Fructose-1,6-bisphosphatase from Rat Liver

A COMPARISON OF THE KINETICS OF THE UNPHOSPHORYLATED ENZYME AND THE ENZYME PHOSPHORYLATED BY CYCLIC AMP-DEPENDENT PROTEIN KINASE

(Received for publication, April 9, 1985)

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A purification procedure for rat hepatic fructose-1,6-bisphosphatase, described earlier, has been improved, resulting in an enzyme preparation with a neutral pH optimum and with both phosphorylatable serine residues present. The subunit $M_r$ was 40,000. Phosphorylation in vitro with cyclic AMP-dependent protein kinase resulted in the incorporation of 1.4 mol of phosphate/mol of subunit and led to an almost 2-fold decrease in apparent $K_m$ for fructose-1,6-bisphosphate. In contrast to yeast fructose-1,6-bisphosphatase, fructose-2,6-bisphosphate had no effect on the rate of phosphorylation or dephosphorylation of the intact enzyme.

The effects of the composition of the assay medium, with regard to buffering substance and Mg$^{++}$ concentration, on the apparent $K_m$ values of phosphorylated and unphosphorylated enzyme were investigated. The kinetics of phosphorylated and unphosphorylated fructose-1,6-bisphosphatase were studied with special reference to the inhibitory effects of adenine nucleotides and fructose-2,6-bisphosphate.

Unphosphorylated fructose-1,6-bisphosphatase was more susceptible to inhibition by both AMP and fructose 2,6-bisphosphate than phosphorylated enzyme, at high and low substrate concentrations. Both ATP and ADP had a similar effect on the two enzyme forms, ADP being the more potent inhibitor. Finally, the combined effect of several inhibitors at physiological concentrations was studied. Under conditions resembling the gluconeogenic state, phosphorylated fructose-1,6-bisphosphatase was found to have twice the activity of the unphosphorylated enzyme.

During the past few years there has been a rapid increase in information on the regulation of fructose-1,6-bisphosphatase (d-fructose 1,6-bisphosphate 1-phosphohydrolase, EC 3.1.3.11 (Fr6-1,6-P$_2$ase)), and this enzyme is thought to play an important role in the control of carbohydrate metabolism in mammalian liver (for a review see Ref. 1). Fr6-1,6-P$_2$ase is inhibited by AMP and fructose 2,6-bisphosphate (Fr6-2,6-P$_2$), which affect the enzyme synergistically, and is activated by magnesium ions and other bivalent cations but inhibited by them at higher concentrations (Ref. 1 and references therein and Ref. 2).

Another effect of Fr6-2,6-P$_2$ (apart from its influence on the kinetics) is to stimulate the phosphorylation in vitro of yeast Fr6-1,6-P$_2$ase, thereby reducing its activity (3, 4), probably as a result of a conformational change of the enzyme. The possibility of a corresponding effect on mammalian Fr6-1,6-P$_2$ase was investigated in the present work.

One central question when dealing with Fr6-1,6-P$_2$ase is how intact the purified enzyme is. It is known that proteolysis at the N-terminal of mammalian Fr6-1,6-P$_2$ase affects its pH optimum and AMP sensitivity (5). It is also generally accepted that rat hepatic Fr6-1,6-P$_2$ase is phosphorylated on a serine residue near the C-terminal (6). Recently there have been reports of another phosphorylatable serine in the same region (7).

The influence of the phosphorylation(s) on the enzymatic activity is the subject of some dispute. Many authors have not detected any kinetic changes (7) while others have observed an increase in $V_{max}$ (8) or a decrease in apparent $K_m$ (9, 10). In our system phosphorylation of rat hepatic Fr6-1,6-P$_2$ase brings about an almost 2-fold decrease of the apparent $K_m$ and also affects the interaction with AMP and Fr6-2,6-P$_2$ (11).

In the present work the influence of Fr6-2,6-P$_2$ on the phosphorylation and dephosphorylation of rat hepatic Fr6-1,6-P$_2$ase was studied. In addition, the effect of AMP and Fr6-2,6-P$_2$ was further investigated, and the study was extended to include ADP and ATP. Also, the influence of magnesium ions and buffer substances was studied, and an attempt was made to explain the discrepancies in the reports concerning the relationship between phosphorylation and activity of rat liver Fr6-1,6-P$_2$ase.

