# Hexose Phosphate Binding Sites of Fructose 6-Phosphate,2-Kinase:Fructose 2,6-Bisphosphatase†

Tomoyuki Tsujikawa,‡ Fusao Watanabe,§ and Kosaku Uyeda*

*Research Service of the Department of Veterans Affairs Medical Center and Biochemistry Department, University of Texas Southwestern Medical Center at Dallas, 4500 South Lancaster Road, Dallas, Texas 75216*

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ABSTRACT: A previous chemical modification study [Kitamura *et al.* (1989) *J. Biol. Chem. 264,* 6344−6438] has shown that *N*-bromoacetylethanolamine phosphate labeled specifically Cys107 of rat liver Fru 6-P,2-kinase:Fru 2,6-Pase and the corresponding Cys of the bovine heart enzyme, leading to inactivation of kinase activity. Since Fru 6-P provided protection against the inactivation, this region of the enzyme was thought to be a Fru 6-P binding site of the kinase enzyme. To examine this possibility, oligonucleotide-directed mutagenesis has been used to alter several residues in expressed rat testis Fru 6-P,2-kinase:Fru 2,6-Pase. The change of Lys100, Lys103, and Asp112 caused at most a 2-fold increase in $K_m^{F6P}$ and a 2−3-fold increase in $K_m^{ATP}$, suggesting that these residues are not involved in the direct binding of Fru 6-P. However, change of Arg102 to Leu and to Lys resulted in a 325× and 22×, respectively, increase in $K_m^{F6P}$, and change of Arg102 to Glu resulted in nearly complete loss of the kinase activity. Change of Cys105 to Ala or Ser increased $K_m^{F6P}$ about 10×. The $V_{max}$ of all these mutated enzymes except the one that changed Arg102 to Glu (R102E) was increased 10% to 85%. The kinetic parameters of Fru 2,6-Pase were not altered by these changes. R102E formed several polymeric forms of the enzyme, including a tetramer. Both R102E and an additional derivative that substituted Lys for Arg102 (R102K) were slightly more susceptible to guanidine inactivation than the wild-type enzyme. These results suggest that Arg102 is essential for binding the 6-phosphate of Fru 6-P and that Cys105 also may play a role at the same site.

Synthesis and degradation of Fru 2,6-$P_2$, the most potent activator of phosphofructokinase, are catalyzed by a bifunctional enzyme, Fru 6-P,2-kinase:Fru 2,6-bisphosphatase (Fru 6-P + ATP ⇆ Fru 2,6-$P_2$ + ADP and Fru 2,6-$P_2$ → Fru 6-P + $P_i$). Several isozymic forms of the enzyme from mammalian tissues have been characterized. They are all homodimers, consisting of subunits with an $M_r$ ranging from 54 000 to 60 000. The primary structures of these isozymes revealed that the amino acid sequences of the catalytic domains are well conserved but that C- and N-termini where regulatory domains occur are quite variable (Rider *et al.* 1987; Algaier & Uyeda, 1988; Lively *et al.*, 1988; Crepin *et al.*, 1989; Sakata & Uyeda, 1990; Sakata *et al.*, 1991). It is known that the kinase domain resides in the amino-terminal half and the phosphatase domain in the carboxyl-terminal half of the enzyme.

The kinetics of the reaction catalyzed by Fru 6-P,2-kinase follow ternary complex formation (Kitajima *et al.*, 1984). The substrate binding residues and the residues involved in the catalysis are not known and are being actively investigated. Thus far, Fru 6-P binding sites of Fru 6-P,2-kinase have been studied by chemical modification and site-directed mutagenesis. When the bovine heart and rat liver Fru 6-P,2-kinase:Fru 2,6-Pase[1] (Sakakibara *et al.*, 1984; Kitamura *et al.*, 1989) are reacted with *N*-bromoacetylethanolamine phosphate, the kinase activity is inactivated completely (>98%), and Fru 6-P protects the kinase activity against the inactivation. This affinity labeling reagent reacts with Cys107 of the liver enzyme and Cys105 of the heart enzyme (Kitamura *et al.*, 1989). The amino acid sequence around this Cys is well conserved in all the bifunctional enzymes. In addition, the same reagent alkylated Cys196 of the heart enzyme (Kitamura *et al.*, 1989) and inactivated the kinase activity.

