Differences in Kinetic Properties of Phospho and Dephospho Forms of Fructose-6-phosphate, 2-kinase and Fructose 2,6-Bisphosphatase*

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Fructose-6-P, 2-kinase (ATP:D-fructose-6-phosphate 2-phosphotransferase) (1–3) catalyzes the synthesis of D-fructose-2,6-P₂ by the reaction: D-fructose-6-P + ATP → D-fructose-2,6-P₂ + ADP. The degradation of fructose-2,6-P₂ is catalyzed by fructose 2,6-bisphosphatase (4–7) (β-D-fructose-2,6-P₂ → D-fructose-6-P + P). These two enzyme activities appear to reside on a single protein based on the observation that they co-elute by chromatography on ion exchanger (6, 7) and blue Sepharose (6). This bifunctional enzyme is a dimer with molecular weight of 100,000 (8) or 85,000–90,000 (2).

The activities of fructose-6-P, 2-kinase and fructose 2,6-bisphosphatase are regulated in part by cAMP-dependent protein kinase-catalyzed phosphorylation-dephosphorylation reactions. Administration of glucagon to hepatocytes results in rapid inactivation of fructose-6-P,2-kinase and activation of fructose-2,6-bisphosphatase (4). When isolated fructose-6-P,2-kinase is phosphorylated by cAMP-dependent protein kinase in vitro, the enzyme is inactivated (8–10). This inactivation is due to the fact that phosphoenzyme shows an increased Km for fructose-6-P. The extent of the change in the Km, however, is a matter of controversy. El-Maghrabi et al. (9) showed a shift in the value from 0.05 to 3 mM while van Schaftingen et al. (10) reported 0.065 to 0.27 mM. Furthermore, the latter workers reported a decrease in Vmax value by phosphorylation.

In contrast to fructose-6-P,2-kinase, fructose 2,6-bisphosphatase is activated after the phosphorylation (5–7). We have shown that this activation resulted from a decreased Km for its substrate, fructose-2,6-P₂, without any change in Vmax (5). In contrast, Van Schaftingen et al. (6) presented evidence that it is due to increased Vmax rather than any change in Km for the substrate. In addition to these differences there are significant differences in various kinetic parameters including Km value of both enzymes for their respective substrates. For example, Van Schaftingen et al. (6) reported the Km for fructose-2,6-P₂ as 0.1 μM, while El-Maghrabi et al. (7) and we (5) presented the values of 15 and 7 μM, respectively. Thus, there are considerable disagreements among these workers which are due to a number of factors including purity of the enzymes, assay conditions, and the state of phosphorylation.

El-Maghrabi et al. (11) have succeeded in the preparation of the enzyme in a homogeneous form with the use of phosphocellulose columns. However, in our hands the majority (over 90%) of the enzyme was not retained by the column resulting in extremely poor yield. Thus, we have prepared a new affinity column containing immobilized fructose-6-P, and with the use of this column we have obtained the enzyme in homogeneous form in good yield. In this communication, we present purification, phosphate contents of the enzyme, and differences in the kinetic properties of phospho and dephospho forms of fructose-6-P,2-kinase and fructose 2,6-bisphosphatase.

EXPERIMENTAL PROCEDURES

[γ-32P]ATP (3000 Ci/mmole) was purchased from Amershams Corp. The catalytic subunit of cAMP-dependent protein kinase and polyethylene glycol (M₀ = 8000 and Mₘ = 300) was purchased from Sigma. Epoxy-activated Sepharose 6B was obtained from Pharmacia, and Blue Sepharose was a product of Pierce Chemical Co. Fructose 2,6-[32P]P₂ was prepared as before (5). All other chemicals were of reagent grade and obtained from commercial sources. Muscle phosphofructokinase was prepared as described before (12).

