Molecular Cloning of the DNA and Expression and Characterization of Rat Testes Fructose-6-phosphate,2-kinase:Fructose-2,6-bisphosphatase*

Junichiro Sakata, Yumiko Abe, and Kosaku Uyeda

From the Preclinical Science Unit, Department of Veterans Affairs Medical Center, and the Department of Biochemistry, University of Texas Southwestern Medical Center, Dallas, Texas 75216

We have isolated and sequenced two overlapping cDNA fragments which could encode the complete amino acid sequence of rat testis fructose-6-phosphate,2-kinase:fructose-2,6-bisphosphatase. Northern blot analysis revealed that the major 2-kilobase mRNA isolated from rat testis hybridized with a cDNA fragment. A full length cDNA, which encoded a protein of 468 amino acids, was constructed and expressed in Escherichia coli. The expressed protein, purified to homogeneity, showed a M, of 55,000 by gel electrophoresis under denaturing conditions, compared to the deduced M, of 54,023. Fru-6-P,2-kinase:Fru-2,6-bisphosphatase with the same M, 55,000 was also present in rat testis extract. The active enzyme was a dimer as judged by molecular sieve filtration. The expressed enzyme was bifunctional with specific activities of 90 and 22 milliunits/mg of the kinase and the phosphatase activities, respectively. Various kinetic constants of the expressed fructose 6-P,2-kinase were K_m^Fru = 85 μM and K_m^KP = 270 μM, and those of fructose 2,6-bisphosphatase were K_m^Fru 2,6-P_2 = 21 μM and K_m^KP = 3.4 μM. The enzyme was phosphorylated by Fru-2,6(2-32P)P and also by protein kinase C, but not by CAMP-dependent protein kinase, which is in contrast to the liver and heart isozymes.

Fructose-2,6-P_2 is the most potent activator of phosphofructokinase, a key regulatory enzyme of glycolysis (see Ref. 1 for review). The synthesis and degradation of Fru-2,6-P_2 are catalyzed by a bifunctional enzyme Fru-6-P,2-kinase and Fru-2,6-bisphosphatase (Equations 1 and 2) (2–8) as follows.

\[
\text{Fru-6-P + ATP} \rightarrow \text{Fru-2,6-P}_2 + \text{ADP} \quad (1)
\]

\[
\text{Fru-2,6-P}_2 + \text{H}_2\text{O} \rightarrow \text{Fru-6-P} + \text{P}, \quad (2)
\]

Three major isozymic forms have been purified to apparent homogeneity from liver (9, 10), skeletal muscle (11, 12), and heart (13), and their properties have been extensively characterized. In addition to these major isozymes, minor isozymic forms that are not well characterized occur in these tissues (14). Kinase activities of the major isozymes are similar (50–60 milliunits/mg) when assayed under identical conditions, but Fru-2,6-bisphosphatase activities are significantly different (33–154 milliunits/mg) (12, 13). The cellular concentration of Fru-2,6-P_2 is determined by the relative activities of the kinase and the phosphatase. The most important regulatory mechanism of these opposing activities is by phosphorylation and dephosphorylation of the enzymes. The liver enzyme is phosphorylated by CAMP-dependent protein kinase which results in inhibition of the kinase and activation of the phosphatase (15–17). Such a direct reciprocal relationship has been demonstrated in isolated hepatocytes treated with glucagon (18). This is consistent with the liver’s role in inhibiting phosphofructokinase (and glycolysis) by decreasing the level of Fru-2,6-P_2 in order to maintain glucose homeostasis under hormonal stimulation or starvation.

Rat skeletal muscle Fru-6-P,2-kinase:Fru-2,6-bisphosphatase has been obtained in homogeneous form (12), and its tryptic peptide map is identical to that of rat liver isozyme except for two minor peptides (12). The muscle enzymes from pigeon breast (11) and rat (12) are not phosphorylated by cAMP protein kinase because the phosphorylation target Ser^85 of the liver enzyme is replaced by Ala in the muscle enzyme (12). Consequently, the regulatory mechanism of the muscle enzyme is not understood.

In contrast to the liver enzyme, the heart isozyme is phosphorylated by both CAMP-dependent protein kinase and protein kinase C, both resulting in activation rather than inhibition of the kinase activity (19, 20). Consistent with these in vitro results is the observation that epinephrine raises the Fru-2,6-P_2 level in perfused heart (21), whereas the hormone decreases the Fru-2,6-P_2 level in liver (23). The amino acid sequence of bovine heart enzyme has been determined (22), and the sequence shows that the phosphorylation sites for both protein kinases are located near each other in the C-terminal region (Ser^466 and Thr^471). On the other hand, the phosphorylation site (by protein kinase A) of the rat liver enzyme is near the N terminus (24).