EXPERIMENTAL PROCEDURES

Materials—Bovine serum albumin, cyclic AMP, AMP, ADP, ATP, dithiothreitol, NADP, Fr6-1,6-P$_2$, Fr6-2,6-P$_2$, AMP deaminase and Coomassie Brilliant Blue G-250 were from Sigma. [$^{32}$P]ATP was bought from New England Nuclear, Sephadex G-50 was purchased from Pharmacia, Uppsala, and DE52 cellulose and CM52 cellulose from Whatman. Glucose-6-phosphate dehydrogenase and phosphoglucone isomerase were bought from Boehringer Mannheim and glucagon from Lilly.

Purification of Fr6-1,6-P$_2$ase—The purification of Fr6-1,6-P$_2$ase was based on the method described by Rico et al. (8). Unless otherwise stated, steps were performed as rapidly as possible, and the temperature was kept between 0 and 4 °C. Livers from five male Sprague-Dawley rats (50 g), fed ad libitum on a normal diet, were homogenized in 3 volumes of 0.25 M sucrose, 20 mM potassium phosphate buffer, pH 7.4, 1 mM EDTA, 1 mM EGTA; 1 mM benzamidine hydrochloride, 1 mM phenylmethylsulfonyl fluoride in a Turmix blender for 2 min. The homogenate was centrifuged at 31,000 × g for 90 min, and the supernatant was filtered through glass wool. The sample was then heated in 40-nl portions in a 70 °C water bath with vigorous shaking for 3 min. In some cases samples were separated into 0.1 M sucrose or 0.5 M sucrose, pH 7.4, 1 mM EDTA, 1 mM EGTA, 1 mM benzamidine hydrochloride, 1 mM phenylmethylsulfonyl fluoride in a Turmix blender for 2 min. The homogenate was centrifuged at 31,000 × g for 90 min, and the supernatant was filtered through glass wool. The sample was then heated in 40-nl portions in a 70 °C water bath with vigorous shaking for 3 min.

The abbreviations used are: EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetracetic acid; MES, 2-(N-morpholino)ethanesulfonic acid.

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Kinetics of Rat Liver Fructose-1,6-bisphosphatase

for 5 min, after which it was rapidly cooled on ice. The denatured proteins were removed by centrifugation at 31,000 \times g for 90 min. A saturated solution of (NH₄)₂SO₄ was added to give 50% saturation. After 30 min the precipitates were collected by centrifugation at 20,000 \times g for 30 min. The sample was dissolved in 6 ml of 20 mM potassium phosphate buffer, pH 7.4, 1 mM dithiothreitol (buffer A), and the (NH₄)₂SO₄ was removed by gel filtration on Sephadex G-50.

The void volume was applied to a column (4.4 \times 13 cm) of DEAE-cellulose (DE52) equilibrated with buffer A. The column was washed with this buffer and then eluted with a 1000-ml linear gradient of potassium phosphate buffer (20-160 mM), pH 7.4, with 0.1 mM dithiothreitol.

The fractions with enzymatic activity were pooled, concentrated by ultrafiltration, and the buffer was changed to 5 mM malonate, pH 5.8, 1 mM dithiothreitol (buffer B) by gel filtration on Sephadex G-50. The enzyme eluted with the void volume was pooled, and 15 ml of CM-cellulose (CM200) equilibrated in buffer B was added. The pH was adjusted to 5.8. If the enzyme was not completely bound to the gel after 30 min of stirring, 5 ml of gel was added and the pH was lowered further to 5.5. The gel was packed in a column, washed with 5 mM malonate buffer, pH 5.5, 1 mM dithiothreitol, and thereafter eluted with 5 mM malonate buffer, pH 6.0, 1 mM dithiothreitol, 2 mM Fru-1,6-P₂. If necessary the pH was raised to 7.0 to elute the enzyme. Finally the enzyme was concentrated by dialysis against polyethylene glycol to 0.5 mg/ml and stored at -70 °C, with retained activity for at least 6 months.

This purification procedure yielded an enzyme preparation which was essentially pure as judged by electrophoresis in sodium dodecyl sulfate using the slab gel system described by Laemmli (12) modified as described in Ref. 13, with a specific activity of approximately 15 units/mg at 30 °C. (One unit of enzyme activity is defined as the amount needed to catalyze the formation of 1 μmol of \( \text{P} \) per min at pH 7.5 divided by that at pH 9.2, was between 2.9 and 3.1 throughout the preparation.