Bazan *et al.* (1989) have proposed a computer-generated model for the Fru 6-P 2-kinase domain based on the known 3-D structure for bacterial phosphofructokinase (Shirakihara & Evans, 1988) which catalyzes a similar reaction. This alignment of the amino acid sequences of PFK and Fru 6-P,2-kinase proposes some similar amino acid residues in the substrate binding region. The proposed sequence alignment is

| | |
|---|---|
| *E. coil* PFK | $^{71}$A R F P E F R D E N I R A$^{93}$ |
| rat testis Fru 6-P,2-kinase | $^{101}$I R K Q C A − L A A L N D$^{112}$ |
| rat liver Fru 6-P,2-kinase | $^{103}$I R K Q C A − L A A L K D$^{112}$ |

Arg72 of *E. coli* PFK binds the α-phosphate of ADP (Shirakihara & Evans, 1988) and corresponds to Arg104 of rat liver or Arg102 of rat testis Fru 6-P,2-kinase according

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* Address correspondence to this author at the Department of Veterans Affairs Medical Center, 4500 S. Lancaster Rd., Dallax, TX 75216. Telephone: (214) 372-7028. FAX: (214) 372-9534.

‡ Present address: Shiga Medical School, Shiga, Japan.

§ Present address: Osaka Medical College, Osaka, Japan.

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[1] Abbreviations: Fru 6-P,2-kinase:Fru 2,6-Pase, fructose 6-phosphate,2-kinase:fructose 2,6-bisphosphatase; WT, wild type; RT2K, rat testis Fru 6-P,2-kinase:Fru 2,6-Pase.

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to the proposed alignment. This is in contrast to the above-mentioned results of chemical modification with *N*-bromoacetylethanolamine phosphate in which the Cys107 of rat liver enzyme is presumed to bind Fru 6-P since the reagent reacts specifically with Cys107. Two additional arginines may be involved in binding Fru 6-P. (1) Arg195 is proposed to bind Fru 6-P, which is also three amino acids distant from the target Cys198 in the liver Fru 6-P,2-kinase or Cys196 in the heart enzyme. A change of Arg195 to Ala increases $K_m^{F6P}$ by 3000-fold (Li *et al.*, 1992), thus confirming the role for this region of the bifunctional enzyme as one of the Fru 6-P binding sites. (2) Treatment of rat liver Fru 6-P,2-kinase:Fru 2,6-Pase with phenylglyoxal inactivates the kinase, and one of the Arg-labeled residues corresponds to Arg225 (Rider & Hue, 1992). Since Fru 6-P protects against the inactivation, it is suggested that Arg225 may be involved in Fru 6-P binding. Since this Arg residue does not occur in the same region of rat testis enzyme, further work is necessary to confirm this idea.

In this paper, we have examined by site-directed mutagenesis several amino acid residues surrounding Cys105 of rat testis enzyme to determine whether the region binds Fru 6-P or ADP. This site corresponds to Cys107 to Cys105 of rat liver and bovine heart enzymes, respectively.