All the isozymes thus far studied extensively are from those tissues which metabolize a variety of substrates and therefore are not limited to glucose (or glycogen) for energy and other functions. Little is known about the nature and the types of Fru-6-P,2-kinase:Fru-2,6-bisphosphatase in those glycolytic tissues such as brain or testis in which glucose is the primary source of energy. In this report we present the results of molecular cloning, DNA and amino acid sequence determinations, expression, and characterization of rat testis Fru-6-P,2-kinase:Fru-2,6-bisphosphatase.

(Received for publication, March 25, 1991)
EXPERIMENTAL PROCEDURES

Materials

The lambda Zap II rat testis library was purchased from Stratagene (LaJolla, CA). The cDNA encoding human liver Frus-P-2,kinase:Fru-2,6-bisphosphatase was prepared as described (25). Restriction enzymes and bacteriophage T4 DNA ligase were from New England Biolabs. T7 DNA polymerase and sequencing kit were purchased from Pharmacia LKB Biotechnology Inc. The pT7-7 RNA polymerase/promoter plasmid was a gift of Dr. Stan Tabor (Harvard Medical School). Rat liver Frus-P-2,kinase:Fru-2,6-bisphosphatase was purified according to the procedure of Sakakibara et al. (9). Fructose-2,6-(2-32)P]P was prepared as described (6). Protein kinase C was a gift of Dr. Carlos N. Coyle (Department of Pharmacology, University of Texas Southwestern Medical Center at Dallas). All other materials were reagent grade and obtained from commercial sources.

Methods

Isolation and DNA Sequence Determination of Rat Testis Frus-P-2,kinase:Fru-2,6-bisphosphatase—The lambda Zap II recombinant cDNA rat testis library plated on Escherichia coli XLI-Blue was screened initially with the cDNA probe encoding human liver Frus-P-2,kinase:Fru-2,6-bisphosphatase (25), and later a DNA fragment of a rat testis clone RT6A (see Fig. 1) was used to rescreen the same library. The repetitive nylon filters were prehybridized for 4 h at 65°C in 1% sodium dodecyl sulfate/1 M NaCl containing fish DNA at 100 μg/ml and hybridized in the same solution containing a 32P-labeled probe at the same temperature overnight. The probes were radiolabeled with [α-32P]dCTP by random oligo-labeling (26). The filters were washed several times with 0.3 M NaCl, 30 mM sodium citrate, 0.2% sodium dodecyl sulfate at 60°C and subjected to autoradiography. For sequencing the cDNA inserts were recovered from the lambda Zap II phages by in vitro excision method using helper phage R408 (27), and the alkali-denatured double-stranded DNAs were sequenced by the dideoxy chain termination method (38) using universal, reverse, or custom primers.

RNA Blot Hybridization Analysis—Poly(A)+ RNA samples were prepared from fresh rat testis using an RNA isolation kit (Invitrogen, San Diego, CA). The RNAs were electrophoresed through formaldehyde/agarose gels (29), transferred to nitrocellulose filters (Schleicher and Schuell), and hybridized at 65°C in 0.5% sodium dodecyl sulfate, 1 M NaCl, and 0.1 M sodium citrate with the random primer labeled RT6A cDNA, or RT014K DNA. RT014K DNA was prepared by polymerase chain reaction (30) using RT6S cDNA as a template and synthetic primer of 20 mer from nucleotides 1-140 in Fig. 1. The expression plasmid pT7-7/RT6S was transformed into E. coli BL21(DE3), where the gene for T7 RNA polymerase is located in the chromosome under control of the inducible lacUV5 promoter. The culture was grown at 37°C in 2% tryptone, 1% yeast extract, 0.5% NaCl, 0.2% glycine, 50 mM potassium phosphate, pH 7.5, and 50 μg/ml ampicillin. When the culture OD600 reached 0.5, T7 polymerase was induced by adding 0.5 mM isopropyl-β-d-thiogalactoside.

Preparation of Rat Testis Tissue Extract—Rat testis extract containing the Frus-P-2,kinase:Fru-2,6-bisphosphatase activity was prepared and further purified with a Blue- Sepharose column as described (14).

