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AN ENZYME THAT CATALYZES HYDROLYSIS OF FRUCTOSE-2,6-BISPHOSPHATE

Eisuke Furuya, Motoko Yokoyama and Kosaku Uyeda

From the Biochemistry Department at the University of Texas Health Science Center, 5323 Harry Hines Boulevard, Dallas, Texas 75235 and the Department of Cellular Regulation, Veterans Administration Medical Center, 4500 South Lancaster Road, Dallas, Texas 75216

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The enzyme which catalyzes the hydrolysis of fructose-2,6-P_2 has been partially purified from rat liver. The product has been identified as fructose-6-P. The enzyme requires Mg^{++}, and the $K_{0.5}$ for fructose-2,6-P_2 varies from 25 - 100 μM, depending upon the preparation. After treatment with cAMP dependent protein kinase the enzyme is activated and the $K_{0.5}$ for the substrate is lowered to 7 μM.

INTRODUCTION

Fructose-2,6-P_2 has been shown to be an important activator of phosphofructokinase (1-6). The synthesis of fructose-2,6-P_2 is catalyzed by fructose-6-P,2-kinase (7-10). Administration of glucagon and epinephrine to hepatocytes results in rapid inactivation of fructose-6-P,2-kinase and a concomittant decrease in the fructose-2,6-P_2 level (11). This enzyme has also been shown to be inactivated in vitro by cAMP dependent protein kinase (12,13) as well as phosphorylase kinase and activated by phosphatase (12).

We have reported the presence of enzymic activity in the extracts of hepatocytes and liver which catalyzes the degradation of fructose-2,6-P_2 (11). More recently we have shown that the degradative enzyme is activated in hepatocytes by glucagon or epinephrine (14). In this communication we report that the enzyme catalyzes the hydrolysis of

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fructose-2,6-P_2 to fructose-6-P and that it is activated by cAMP dependent protein kinase.

MATERIALS AND METHODS

[^{32}P]-ATP (3000 mc/mmol) was purchased from Amersham. [U^{14}C] Fructose-6-P (1.5 x 10^6 cpm/µmol) was prepared as described previously (15). [6-^{32}P] Fructose-6-P (3 x 10^7 cpm/µmol) was prepared with the same procedure as for [^{14}C] fructose-6-P except unlabeled fructose and [^{32}P] ATP were used. Fructose-2,6-P_2 was prepared as described previously (16). All other chemicals were reagent grade and obtained from commercial sources. The catalytic subunit of cAMP dependent protein kinase was a purchased from Sigma Chemical Company, St. Louis, MO.

Preparation of [^{14}C] Fructose-2,6-P_2 and [6^{32}P] Fructose-2,6-P_2. [^{14}C] Fructose-2,6-P_2 was prepared in a reaction mixture containing [U^{14}C] Fructose-6-P (8.7 x 10^5 cpm, 0.6 µmol), ATP (3 µmol), $MgCl_2$ (6 µmol) and Tris Cl (50 µmol) pH 7.4 in a final volume of 0.6 ml. The reaction was initiated with the addition of 2 units of fructose-6-P,2-kinase (7) and incubated for 1 hr at 30°. At the end of the incubation the reaction mixture was directly spotted onto a Whatman 3 MM paper, and the paper developed in ethyl alcohol:conc. NH_4OH:H_2O (15:9:1) for 18 hrs at 25°. The paper was dried and the spot containing ^{14}C fructose-2,6-P_2 was eluted with 0.1 M cylohexylamine and lyophilized. [U^{14}C] Fructose-2,6-P_2 was obtained in 80% yield. [6^{32}P] Fructose-2,6-P_2 was prepared in the same reaction mixture as above except [6^{32}P] fructose-6-P instead of [^{14}C] fructose-6-P was used.

Assay for Fructose-2,6-Bisphosphatase (Fructose-2,6-Pase). The assay mixture contained in a final volume of 0.1 ml: 100 mM Tris Cl, pH 8.0, 0.5 mM $MgCl_2$ and 10 µM fructose-2,6-P_2. The reaction was initiated with addition of the enzyme and incubated at 30°. At various intervals 10µl aliquots of the reaction mixture were transfered to 90 µl of 100 mM Tris Cl, pH 8 and heated at 80° for 1 min to stop the reaction. An aliquot was then assayed for fructose-2,6-P_2 as described previously (6). One unit of the enzyme activity is defined as that amount of the enzyme which catalyzes the hydrolysis of one µmol of fructose-2,6-P_2 per min under these conditions.