## EXPERIMENTAL PROCEDURES

[$\gamma$-$^{32}$P]ATP and [$\alpha$-$^{35}$S]dATP were purchased from Amersham Corp. (Arlington Heights, IL). Rabbit muscle phosphofructokinase was prepared as described (Uyeda *et al.*, 1978). The cDNA encoding rat testis Fru 6-P,2-kinase: Fru 2,6-Pase was prepared as described (Sakata *et al.*, 1991). Restriction enzymes, T4 DNA ligase, and T4 polynucleotide kinase were purchased from the New England BioLabs (Beverly, MA). Muta-Gene M13 *in vitro* mutagenesis kit was purchased from BioRad Laboratories (Hercules, CA). Fructose 2,6-[2-$^{32}$P]$P_2$ was prepared as described (Furuya *et al.*, 1982). The pT7-7 RNA polymerase/promoter plasmid (Tabor & Richardson, 1985) was a gift of Dr. Stan Tabor (Harvard Medical School). The Sequenase Version 2 sequencing kit was purchased from U.S. Biochemicals (Cleveland, OH). All other chemicals were reagent grade and obtained from commercial sources.

*Site-Directed Mutagenesis.* Plasmid RT2K/pT7-7, containing the rat testis Fru 6-P,2-kinase:Fru 2,6-Pase (RT2K) gene cloned in a pT7-7 vector (Sakata *et al.*, 1991), was digested with *Xba*I and *Hin*dIII, and the isolated 1.7 kb fragment was ligated into the *Xba*I–*Hin*dIII site of M13mp18 (M13/RT2K). The ligation mixture was used to transform *E. coli* JM109-competent cells. The phage harboring M13/RT2K was harvested and transfected into *E. coli* CJ236 (dut$^-$ung$^-$). The purified recombinant MT13/RT2K phage was used to prepare uracil-containing single-stranded template. Synthetic oligonucleotides primers (corresponding to the complementary strand of nucleotides 326–382 of RT2K) used for constructing various mutants are shown in Table 1. The oligonucleotide-directed *in vitro* mutagenesis was performed as described by Kunkel (1985) using the Muta-Gene M13 *in vitro* mutagenesis kit. The synthesized double-stranded DNA was used to transform *E. coli* MV1190-competent cells. Mutant derivatives were identified by DNA sequencing (Sanger *et al.*, 1977), and the DNAs were digested with *Nde*I and *Hin*dIII. The DNA fragments containing the mutated RT2K genes were subcloned into the *Nde*I–*Hin*dIII sites of RT2K/pT7-7 and expressed in *E. coli* as before (Tominaga *et al.*, 1993). The WT and mutant Fru 6-P,2-kinase:Fru 2,6-Pase enzymes were purified as described previously (Tominaga *et al.*, 1993).

Table 1: Oligonucleotides Used for Mutagenesis[a]

| mutants | oligonucleotide sequence |
|---|---|
| K100L | 5′-CTTCCTGATAGCAGGCCCTC-3′ |
| R102L | 5′-ACACTGCTTCAGGATTTTCAG-3′ |
| R102K | 5′-ACACTGCTTCTTGATTTTCAG-3′ |
| R102E | 5′-ACACTGCTTCTCGATTTTCAG-3′ |
| K103L | 5′-GGCACACTGCAGCCTGATTTT-3′ |
| C105A | 5′-TGCCAAGGCAGCCTGCTTCCT-3′ |
| C105S | 5′-TGCCAAGGCAGACTGCTTCCT-3′ |
| D112A | 5′-CTTCCGGACGGCATTGAGGGC-3′ |

[a] The wild-type sequence (5′-CTTCCGGACGTCATTGAGGGCTGCCAAGGCACACTGCTTCCTGATTTTCAGGCCCTC-3′) is complementary to 326–382 of the rat testis Fru 6-P, 2-kinase:Fru 2.6-Pase gene (Sakata *et al.*, 1991).

*Assay Method for Fru 6-P,2-Kinase.* This assay was based on the determination of Fru 2,6-$P_2$ and is the same as described previously (Furuya & Uyeda, 1981) with slight modification. The reaction mixture in a final volume of 50 $\mu$L contained 100 mM Tris/HCl (pH 7.5), 0.1 mM EDTA, 10 mM $MgCl_2$, 2 mM ATP, and 2 mM Fru 6-P. The mixture was incubated at 30 °C for 10 min. At the end of the reaction, 0.1 N NaOH (50 $\mu$L) was added, and the mixture was heated for 90 s at 80 °C. Suitable aliquots were assayed for Fru 2,6-$P_2$ as described by Uyeda *et al.* (1981). One unit of enzyme activity is defined as the amount of enzyme that catalyzes the formation of 1 $\mu$mol of Fru 2,6-$P_2$/min under these conditions.