Assembly Methods for Frus-P-2,kinase—The reaction mixture contained in a final volume of 0.1 ml, 100 mM Tris-HCl, pH 7.5, 2 mM dithiorethiol, 0.1 mM EDTA, 5 mM ATP, 1 mM Frus-P-2, 5 mM potassium phosphate, and 10 mM MgCl2. The mixture was incubated at 39°C and at timed intervals 10 μl aliquots were transferred into 90 μl of 0.1 N NaOH, and the diluted solution was heated for 1 min at 90°C to stop the reaction. Suitable aliquots of the heated reaction mixture were then assayed for Frus-P-2, as described by Uyeda et al. (33). One unit of activity is defined as the amount of enzyme that catalyzes the formation of 1 μmol of phosphate per min under these conditions.

Assembly Methods for Frus-2,6-bisphosphatase—The reaction mixture contained in a final volume of 0.1 ml, 100 mM Tris-HCl, pH 7.5, 2 mM dithiorethiol, 0.1 mM EDTA, 5 mM MgCl2, 5 μM Na2ATP, 0.4 μM of salicylated-glycine-6-P dodehydrogenase, 1 unit of phosphoglucose isomerase, and 20 μM Frus-2,6-[2-32P]P (5.2 x 10⁴ cpm/nmol) (34). The reaction was initiated with the addition of enzyme and incubated at 30°C. At timed intervals aliquots were transferred into 100 μl of 0.1 N NaOH, and the solution was heated at 80°C for 2 min. H2O (1 ml) was added to the heated reaction mixture, and the sample was adsorbed on a Dowex 1-C1 column (0.5 x 4 cm) equilibrated with 0.02 N NaOH. The column was washed with 1 ml of 0.02 N NaOH followed with a 0.15 M NaCl in 0.02 N NaOH. [32P]Phosphate was eluted with 5 ml of the same solution; a 2-ml portion was diluted in 10 ml of Aquasol (Du Pont-New England Nuclear) and was counted in a scintillation counter.

RESULTS

Isolation and Characterization of cDNA Clones—Initial screening of the Stratagene rat testis library with the DNA encoding human liver Frus-P-2,kinase:Fru-2,6-bisphosphatase (25) yielded a positive clone containing a 1.5-kb insert designated RT6A. Rescreening the same library with RT6A DNA produced a clone containing a 0.65-kb insert, which was designated RT6B. The relationship between these two clones is shown in the restriction map in Fig. 1. The nucleotide sequence data (Fig. 2) showed that the 3' end of RT6A (from nucleotide 1 to nucleotide 650) overlapped with the first 402 nucleotides of the 5' end of RT6A. RT6A contained 34 nucleotides of 5'-untranslated sequence followed by an initiation codon. This initiation site and the surrounding nucleotide sequence had the general consensus structure of a eukaryotic initiation site. The composite nucleotide sequences of these clones was 1773 nucleotides in length and, in addition to 5'- and 3'-noncoding sequences, contains an open reading frame of 1404 nucleotides. The deduced amino acid sequence of Frus-P-2,kinase:Fru-2,6-bisphosphatase of rat testis is shown in Fig. 2. The specified protein contained 468 amino acid residues and the calculated molecular weight was 54,023.

mRNA in Rat Testis—To analyze Frus-P-2,kinase:Fru-2,6-bisphosphatase isozyme distribution in rat testis, Northern blot analyses of mRNAs isolated from testis were performed using two DNA probes. A DNA RT014K (nucleotides 1-140; Fig. 2), specific for mRNA of the testis enzyme, and a RT6A DNA (Fig. 1), having sequence homology with both liver and heart type enzyme mRNAs (22, 25), were labeled with random priming and used as probes to detect complementary mRNA sequences under high stringency conditions. The Northern blot analysis detected two species of mRNA in rat testis (Fig. 3). A 3.3-kb mRNA hybridized with the RT6A probe (lane 1) but not with RT014 (lane 2). However, a 2-kb mRNA hybridized with both probes indicating that the 2-kb mRNA was specific for rat testis enzyme. Furthermore, judging from the intensity of the bands, the 2-kb mRNA was considerably more abundant than the 3.3-kb mRNA.

