Purification and Characterization of a Novel Xylulose 5-Phosphate-activated Protein Phosphatase Catalyzing Dephosphorylation of Fructose-6-phosphate,2-kinase:Fructose-2,6-bisphosphatase*

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We have shown previously (Nishimura, M., Fedorov, S., and Uyeda, K. (1994) J. Biol. Chem. 269, 26100–26106) that the administration of high concentrations of glucose stimulates dephosphorylation of Fru-6-P,2-kinase: Fru-2,6-bisphosphatase in perfused liver, and xylulose (Xu) 5-P activates the dephosphorylation reaction. To characterize the protein phosphatase, we have purified the Xu 5-P-activated protein phosphatase to homogeneity from livers of rats injected with high glucose. Several protein phosphatases in the livers were separated by DEAE-cellulose chromatography, but only one peak of protein phosphatase was inhibited by okadaic acid (IC50 = 1–3 nm) and did not require Mg2+ or Ca2+, suggesting that the enzyme was type 2A. The enzyme was a heterotrimer (Mr = 150,000) and consisted of structural (A, 65 kDa), catalytic (C, 36 kDa), and regulatory (B, 52 kDa) subunits. Amino acid sequences of five tryptic peptides derived from the B subunit showed similarity with those of the B1 isoform of rat protein phosphatase 2A, but five out of 73 residues were different. The protein phosphatase catalyzed dephosphorylation of Fru-6-P,2-kinase:Fru-2,6-Pase, phosphorylase a, and pyruvate kinase, and the Km values were 0.8 μM, 3.7 μM, and 2.2 μM, respectively. Among these substrates dephosphorylation of only the bifunctional enzyme was activated by Xu 5-P, and the Ka value for Xu 5-P was 20 μM. Xu 5-P was the only sugar phosphate which activated the PP2A among all the sugar phosphates examined.

These results demonstrated the existence and isolation of a unique heterotrimeric protein phosphatase 2A in rat liver which catalyzed the dephosphorylation of Fru-6-P,2-kinase:Fru-2,6-Pase and was activated specifically by Xu 5-P. The Xu 5-P-activated protein phosphatase 2A explains the increased Fru 2,6-P2 level in liver after high glucose administration.

Many cellular processes and signaling transductions are controlled by reversible phosphorylation of proteins. Fru 2,6-P2 is the most potent activator of phosphofructokinase and plays an important role in regulation of glycolysis, especially in liver (reviewed in Ref. 1). Synthesis and degradation of Fru 2,6-P2 are catalyzed by a bifunctional enzyme, Fru-6-P,2-kinase:Fru-2,6-bisphosphatase. Liver Fru-6-P,2-kinase:Fru-2,6-Pase is phosphorylated by cAMP-dependent protein kinase (2–4). When blood glucose level falls, glucagon level increases which raises the cAMP level in hepatic cells. The elevated cAMP activates cAMP-dependent protein kinase, which phosphorylates Fru-6-P,2-kinase:Fru-2,6-Pase, leading to the inhibition of Fru-6-P,2-kinase and the activation of Fru-2,6-Pase. This results in a rapid decrease in Fru 2,6-P2, inhibition of phosphofructokinase and glycolysis, and activation of gluconeogenesis.

Mechanism for regulation of the dephosphorylation of Fru-6-P,2-kinase:Fru-2,6-Pase remains unclear. Pelech et al. (5) sought to identify the nature of protein phosphatases in liver involved in dephosphorylation of some of the known regulatory enzymes of carbohydrate metabolism, such as the bifunctional enzymes, phosphofructokinase, and fructose-1,6-bisphosphatase. In rat liver extract, they detected four classes of protein phosphatase (PP1, PP2A, PP2B, and PP2C) by DEAE chromatography and determined their activities toward the substrates in vitro. All of these protein phosphatases catalyzed the dephosphorylation of all the substrates, but they concluded, based on the differences in hydrolysis rates, that PP2A and PP2C appear to be the major phosphatases toward the glycolytic/gluconeogenic enzymes in liver but could not assess the significance of these results in vivo. Previous work from this laboratory has shown that perfusion of the liver with high concentrations of glucose results in an increased Fru 2,6-P2 level which is due to dephosphorylation of the bifunctional enzyme (6). This is in contrast to the earlier view (7) which attributed to the increased Fru 6-P concentration, which is the substrate for the kinase. This dephosphorylation of the bifunctional enzyme was a result of activation of a protein phosphatase, and the activation appears to be caused by two factors (6). One factor is Xu 5-P, and the other is yet unidentified but may involve a covalent modification of the protein phosphatase. These results suggested the existence of a specific protein phosphatase in liver which dephosphorylates the bifunctional enzyme and which is activated by Xu 5-P in response to the high glucose.