RESULTS AND DISCUSSION

Purification of Fructose-2,6-Pase. Fresh livers (29 g) from rats were homogenized at 4° with a Polytron homogenizer in 3 vol of 20 mM Na-phosphate, pH 7.0, 0.15 M NaF, 0.1 mM EDTA and 10 µg/ml phenylmethylsulfonyl fluoride and the homogenate was centrifuged at 20,000 x g for 20 min. Solid ammonium sulfate was added to the supernatant solution to 50% saturation and after 15 min, the precipitate was removed by centrifugation. Ammonium sulfate was then added to the supernatant solution to 70% saturation and the precipitate was collected by centrifugation. The precipitate was dissolved in 40 ml of the homogenizing medium and 50% polyethyleneglycol was added to 10% saturation. The precipitate formed in 15 min was removed by centrifugation. Polyethyleneglycol (50%) was then added to the supernatant solution to 18% saturation and the precipitate was collected by centrifugation and dissolved in 15 ml 10 mM Na phosphate, pH

Table I. Purification of Fructose-2,6-Pase

Fractionation	Total Activity (milliunits)	Specific Activity (milliunits/mg)	Total Protein (mg)
Extract	145	0.095	1,968
Ammonium Sulfate	68	0.40	255
Polyethylene Glycol	88	0.85	104
DEAE-Cellulose Chromatography	56	8.0	7

7.3, 1 mM dithiothreitol and 1.0 mM EDTA. The enzyme solution was adsorbed onto a DEAE cellulose (DE52) column (1.5 x 15 cm) and the column was washed with the same buffer. The enzyme was then eluted with 20 mM Na phosphate, pH 7.3 and 1 mM dithiothreitol. The enzyme usually appeared after the void volume of the column and it was concentrated to 2 ml with a Diaflow concentrator (Amicon) using a PM-30 membrane. The enzyme was stored frozen at -70°. A typical example of the partial purification of fructose-2,6-Pase is shown in Table I.

The enzyme is relatively unstable even at -70°. The enzyme preparation does not contain fructose-1,6-Pase or phosphofructokinase.

The Reaction Product. The product of the reaction catalyzed by fructose-2,6-Pase was determined by incubating [6^{32}P] fructose-2,6-P_2 or [U^{14}C] fructose-2,6-P_2 with the enzyme and the product was identified by paper chromatography. As shown in Figure 1A, two ^{32}P labeled spots were detected; one corresponding to fructose-6-P and the other fructose-2,6-P_2. In order to identify the product as fructose-6-P more conclusively, this fructose-6-P spot was eluted and the eluate was reacted either with phosphofructokinase in the presence of ATP or phosphoglucose isomerase and glucose-6-P dehydrogenase in the presence of NADP. The reaction products were then subjected to the same chromatography. As shown in Figure 1B and C, the ^{32}P spots corresponding to fructose-1,6-P_2 and 6-P-gluconate, respectively were detected. The same results were obtained with [^{14}C] fructose-2,6-P_2 (data not shown). Moreover, when the ^{14}C-labeled product was treated with alkaline phosphatase, it yielded a product which showed