Subcloning and Expression—The overall procedure of constructing the plasmid for direct expression of rat testis Frus-P-2,kinase:Fru-2,6-bisphosphatase is summarized in Fig. 4.
with synthetic oligoDNA having NdeI-BglII cohesive ends, to synthesize the full length cDNA encoding the testis Fru-6-P,2-kinase:Fru-2,6-bisphosphatase, which was designed to match the reading frame of the bacteriophage T7 direct-expression system (31). Insertion of this fragment into the NdeI-PstI-digested pT7-7 yielded a construct, pT7-7/RT2K, that contained the full length cDNA of the testis enzyme as approximately 4.4 kb. The synthetic fragment coding amino acids 1–15 was formed by the annealing of two complementary oligonucleotides: 5' TATGGCGTC-3' and 3' GTGGATGACCATCCACGGGAACTGACCCAGAATCCCCTGAAGAA-5'.

The constructed cDNA of the testis enzyme was expressed rapidly and appeared to reach the maximum value in 2 h. The expression vector, pT7-7/RT2K, containing the full length cDNA of testis enzyme was harvested at 2.5 h after induction. The mRNA of testis enzyme was purified from rat testis. On the basis of the specific catalytic activity of the corresponding protein band was absent in the zero time sample. The mRNA was purified from the expression vector and then ligated with the T7 RNA polymerase promoter.

![Northern blot analysis of poly(A)⁺ RNA isolated from rat testis.](image)

**Fig. 3.** Northern blot analysis of poly(A)⁺ RNA isolated from rat testis. Poly(A)⁺ RNAs (10 µg) from rat testis were separated by electrophoresis and hybridized with ³²P-labeled RT6A cDNA having homology with mRNAs of liver and heart type enzymes (22, 25) (lane 1) and RT014k DNA (nucleotides 1–140) (Fig. 2) specific for mRNA of testis enzyme (lane 2). The migration of RNA markers (7.46–1.35 kb) is indicated.

![Schematic description of the construction of an expression vector, pT7-7/RT2K, containing the full length testis Fru-6-P,2-kinase:Fru-2,6-bisphosphatase cDNA.](image)

**Fig. 4.** Schematic description of the construction of an expression vector, pT7-7/RT2K, containing the full length testis Fru-6-P,2-kinase:Fru-2,6-bisphosphatase cDNA. Plasmid pBluescript/RT2K contains the full coding region of the new isozyme. The synthetic fragment coding amino acids 1–15 was formed by the annealing of two complementary oligonucleotides: 5'-TATGGCGTC-3' and 3'- GTGGATGACCATCCACGGGAACTGACCCAGAATCCCCTGAAGAA-5'. The bars show rat testis Fru-6-P,2-kinase:Fru-2,6-bisphosphatase cDNA. ATG and TGA indicate the initiator and terminator codons, respectively, of the enzyme. 

![Nucleotide and deduced amino acid sequences.](image)

**Fig. 2.** Nucleotide and deduced amino acid sequences. The nucleotide sequence begins with the 5' end of the DNA insert; nucleotides representing the proposed coding region are capitalized. Numbers on the left refer to the nucleotide sequence. Fragment RT6S contained bases 1 to 651, and fragment RT6A contained bases 248 to 1739. Numbers on the right refer to the deduced amino acid sequence given in one-letter code below the nucleotide sequence.

To synthesize the full length cDNA encoding the testis Fru-6-P,2-kinase:Fru-2,6-bisphosphatase, plasmidScript-containing fragment RT6S was digested with SauI and PstI. The cDNA fragment RT6A was digested with the same restriction enzymes, isolated, and introduced into the SauI-PstI-digested plasmidScript/RT2K ("pPlasmidScript/RT2K"). The full length cDNA of the Fru-6-P,2-kinase:Fru-2,6-bisphosphatase was amplified and digested with BglII and PstI and then ligated with synthetic oligoDNA having Ndel-BglII cohesive ends, which was designed to match the reading frame of the bacteriophage T7 direct-expression system (31). Insertion of this fragment into the Ndel-PstI-digested pT7-7 yielded a construct, pT7-7/RT2K, that contained the full length Fru-6-P,2-kinase:Fru-2,6-bisphosphatase cDNA attached directly to the translation initiation codon of pT7-7. The constructed cDNA was validated by dideoxynucleotide sequencing and restriction analysis. This construct was stable in E. coli. The proteins expressed in these cells were analyzed by polyacrylamide gel electrophoresis under denaturing conditions. As shown in Fig. 5, the time course for the expression of the testis enzyme indicated that the enzyme (arrow) was rapidly and appeared to reach the maximum value in 2 h. The corresponding protein band was absent in the zero time sample. On the basis of the specific catalytic activity of the purified testis Fru-6-P,2-kinase:Fru-2,6-bisphosphatase, the transformant of E. coli carrying the DNA produced the enzyme as approximately 3% of the soluble protein in cells harvested at 2.5 h after induction.
**Purification of expressed Fru-6-P,2-kinase:Fru-2,6-bisphosphatase**—A 2-liter culture of BL21(DE3) containing pT7-7/RT2K was grown and protein synthesis was induced with 0.5 mM isopropyl β-D-thiogalactoside. At 0 (lane 2), 0.5 (lane 3), 1 (lane 4) and 2 h (lane 5) after induction, 1 ml of the culture was centrifuged and processed for polyacrylamide gel electrophoresis as described under “Methods.” Lane 1 contains standard marker proteins (from top): phosphorylase b (MW 92,000), bovine serum albumin (MW 68,000), ovalbumin (MW 45,000), carbonic anhydrase (MW 31,000) and soy bean trypsin inhibitor (MW 21,500). The arrow indicates the position of the expressed protein (MW 55,000).