In the present study, we purified the Xu 5-P-activated protein phosphatase to homogeneity from rat liver and investigated the properties of the protein phosphatase.

**EXPERIMENTAL PROCEDURES**

Materials—[γ-32P]ATP (3000 Ci/mmol) was purchased from Amersham Corp. The catalytic subunit of cAMP-dependent protein kinase (PP2A) was purified as described (8). HPLC, high performance liquid chromatography.
kinase was purchased from Promega (Madison, WI). Rat liver pyruvate kinase was purified according to the procedure of Kohl and Cottam (8). Rat liver Fru-6-P-2-kinase:Fru-2,6-Pase and a mutant enzyme of rat testis Fru-6-P-2-kinase:Fru-2,6-Pase (RLN-RT2K) were overexpressed in Escherichia coli using the same method as that of the rat testis wild type enzyme (9). The RLN-RT2K is a mutant enzyme in which NH2-terminal amino acids (1–60) of the rat testis enzyme was replaced with amino acids of the liver isozyme (1–63). It has kinetic properties similar to the liver isozyme. These bifunctional enzymes were purified essentially with the same procedure as reported (9), except that reactive Green-5 (Sigma) was used instead of reactive yellow-3 and that Sephacyr S-200 (Pharmacia Biotech Inc.) size exclusion chromatography was omitted in a final purification step. DEAE-cellulose was purchased from Whatman (DE-52). d-Aminoaceyl-Sepharose 4B was obtained from Sigma. CNBr-activated Sepharose 4B was purchased from Pharmacia and coupled with thiophosphorylated RLN-RT2K according to the manufacturer’s recommendation. Phosphorylase b and phosphorylase kinase were purchased from Life Technologies, Inc. The rabbit polyclonal antibody against the catalytic subunit of PP2A was obtained from Promega. All other chemicals were analytical grade and purchased from commercial sources.

Rats—Male Sprague-Dawley rats, weighing 200–250 g (Sasco Co., Omaha, NE), were used. Rats were fed ad libitum with the standard NIH diet and fasted for 24 h before use.

Phosphorylated Substrates—Pure rat liver Fru-6-P-2-kinase:Fru-2,6-Pase, pyruvate kinase, and rat testis mutant Fru-6-P-2-kinase:Fru-2,6-Pase (RLN-RT2K) were phosphorylated in a reaction mixture containing 50 mM Tris-phosphate (pH 7.5), 0.2 mM [γ-32P]ATP (1000 cpm/pmol), 10 mM MgCl2, 0.5 mM EDTA, 2 mM dithiothreitol, and 5.6 μg of the catalytic subunit of CAMP-dependent protein kinase. The reaction mixture was incubated at 30°C for 60 min and then precipitated with 75% ammonium sulfate. The pellets were washed with 75% ammonium sulfate solution several times and dissolved in buffer containing 50 mM Tris-phosphate (pH 7.5), 0.5 mM EDTA, and 2 mM dithiothreitol. The solution was desalted by dialysis and concentration several times using a Centricon-30 concentrator (Amicon, Beverly, MA). [γ-32P]Labeled phosphorylase a was prepared by essentially the same procedure, except that phosphorylase b was incubated in a reaction mixture containing 100 mM glyceraldehyde 3-phosphate (pH 8.2), 0.1 mM CaCl2, 10 mM magnesium acetate, 0.2 mM ATP and 10 mM MgCl2 as a phosphate donor. The specific activities of the substrates were approximately 106 cpm/nmol. Thiophosphorylated RLN-RT2K was prepared by the same procedure as [γ-32P]-phosphorylated RLN-RT2K except that ATPγS was used instead of [γ-32P]ATP.