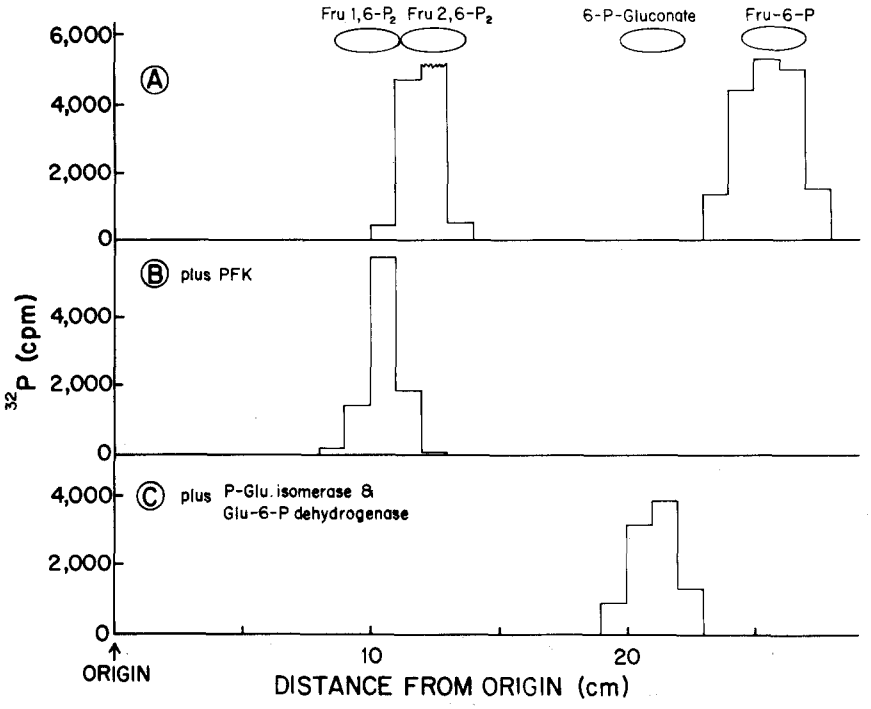


Figure 1. Identification of the Products. (A) The reaction mixture contained Tris Cl 100 mM (pH 7.8), $MgCl_2$ 0.2 mM, [$6^{32}P$] fructose-2,6-P_2 (3.2×10^5 cpm, 4 nmoles) in 50 µl. The reaction was initiated with fructose-2,6-Pase (0.07 units) and incubated at 30°. After 15 min the same amount of the enzyme was added and the incubation continued for 15 min. At the end of the period, 0.2 µmole of fructose-6-P was added and the reaction mixture was directly spotted on a Whatman 3 MM paper and the paper chromatography was performed as described under "Methods". (B) and (C) The ^{32}P spot corresponing to fructose-6-P in the sample identical to (A) was eluted with H_2O, concentrated and treated as follows. (B) The phosphofructokinase (PFK) reaction mixture (100 µl) contained 50 mM Tris/P pH 8, 1 mM ATP, 3 mM $MgCl_2$, the ^{32}P eluate (17,000 cpm) and PFK (1 unit). (C) The reaction mixture (100 µl) contained 50 mM Tris Cl (pH 7.4), 0.5 mM NADP, 3 mM $MgCl_2$, P-glucose isomerase (P-Glu) (2 units) and glucose-6-P dehydrogenase (Glu-6-P) (1 unit). After 15 min incubation at 30° both reaction mixtures were spotted on a paper and the paper was developed as in (A). The papers were cut in 1 cm sections, eluted with 0.1 M $(NH_4)_2CO_3$ and counted. Circles show the marker compounds which were detected with phosphate spray (14).

the same mobility as fructose in three solvent systems. Thus, these results strongly suggest that the enzyme catalyzes the hydrolysis of fructose-2,6-P_2 to yield fructose-6-P. This reaction requires 0.1 mM Mg^{++}.

Activation by Protein Kinase. As shown in Table II, fructose-2,6-Pase is activated by catalytic subunit of cAMP dependent protein kinase. The extent of the activation varied from 3- to 15-fold depending upon the preparation of fructose-2,6-Pase, probably due to a variation in the amount of

Table II. Activation of Fructose-2,6-Pase by Protein Kinase

Addition	Fructose-2,6-Pase Activity
	(milliunits/mg)
1. None	5.3
2. Protein Kinase	18.0
3. Protein Kinase Plus Protein Kinase Inhibitor 44 µg	10
100 µg	5.8

The reaction mixture (1) contained in 50 µl, 10 mM Na phosphate (pH 7.3), 1.0 mM ATP, 2.0 mM $MgCl_2$ and fructose-2,6-Pase, 45 µg. The reaction mixture was incubated at 30° for 30 min. (3) contained the indicated amounts of protein kinase inhibitor. In (2) and (3) the reaction was initiated with addition of protein kinase (20 units, 1 µg).

the dephospho form of the enzyme. The inclusion of protein kinase inhibitor in the reaction prevents this activation by the protein kinase.