### TABLE I

**Purification of expressed Fru-6-P,2-kinase:Fru-2,6-bisphosphatase**

| Protein            | Volume  | Total Protein | Total Activity | Specific Activity | Recovery |
|--------------------|---------|---------------|----------------|-------------------|----------|
| Lysate             | 60      | 66            | 200            | 200               | 100      |
| DEAE-cellulose     | 40      | 17            | 156            | 9.2               | 78       |
| Blue-Sepharose     | 20      | 3.2           | 107            | 33.4              | 54       |
| Superose 12        | 1.6     | 0.5           | 46             | 92                | 23       |
| * mU, milliunits. |

* Purified from a 2-liter culture of BL21(DE3) containing pT7-7/RT2K.

**Phosphorylation of Fru-2,6-bisphosphatase with Fru-2,6-P2—**The rate of phosphorylation of Fru-2,6-bisphosphatase by its substrate [2-32P]Fru-2,6-P2 and the rate of dephosphorylation of [32P]-phospho enzyme were similar to the rates of rat liver enzyme (Fig. 7). The extent of phosphate incorporation, however, was lower (0.4 mol of P incorporated per mol subunit) than that (0.55 mol of P per mol) of the liver Fru-2,6-bisphosphatase.

To confirm the existence of the same enzyme in rat testis in vivo, Fru-6-P,2-kinase:Fru-2,6-bisphosphatase in tissue extracts was phosphorylated with [2-32P]Fru-2,6-P2 and subjected to polyacrylamide gel electrophoresis along with expressed phosphorylated enzyme. The results shown in Fig. 8 demonstrate that a [32P]-labeled enzyme with identical M, was phosphorylated rat testis Fru-6-P,2-kinase:Fru-2,6-bisphosphatase should contain 468 amino acids, and the calculated M, of the subunit of rat testis Fru-6-P,2-kinase:Fru-2,6-bisphosphatase was 54,023. The polyacrylamide gel electrophoresis under denaturing conditions showed that the expressed testis enzyme had the subunit M, as 55,000 (Fig. 6). Based on the elution position of the enzyme from a Superose column, M, of the active enzyme was estimated as 11 × 10^6 indicating that the enzyme was a dimer.

### TABLE II

**Comparison of the kinetic constants of rat testis (expressed), liver, muscle, and bovine heart Fru-6-P,2-kinase:Fru-2,6-bisphosphatase**

|        | Fru-6-P,2-kinase | Fru-2,6-bisphosphatase |
|--------|-----------------|------------------------|
| Rat    |                  |                        |
| Testis | K<sub>Fru-6-P</sub> (μM) | 85 ± 5                  |
|        | K<sub>ATP</sub> (μM)    | 270 ± 43                |
|        | V<sub>max</sub> (milliunits/mg) | 90 ± 5                  |
| Liver  |                 |                        |
|        | K<sub>Fru-6-P</sub> (μM) | 21 ± 4.7               |
|        | K<sub>ATP</sub> (μM)    | 3.4 ± 0.2               |
|        | V<sub>max</sub> (milliunits/mg) | 22 ± 3.3              |
| Muscle |                 |                        |
|        | K<sub>Fru-6-P</sub> (μM) | >1                      |
|        | K<sub>ATP</sub> (μM)    | 4.7                    |
|        | V<sub>max</sub> (milliunits/mg) | 45                   |
| Bovine |                 |                        |
|        | K<sub>Fru-6-P</sub> (μM) | 16                      |
|        | K<sub>ATP</sub> (μM)    | 56                     |
|        | V<sub>max</sub> (milliunits/mg) | 33                   |