Preparation of Immobilized Fructose-6-P—A general procedure of Andeson et al. (13) was used for immobilization of fructose-6-P to Sepharose 6B. Fructose-6-P (750 mg) was dissolved in 10 ml of formamide, and epoxy-activated Sepharose (6 g) was added to the solution. The reaction was initiated with the addition of 20 mg of ZnCl₂, and the mixture was incubated at 40 °C. After 20 h additional fructose-6-P (500 mg) in 3 ml of formamide was added, and the incubator was continued for an additional 4 h. The reaction mixture was defrayed in part by the payment of page charges.
was filtered and the Sepharose was washed with acetone (300 ml) which was followed with H$_2$O (600 ml). The Sepharose was then suspended in 20 ml of 1 M 2-aminoethanol and incubated overnight at room temperature. The resin was washed with H$_2$O, 7 M urea containing 2 M KCl, and again with H$_2$O. Under these conditions usually 0.1 to 0.5 $\mu$mol of fructose-6-P per ml of packed Sepharose was coupled as judged by phosphate determination after combustion (14).

**Assay Method for Fru-6-P,2-kinase**—The activity of Fru-6-P,2-kinase was assayed as described previously (1) with slight modification. The reaction mixture contained in 0.1 ml: 100 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 1 mM fructose-6-P, and 10 mM MgCl$_2$. The mixture was incubated at 30 °C, and at various time intervals, aliquots (10 $\mu$l) were transferred to 90 $\mu$l of 50 mM Tris-HCl, pH 8, and the diluted solution was heated for 1 min at 90 °C to stop the reaction. Suitable aliquots of the heat-treated reaction mixture were then assayed for fructose-2,6-P$_2$ as described (15). One unit of the activity is defined as the amount of enzyme that catalyzes the formation of 1 $\mu$mol of fructose-2,6-P$_2$ per min under these conditions.

**Assay Method for Fructose 2,6-Bisphosphatase**—The reaction mixture contained in a final volume of 0.1 ml: 100 mM Tris-HCl, pH 7.5, 5 mM MgCl$_2$, 50 $\mu$M NADP, 0.4 unit of desalted glucose-6-P dehydrogenase, and 1 unit of phosphoglucomutase, and 20 $\mu$mol fructose-2,6-[32P]P$_2$ (8.2 x $10^{3}$ cpm/nmol). The reaction was initiated with the addition of the enzyme, and the reaction mixture was incubated at 30 °C. At given time intervals, aliquots were removed, transferred into 100 $\mu$l of 0.1 N NaOH, and the solution was heated at 100 °C for 2 min. F$_{2,6}$P$_2$ (1 $\mu$l) was added to the heated reaction mixture and adsorbed on a Dowex-1-$Cl^-$ column (0.5 x 4 cm) which had been equilibrated with 0.02 N NH$_4$OH. The column was washed with 1 ml of 0.02 N NH$_4$OH and 1 ml of 0.15 N NaCl in 0.02 N NH$_4$OH. [32P]Phosphate was then eluted with 5 ml of the same solution, and a portion (2 ml) of the eluate was diluted in 10 ml of Aquasol (New England Nuclear) and counted in a scintillation counter. One unit of the activity is defined as the amount of enzyme that catalyzes the formation of 1 $\mu$mol of phosphate per min under those conditions.

**Other Methods**—Protein concentration was routinely determined with the Bradford method (16) using crystalline bovine serum albumin as a standard. However, for more precise measurement such as for the determination of phosphate content of Fru-6-P,2-kinase, the protein concentration was determined with an analytical ultracentrifuge equipped with interference optics (17). Phosphate contents of the enzyme were determined after repeated washing by precipitation with trichloroacetic acid and solubilization according to the procedure of Itaya and Uti (18) and after ashing (14).

**RESULTS**

**Purification of Fru-6-P,2-kinase**

We have modified our original purification procedure of Fru-6-P,2-kinase (1) and added additional fractionation steps to achieve complete purification. The key step in this procedure is the use of an affinity chromatography on the fructose-6-P column. El-Maghrabi et al. (11) have employed P-cellulose in their preparation of the enzyme. However, in our hands, 90% of the enzyme was not retained by the resin and appeared with other proteins. This resulted in poor yield with no purification. We have synthesized a new affinity column of immobilized fructose-6-P-agarose (see under "Experimental Procedures") and used it in later stages of the purification.