Protein Phosphatase Assay—The reaction mixture, in a final volume of 50 μl, contained 20 mM MOPS (pH 7.0), 1 mM dithiothreitol, 25 μg of bovine serum albumin, and the [γ-32P]phosphate-labeled substrates (4–6 μl for standard assay) for 4–20 min at 30°C. Since large amounts of the rat rat testis Fru-6-P-2-kinase:Fru-2,6-Pase enzyme (RLN-RT2K) are available in our laboratory, it was used as a substrate to test the purification of the protein phosphatase. An aliquot (15 μl) was removed from the reaction mixture at a given time and precipitated by adding 30 μl of cold trichloroacetic acid (10% final concentration) and 30 μl of 6% bovine serum albumin as a carrier. The precipitate was removed by centrifugation at 10,000 × g for 5 min, and the supernatant was stored at –20°C until assayed. Peptide Sequencing—Approximately 300 pmol of the purified Xu 5-P-activated protein phosphatase were run on an SDS-polyacrylamide gel and transferred onto a Millipore poly(vinylidene difluoride) membrane according to the method of Matsudaira (10). The appropriate region of the membrane was excised and digested in situ with trypsin. The resulting peptides were separated with reverse-phase HPLC (11), and the NH2-terminal amino acid sequence was performed on an Applied Biosystems model 470A gas phase sequencer coupled to an on-line model 120A high performance liquid chromatograph (12).

Other Methods—Protein concentration was determined using the Bradford (13) method with bovine serum albumin as a standard. Polyacrylamide gel electrophoresis was performed in 8% acrylamide containing 0.1% sodium dodecyl sulfate according to the procedure of Laemmli (14). Immunoblot analysis of catalytic subunit of PP2A was carried out as follows. After electrophoresis and blotting onto a nitrocellulose (Schleicher & Schuell), blots were blocked with TBS-T (20 mM Tris-HCl (pH 7.6), 137 mM NaCl, 0.1% (v/v) Tween-20) containing 5% non-fat dry milk and incubated with rabbit polyclonal antibodies against catalytic subunit (Promega) in TBS-T for 1–2 h. Fab fragment of horseradish peroxidase-conjugated goat anti-rabbit antibodies (Bios-Rad) (diluted 1:3000) and enhanced chemiluminescence (Amersham) were used to detect bound antibody.