These values for testis enzymes were the average ± S.D. of three to six determinations. The values for liver, muscle, and heart enzymes were from Refs. 12 and 13, respectively.
phosphate. The reaction was initiated with addition of 1 pM Fru-2,6-P,2-kinase:Fru-2,6-bisphosphatase, pressed testis versus native liver Fru-2,6-bisphosphatase. In

intervals, 204 aliquots were removed and mixed with 180 ml of cold

mM dithiothreitol, pressed on a 0.45-pm Millipore filter (HA), and washed with 15 ml of

10% trichloroacetic acid containing 0.1% bovine serum albumin,

"C. At various time intervals, 20-ml aliquots were removed and mixed with 180 ml of cold 0.1 N NaOH containing 0.1 mg/ml bovine serum albumin. Aliquots (40 ml) of alkali-treated samples were pipetted into 0.5 ml of cold 10% trichloroacetic acid containing 0.1% bovine serum albumin, filtered on a 0.45-μm Millipore filter (HA), and washed with 15 ml of cold trichloroacetic acid. The filters were counted in liquid scintillation fluid.

detectable in testis extract, suggesting that the enzyme is present in vivo.

Phosphorylation by Protein Kinase C—The rat testis Fru-6-P,2-kinase:Fru-2,6-bisphosphatase could not be phosphorylated by cAMP-dependent protein kinase, unlike the rat liver or bovine heart isozymes. However, the testis enzyme was phosphorylated by protein kinase C and the phosphorylation required the presence of phosphatidylinserine, Ca²⁺, and diolein (data not shown). The phosphorylation site was not determined, but based on a computer search for a potential site for protein kinase C, the likely target serine was localized in serine 28 or threonine 443.

**DISCUSSION**

The cDNA clone for rat testis Fru-6-P,2-kinase:Fru-2,6-bisphosphatase reported herein encoded an enzymatically active form of the enzyme. Our previous results, based on ion-exchange chromatography and immunoreaction, showed the existence of a heart-type isozyme and an unidentified form of the isozyme which is more abundant in rat testis. The present results indicate that the cloned enzyme is the testis-specific isozyme. This conclusion was reached based on the following observations: (a) The Northern blot analysis (Fig. 3) demonstrated that the 2.0-kb mRNA specified the testis enzyme as determined by the testis enzyme DNA probe. (b) The expressed enzyme showed the same characteristics as the major enzyme present in rat testis extract. Earlier we detected a second isozyme in testis which appears to be a heart-type isozyme, since the chromatographic behavior and immunoreaction are similar to those of a heart-type isozyme.

Various kinetic constants of the expressed testis enzyme are different from other major isozymes of liver, skeletal muscle, and heart isozymes. However, some of the isozymes have been reported to have comparable or higher kinase activity than the testis enzyme. The specific activities of those preparations range from 100 to 180 milliunits/mg for beef heart (13, 39, 40) and pigeon muscle (41). However, some of these differences may be due to different assay conditions and to different isozymic forms in the tissue. For example, we have shown that the major isozyme (M, 58,000) in bovine heart has the specific kinase activity of 61 milliunits/mg, but the minor isozyme (M, 54,000) shows 175 milliunits/mg. It is interesting that the testis enzyme has the highest Fru-6-P,2-kinase activity and the lowest Fru-2,6-bisphosphatase activity. Since the Fru-2,6-P₂ level in cells is controlled by the relative activities of kinase/phosphatase, the high ratio of these two activities in testis may reflect the importance of Fru-2,6-P₂ in a glycolytic tissue such as testis.

One of the important regulatory mechanisms of liver and heart Fru-6-P,2-kinase:Fru-2,6-bisphosphatase is phosphorylation and dephosphorylation as discussed in the introduction. It is possible that the testis enzyme also is regulated by the same mechanism. The testis enzyme was not phosphorylated by CAMP-dependent protein kinase, unlike the liver and heart isozymes, but similar to rat skeletal muscle enzyme. However, it was phosphorylated by protein kinase C, and in this respect the enzyme was similar to the bovine heart isozyme. The significance of the phosphorylation of the testis enzyme is not certain. The testis enzyme contained three potential phosphorylation sites for protein kinase C, namely Ser¹, Ser², and Thr³⁵. Because of the opposite effects on the enzyme activities depending upon the location of the phosphorylation site(s), i.e. N or C terminus of the enzyme (19), it is of great interest to distinguish these sites in the testis enzyme. Currently we are ascertaining the location of the phosphorylation site and determining its effect on the activities of the bifunctional enzyme. These studies may elucidate the regulatory mechanism of phosphofructokinase and glycolysis in testis tissue.