*Assay Method for Fru 6-P,2-Phosphatase.* This assay measures the formation of Fru 6-P fluorometrically coupled to NADPH formation and was described previously (Tominaga *et al.*, 1993). The reaction mixture (in a final volume of 1.0 mL) contained 100 mM Tris/HCl (pH 7.5), 0.2 mM EDTA, 100 $\mu$M NADP, 0.4 unit of Glu-6-P dehydrogenase, 1 unit of phosphoglucose isomerase, and varying amounts of Fru 2,6-$P_2$. The enzyme was desalted by column centrifugation (Penefsky, 1977) in 15 mM Tris/sulfate (pH 7.5), 0.5 mM EDTA, and 5 mM dithiothreitol. The reaction was initiated with the addition of enzyme and was followed by 25 °C by measuring the NADPH formation at 452 nm emission and excitation at 350 nm using an Aminco-Bowman Series 2 luminescence spectrometer.

*Fru 6-P Inhibition Assay for Fru 2,6-Bisphosphatase.* The assay is the same as described previously (Kitajima *et al.*, 1984) with slight modification. The reaction mixture contained 100 mM Tris/HCl (pH 7.5), 5 mM $MgCl_2$, and 0.5 $\mu$M Fru 2,6-[2-$^{32}$P]$P_2$ ($2.0 \times 10^5$ cpm) in a final volume of 0.1 mL. The reaction was initiated with addition of the enzyme, and the mixture was incubated at 30 °C. At given intervals, aliquots were removed and transferred into 100 $\mu$L of 0.1 N NaOH, and the solution was heated at 80 °C for 2 min to stop the reaction. $H_2O$ (1 mL) was added to the heated reaction mixture, and the sample was adsorbed on a Dowex 1-Cl$^-$ column ($0.5 \times 4$ cm) which had been equilibrated with 0.02 N $NH_4OH$. The column was washed with 1 mL of $NH_4OH$ followed by 1 mL of 0.15 M NaCl in 0.02 N $NH_4OH$. [$^{32}$P]Phosphate was then eluted with 7 mL

Table 2: Kinetic Constants of Fru 6-P,2-Kinase Activity of Rat Testis Fru 6-P,2-Kinase:Fru 2,6-Pase and the Mutant Enzymes[a]

| enzyme | $K_m^{F6P}$ ($\mu$M) | $K_m^{ATP}$ ($\mu$M) | $V_{max}$ (milliunits/mg) | $V_{max}/K_m^{F6P}$ | $V_{max}/K_m^{ATP}$ |
|---|---|---|---|---|---|
| wild type | 40 | 100 | 100 | 2.5 | 1.0 |
| R102L | $13 \times 10^3$ | 128 | 156 | 0.012 | 1.2 |
| R102K | 870 | 185 | 185 | 0.21 | 1.0 |
| R102E | $>150 \times 10^3$ | | <25 | | |
| K100L | 107 | 254 | 114 | 1.1 | 0.45 |
| K103L | 65 | 278 | 114 | 1.8 | 0.41 |
| C105A | 360 | 83 | 130 | 0.36 | 1.6 |
| C105S | 400 | 376 | 183 | 0.46 | 0.49 |
| D112A | 47 | 233 | 112 | 2.4 | 0.48 |

[a] $K_m^{F6P}$s were determined at 2 mM ATP and other experimental conditions as described under Experimental Procedures. $K_m^{ATP}$s were determined with the following Fru 6-P concentrations: 100 mM for R102L, R102E, and R102K; and 2 mM for all the other enzymes.

of the same solution. Aliquots (2 mL) of the eluate were mixed with 10 mL of Optifluor (Packard) and counted in a scintillation counter.