The preparation of extract from fresh rat liver (usually 40–45 livers) was as described previously (1). Polyethylene glycol ($M_r$ = 8000) was added to the extract to bring its concentration to 6% and after 30 min the precipitate was removed by centrifugation. Sufficient polyethylene glycol was then added to the supernatant solution to bring its concentration to 15%. After 30 min the precipitate was collected by centrifugation and dissolved in 350 ml of 50 mM Tris/phosphate, pH 8.0, 0.2 mM ATP, 0.2 mM EDTA, 1 mM dithiothreitol, 1% polyethylene glycol ($M_r$ = 300) (Buffer A). The enzyme was then adsorbed on a DEAE-cellulose column (5 x 30 cm) which had been equilibrated with Buffer A. The column was washed with the same buffer mixture followed with Buffer A containing 0.12 M NaCl until absorbance at 280 nm is less than 0.1. The enzyme was eluted from the column with Buffer A containing 0.3 M NaCl. The eluted enzyme fractions (300 ml) were pooled and adsorbed on a Blue Sepharose column (1.5 x 15 cm) which had been equilibrated with the same solution. The column was extensively washed with 500 ml of the buffer, and the enzyme was eluted with a linear gradient consisting of 200 ml each of 0.3 to 2 M NaCl in Buffer A. The enzyme was usually eluted at 0.8 M NaCl. The enzyme fractions were pooled (200 ml) and concentrated to 15 ml with an Amicon concentrator equipped with a YM-10 membrane. The concentrated enzyme was desalted on a Sephadex G-50 column (1.5 x 25 cm) in a solution containing 50 mM Tris/phosphate, pH 7.5, 0.2 mM EDTA, 2 mM dithiothreitol, 1% polyethylene glycol (Buffer B). The desalted enzyme (15 ml) was then adsorbed on a fructose-6-P-Sepharose 4B column (1.5 x 7 cm) which had been equilibrated with Buffer B. The column was washed extensively with approximately 100 ml of Buffer B until the absorbance at 280 nm was less than 0.02 and then the enzyme was eluted with Buffer B containing 10 mM fructose-6-P. The flow rate was 10 ml/h, and 3-ml fractions were collected. An example of the elution pattern is shown in Fig. 1.

The enzyme fractions (42 to 60, Fig. 1) were pooled and concentrated to 1 ml with the Amicon concentrator as above. The concentrated enzyme was applied on a Sephadex G-100 (2.5 x 40 cm) column which had been equilibrated with Buffer B, eluted with the same buffer at a flow rate of 10 ml/h, and 3-ml fractions were collected. The major enzyme fractions (22 ml) were then pooled, concentrated, and stored at −80 °C. A typical example of this purification procedure is summarized in Table I.

**General Comments on the Purification**

Fru-6-P,2-kinase can be eluted from the fructose-6-P-Sepharose affinity column with 0.5, 5, or 10 mM fructose-6-P, and the purity of the enzyme in these eluates is similar (Fig. 2, lanes 8–10). However, the enzyme concentration in the 0.5 mM eluate is considerably more diluted than those eluted in higher concentrations of the ester. Fig. 2 shows a polyacrylamide gel electrophoresis in sodium dodecyl sulfate of various fractions obtained during the purification. As can be seen in the figure, the enzyme preparation after Sephadex G-100 filtration is homogeneous. The yield of the enzyme varied from 20–28%.

