of protein kinase may explain why previous attempts to phosphorylate rabbit hepatic Fru-P_2ase were unsuccessful (22). Mendicino *et al.* (23) suggested that swine kidney Fru-P_2ase also can be phosphorylated. With physiological Fru-P_2ase concentrations, it can be calculated that the rate of phosphorylation is 2% that of the optimal rate of histone phosphorylation. The phosphorylation of the trypsin-sensitive site of muscle glycogen synthase (24) and the phosphorylation of liver pyruvate kinase (2) occur, in our experience, at a rate similar to that of Fru-P_2ase.

The ability of the catalytic subunit to catalyze phosphorylation of hepatic Fru-P_2ase but not muscle Fru-P_2ase suggests some specificity for the phosphorylation reaction. Supporting this is our recent observation that the catalytic subunit does not catalyze phosphorylation of purified preparations of muscle aldolase, muscle phosphorylase, or liver phosphoenolpyruvate carboxykinase (unpublished data).

The cAMP-dependent protein kinase may also be involved

in the *in vivo* phosphorylation of Fru-P_2ase (Figs. 6 and 7). Physiological concentrations of this kinase will catalyze phosphorylation of Fru-P_2ase *in vitro* when the substrate is at concentrations one might expect *in vivo* (Fig. 2).‡ However, other kinases have been shown to catalyze phosphorylation of hepatic glycogen synthase (25), although one such cAMP-independent kinase did not catalyze phosphorylation of hepatic Fru-P_2ase (26).

Phosphorylation of Fru-P_2ase is associated with a 40% increase in enzyme activity measured in the absence of EDTA. This effect is relatively small, and it is possible that optimal assay conditions for demonstrating it have not yet been found. Hepatic Fru-P_2ase is affected by a number of substances (15, 27, 28) whose interaction with Fru-P_2ase may be affected by phosphorylation of the enzyme. Alternatively, hepatic Fru-P_2ase, as currently prepared, may have altered regulatory properties which would tend to obscure somewhat a phosphorylation-induced activity change.

Recent reports indicate that hormones may regulate gluconeogenesis by controlling flux through the enzymes of the substrate cycle between pyruvate and phosphoenolpyruvate and between fructose 6-phosphate and fructose 1,6-bisphosphate (1, 5–7). The phosphorylation of pyruvate kinase (2, 9), phosphofructokinase (10), and now Fru-P_2ase (29) suggests that the activity of these enzymes may be controlled, at least in part, by a phosphorylation/dephosphorylation mechanism. These three enzymes share several features. They are tetrameric enzymes that behave allosterically with regard to substrate or effectors (30). They are inhibited by ATP and are affected by fructose bisphosphate (30). They can be phosphorylated *in vitro*, with 4 mol of phosphate being incorporated per mol of enzyme (9, 12, 29). In the case of pyruvate kinase and phosphofructokinase, the effect of phosphorylation on enzyme activity is overcome by fructose bisphosphate (2, 9, 10). The level of this intermediate, which is controlled by the activity of Fru-P_2ase and phosphofructokinase, is decreased upon addition of glucagon to intact hepatocytes (1). Injection of glucagon or insulin *in vivo* acutely affected Fru-P_2ase activity in rat liver (8). Thus, the possible hormonal regulation of Fru-P_2ase by phosphorylation may be an important factor in the hormonal regulation of substrate cycling of gluconeogenesis. In view of the present demonstration that Fru-P_2ase is phosphorylated *in vivo* (Figs. 6 and 7), the possibility that the hormone-induced activity changes are related to phosphorylation/dephosphorylation of Fru-P_2ase is an attractive hypothesis.

The authors are particularly grateful to Dr. B. L. Horecker for providing the specific antiserum to Fru-P_2ase in these studies. We thank Ms. J. Pilkis, Ms. N. Nasr, and Mr. Keith Anderson for their technical assistance. This research was supported by National Institutes of Health Grants AM 18270 and AM 15988, Vanderbilt University Diabetes-Endocrinology Center Grant AM 17026, and by a grant to S.J.P. from the American Diabetes Association. J.P.R. was supported by a Fellowship from "Ministere des Affaires Etrangeres," France. S.J.P. and J.D.C. are Career Investigators of the Howard Hughes Medical Institute.

‡ The *in vivo* concentration of Fru-P_2ase is estimated to be 2–4 μM, assuming a specific activity of 40 units/mg of protein, a molecular weight of 144,000, and 0.5 ml of water per g of wet liver.