*Other Methods.* Polyacrylamide slab gel electrophoresis was performed with the Phast System (Pharmacia). Protein concentration was determined by the Bradford method (Bradford, 1976) using bovine serum albumin as a standard.

## RESULTS

*Expression and Purification of Various Mutant Enzymes of Rat Testis Fru 6-P,2-Kinase:Fru 2,6-Pase.* Previously we reported a purification procedure for the wild-type rat testis bifunctional enzyme as well as N-terminal deletion mutant enzymes (Tominaga *et al.*, 1993). The mutated enzymes described here were purified using the same purification procedure as that for the wild-type enzyme. All the mutant enzymes were purified to apparent homogeneity as judged by polyacrylamide gel electrophoresis.

*Steady-State Fru 6-P,2-Kinase Kinetics.* The kinetic constants for the wild-type Fru 6-P,2-kinase and the mutant enzymes are presented in Table 2. Among these mutated enzymes, R102 derivative mutant enzymes showed most the dramatic increase in $K_m$ for Fru 6-P; the $K_m$ values of R102L, R102K, and R102E increased 325-fold, 24-fold, and 3750-fold, respectively, compared to that of the wild-type enzyme. The $V_{max}$ values of R102L and R102K increased 30−40% over that of the wild-type enzyme, but the kinase activity of R102E was too low to determine any of the kinetic constants accurately. $V_{max}/K_m^{F6P}$ values of these mutants decreased 10−150-fold. On the other hand, the $K_m^{ATP}$ of R102L increased only 2-fold, but that of R102K was the same as that of the wild-type enzyme. These results demonstrate the importance of positive charges in the amino acid side chain and suggest an ionic interaction between Arg102 with the 6-phosphate of Fru 6-P. C105A and C105S also showed a 9−10-fold increase in the $K_m$ for Fru 6-P, while K100L and K103L showed only slight increases (1.5−2.6-fold) in the $K_m^{F6P}$. $V_{max}/K_m^{Fru6P}$ values of the Cys mutants were 5−7-fold lower than that of the wild-type enzyme. All mutant enzymes except the C105A showed a 1.3−3.8-fold increase in the $K_m$ for ATP. These results suggested the importance of Arg102 and Cys105 as binding sites for Fru 6-P, and the confirmed the earlier report for the involvement of Cys105 in hexose-P binding as demonstrated with affinity labeling with *N*-bromoacetylethanolaminephosphate (Sakakibara *et al.*, 1984; Kitamura *et al.*, 1989).

Table 3: Kinetic Constants of Fru 2,6-Pase Activities of Rat Testis Fru 6-P,2-Kinase:Fru 2,6-Pase and the Mutant Enzymes[a]

| enzyme | $K_m^{F26P2}$ ($\mu$M) | $V_{max}$ (milliunits/mg) | $V_{max}/K_m$ | $K_i^{F6P}$ |
|---|---|---|---|---|
| wild type | 0.031 | 17 | 548 | 0.48 |
| R102L | 0.043 | 17 | 391 | 0.56 |
| R102K | 0.035 | 20 | 571 | 0.52 |
| R102E | 0.18 | 18 | 90 | 0.51 |
| K100L | 0.020 | 11 | 555 | |
| K103L | 0.021 | 11 | 500 | |
| C105A | 0.022 | 12 | 536 | |
| C105S | 0.029 | 15 | 503 | |
| D112A | 0.027 | 16 | 600 | |

[a] Fru 2,6-Pase activity was assayed as described under Experimental Procedures, and $K_i^{F6P}$ was determined in the presence of varying concentrations of Fru 6-P.