![Fig. 1. Elution of Fru-6-P,2-kinase from Fru-6-P-Sepharose affinity column.](https://example.com/f1.png)
Fructose-6-P,2-kinase

The purification was performed as described under "Results" starting with 555 g of fresh rat livers. The numbers in parentheses represent total activity of Fru-2,6-bisphosphatase in milliunits.

| Fractionation step | Volume | Total activity | Total protein | Specific activity | Recovery |
|--------------------|--------|----------------|---------------|------------------|----------|
| Extract            | 1,720  | 1,490 (1,240)  | 43,000        | 0.032            | 100      |
| Polyethylene glycol| 385    | 1,250 (1,040)  | 12,940        | 0.100            | 89       |
| DEAE-cellulose     | 300    | 1,270 (826)    | 1,650         | 0.770            | 90       |
| Blue Sepharose     | 15     | 725 (602)      | 44            | 16.6             | 52       |
| Fruuctose-6-P-Sepharose | 78 | 375            | 7.3           | 51               | 27       |
| Sephadex G-100     | 22     | 290 (232)      | 5.1           | 57               | 21       |

The enzyme is stable at 0 or −80 °C for at least one month, but repeated freezing-thawing results in significant loss of the activity.

Ratio of Fru-6-P,2-kinase to Fru-2,6-bisphosphatase

We have determined both enzyme activities in all the fractions from the extract to Sephadex G-100 eluate except the affinity column chromatography and found that the ratio of Fru-6-P,2-kinase to Fru-2,6-bisphosphatase activities remain constant at approximately 1.2 (Table I). Fru-2,6-bisphosphatase activity in the eluate of Fru-6-P-Sepharose column could not be determined because of the presence of high concentration of fructose-6-P which is a potent inhibitor of this enzyme. The observation that the ratio of two activities is the same in all these fractions supports the idea that both enzyme activities reside on the same protein (6, 7), although these investigators did not determine both activities in crude fractions. The ratio of these two activities, however, differs considerably from our results. Van Schaftingen et al. (6) reported the value for Fru-6-P,2-kinase/Fru-2,6-bisphosphatase of 0.2 (Figs. 1 and 2 of Ref. 6) in their preparation of partially purified enzymes while El-Maghrabi et al. (7) estimated it as 3 (Fig. 2 of Ref. 7) for their preparation.

Phosphate Contents

The phosphate content of the isolated Fru-6-P,2-kinase, termed henceforth "native" or dephosphoenzyme, was determined as 0.2 ± 0.01 mol of phosphate/mol of subunit (M, = 55,000 (1)). When the native enzyme was incubated with catalytic subunit of cAMP-dependent protein kinase in the presence of $[^{32}P]ATP$, 0.76 mol of phosphate/mol of subunit is incorporated (Fig. 3). Since the native enzyme contains 0.2 mol of phosphate/mol of subunit, the phosphorylation resulted in fully phosphorylated Fru-6-P,2-kinase (0.96 mol of phosphate/mol of subunit). As shown in the figure, the time course of the phosphorylation and inactivation of Fru-6-P,2-kinase catalyzed by the protein kinase is very similar. When the $[^{32}P]$-labeled enzyme was subjected to gel electrophoresis its radioautogram showed only a single radioactive band which corresponded to the enzyme (not shown) indicating that all the $[^{32}P]$phosphate was incorporated into Fru-6-P,2-kinase.

In the subsequent kinetic studies, the native and the phospho-Fru-6-P,2-kinase containing 0.2 and 0.96 mol of phosphate/mol of subunit, respectively, were used.

![Figure 2](http://...)

**Fig. 2.** Polyacrylamide gel electrophoresis in sodium dodecyl sulfate in various fractions of Fru-6-P,2-kinase. Aliquots of the fractions were subjected to the gel electrophoresis in 0.1% sodium dodecyl sulfate. Lanes 1 and 12, standard marker proteins (from top): myosin (200,000), β-galactosidase (116,000), phosphorylase b (92,500), bovine serum albumin (68,000), and ovalbumin (45,000). 2, crude extract; 3, polyethylene glycol (6%) supernatant; 4, polyethylene glycol precipitate; 5, DEAE-cellulose chromatography; 6, Blue Sepharose; 7, Sephadex G-50 eluate; 8, 0.5 mM; 9, 5 mM; 10, 10 mM fructose-6-P eluate from Fru-6-P-Sepharose; 11, Sephadex G-100 eluate.