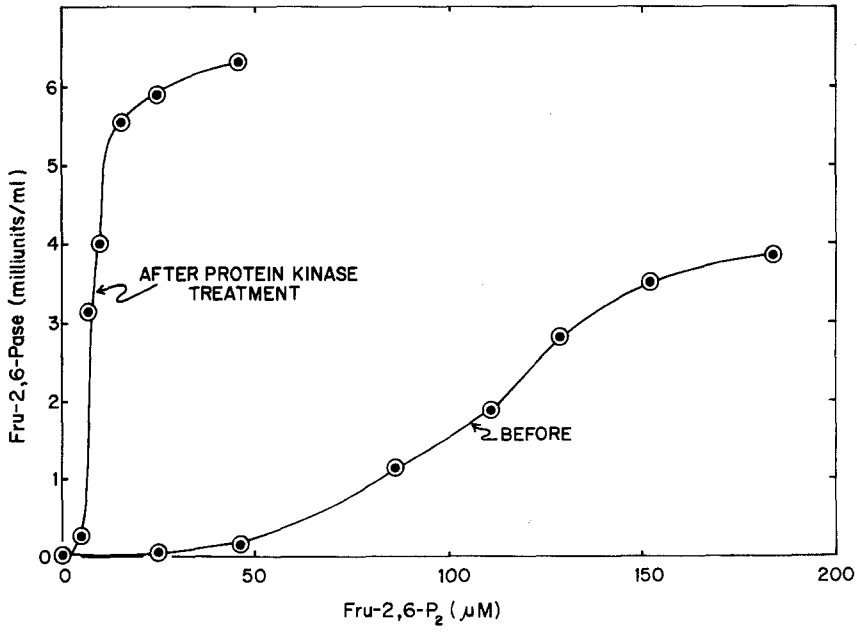
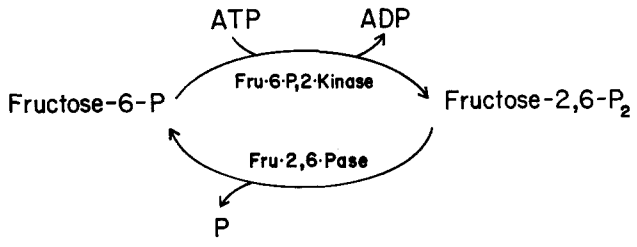


Figure 2. Activity of fructose-2,6-Pase before and after Protein Kinase Treatment at Varying Fructose-2,6-P_2 Concentrations. The protein kinase treatment was as described in Table II except fructose-6-P,2-kinase (180 µg) and protein kinase (30 µg) were used. The activities of fructose-2,6-Pase before and after the treatment were determined as described under "Methods" except the fructose-2,6-P_2 concentration was varied.



SCHEME I

Since the enzyme preparation was impure, attempts to demonstrate the phosphorylation was not made.

In order to understand the mechanism of the activation, saturation curves for fructose-2,6-P_2 of protein kinase treated and untreated fructose-2,6-Pase were determined and the results are shown in Figure 2. Depending upon the preparation of untreated fructose-2,6-Pase, the $K_{0.5}$ for fructose-2,6-P_2 varies from 25 to 110 μM. Upon phosphorylation by the protein kinase, the $K_{0.5}$ for the substrate is lowered to approximately 7 μM. These results suggest that the enzyme (dephospho) is essentially inactive under in vivo concentration of fructose-2,6-P_2 (less than 20μM), but becomes very active by the phosphorylation catalyzed by the protein kinase.

Thus, these results as well as the previous results (7) indicate that a cycle between fructose-6-P and fructose-2,6-P_2 appears to occur in liver in which the synthesis of fructose-2,6-P_2 catalyzed by fructose-6-P, 2-kinase and the hydrolysis is catalyzed by fructose-2,6-Pase (Scheme I). Moreover, both enzymes are controlled by phosphorylation. Fructose-6-P, 2-kinase is inactivated by phosphorylation while fructose-2,6-Pase is activated by the same covalent modification. Thus, the decrease in the fructose-2,6-P_2 concentration in hepatocytes in response to glucagon is attributable to simultaneous decreased synthesis by inactivation of fructose-6-P,2-kinase and increased degradation by the activation of fructose-2,6-Pase by the cAMP dependent process.

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