RESULTS
Purification of Xu 5-P-Stimulated Protein Phosphatase—All operations were performed at 2–5°C. Sprague-Dawley rats fasted over 24 h were given 5 ml of 5% (w/v) glucose via intraperitoneal administration. We established previously (6) that glucose administration was necessary for maximum activation of the protein phosphatase (7). After 1 h, livers were excised under pentobarbiturate anesthesia (50 mg/kg, intraperitoneal) and homogenized in 3 volumes of extraction buffer (0.25 mM sucrose, 10 mM Tris-HCl (pH 7.1), 0.1 mM EDTA, 2 mM dithiothreitol, 1 mM benzamidine, 1 mM leupeptin, 0.1 mM phenylmethylsulfonyl fluoride). The homogenate was centrifuged for 20 min at 28,000 × g, and the pellets were discarded. The extract was mixed with 400 ml of DEAE-cellulose equilibrated with buffer A (20 mM Tris-HCl, pH 7.1, 0.1 mM EDTA, 2 mM dithiothreitol), and the mixture was stirred for 1.5 h. The DEAE-cellulose was washed with 4 liters of buffer A containing 0.05 mM NaCl over a funnel, and the enzyme was eluted with 2 liters of buffer A containing 0.5 mM NaCl. Fractions of 400 ml each were collected, and the fractions containing the enzyme activity were pooled. Usually the first three fractions contained most of the enzyme. The enzyme in the pooled fractions was precipitated with 30% saturation of ammonium sulfate, and the pellet was discarded. Sufficient ammonium sulfate was added to the enzyme solution to bring it to 50% saturation, and the pellet was dissolved in buffer B (20 mM Tris-HCl (pH 7.1), 0.1 mM EDTA, 2 mM dithiothreitol, and 10% (w/v) glycerol). The enzyme solution was dialyzed overnight against buffer B (4 liters) and applied onto a DEAE-cellulose column (3 × 40 cm) equilibrated with buffer B. The column was washed with at least 3 volumes of buffer B containing 0.05 mM NaCl and developed with a total volume of 1600 ml of a linear gradient from 0.05 mM to 0.5 mM NaCl in buffer B. The enzyme fractions were pooled, diluted 2-fold with buffer B, and applied to a d-aminoxyethyl-Sepharose 4B (1.5 × 10 cm) that had been equilibrated with buffer B. The column was washed with 200 ml of buffer B containing 0.15 M NaCl and developed with a total volume of 200 ml of a linear gradient from 0.15 M to 0.45 M NaCl in buffer B. The enzyme fractions were pooled and concentrated to about 10 ml with an Amicon concentrator equipped with a YM-30 membrane. The solution was loaded onto a G-50 column (2 × 25 cm), and the eluate was directly applied to Toyopearl DEAE-650 M (2 × 14 cm). The column was washed with buffer B containing 0.1 M NaCl and developed with a total 500-ml linear gradient from 0.1 M to 0.4 M NaCl in buffer B. The enzyme fractions were pooled and concentrated with an Amicon concentrator to approximately 2 ml. The solution was diluted 2-fold with buffer B and applied to a DEAE-5PW HPLC column (0.7 × 7 cm, Tosoh Assays, Montgomeryville, PA). The active fractions were pooled and concentrated with a Centricon-30 concentrator (Amicon) to about 1 ml. The solution was desalted with a G-50 column (1.5 × 10 cm), and the protein peak was applied to thiophosphorylated RLN-RT2K (0.7 × 5 cm) equilibrated with buffer B. The column was washed with buffer B containing 0.03 M NaCl and developed with a total 50-ml linear gradient from 0.03 M to 0.2 M NaCl in buffer B. The enzyme fractions were pooled and concentrated with a Centriline-30 concentrator (Amicon) to less than 1 ml. The solution was applied to two serially connected TSK G3000SWXL (1 × 30 cm,
TosoHaas) which had been equilibrated with buffer B. The active fractions were pooled and concentrated with a Centri-
con-30 concentrator. A summary of the purification is provided in Table I. Approximately 300 μg of protein phosphatase were obtained from 350 g of rat liver. The yield (3%) of the enzyme was based on the total protein phosphatase activity in the liver extract which included nonspecific and the Xu 5-P-activated enzyme. We estimated the latter protein phosphatase to represent approximately 25% of the total protein phosphatase in the extract.

Separation of Xu 5-P-Activated Protein Phosphatase—Upon DEAE-cellulose chromatography of crude extract of liver, at least three major peaks of protein phosphatase were eluted (Fig. 1). Judging from the elution patterns, three major peaks corresponded closely to the three peaks of protein phosphatases reported by Pelech et al. (5). However, none of these protein phosphatases was activated by Xu 5-P. Xu 5-P-activated protein phosphatase eluted at 0.35 M NaCl after the third major peak, and its activity was detected clearly in the presence of Xu 5-P.

M₆ and Subunit Composition—The elution from a size exclusion column TSK G3000SW_A (TosoHaas) indicated that the Mr of Xu 5-P-activated protein phosphatase was approximately 150,000. SDS-polyacrylamide gel electrophoresis under denaturing conditions (Fig. 2, lane 2) showed that the enzyme consisted of three subunits of 65, 52, and 36 kDa in nearly equimolar ratio since a densitometric scan of the Coomassie Blue-stained gel indicated the ratio of 1:0.7:0.7 (data not shown). No other protein bands were visible. Known serine/threonine protein phosphatases comprise at least two groups of protein phosphatases with distinct biochemical properties. These are protein phosphatase type 1 (PP1) and type 2 (PP2) which can be further divided into three subspecies, PP2A, PP2B, and PP2C (15). Because the pattern of the SDS-gel of the Xu 5-P-stimulated protein phosphatase was very similar to that of the heterotrimERIC PP2A, immunoblotting was performed using an antibody against the catalytic subunit of PP2A. As shown in Fig. 2 (lane 3), the antibody strongly reacted with the 36-kDa band, suggesting that the Xu 5-P-stimulated protein phosphatase is PP2A.