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![Figure 3](http://...)

**Fig. 3.** $[^{32}P]$Phosphate incorporation and inactivation of Fru-6-P,2-kinase. The reaction mixture contained in a final volume of 0.3 ml, 50 mM Tris/phosphate, pH 7.5, 0.2 mM EDTA, 2 mM dithiothreitol, 0.1 mM $[^{32}P]ATP$ (4000 cpm/pmol, 5 mM MgCl₂, and the reaction was initiated with 900 units of catalytic subunit of protein kinase. The reaction mixture was incubated at 30 °C. At the indicated time intervals, 10-μl aliquots were removed for Fru-6-P,2-kinase assay, and the incorporation of $[^{32}P]$phosphate into the enzyme was determined as described (23).
Kinetic Properties of Native and Phospho-Fru-6-P,2-kinase

Fructose-6-P—When the initial velocity as a function of fructose-6-P at constant ATP (5 mM) was determined, both phospho- and dephospho-Fru-6-P,2-kinase exhibit sigmoidal kinetics (Fig. 4). However, phosphoenzyme shows a higher $K_{\text{m}}$ for fructose-6-P compared to that for dephospho form with the same $V_{\text{max}}$. The same results were obtained at more physiological concentration of ATP (3 mM). Hill coefficients estimated from Hill plots of the data in Fig. 4 are approximately 1.3 and 2.1 for the native and phospho-Fru-6-P,2-kinase, respectively.

ATP—The initial velocity as a function of varying ATP concentrations at two different concentrations of fructose-6-P (40 μM and 5 mM) was determined. As shown in Fig. 5, the ATP saturation curves of the native and phosphofructose-6-P,2-kinase were hyperbolic and the apparent $K_{\text{m}}$ values were 0.25 and 0.5 mM, respectively. At higher concentrations of fructose-6-P (5 mM), the results are similar to those at 40 μM ATP except the values for $K_{\text{m}}$ increased to 0.31 and 0.76 mM for native and phosphoenzyme, respectively (Table II).

Kinetics of Native and Phospho-Fru-2,6-bisphosphatase

Fructose-2,6-P$_2$—Fig. 6 shows the initial velocity of native and phospho-Fru-2,6-bisphosphatase as a function of fructose-2,6-P$_2$ concentration. The phosphoenzyme exhibits normal Michaelis-Menten kinetics with an apparent $K_{\text{m}}$ of 0.5 μM. The native Fru-2,6-bisphosphatase, however, exhibits a biphasic saturation curve in which the curve is hyperbolic below 5 μM fructose-2,6-P$_2$, but approaches $V_{\text{max}}$ much more slowly at higher concentrations of the substrate. The double reciprocal plot shows a concave downward deviation (Fig. 6, inset). A Hill coefficient estimated from a Hill plot is approximately 0.6. These results could be interpreted as the native Fru-2,6-bisphosphatase exhibiting a negative cooperativity for the substrate.

Inhibition by Fructose-6-P—Both native and phospho-Fru-2,6-bisphosphatases are inhibited by fructose-6-P (Fig. 7).

![Graph of initial reaction velocity (V0) of native (○) and phospho (○) Fru-6-P,2-kinase with respect to Fru-6-P concentration. The assay conditions are as described under “Experimental Procedures” except the ATP concentration was 5 mM.](http://www.jbc.org/)

![Graph of initial reaction velocity of native (○) and phospho (○) Fru-6-P,2-kinase with respect to ATP concentration. The assay conditions were the same as in Fig. 4 except 40 mM Fru-6-P and varying concentrations of ATP were used. The inset is a double reciprocal plot of the data.](http://www.jbc.org/)

![Graph of initial reaction velocity of native (○) and phospho (○) Fru-2,6-bisphosphatase with respect to Fru-2,6-P$_2$. The assay conditions are as described under “Experimental Procedures.” The inset is a double reciprocal plot of the data.](http://www.jbc.org/)