The critical step in this method of purification of Fru-1,6-P₂ase was the heating of the cell sap. In experiments designed to elucidate the best conditions for this, the cell sap was heated for various temperatures, rapidly cooled, and then the pH ratio was determined with the value of 37,000 obtained earlier with a different technique (9). The enzyme was partially denatured under these conditions before heating. The samples were then rapidly cooled on ice, and the pH ratio when heated to temperatures of 65-70 °C. Cell sap incubated at lower temperatures yielded enzyme with an alkaline pH maximum.

The subunit molecular weight after electrophoresis in sodium dodecyl sulfate was estimated at \( M_r \) 40,000. This value is comparable with the value of 37,000 obtained earlier with a different technique (9).

Assay of Fru-1,6-P₂ase—The activity of Fru-1,6-P₂ase was estimated by a coupled assay with phosphohexose isomerase and glucose-6-phosphate dehydrogenase, essentially as described in Ref. 9. The reaction mixture consisted of 2.5 mM potassium phosphate buffer, pH 7.2, 25 mM mercaptoethanol, 1 mg/ml bovine serum albumin, 5 mM MgSO₄, 0.4 mM NADP, 0.7 unit of glucose-6-phosphate dehydrogenase, 0.35 unit of phosphohexose isomerase, unless otherwise stated. The final volume was 600 μl. About 6 milliliters of Fru-1,6-P₂ase in 10 μl of 25 mM histidine HCl buffer, pH 7.0, 1 mM dithiothreitol, 0.1 mM Fru-1,6-P₂, 30% glycerol was incubated with this mixture for 5 min at 30 °C. During preincubation the Fru-1,6-P₂ was added with the sample was consumed. When measured under strongly inhibitory conditions, it was sometimes necessary to increase the sample volume to 25 μl. For determination of the pH ratio, this mixture was titrated to pH 9.2. When Fru-2,6-P₂, AMP, ADP, ATP, or other effectors were present they were preincubated together with the enzyme for 1 min. The reaction was started with the addition of Fru-1,6-P₂, giving final concentrations of up to 33 μM. The reaction took place at 30 °C, and the velocity was measured between 1 and 2 min after initiation.

Activity at Near-physiological Concentrations of Effectors—In some experiments the concentrations of various inhibitors were chosen so as to mimic the physiological ones. The concentrations of adenine nucleotides in livers from fed and starved rats were those reported in Ref. 14, the amount of potassium phosphate was chosen from those quoted in Ref. 15, and the concentrations of Fru-2,6-P₂ related to that used in Ref. 16. The mixture simulating the nutritional state of livers from fed rats contained 2.45 mM ATP, 0.76 mM ADP, 130 μM AMP, 5 mM potassium phosphate, 8.34 mM Mg²⁺, and 15 μM or no Fru-2,6-P₂. In the mixture simulating starvation, 1.86 mM ATP, 0.825 mM ADP, 184 μM AMP, 5 mM potassium phosphate, 7.85 mM Mg²⁺, and 2 μM or no Fru-2,6-P₂ were included. The activities of phosphorylated and unphosphorylated Fru-1,6-P₂ase were determined in the two different solutions with or without Fru-2,6-P₂ at concentrations of Fru-1,6-P₂ ranging from 5.3-83 μM.

Purification of Protein Kinase—The catalytic subunit of cyclic AMP-dependent protein kinase from pig muscle was prepared and assayed essentially as described by Bechtel et al. (17), with the modifications described in Ref. 9. (One unit of catalytic subunit is defined as the amount of enzyme which catalyzes the incorporation of 1 pmol of phosphate into mixed histone/min.)

Phosphorylation of Fru-1,6-P₂ase—Fru-1,6-P₂ase (150 μg) was phosphorylated with 2.6 \times 10⁴ units of catalytic subunit of cyclic AMP-dependent protein kinase for 90 min at 30 °C in the presence of 25 mM MES, pH 6.9, 4 mM magnesium acetate, 2 μM cyclic AMP, 1 mM dithiothreitol, and 0.5 mM [³²P]ATP (specific activity about 30,000 Ci/mM) in a total volume of 5 ml. The phosphorylation was interrupted by gel filtration on a 10-ml Sephadex G-50 column, equilibrated and eluted with 25 mM histidine HCl buffer, pH 7.0, 1 mM dithiothreitol, 0.1 mM Fru-1,6-P₂, 30% glycerol. If the phosphorylation reaction was determined to be complete, the previously described chromatofocusing procedure (11), intended to remove unphosphorylated enzyme, was omitted. Another sample was treated identically except for the addition of [³²P]ATP.