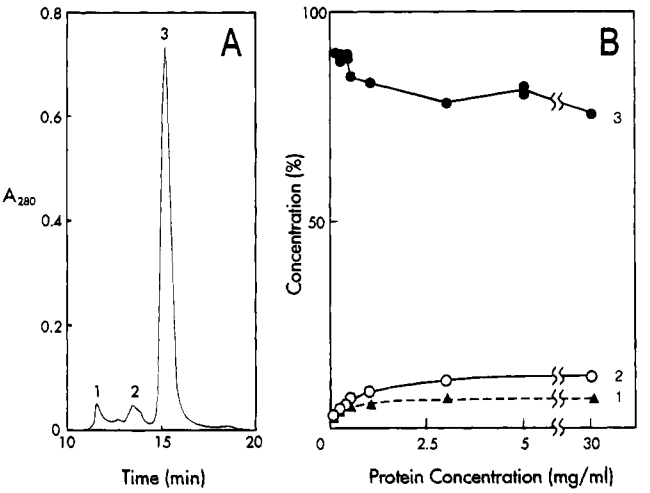


FIGURE 1: Molecular sieve filtration of R102E and distribution of polymers at varying protein concentrations. (A) The R102E enzyme (50 $\mu$L, 5 mg/mL) was applied to a TSK gel G3000 SW (7.8 mm × 30 cm) (TosoHaas) and eluted with buffer A at 0.5 mL/min, using a Dionex HPLC system. (B) The relative concentrations of enzyme peaks were estimated from the areas under the peaks. The protein concentrations represent those in the samples loaded onto the column.

*Steady-State Fru 2,6-Pase Kinetics.* The kinetic constants of the wild-type Fru 2,6-Pase and the mutant enzymes are shown in Table 3. Since each mutation was introduced in the Fru 6-P,2-kinase domain, one would not expect Fru 2,6-Pase activity to be affected. Indeed the $K_m^{F26P2}$ values of all but one mutant enzyme were found to be similar to that of the wild-type enzyme. The R102E enzyme showed a 6-fold increase in its $K_m^{F26P2}$. The reason for this is unclear, but it might be related to the tendency of this mutant enzyme to aggregate as discussed below. The $V_{max}$ values of all enzymes except K100L, K103L, and C105A were similar. With the exception of R102E, the specificity constants ($V_{max}/K_m$) were also very similar to the wild-type enzyme. $K_i^{F6P}$ values of the R102 enzymes also were not changed.

*Polymerization of R102E.* When the R102E enzyme was subjected to molecular sieve filtration on a TSK G3000W column (TosoHaas, Penn), one major and at least two minor peaks of the enzyme were eluted from the column (Figure 1). Neither the wild-type enzyme nor any of the other mutant enzymes contained any significant amounts of these higher polymers under the same conditions. Table 4 summarizes the specific activities and the relative concentrations of these multiple forms of the enzymes and their molecular weights, which were estimated based on the elution times of a dozen

Table 4: Characteristics of Multiple Forms of R102E

| peak no. | $M_r$ | concn (%) | sp act (milliunits/mg) |
|---|---|---|---|
| 1 | 207 000 | 6.2 | 4 |
| 2 | 127 000 | 8.2 | 22 |
| 3 | 82 000 | 83 | 47 |

marker proteins from the same column. The major enzyme, representing 83% of the mixture, was a dimer since the wild-type enzyme showed the same elution time (15.8 min). The estimated $M_r$ of the dimer was 82 000, which was considerably smaller than the theoretical value of 110 000. The lower $M_r$ suggests that the dimeric form of the enzyme was a compact molecule compared to the marker proteins. Peak 1 enzyme ($M_r$ = 207 000) was probably a tetramer, and peak 2 enzyme ($M_r$ = 127 000) might be either a trimer or a partially unfolded dimer. The Fru 2,6-Pase activities of these polymeric forms of the enzymes were considerably less ($^1/_{10}$ for tetramer; $^1/_2$ for peak 2) than the dimer. These polymers underwent protein concentration-dependent dissociation (Figure 1B); below 0.5 mg/mL, both the tetramer and the peak 2 protein gradually decreased and the dimer concentration increased, suggesting that the polymers are in equilibrium with the dimeric forms. Freezing caused a significant increase in the tetrameric form of R102E to as much as 70%. Similar aggregation to a tetramer was observed with frozen samples of R102K but was not observed with the other enzymes.