Effect of Cations and Inhibition with Okadaic Acid—Serine/threonine protein phosphatases are known to be characterized by their dependencies on cations and response to inhibitors (15). The Xu 5-P-stimulated protein phosphatase activity was not dependent on Mg²⁺, Ca²⁺, or Mn²⁺ ions (data not shown), thus excluding PP2B and PP2C.

Okadaic acid is a potent inhibitor of PP2A (IC₅₀ = 1 nM) but requires much higher concentrations to inhibit PP1 (IC₅₀ = 15-40 nM). It is, therefore, useful in distinguishing PP1 and PP2A (16). The Xu 5-P-activated protein phosphatase activity was inhibited 45 and 53% by 1 nM okadaic acid in the absence and presence of Xu 5-P, respectively (Fig. 3). These results indicated that the Xu 5-P-activated protein phosphatase was PP2A, consistent with the immunoblotting analysis.

Kinetic Properties of Xu 5-P-Activated Protein Phosphatase—The concentration of Xu 5-P which produced a half-maximal activation was 10 μM (data not shown), which is similar to the values we reported previously with crude liver extract (6). When rat liver Fru-6-P,2-kinase:Fru-2,6-Pase was used as a substrate, the Xu 5-P-activated protein phosphatase showed a Kₘ value of 0.8 μM and Vₘₐₓ = 280 mU/mg in the absence of Xu 5-P (Table II). Addition of 50 μM Xu 5-P increased the Vₘₐₓ by more than 2-fold without affecting the Kₘ.

### Table I

| Step | Volume (ml) | Total Protein (mg) | Total Activity (milliunits) | Specific Activity (milliunits/mg) | Activation by Xu 5-P (%) | Yield (%) |
|------|------------|--------------------|----------------------------|----------------------------------|-------------------------|----------|
| 1. Extract | 1080 | 12960 | 1728 | 0.13 | 1.26 | 100 |
| 2. DEAE-cellulose (batchwise) | 1270 | 4572 | 1524 | 0.33 | 1.24 | 88 |
| 3. Ammonium sulfate | 62 | 899 | 992 | 1.1 | 1.30 | 57 |
| 4. DEAE-cellulose | 234 | 187 | 304 | 1.6 | 2.06 | 18 |
| 5. Aminohexyl-Sepharose 4B | 58 | 87 | 290 | 3.3 | 3.30 | 17 |
| 6. DEAE-650 M | 46 | 18 | 179 | 9.8 | 2.12 | 10 |
| 7. DEAE-5PW | 10 | 6 | 120 | 20 | 2.10 | 7 |
| 8. Thiophosphorylated RN-LRT2K-Sepharose 4B | 1.5 | 0.8 | 56 | 70 | 2.11 | 3 |
| 9. G-3000SW | 0.5 | 0.3 | 51 | 170 | 2.15 | 3 |

![Fig. 1. DEAE-Sepharose chromatography of Xu 5-P-activated protein phosphatase.](image)

![Fig. 2. SDS-polyacrylamide gel electrophoresis of Xu 5-P-activated protein phosphatase.](image)
When phosphorylase a and pyruvate kinase were used as substrates, the $K_m$ value was five and three times, respectively, higher than that with rat liver bifunctional enzyme as a substrate, while the $V_{\text{max}}$ remained the same. Interestingly, addition of Xu 5-P (50 $\mu$m) had no effect on either $K_m$ or $V_{\text{max}}$ when phosphorylase a or pyruvate kinase was used as a substrate. Although the Xu 5-P-activated protein phosphatase catalyzed the dephosphorylation of these substrates, the activation by Xu 5-P occurred only with Fru-6-P,2-kinase:Fru-2,6-Pase as a substrate.

To examine the specificity for Xu 5-P, effects of various sugar phosphates including the major intermediates of the pentose phosphate pathway were examined at near physiological concentrations (17). These compounds included glyceroldehyde 3-phosphate (50 $\mu$m), erythrose 4-phosphate (5 $\mu$m), ribose 5-phosphate (50 $\mu$m), ribulose 5-phosphate (50 $\mu$m), 6-phosphogluconate (100 $\mu$m), glucose 6-phosphate (200 $\mu$m), Fru 6-P (50 $\mu$m), and sedoheptulose 7-phosphate (100 $\mu$m). None of these sugar phosphates activated more than 1.2 times while under the same conditions Xu 5-P activation was 2.2 times.