A comparison of the amino acid sequence of rat testis Fru-6-P,2-kinase:Fru-2,6-bisphosphatase with those of rat liver (42, 43), rat heart (this paper), and bovine heart (22) enzymes is presented in Fig. 9. To maximize the alignment of these sequences one amino acid gap had to be added to the testis and two gaps to the liver enzyme sequences. During the course of this work, we have also isolated from the same library a cDNA clone which encoded for rat heart Fru-6-P,2-kinase:Fru-2,6-bisphosphatase. The deduced amino acid sequence of this clone corresponded to Pro³⁸² to the C terminus of the sequence of the bovine heart enzyme, and those sequences of the heart enzymes were 94% identical. The rat testis enzyme showed 74, 67, and 64% similarity with rat liver, rat heart, and bovine heart enzymes, respectively. The major differences among these sequences were in the N and C termini where the regulatory phosphorylation sites are located. Although the C termini of the testis and the liver

---

2 R. Sakakibara and K. Uyeda, unpublished results.
Testis Fru-6-P,2-kinase/Fru-2,6-bisphosphatase

residues, Lys\textsuperscript{177} and Lys\textsuperscript{296} of the liver enzyme are also conserved in both the testis and heart enzymes. It is noteworthy that all of these substrate binding sites are for Fru-6-P,2-kinase and localized in the N-terminal half of the molecule. On the other hand, the binding sites of Fru-2,6-P\textsubscript{2}, the substrate, and for Fru-6-P, the inhibitor of Fru-2,6-bisphosphatase, have not been identified. Apparently these reagents that react with the same sugar phosphate binding sites of Fru-6-P,2-kinase do not react with Fru-2,6-bisphosphatase sites, which may suggest that the active site is buried. The only site identified thus far is the active site, His\textsuperscript{286}. The phospho-His\textsuperscript{286} as the reaction intermediate of Fru-2,6-bisphosphatase (38) and its neighboring amino acids (Ile\textsuperscript{284}, Glu\textsuperscript{286} of the liver enzyme) are also identical in all these enzymes. Thus, the additional ligand binding sites of Fru-2,6-bisphosphatase as well as other sites of Fru-6-P,2-kinase need to be elucidated.

Acknowledgments—We wish to thank Nami Lee and Dawn Hancock for expert technical assistance and Dr. Sarah McIntire for her advice in manuscript preparation. We also thank Dr. Melanie H. Cobb (University of Texas Southwestern Medical Center at Dallas, Department of Pharmacology) for protein kinase C.