When the purpose of the phosphorylation experiments was to determine the possible effect of Fru-1,6-P₂ on the rate of phosphorylation, the volume of the reaction mixture was decreased to 4 μl. When included, Fru-2,6-P₂ had a concentration of 1-360 μM. The reaction was terminated by precipitation with 2 ml of ice-cold 10% (w/v) trichloroacetic acid with 50 mM phosphoric acid added and in the presence of 1 mg of bovine serum albumin. The precipitate was washed twice with 0.2 ml of 0.1 M NaOH, and the sample was reprecipitated four times. The final pellet was dissolved in 0.5 ml of 0.5 M NaOH, and the radioactivity was measured as Cerenkov radiation (18).

Dephosphorylation of Fru-1,6-P₂ase—Phosphorylated Fru-1,6-P₂ase (28 pmol of subunit) was dephosphorylated using 7.8 milligrams of a low molecular weight phosphoprotein phosphatase prepared from rat liver as described in Ref. 19. (One unit is the amount of enzyme which releases 1 nmol of [³²P]phosphate from [³²P]phosphohistone from rat liver at 30 °C for 10 min in a final volume of 240 μl.) Fru-2,6-P₂, when present, had a final concentration of 1-150 μM. After incubation, the remaining protein-bound [³²P]phosphate was determined as above.

Preparation of Extracts of Hepatocytes—Hepatocytes were isolated by perfusion with collagenase of livers from male Sprague-Dawley rats, essentially as described by Blair et al. (20) except for the incubation with labeled orthophosphate, which was omitted. The cells were incubated in a suspension of 75 mg/ml with 1 × 10⁻⁶ M glucose for 20 min. This treatment was shown to decrease the activity ratio of 850 μM AMP to 0.5 and 5 min survival of 850 μM AMP was maintained between 0.70-0.85 (20). After incubation, 2-m aliquots were withdrawn, and the cells were precipitated by centrifugation at 190 × g for 1 min at room temperature, the supernatant was discarded, and the cells were frozen in liquid nitrogen.

The hepatocytes were thawed within 15 s in 1 ml of 20 mM potassium phosphate buffer, pH 7.5, 12.5 mM KCl, 125 mM NaF, 10 mM EDTA at 70 °C and then incubated at the same temperature for 1 min. In separate experiments 850 μM AMP or 70 μM Fru-2,6-P₂ was added before heating. The samples were then rapidly cooled on ice, and the precipitates were removed by centrifugation.

Removal of AMP—Aliquots of 35 μl of the hepatocyte extract were preincubated with 95 μl (1 unit) of AMP deaminase or the same volume of the deaminase carrier solution (86% glycerol, 0.33 M KCl) for 4 min at 30 °C in the reaction mixture was used as described by Han et al. (21). In some cases Fru-2,6-P₂ or AMP was added simultaneously. The reaction of Fru-1,6-P₂ase was initiated by addition of substrate, and the final volume was 600 μl.

Other Methods—The protein determinations were made according to the method of Bradford (22), with bovine serum albumin as standard.

RESULTS

General Properties of the Enzyme

The enzyme obtained after purification generally had a neutral pH optimum with a pH ratio of about 3 and is...
was slight activation at the lower substrate concentration and maintain full activity, and the activity ratio became identical and unphosphorylated enzyme and the degrees of inhibition phosphorylated and phosphorylated Fru-1,6-P_2ase were inhibited with a pH ratio below 1.

Parent apparent type A, but it did not change upon phosphorylation. Also, for both forms. The apparent ratio of 1.6 (11), as was the degree of inhibition by both AMP and Fru-2,6-P_2. Unphosphorylated enzyme regarding the degree of inhibition by AMP there was no difference between phosphorylated and unphosphorylated enzyme. (After 1 min 50% of potassium phosphate buffer, pH 7.0, 1 mM dithiothreitol, 0.1 M magnesium concentrations between 1.5 and 5 mM. In some cases where the heating step had not been performed swiftly enough, the result was a form of Fru-1,6-P_2ase with a pH ratio below 1 (type B). It was possible to incorporate about the same amount of phosphate into each mol of subunit of this enzyme type as in type A. The apparent K_v values was of the same order of magnitude as that of the unphosphorylated type A, but it did not change upon phosphorylation. Also, there was no difference between phosphorylated and unphosphorylated enzyme regarding the degree of inhibition by AMP and Fru-2,6-P_2.