Amino Acid Sequence of 52-kDa Subunit—PP2A are heterotrimers composed of structural (A, 65 kDa), catalytic (C, 36 kDa), and regulatory (B, 55 or 75 kDa) subunits, which determine the substrate specificity, are most variable (18). A band corresponding to the B subunit (52 kDa; Fig. 2) of the Xu 5-P-activated protein phosphatase after SDS-polyacrylamide gel electrophoresis was digested by trypsin, and the amino acid sequences of five tryptic peptides were determined. Based on the limited sequence analysis of these peptides (Fig. 4), B subunit of Xu 5-P-activated protein phosphatase was similar to B($\alpha$) subunit of rat PP2A (19). However, there were five amino acid residues of 73 which differed from the B($\alpha$) sequence, and three of these five residues were nonconservative substitutions.

**DISCUSSION**

In the present study we have purified Xu 5-P-activated protein phosphatase to apparent homogeneity from rat liver administered with high glucose. Based on the cation dependencies, the dose response to okadaic acid, and the reaction to antibody, this protein phosphatase is likely to be an isozyme of PP2A. Although PP2A is the most abundant isozyme among protein phosphatases, this PP2A is new because no other PP2A holoenzyme (or other protein phosphatase) reported thus far is activated by a sugar phosphate. The enzyme is a heterotrimer, consisting of subunits with $M_r$ = 65,000, 52,000, and 36,000. The known PP2A holoenzymes are heterotrimeric complexes consisting of a catalytic subunit (C, $M_r$ = 36,000–38,000), a structural subunit (A, $M_r$ = 60,000–65,000), and a third subunit termed B or phoshpatase regulatory (PR) subunit ($M_r$ = 54,000–55,000 or 72,000–74,000) (15, 18, 20). Molecular donor thus far has identified two isozymes (\(\alpha\) and \(\beta\)) of both A (21, 22) and C (23, 24) subunits, and the amino acid sequences of these subunits are highly conserved during evolution (25). Because of the highly conserved nature of these subunits, we assigned the 65-kDa subunit of the PP2A as a subunit of Xu 5-P-activated protein phosphatase and the 36-kDa subunit as the C subunit based on its $M_r$ and immunoreactivity toward the catalytic subunit. Multiple families and isozymes of the regulatory subunits (B), however, increase the diversity and complexity of the PP2A enzyme. Various holoenzymes containing different B subunits are thought to play important roles in diverse physiological functions by determining substrate specificity and by regulating catalytic activities of PP2A (26–29). While the same A and C subunits are present in different cells, the B subunits comprise at least three distinct families of proteins. These B subunits include $M_r$ = 55,000 (B/PR55), $M_r$ = 54,000 (B'), and $M_r$ = 72,000–74,000 (B''/PR72). Biochemical and immunological analyses have shown that these regulatory subunits are distinct polypeptides (15, 18, 20). Three cDNAs for B/PR55 (\(\alpha\), \(\beta\), and \(\gamma\)) have been cloned from mammalian sources (19, 30, 31), and these isozymes of B subunits have been shown to be also highly conserved during evolution (18). The amino acid sequences of a limited number of tryptic peptides derived from the B subunit of Xu 5-P-activated PP2A revealed that, although it is closest to the rat neuronal B\(\alpha\) subunit (19), several amino acids are different. Moreover, since this B subunit was smaller (52 kDa) than any of the known B subunits, it is possible that this is a new and unique subunit.

A question may be raised whether Xu 5-P-activated PP2A could account for the dephosphorylation of the bifunctional enzyme in vivo. The concentration of the bifunctional enzyme in rat liver is 0.4 $\mu$m dimer (32), containing 0.8 $\mu$m phosphorylation sites, which is the same as the $K_m$ of the PP2A for this substrate. The PP2A activity ($V_{\text{max}}$) in rat liver is 2.4 and 6 milliunits/g of liver in the absence and the presence of Xu 5-P, respectively. Thus, there is sufficient Xu 5-P-activated PP2A revealed that, although it is closest to the rat neuronal B\(\alpha\) subunit (19), several amino acids are different. Moreover, since this B subunit was smaller (52 kDa) than any of the known B subunits, it is possible that this is a new and unique subunit.