![Effect of Fru-6-P on native (○) and phospho (○) Fru-2,6-bisphosphatase (Fru-2,6-Pase). The Fru-2,6-P$_2$ concentration was 20 μM. Other details are described under “Experimental Procedures.”](http://www.jbc.org/)

**TABLE II**

Comparison of kinetic constants of native and phosphofructose-6-P,2-kinase and fructose 2,6-bisphosphatase

|                | Native | Phospho |
|----------------|--------|---------|
| Phosphate content (mol/mol subunit) | 0.20   | 0.97    |
| Fructose-6-P,2-kinase |        |         |
| $K_{\text{m}}^{\text{F}-6-P}$ (μM) | 16     | 50      |
| $K_{\text{f}}^{\text{P}}$ (μM) at Fru-6-P (40 μM) | 0.25 | 0.53    |
| $K_{\text{f}}^{\text{P}}$ (μM) at Fru-6-P (5 mM) | 0.31 | 0.76    |
| Fructose 2,6-bisphosphatase |        |         |
| $K_{\text{m}}^{\text{F}-6-P}$ (μM) | 0.5    |         |
| $K_{\text{m}}^{\text{F}-6-P}$ (μM) | 3.5    | 10      |
The native enzyme is more sensitive to inhibition by fructose-6-P than the phosphoenzyme, and the apparent $K_v$ values are estimated as approximately 3.5 and 10 $\mu$M, respectively (Table II), when assayed at 20 $\mu$M fructose-2,6-P$_2$.

**DISCUSSION**

Fructose-6-P,2-kinase: Fructose-2,6-bisphosphatase has been purified to homogeneity in good yield. The key step is the use of affinity chromatography on an immobilized fructose-6-P-Sepharose column. In addition, ATP was also included in all solutions during the early stages of the purification because we found that fructose-6-P,2-kinase is especially susceptible to proteolytic digestion and ATP protects against the attack. The specific activities of Fru-6-P,2-kinase and Fru-2,6-bisphosphatase of our pure enzyme are 55-70 and 45 milliunits/mg, respectively. El-Maghrabi et al. (11) have reported earlier a specific activity of 50 milliunits/mg for their preparation of the kinase.

The ratio of Fru-6-P,2-kinase to Fru-2,6-bisphosphatase activities of our preparation of the enzyme with the phosphate content of 0.2 mol/mol of subunit is approximately 1.2. This value is significantly different from those reported by others. Van Schaftingen et al. (6) and El-Maghrabi et al. (7) reported the ratio as 0.2 and 3, respectively. These differences are probably due to the differences in assay conditions, phosphate contents of the enzyme, etc. They may also vary depending upon whether the enzyme is partially digested by a protease.

We have taken special care to include ATP as a stabilizing agent against proteolytic attack. The phosphorylation state of the enzyme, as discussed below, also greatly affects the activities of both enzymes in opposite direction, i.e., increasing phosphate content results in decreasing kinase activity and increasing phosphatase activity. Since the phosphate contents of their enzyme preparations are not known, we cannot compare these results.

The experiments presented here show some important aspects of changes in regulatory properties of Fru-6-P,2-kinase and Fru-2,6-bisphosphatase induced by phosphorylation. The apparent $K_v$ of Fru-6-P,2-kinase for fructose-6-P is increased at least 3-fold from 16 to 50 $\mu$M (Fig. 4). Moreover, the positive cooperativity for this substrate appears to be increased as the result of the phosphorylation. In contrast, the phosphorylation of Fru-2,6-bisphosphatase alters its kinetic behavior from negative cooperativity for Fru-2,6-P$_2$ to normal Michaelis-Menten type saturation curve. In view of the bifunctional nature of the enzyme, these results could be interpreted as the native enzyme (dephospho) having one conformational state which shows little cooperativity with fructose-6-P, but strong negative cooperativity with Fru-2,6-P$_2$. The phosphorylation of the enzyme then induces the changes in the conformation such that it now shows positive cooperativity with fructose-6-P, but no cooperativity with respect to Fru-2,6-P$_2$. Efforts are now underway to investigate the proposed differences in these conformational states in order to explain the interesting kinetic behaviors.