Unless otherwise stated, the measurements were made with the intact type A enzyme.

**Effect of Fru-2,6-P_2 on the Rates of Phosphorylation and Dephosphorylation of Fru-1,6-P_2ase**

Fru-2,6-P_2 when present in concentrations up to 360 μM had no effect on the rate of phosphorylation of type A Fru-1,6-P_2ase. On the other hand, it increased the rate of phosphorylation of type B by 10–20% at a concentration of 15 μM.

Fru-2,6-P_2 had no detectable effect on the rate of dephosphorylation of either of the two types. (After 1 min 50% of the radioactivity was removed in the absence of Fru-2,6-P_2 and 48% with 150 μM Fru-2,6-P_2 present.)

**Influence of Buffer Substance on the Activity of Fru-1,6-P_2ase**

The normal reaction mixture consisted of 2.5 mM potassium phosphate buffer, pH 7.5, and the sample added was in 25 mM histidine/Cl buffer, pH 7.0, 1 mM dithiothreitol, 0.1 mM Fru-1,6-P_2, 30% glycerol, giving a final concentration of histidine of 0.5–1 mM in the assay medium. In this environment unphosphorylated and phosphorylated Fru-1,6-P_2ase were found to have apparent K_v values for Fru-1,6-P_2 of 15 and 10 μM, respectively. At 25 mM histidine the apparent K_v values of the two forms were altered and became 8 μM for both, and V_max was increased slightly (by less than 10%). The degree of inhibition by both Fru-2,6-P_2 and AMP was lowered considerably (data not shown). Experiments were also performed after both enzyme forms had been dialyzed against 25 mM potassium phosphate buffer, pH 7.0, 1 mM dithiothreitol, 0.1 mM Fru-1,6-P_2, 30% glycerol. Their apparent K_v values did not differ from those found in the presence of 1 mM histidine (data not shown).

**Dependence of Fru-1,6-P_2ase Activity on Magnesium Ions**

The effect of 0.5–5.0 mM Mg_2+ on the activity of Fru-1,6-P_2ase is illustrated in Fig. 1. At Mg_2+ concentrations between 1.5–5.0 mM the ratio of activity at 12.5 μM Fru-1,6-P_2 to that at 33 μM Fru-1,6-P_2 remained constant. At 1 mM Mg_2+ there was slight activation at the lower substrate concentration and 0.5 mM obviously was too low a concentration of Mg_2+ to maintain full activity, and the activity ratio became identical for both forms. The apparent K_v values for phosphorylated and unphosphorylated enzyme and the degrees of inhibition

**Fig. 1. Effect of Mg_2+ ions on the activity of unphosphorylated (uchsia, □) and phosphorylated (ocol, ●) Fru-1,6-P_2ase (F1,6-P_2ase) at 33 and 12.5 μM Fru-1,6-P_2, respectively. (uchsia, ■) and unphosphorylated and (ocol, □) phosphorylated enzyme in the presence of 25 μM AMP and 2 μM Fru-2,6-P_2 at 12.5 μM Fru-1,6-P_2.**

**Effect of pH on the Activity of Fru-1,6-P_2ase**

The effect of pH on the rate of the Fru-1,6-P_2ase reaction was studied in 2.5 mM potassium phosphate buffer, titrated with HCl to the different pH values. As seen in Fig. 2, the pH optimum was the same, about 7.5, for both the phosphorylated and unphosphorylated forms. The phosphorylated enzyme had a markedly broader pH profile. Similar pH profiles were obtained when the two enzyme forms were assayed at 12.5 μM Fru-1,6-P_2 instead of 33 μM (not included in the figure).

At pH 7.0 and 7.5, Fru-2,6-P_2 and AMP inhibited the unphosphorylated more than the phosphorylated enzyme, when measured at 33 μM Fru-1,6-P_2, as shown in Fig. 2. Similar results were obtained at 12.5 μM Fru-1,6-P_2. Obviously, both AMP and Fru-2,6-P_2 exert their most potent inhibitory effect(s) at neutral pH, both at high and low substrate concentrations.