*Denaturation in Guanidine.* The above results of R102E and R102K may suggest alteration in the subunit−subunit interaction as a result of Arg102 substitution. Denaturation of R102E, R102K and the wild-type enzyme in guanidine was determined by following Fru 2,6-Pase activity. The results showed that these mutants were slightly more sensitive to guanidine denaturation (data not shown), and 50% inactivation of R102E and R102K occurred at 0.87 and 0.89 M guanidine, respectively, compared to 0.93 M for wild type.

## DISCUSSION

Hartman *et al.* (1973) showed that *N*-bromoacetylethanolamine phosphate alkylates essential histidine and lysine residues of aldolase. The previous study (Kitamura *et al.*, 1989) from this laboratory demonstrated that it also alkylates two cysteine residues, Cys107 of rat liver and bovine heart Fru 6-P,2-kinase;Fru 2,6-Pase (or Cys105 of bovine heart enzyme) and Cys196 of the heart enzyme. The results of the site-directed mutagenesis presented in this paper support the conclusion that this reagent reacts with or near the Fru 6-P binding site of Fru 6-P,2-kinase domain of the bifunctional enzymes. The change of Cys to Ala or Cys to Ser resulted in a 8−10-fold increase in the $K_m$ for Fru 6-P without affecting $V_{max}$. More importantly, however, the change of Arg102 to Leu and Arg102 to Lys, which is three amino acid residues to the N-terminal side of this Cys, affected the $K_m$ for Fru 6-P from 22- to 325-fold without affecting $V_{max}$. The Arg102 to Glu substitution resulted in over 75% loss of the kinase activity. These changes in the kinetic properties of the mutated enzymes were not due to global structural changes for the following reasons: (a) Only the $K_m$ for Fru 6-P of Fru 6-P,2-kinase was specifically altered without affecting Fru 2,6-Pase kinetic properties. (b) Intrinsic tryptophan fluorescence spectra of the Arg102 mutant enzymes were identical to that of the wild-type enzyme (data not shown). (c) With the exception of the R102E mutant, no derivative altered the elution pattern from the ion exchange or the affinity chromatography during purification. The R102E enzyme did not bind to an affinity column using Yellow-3 (Sigma, St. Louis, MO) as tightly as the others, and this may be the result of nearly complete loss of Fru 6-P,2-kinase activity and the extremely large $K_m^{F6P}$. (d) The denaturation curves in guanidine were also similar. Furthermore, Arg102 seems to be a specific residue since the mutations of Lys100 and the adjacent Lys103 had only 1.5−2.5-fold changes in $K_m^{F6P}$. Thus, we conclude that Arg102 probably binds the 6-phosphoryl group of Fru 6-P. However, we cannot rule out the possibility that Arg102 may form a hydrogen bond with one of the OH groups of Fru 6-P. Cys105 may not be essential for direct interaction with Fru 6-P since the substitution with hydrophobic (Ala) and hydrophilic (Ser) amino acids caused a similar (9−10×) increase in the apparent $K_m$ for Fru 6-P. These results suggest that even though Cys105 may be a component of the Fru 6-P binding site, it does not interact in an essential manner with the substrate. It may, for example, provide a proper orientation of the side chains of the other amino acid residues at the binding site.