There are considerable differences in the $K_v$ for the substrates between our results and those reported by others. For example, Van Schaftingen et al. (10) and El-Maghrabi et al. (9) have reported $K_v$ for fructose-6-P as 65 and 50 $\mu$M, respectively, for native Fru-6-P,2-kinase and 270 $\mu$M and 3 $\mu$M, respectively, for phosphoenzyme. All these values are at least 3 to 4 times larger than those reported here. Since the phosphate content of their enzyme preparations is not known, it is difficult to compare the results. However, we have been unable to observe these high values especially for the phosphoenzyme in any of our enzyme preparations partially purified or pure. Moreover, the extent of the shift in $K_v$ from dephospho- to phospho-Fru-6-P,2-kinase is usually 3 times, so the shift of 60 times (50 $\mu$M to 3 $\mu$M) reported by El-Maghrabi et al. (9) appears to be too large. In addition, Van Schaftingen et al. (6) reported that the phosphorylation of Fru-2,6-bisphosphatase results in an altered $V_{\text{max}}$ without any change in $K_v$. This conclusion is based on the assumption that fructose-2,6-bisphosphatase activity was measured only in a limited range of the substrate concentration (less than 2 $\mu$M). Our results demonstrate that phospho-Fru-2,6-bisphosphatase shows the same $V_{\text{max}}$, as the native enzyme when assayed at much higher concentrations of the substrate (Fig. 6). It is difficult to comment on the $K_v$ because the native enzyme shows the negative cooperativity with respect to fructose-2,6-P$_2$.

It is interesting to note that the differences in the activities between dephospho and phospho forms of Fru-6-P,2-kinase and Fru-2,6-bisphosphatase occur in the concentration ranges of fructose-6-P and fructose-2,6-P$_2$ which are known to occur in vivo. The concentrations of fructose-6-P in the livers of starved and fed rats are 20 and 47 $\mu$M, respectively (19), while fructose-2,6-P$_2$ concentrations are 1 and 15 $\mu$M, respectively (20,21). If our in vitro results could be extrapolated to in vivo conditions, Fru-6-P,2-kinase (native) in fed liver is fully active (Fig. 4), and native Fru-2,6-bisphosphatase (at 15 $\mu$M Fru-2,6-P$_2$) is approximately 70% as active as the phospho form (Fig. 6). However, since the phosphatase is completely inhibited by 50 $\mu$M fructose-6-P (Fig. 7), the enzyme would be inactive in vivo. In the starved states Fru-6-P,2-kinase (phospho) is completely inactive at 15 $\mu$M fructose-6-P (Fig. 4). On the other hand, Fru-2,6-bisphosphatase (phospho) has 70-80% of its maximum activity, but fructose-6-P (15 $\mu$M) inhibits the activity nearly 60% resulting in a net activity of about 30% of the maximum activity. These calculations are based on the assumption that the enzyme is fully phosphorylated by starvation and that it is the same as our native enzyme in the fed states. This assumption appears to be valid because the phosphorylation of the enzyme by cAMP-dependent protein kinase always reaches completion at least in vitro. Thus, the above calculation suggests that in the fed state, only Fru-6-P,2-kinase is active; consequently, only the synthesis of fructose-2,6-P$_2$ occurs and the degradation is completely shut down because of strong inhibition of Fru-2,6-bisphosphatase by fructose-6-P. In the starved states, the synthesis is shut down because of low fructose-6-P and its phosphorylated state of Fru-6-P,2-kinase, while the degradation proceeds at 30% of the maximum activity of Fru-2,6-bisphosphatase. In support of this conclusion is the observation that administration of glucagon to hepatocytes from fed rats results in rapid inactivation of Fru-6-P,2-kinase and activation of Fru-2,6-bisphosphatase resulting in a rapid decrease in fructose-2,6-P$_2$ to a basal level (22). Thus, the most important factors in regulation of Fru-6-P,2-kinase and Fru-2,6-bisphosphatase are phosphorylation of these enzymes and the concentrations of fructose-6-P and fructose-2,6-P$_2$.