**Effect of Phosphorylation on the Activity of Fru-1,6-P_2ase**

In Fig. 3 it is shown how different proportions of unphosphorylated and phosphorylated Fru-1,6-P_2ase were inhibited.
by Fru-2,6-P₂ and AMP. The enzyme mixture became more susceptible to both inhibitors with increasing proportions of the unphosphorylated form, except at a high concentration of Fru-2,6-P₂ and low concentration of Fru-1,6-P₂, thereby confirming the results shown in Fig. 2. The relationship was roughly linear for all concentrations used of both effectors.

**Inhibition of Fru-1,6-P₂ase by Adenine Nucleotides**

**AMP**—There was a small difference between the activities of phosphorylated and unphosphorylated Fru-1,6-P₂ase, when measured in the presence of AMP at 33 μM Fru-1,6-P₂, as seen in Fig. 4A. The maximal difference was found at 50 μM AMP, where the unphosphorylated enzyme had kept about 45% and the phosphorylated 65% of its initial activity. At higher concentrations of the nucleotide the difference was decreased, and about 20% of the activity remained at a concentration of 130 μM AMP.

**ADP**—In contrast to AMP, ADP at concentrations of up to 2 mM affected the phosphorylated and unphosphorylated forms equally when measured at 33 μM Fru-1,6-P₂, with only about 30% of the activity remaining at the highest concentration of ADP, as shown in Fig. 4B. At 12.5 μM Fru-1,6-P₂ and low concentrations of ADP there was a small difference in activity between the two forms, probably reflecting their difference in apparent Kᵢ. At 1 mM or higher concentrations of ADP, the difference could be considered negligible.

**ATP**—Both enzyme forms were inhibited to an equal degree by ATP, with about 65% of the activity remaining in the presence of 2 mM ATP and 33 μM Fru-1,6-P₂ (Fig. 4C). When measured at 12.5 μM Fru-1,6-P₂ the difference in activity reflected the difference in apparent Kᵢ of the two enzyme forms. In the concentration range of ATP used both were inhibited by the same percentage, but the shape of the curve closely resembled those for AMP and ADP.

When the effect of any of the three adenosine nucleotides was tested on preparations of Fru-1,6-P₂ase with an alkaline activity maximum (type B) the activity was decreased but to a lesser extent, with about 90% activity remaining at 2 mM ATP and 50% at 2 mM ADP for both the phosphorylated and unphosphorylated forms. At 100 μM AMP 40% of the activity remained for the phosphorylated and 30% for the unphosphorylated form. The same decrease in inhibition was observed with phosphorylated and unphosphorylated enzyme of type A after repeated freezing and thawing.

**Activators of Fru-1,6-P₂ase**

Fru-1,6-P₂ase from bovine liver (23) and rabbit muscle (24) have been reported to be activated by citrate and histidine resulting in an increased Vₘₐₓ. In similar experiments rat liver enzyme was assayed in the presence of 3 mM citrate, either in the standard reaction mixture containing 1 mM histidine or after removal of the histidine by dialysis. No significant difference in Vₘₐₓ was found, indicating that these substances did not activate rat liver Fru-1,6-P₂ase.

**Activity of Phosphorylated and Unphosphorylated Fru-1,6-P₂ase at Near-physiological Concentrations of Effectors**

The total effect of all the substances present was a substantial decrease in the Vₘₐₓ compared with the Vₘₐₓ of the uninhibited enzyme (Fig. 5). At both simulated nutritional states the activity of phosphorylated Fru-1,6-P₂ase leveled off
The activity of Fru-1,6-P₂ase measured at 83 μM Fru-1,6-P₂ in extract from glucagon-treated hepatocytes compared with that of purified phosphorylated Fru-1,6-P₂ase

The experimental conditions were as described under "Experimental Procedures" and the additions were those given in the table.

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* ND, not determined.

The product is usually an enzyme form lacking both the C- and the N-terminal, with an alkaline pH optimum and which is impossible to phosphorylate (25). This form we have named type C. Ever since phenylmethylsulfonyl fluoride was first included in the homogenization solution, this form has not been isolated in our laboratory.