REFERENCES

1. Uyeda, K., Furuya, E., Richards, C. S., and Yokoyama, M. (1982) Mol. Cell. Biochem. 48, 97–120
2. Furuya, E., and Uyeda, K. (1988) J. Biol. Chem. 256, 7109–7112
3. El-Maghrabi, M. R., Claus, T. H., Piliks, J., and Piliks, S. J. (1981) Biochem. Biophys. Res. Commun. 101, 1071–1077
4. Van Schaftingen, E., and Hers, H. G. (1981) Biochim. Biophys. Res. Commun. 101, 1078–1084
5. Van Schaftingen, E., Davies, D. R., and Hers, H. G. (1982) Eur. J. Biochem. 124, 143–149
6. Furuya, E., Yokoyama, M., and Uyeda, K. (1982) Biochim. Biophys. Res. Commun. 105, 264–270
7. El-Maghrabi, M. R., Claus, T. H., Piliks, J., Fox, E., and Piliks, S. J. (1982) J. Biol. Chem. 257, 7603–7607
8. Rider, M. H., Foret, D., and Hue, L. (1986) Biochem. J. 231, 193–196
9. Sakakibara, R., Kitajima, S., and Uyeda, K. (1984) J. Biol. Chem. 259, 41–46
10. El-Maghrabi, M. R., Pate, T. M., Murray, K. J., and Piliks, S. J. (1984) J. Biol. Chem. 259, 13096–13103
11. van Schaftingen, E., and Hers, H. G. (1986) Eur. J. Biochem. 159, 359–365
12. Kitamura, K., Uyeda, K., Kangawa, K., and Matsuo, H. (1989) J. Biol. Chem. 264, 9799–9806
13. Kitamura, K., and Uyeda, K. (1988) J. Biol. Chem. 263, 9027–9033
14. Taniyama, M., Kitamura, K., Thomas, H., Lawson, J. W., and Uyeda, K. (1988) Biochem. Biophys. Res. Commun. 157, 949–954
15. Furuya, E., Yokoyama, M., and Uyeda, K. (1982) Proc. Natl. Acad. Sci. U. S. A. 79, 325–329
16. El-Maghrabi, M. R., Claus, T. H., Piliks, J., and Piliks, S. J. (1982) Proc. Natl. Acad. Sci. U. S. A. 79, 315–319
17. van Schaftingen, E., and Hers, H. G. (1981) Biochem. Biophys. Res. Commun. 103, 362–368
18. Richards, C. S., Yokoyama, M., Furuya, E., and Uyeda, K. (1982) Biochem. Biophys. Res. Commun. 104, 1073–1079
19. Kitamura, K., and Uyeda, K. (1987) J. Biol. Chem. 262, 679–681
20. Kitamura, K., Kangawa, K., Matsuo, H., and Uyeda, K. (1988) J. Biol. Chem. 263, 16790–16801
21. Narabayashi, H., Lawson, J. W. R., and Uyeda, K. (1985) J. Biol. Chem. 260, 9760–9768
22. Sakata, J., and Uyeda, K. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 4951–4955
23. Richards, C. S., Yokoyama, M., Furuya, E., and Uyeda, K. (1982) Biochem. Biophys. Res. Commun. 104, 1073–1079
24. Murray, K. J., El-Maghrabi, M. R., Kountz, P. D., Sukas, T. J., Soderling, T. R., and Piliks, S. J. (1984) J. Biol. Chem. 259, 7693–7698
25. Algaer, J., and Uyeda, K. (1988) Biochem. Biophys. Res. Commun. 153, 328–333
Testis Fru-6-P,2-kinase:Fru-2,6-bisphosphatase

26. Feinberg, A. P., and Vogelstein, B. (1983) Anal. Biochem. 132, 6-13
27. Dotto, G. P., Horiuchi, K., and Zinder, N. D. (1984) J. Mol. Biol. 172, 507-521
28. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 5463-5467
29. Lehrach, H., Diamond, D., Wozney, J. M., and Boedtker, H. (1977) Biochemistry 16, 4743-4750
30. Saiki, R. K., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn, G. T., Mullis, K. B., and Erlich, H. A. (1988) Science 239, 487-491
31. Tabor, S., and Richardson, C. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 1074-1078
32. Studier, F. W., and Moffatt, B. A. (1986) J. Mol. Biol. 189, 113-130
33. Uyeda, K., Furuya, E., and Luby, L. J. (1981) J. Biol. Chem. 256, 8394-8399
34. Kitajima, S., Sakakibara, R., and Uyeda, K. (1984) J. Biol. Chem. 259, 6896-6903
35. Laemmli, U. K. (1970) Nature 227, 680-685
36. Bradford, M. M. (1976) Anal. Biochem. 72, 248-254
37. Kitajima, S., Thomas, H., and Uyeda, K. (1985) J. Biol. Chem. 260, 13995-14001
38. Stewart, H. B., El-Maghrabi, M. R., and Pilkis, S. J. (1985) J. Biol. Chem. 260, 12935-12941
39. Rider, M. H., Foret, D., and Hue, L. (1985) Biochem. J. 231, 193-196
40. Rider, M. H., and Hue, L. (1986) Biochem. J. 240, 57-61
41. Van Schaftingen, E., and Hers, H. G. (1986) Eur. J. Biochem. 159, 359-365
42. Darville, M. I., Crepin, K. M., Vandekerckhove, J., Van Damme, J., Octave, J. N., Rider, M. H., Marchand, M. J., Hue, L., and Rousseau, G. G. (1987) FEBS Lett. 224, 317-321
43. Lively, M. O., El-Maghrabi, M. R., Pilkis, J., D’Angelo, G., Colesia, A. D., Ciavola, J.-A., Fraser, B. A., and Pilkis, S. J. (1988) J. Biol. Chem. 263, 839-849
44. Rossman, M. G., Liljas, A., Branden, C. I., and Banaszak, L. (1975) in The Enzymes (Boyer, P., ed) 3rd Ed., vol. 11, pp. 61-102, Academic Press, New York
45. Kitamura, K., Uyeda, K., Hartman, F. C., Kangawa, K., and Matsuo, H. (1989) J. Biol. Chem. 264, 6344-6348