**REFERENCES**

1. Furuya, E., and Uyeda, K. (1981) *J. Biol. Chem.* 256, 7109-7112
2. El-Maghrabi, M. R., Claus, T. H., Pilkis, J., and Pilkis, S. J. (1981) *Biochem. Biophys. Res. Commun.* 101, 1071-1077
3. Van Schaftingen, E., and Hers, H.-G. (1981) *Biochem. Biophys. Res. Commun.* 101, 1078-1083
4. Richards, C. S., Yokoyama, M., Furuya, E., and Uyeda, K. (1982) *Biochem. Biophys. Res. Commun.* 104, 1073-1079
5. Furuya, E., Yokoyama, M., and Uyeda, K. (1982) *Biochem. Biophys. Res. Commun.* 105, 264-270

---

1. R. Sakakibara and K. Uyeda, manuscript in preparation.
6. Van Schaftingen, E., Davies, D. R., and Hers, H. G. (1982) *Eur. J. Biochem.* **124**, 142–149
7. El-Maghrabi, M. R., Claus, T. H., Pilkis, J., and Pilkis, S. J. (1982) *J. Biol. Chem.* **257**, 7603–7607
8. Furuya, E., Yokoyama, M., and Uyeda, K. (1982) *Proc. Natl. Acad. Sci. U. S. A.* **79**, 325–329
9. El-Maghrabi, M. R., Claus, T. H., Pilkis, J., and Pilkis, S. J. (1982) *Proc. Natl. Acad. Sci. U. S. A.* **79**, 315–319
10. Van Schaftingen, E., and Hers, H. G. (1981) *Biochem. Biophys. Res. Commun.* **103**, 362–368
11. El-Maghrabi, M. R., Fox, E., Pilkis, J., and Pilkis, S. J (1982) *Biochem. Biophys. Res. Commun.* **106**, 794–802
12. Uyeda, K., Miyatake, A., Luby, L. J., and Richards, E. G. (1978) *J. Biol. Chem.* **253**, 8319–8327
13. Anderson, K., Biquater, R., Canogorcerie, G., McKenzie, R., and Ottosson, T. (1983) *Reactive Polymers*, in press
14. Ames, B. N. (1966) *Methods Enzymol.* **8**, 116–118
15. Uyeda, K., Furuya, E., and Luby, L. J. (1981) *J. Biol. Chem.* **256**, 8394–8399
16. Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
17. Babul, J., and Stellwagen, E. (1969) *Anal. Biochem.* **28**, 216–221
18. Itaya, K., and Ui, M. (1966) *Chem. Acta* **14**, 361–366
19. Lawson, J. W. R., Guyan, R. W., Cornell, N., and Veech, R. L. (1976) in *Glucose Homeostasis* (Mehlman, M. A., and Hanson, R., eds) John Wiley and Sons, New York
20. Uyeda, K., Furuya, E., Richards, C. S., and Yokoyama, M. (1982) *Mol. Cell Biochem.* **48**, 97–120
21. Kuwajima, M., and Uyeda, K. (1982) *Biochem. Biophys. Res. Commun.* **104**, 84–88
22. Richards, C. S., Yokoyama, M., Furuya, E., and Uyeda, K. (1982) *Biochem. Biophys. Res. Commun.* **104**, 1073–1079
23. Corbin, J. D., and Reimann, E. M. (1974) *Methods Enzymol.* **38**, 287–299
Differences in kinetic properties of phospho and dephospho forms of fructose-6-phosphate, 2-kinase and fructose 2,6-bisphosphatase.
R Sakakibara, S Kitajima and K Uyeda

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