Concentrations of Fru-2,6-P₂ between 1 and 360 μM had no effect on the rate of phosphorylation of type A of Fru-1,6-P₂ase, while it increased the rate of phosphorylation of type B by up to 20% at 15 μM Fru-2,6-P₂, probably due to conformational changes induced by removal of the N-terminal. These results are in contrast to those reported by Mekk and Nimmo (10), who detected a slight decrease in the velocity of phosphorylation in the presence of 100 μM Fru-2,6-P₂. These authors have worked with an enzyme form with a neutral pH optimum, into which they can incorporate 1 mol of phosphate/mol of subunit. Judging from the kinetic data presented, the N-terminal of the enzyme appears to be intact, but since it is not fully phosphorylated, the C-terminal might not be completely preserved. The observed effects required much higher concentrations of Fru-2,6-P₂ than those used for yeast Fru-1,6-P₂ase, where the incorporation of phosphate was roughly doubled in the presence of 3 μM Fru-2,6-P₂ (4).

The binding of Fru-2,6-P₂ to phosphorylated Fru-1,6-P₂ase did not make the enzyme more susceptible to low molecular weight phosphoprotein phosphatase.

The dual role of Mg²⁺ as both an activator and inhibitor of Fru-1,6-P₂ase makes direct comparisons between kinetic studies performed in different laboratories difficult. Generally the concentration of free Mg²⁺ has varied between 1 and 5 mM.

François et al. (26) report that the binding of Mg²⁺ to rabbit liver Fru-1,6-P₂ase had little effect on its Kₘ but they found a 2.5-fold decrease in the binding of both AMP and Fru-2,6-P₂ when the concentration of Mg²⁺ was increased from 0.5–5.0 mM. The difference in binding of AMP and of Fru-2,6-P₂ at 2.0 and 5.0 mM was still about 1.5-fold. Since phosphorylation was not included in the study, no conclusions can be drawn concerning the C-terminal of the enzyme. In contrast, Mekk and Nimmo (27) reported that increasing concentrations of free Mg²⁺ had no effect on the binding of Fru-2,6-P₂ to Fru-1,6-P₂ase.

In our hands there was no difference in the degree of inhibition by AMP and Fru-2,6-P₂ when measured at 1.5 and 5.0 mM Mg²⁺. This suggested that velocity data obtained within this range can be directly compared with regard to the Mg²⁺ effect. At 0.5 mM Mg²⁺ we found no difference in

Effect of Removal of AMP on the Activity of Fru-1,6-P₂ase

Heating of the hepatocyte extract did not lead to degradation of AMP, since the same degree of inhibition was found when AMP was added to the cells at the point of thawing or to the cuvette together with the sample (Table I). Similar results were obtained for Fru-2,6-P₂. The amount of AMP deaminase added was sufficient to degrade the 50 μM AMP included in the tests. The Fru-1,6-P₂ase of the cell extract was inhibited by only about 10%, while purified phosphorylated enzyme, when measured under the same conditions, was inhibited by 45%.

DISCUSSION

When discussing the kinetic and other properties of Fru-1,6-P₂ase, a very important point is whether the N- and C-terminals of the enzyme molecules have been preserved. In order to clarify the discussion we have called the intact form of the molecule, with a neutral pH optimum which could be phosphorylated to 1.4 mol/mol of subunit, type A. The alkaline equally phosphorylatable form lacking its N-terminal end we have designated type B. When the purification of Fru-1,6-P₂ase is performed under conditions which favor proteolysis,
Kinetics of Rat Liver Fructose-1,6-bisphosphatase

apparent $K_m$ between phosphorylated and unphosphorylated Fru-1,6-P$_2$ase, which could indicate that authors who find no change in activity after phosphorylation might have used too low a final concentration of Mg$^{2+}$-

Another factor affecting the apparent $K_m$ is the nature of the buffering substance. In the present study we noted that a change from potassium phosphate at a physiological concentration (15) to histidine altered the apparent $K_m$ of both the phosphorylated and the unphosphorylated enzyme to the same value. In an earlier study (9) it was shown that replacing phosphate by Tris-HCl buffer decreased the apparent $K_m$ for Fru-1,6-P$_2$ for both the phosphorylated and unphosphorylated forms of the enzyme to a very great extent.

Many authors have high concentrations of $K^+$ in the assay mixture (7, 10, 25), and $K^+$ has been reported to activate Fru-1,6-P$_2$ase from pig renal cortex and to diminish its sensitivity to AMP (28), and possibly to affect the rat liver enzyme.

It must be concluded that great care should be taken when comparing kinetic data unless the composition of the assay medium and all other conditions are identical.

In spite of differences in the enzyme preparation and in the assay conditions used, the pH curves of Fru-1,6-P$_2$ase were in good accordance with previous reports concerning the unphosphorylated enzyme (29). Also, the inhibition by both AMP and Fru-2,6-P$_2$ was considerably weaker at pH 8.5 than at 7.5, for both forms, as reported earlier for the unphosphorylated enzyme (30). The pH profiles flattened out with increasing concentrations of both effectors.