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**Fig. 3. Okadaic acid inhibition of Xu 5-P-activated protein phosphatase.** Xu 5-P-activated protein phosphatase activity was determined in the absence (■) and the presence (○) of Xu 5-P (50 $\mu$m) with various concentrations of okadaic acid. Data are the mean of results from three experiments.

**Table II**

| Substrate       | Phosphorylase a | Pyruvate kinase |
|-----------------|-----------------|-----------------|
| -Xu 5-P         | -Xu 5-P         | -Xu 5-P         |
| +Xu 5-P         | +Xu 5-P         | +Xu 5-P         |
| $K_m$ ($\mu$m)  | 0.76 ± 0.11     | 3.7 ± 0.4       | 2.2 ± 0.6       |
| $V_{\text{max}}$ (milliunits/mg) | 280 ± 50 | 330 ± 60 | 340 ± 10 |
|                 | 0.70 ± 0.10     | 4.4 ± 1.7       | 2.4 ± 0.1       |
|                 | 690 ± 50        | 350 ± 50        | 320 ± 50        |
The activation of PP2A by Xu 5-P in rat liver is described in vivo, indicating that PP2A is not always in an active state and others are currently under investigation. It is generally viewed in Ref. 1 that PP2A is activated by specific metabolites. Consequently, the bifunctional enzyme, Fru-6-P,2-kinase:Fru-2,6-Pase, catalyzes synthesis and degradation of Fru-2,6-P2, which is one of the most important activators of phosphofructokinase and glycolysis. Thus, the hormonal control of the bifunctional enzyme is by cAMP-mediated phosphorylation of the bifunctional enzyme.

Dephosphorylation of Fru-6-P,2-kinase:Fru-2,6-Pase is catalyzed by Xu 5-P-activated PP2A described here, which is regulated in part by the Xu 5-P level in liver. This conclusion is based on the following observations: (a) Xu 5-P is an intermediate of both oxidative and nonoxidative parts of the hexose monophosphate shunt pathway and is shown to rise with high glucose administration in liver (17); (b) among the protein phosphatases in the liver extract, only this PP2A was activated specifically by Xu 5-P. The observation that Xu 5-P-activated protein phosphatase also catalyzes dephosphorylation of phosphorylase a and pyruvate kinase in the absence of Xu 5-P may be explained by the possible differences in the locations of these substrates and the PP2A in the cells; and (c) Xu 5-P-activated PP2A is activated by Xu 5-P only with the bifunctional enzyme as a substrate. It is, therefore, tempting to suggest that Xu 5-P serves as a second messenger, sensing the glucose level in circulation and attenuating the effects of cAMP. Thus, it is possible that cAMP and Xu 5-P are the key signals in regulating the relative activities of the protein kinase and the protein phosphatase in a reciprocal manner to maintain glucose homeostasis. Obviously, further investigation is necessary to determine the significance of the roles that Xu 5-P-activated PP2A plays in liver.

In addition to Xu 5-P, another factor is required to completely activate the PP2A induced by high glucose administration in liver (6). The nature of this factor is unknown at present but may involve a covalent modification of the PP2A. There is evidence that the free catalytic subunit or AC form of PP2A can be phosphorylated in vitro by tyrosine kinases such as pp60<sup>+</sup>, the epidermal growth factor, and the insulin receptor (36). It is possible that Xu 5-P-activated PP2A is also regulated by phosphorylation/dephosphorylation, and this possibility requires further investigation.