1. Pilkis, S. J., Riou, J. P. & Claus, T. H. (1976) *J. Biol. Chem.* **251**, 7841–7852.
2. Riou, J. P., Claus, T. H. & Pilkis, S. J. (1976) *Biochem. Biophys. Res. Commun.* **73**, 591–600.
3. Feliu, J. F., Hue, L. & Hers, H. G. (1976) *Proc. Natl. Acad. Sci. USA* **73**, 2762–2766.
4. Blair, J. B., Cimbala, M. H., Foster, J. L. & Morgan, R. H. (1976) *J. Biol. Chem.* **251**, 3756–3762.
5. Clark, M. G., Kneer, N. M., Bosch, A. L. & Lardy, H. A. (1974) *J. Biol. Chem.* **249**, 5695–5703.
6. Dunn, A., Chenoweth, M. & Bever, K. (1976) *Fed. Proc.* **35**, 1427.
7. Rognstad, R. & Katz, J. (1976) *Arch. Biochem. Biophys.* **177**, 337–345.
8. Taunton, O. D., Stifel, F. B., Greene, H. L. & Herman, R. H. (1974) *J. Biol. Chem.* **249**, 7228–7239.
9. Titanji, V. P. K., Zetterqvist, O. & Engstrom, L. (1976) *Biochim. Biophys. Acta* **422**, 98–108.
10. Brand, I. A. & Soling, H. D. (1975) *FEBS. Lett.* **57**, 163–168.
11. Carlson, C. W., Baxter, R. C., Ulm, E. H. & Pogell, B. M. (1973) *J. Biol. Chem.* **248**, 5555–5561.
12. Pogell, B. M. (1966) in *Methods in Enzymology*, ed. Wood, W. A. (Academic Press, New York), Vol. 9, pp. 9–15.
13. Pontremoli, S., Traniello, S., Luppis, B. & Wood, W. A. (1965) *J. Biol. Chem.* **240**, 3459–3463.
14. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275.
15. Tejwani, J. A., Pedrosa, F. O., Pontremoli, S. & Horecker, B. L. (1976) *Arch. Biochem. Biophys.* **177**, 253–264.
16. Sugden, P., Holloday, L., Reimann, E. M. & Corbin, J. (1976) *Biochem. J.* **159**, 409–422.
17. Beavo, J. A., Bechtel, P. J. & Krebs, E. G. (1975) *Adv. Cyclic Nucleotide Res.* **5**, 241–251.
18. Corbin, J. & Reimann, E. H. (1974) in *Methods in Enzymology*, eds. Hardman, J. G., O'Malley, B. W. (Academic Press, New York), Vol. 38, part C, pp. 287–299.
19. Fairbanks, G., Steck, T. H. & Wallach, D. F. H. (1971) *Biochemistry* **10**, 2606–2615.
20. Schaffer, N. K. (1967) in *Methods in Enzymology*, ed. Hirs, C. H. W. (Academic Press, New York), Vol. 11, pp. 702–705.
21. Traniello, S., Pontremoli, S., Tashima, Y. & Horecker, B. L. (1971) *Arch. Biochem. Biophys.* **146**, 161–166.
22. Taketa, K., Sarngadharan, M. G., Watanabe, A., Aoe, H. & Pogell, B. M. (1971) *J. Biol. Chem.* **246**, 5676–5683.
23. Mendicino, J., Beaudreau, C. & Bhattacharryya, R. N. (1966) *Arch. Biochem. Biophys.* **116**, 436–447.
24. Soderling, T. R. (1975) *J. Biol. Chem.* **250**, 5407–5612.
25. Jett, M. F. & Soderling, T. (1977) *Fed. Proc.* **36**, 777.
26. Maragoudakis, M. E. & Hankins, H. (1977) *Biochim. Biophys. Acta* **480**, 122–137.
27. Horecker, B. L., Melloni, E. & Pontremoli, S. (1975) in *Advances in Enzymology and Related Subjects of Biochemistry*, ed. Meister, A. (Wiley, New York), Vol. 42, pp. 193–226.
28. Tejwani, G. A., Pedrosa, F. O., Pontremoli, S. & Horecker, B. L. (1976) *Proc. Natl. Acad. Sci. USA* **73**, 2692–2695.
29. Riou, J. P., Claus, T. & Pilkis, S. J. (1977) *Fed. Proc.* **36**, 728.
30. Clark, M. G. & Lardy, H. A. (1975) in *M.T.P. International Review of Science Biochemistry of Carbohydrates*, ed. Whelan, W. J. (Academic Press, New York), Vol. 5, pp. 223–266.