The susceptibility of a mixture of varying proportions of phosphorylated and unphosphorylated Fru-1,6-P$_2$ase to the inhibitors AMP and Fru-2,6-P$_2$ was roughly linear as might be expected except in the presence of high concentrations of Fru-2,6-P$_2$ and low concentrations of Fru-1,6-P$_2$.

All three adenine nucleotides inhibited Fru-1,6-P$_2$ase. ATP and ADP affected the phosphorylated and unphosphorylated forms equally, while the unphosphorylated form was slightly more sensitive to AMP than the phosphorylated form. The concentration of the effector needed for comparable inhibition was highest for ATP, lower for ADP, and much lower for AMP. The results obtained with type B enzyme were qualitatively the same, but magnitude of the inhibition was much lower. This is in good agreement with the previous finding that increasing proteolysis decreased the degree of inhibition by AMP (5). The degraded type C described in Ref. 25 required even higher concentrations of AMP for a comparable effect (the activity remaining at 250 $\mu$M AMP was 70%, as reported in Ref. 25).

The fact that AMP on its own affects phosphorylated and unphosphorylated Fru-1,6-P$_2$ase differently suggests that the energy charge of the cell may be important. It has been reported that the activity of rabbit liver Fru-1,6-P$_2$ase is inhibited by AMP, regardless of the presence of other nucleotides (31), but since this enzyme lacks the phosphorylatable C-terminal sequence (1, 7), direct comparisons are not possible.

In an attempt to summarize the effect of all inhibitors we performed experiments in which they were present together at presumed physiological concentrations. The greatest uncertainty in the literature appears to be the amounts of adenine nucleotides present in the liver. The concentrations used in our study were those reported by Newsholme and Start (14), as mentioned under "Experimental Procedures." Faupel et al. (32) observed that the amounts of adenine nucleotides are greatly dependent on the amount of stress to which the rat is subjected prior to death. The concentration of ATP decreased from 3.5–2.5 mM, ADP increased from 0.7–1.0 $\mu$M, and AMP increased from 20–100 $\mu$M in a maximally frightened rat compared with one which was put to death while sleeping. This reflects the difficulty in estimating the true physiological concentrations.

According to our results the difference in the concentrations of ATP and ADP between the "starved" and "fed" states does not greatly affect the activity of Fru-1,6-P$_2$ase, but the degree of inhibition of the unphosphorylated enzyme by AMP was 25% at 20 $\mu$M (fed) and 85% at 130 $\mu$M (starved).

Reports on the concentrations of Fru-2,6-P$_2$ which is the other important inhibitor, are more unanimous.

In the reaction mixture corresponding to the starved and fed nutritional states, the activity of phosphorylated Fru-1,6-P$_2$ase was about twice that of the unphosphorylated form when Fru-2,6-P$_2$ was added. The activity of both enzyme forms, however, was very low partly due to an exaggeratedly high AMP concentration. Since the phosphorylated enzyme was inhibited less than the unphosphorylated form at AMP concentrations tested, the difference in activity between the two forms should be maintained or even increased with decreasing amounts of AMP. Also, the distribution of metabolites is not homogeneous in the cell, but as yet there is no reliable information about this compartmentation of the metabolites.

Our results presented in Table I might indicate that the inhibitory effect of AMP is counteracted by another compound present in the cell extract, since the degree of inhibition of purified Fru-1,6-P$_2$ase was so much higher than that of the enzyme added together with the metabolites from the cell. The inhibition caused by Fru-2,6-P$_2$ was the same in this assay system as when purified Fru-1,6-P$_2$ase was used (11). We do not at present know the nature of the compound causing this effect on the AMP inhibition. Citrate and histidine, which activate Fru-1,6-P$_2$ase from other species, had no effect on our enzyme preparation.

In conclusion, phosphorylation of rat hepatic Fru-1,6-P$_2$ase decreases the apparent $K_m$ for Fru-1,6-P$_2$, but also, which is perhaps more important, it makes the enzyme less sensitive to such potent inhibitors as Fru-2,6-P$_2$ and AMP, compounds which greatly vary with the nutritional state of the cell.

REFERENCES
Kinetics of Rat Liver Fructose-1,6-bisphosphatase