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REFERENCES

1. Uyeda, K. (1991) in CRC Review (Kuby, S. A., ed.), vol. 2, pp. 445–456, CRC Press, Boston.
2. Furuya, E., Yokoyama, M., and Uyeda, K. (1982) Proc. Natl. Acad. Sci. U. S. A. 79, 325–329
3. van Schaftingen, E., Davis, D. R., and Hers, H. G. (1981) Biochem. Biophys. Res. Commun. 103, 362–368
4. El-Maghrabi, M. R., Claus, T. H., Pilkis, J., and Pilkis, S. J. (1982) Proc. Natl. Acad. Sci. U. S. A. 79, 315–319
5. Pelech, S., Cohen, P., Fisher, M. J., Pogson, C. I., El-Maghrabi, M. R., and Pilkis, S. J. (1984) J. Biol. Chem. 269, 26100–26106
6. Nishimura, M., Fedorow, S., and Uyeda, K. (1994) J. Biol. Chem. 269, 26100–26106
7. Hue, L., and Rider, M. H. (1987) Biochem. J. 254, 313–324
8. Kohl, E. A., and Cottam, G. L. (1976) Arch. Biochem. Biophys. 176, 671–682
9. Tominaga, N., Minami, Y., Sakakibara, R., and Uyeda, K. (1993) J. Biol. Chem. 268, 15951–15957
10. Matsudaira, P. (1987) J. Biol. Chem. 262, 10035–10038
11. Rowles, J., Slaughter, C., Moonaw, C., Hsu, J., and Cobb, M. H. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 9548–9552
12. Kamibayashi, C., Estes, R., Slaughter, C., and Mumby, M. C. (1991) J. Biol. Chem. 266, 12351–12360
13. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
14. Laemmli, U. K. (1970) Nature 227, 680–685
15. Cohen, P. (1989) Annu. Rev. Biochem. 58, 453–500
16. Cohen, P., and Cohen, P. T. W. (1989) J. Biol. Chem. 264, 21345–21438
17. Casazza, J. P., and Veech, R. L. (1986) Biochem. J. 236, 635–641
18. Mumby, M. C., and Walter, G. (1993) Physiol. Rev. 73, 673–700
19. Pallas, D. C., Welker, W., Jaspers, S., Miller, T. B., Jr., Lane, W. S., and Roberts, T. M. (1992) J. Biol. Chem. 266, 886–893
20. Shenolikar, S., and Nairn, A. C. (1991) Adv. Second Messenger Phosphoprotein Res. 23, 1–121
21. Walter, G., Ferrer, F., Espritu, O., and Carbone-Wiley, A. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 8669–8672
22. Hemmings, B. A., Adams-Pearson, C., Maurer, F., Muller, P., Goris, J., Merlvede, W., Hofsteenge, J., and Stone, S. R. (1990) Biochemistry 29, 3166–3173
23. Green, D. D., Yang, S., and Mumby, M. C. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 4880–4884
24. Stone, S. R., Hofsteenge, J., and Hemmings, B. A. (1987) Biochemistry 26, 7215–7220
25. Orgad, S., Brewis, N. D., Alphay, L., Axton, J. M., Dudai, Y., and Cohen, P. T. W. (1990) FEBS Lett. 275, 44–48
26. Imaoka, T., Imaiz, M., Usui, H., Kinohara, N., and Takeda, M. (1983) J. Biol. Chem. 258, 1526–1535
27. Mumby, M. C., Russell, K. L., Garrard, L. J., and Green, D. D. (1987) J. Biol. Chem. 262, 6257–6265
28. Usui, H., Imaiz, M., Maeta, K., Tsukamoto, H., Azuma, K., and Takeda, M. (1988) J. Biol. Chem. 263, 3752–3761
29. Agostinin, P., Derua, R., Sano, S., Goris, J., and Merlvede, W. (1992) Eur. J. Biochem. 205, 241–248
30. Mayer, R. E., Hendrix, P., Cron, P., Matthies, R., Stone, S. R., Goris, J., Merlvede, W., Hofsteenge, J., and Hemmings, B. A. (1991) Biochemistry 30, 3589–3597
31. Healy, A. M., Zornierowicz, S., Stapelton, A. E., Goeb, M., DePaoli-Roach, A. A., and Pringle, J. R. (1991) Mol. Cell. Biol. 11, 5767–5780
32. Sakakibara, R., Kitajima, S., and Uyeda, K. (1984) J. Biol. Chem. 259, 42–46
33. Dobrowsky, R. T., and Hannun, Y. A. (1992) J. Biol. Chem. 267, 5048–5051
34. Dobrowsky, R. T., Kamibayashi, C., Mumby, M. C., and Hannun, Y. A. (1993) J. Biol. Chem. 268, 15523–15530
35. Law, B., and Rossie, S. (1995) J. Biol. Chem. 270, 12808–12813
36. Chen, J., Martin, B. L., and Brautigan, D. L. (1992) Science 257, 1261–1264