Clearly, Arg102 is not the only basic residue essential in hexose-P binding at the Fru 6-P,2-kinase active site. Crystallography of bacterial PFK has shown that three Arg residues are involved in binding of the 6-phosphate of Fru 6-P (Shirakihara & Evans, 1988). If the hexose-P binding site of the bifunctional enzymes was similar to that of phosphofructokinase, additional basic residues are essential. Previously it was shown that Cys196 of the bovine heart enzyme also reacts with *N*-bromoacetylethanolamine phosphate (Kitamura *et al.*, 1989). It is interesting that by site-directed mutagenesis, Arg195 of rat liver enzymes, which corresponds to Arg193 of heart enzyme, has been shown to be essential for Fru 6-P binding (Li *et al.*, 1992). Similarly to Arg102, Arg195 also is situated three amino acid residues to the N-terminal side of the target Cys in the highly conserved peptide whose sequence in the bifunctional enzymes is ArgIleGluCysTyr. Thus, Arg195 represents the potential second Arg residue linking Fru 6-P. The third basic residue may be Arg225 since chemical modification of rat liver Fru 6-P,2-kinase·Fru2,6-Pase with phenylglyoxal reacts with Arg225 and inactivates the kinase (Rider & Hue, 1992). However, since this Arg residue occurs in the rat liver and bovine heart enzymes but not in rat testis bifunctional enzyme, additional experimentation such as mutagenesis is required to provide more definitive support for this contention.

The sequence alignment proposed by Bazan *et al.* (1989) suggests Arg102 and Lys103 as being involved in ADP binding. The results presented herein strongly suggest that Arg102 is essential in binding Fru 6-P. However, the change of Lys103 to Leu resulted in a slight increase (1.5-fold) in $K_m^{F6P}$ but a 2.8-fold increase in $K_m^{ATP}$, which may imply that this Lys is involved in binding ATP or ADP. This possibility seems unlikely, however, because if Fru 6-P and ATP-Mg were oriented by binding to these two adjacent amino acid residues at the active site, C2-OH, rather than 6-$PO_4$, should be in close proximity for the nucleophilic attack on the terminal phosphate of ATP. Thus, Arg102 and Lys103, if any, seem more likely to be involved in Fru 6-P binding. These results suggest that the active sites of Fru

6-P,2-kinase and phosphofructokinase are different, although some similarity exists, and that the proposed alignment may not be accurate and requires further refinement. An additional difference is that the Fru 6-P binding site of phosphofructokinase spans two different subunits (Shikakihara & Evans, 1988), but as yet there is no direct evidence that the same holds true for Fru 6-P,2-kinase. Furthermore, the alignment between phosphofructokinase and Fru 6-P,2-kinase predicts that Cys160 and Asp162 of the latter enzyme (liver) corresponds to Asp127 and Asp129 of phosphofructokinase which are known to be involved in the catalysis. However, site-directed mutagenesis demonstrated that these residues are not involved in catalysis of the Fru 6-P,2-kinase reaction (Crepin *et al.*, 1993).

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## REFERENCES

Algaier, J., & Uyeda, K. (1988) *Biochem. Biophys. Res. Commun. 153*, 328−333.
Bazan, J. F., Fletterick, R. J., & Pilkis, S. J. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 9642−9646.
Bradford, M. M. (1976) *Anal. Biochem. 72*, 248−254.
Crepin, T. M., Darville, M. I., Michel, A., Hue, L., & Rousseaue, G. G. (1989) *Biochem. J. 264*, 151−160.
El-Maghrabi, M. R., Pate, T. M., Pilkis, J., & Pilkis, S. J. (1984) *J. Biol. Chem. 259*, 13104−13110.
Furuya, E., & Uyeda, K. (1981) *J. Biol. Chem. 256*, 7109−7112.
Furuya, E., Yokoyama, M., & Uyeda, K. (1982) *Biochem. Biophys. Res. Commun. 105*, 164−270.
Hartman, F. C., Suh, B., Welch, M. H., & Barker, R. (1973) *J. Biol. Chem. 248*, 8233−8239.
Kitajima, S., Sakakibara, R., & Uyeda, K. (1984) *J. Biol. Chem. 259*, 6896−6903.
Kitajima, S., Thomas, H., & Uyeda, K. (1985) *J. Biol. Chem. 260*, 13995−14002.
Kitamura, K., Uyeda, K., Hartman, F. C., Kangawa, K., & Matsuo, H. (1989) *J. Biol. Chem. 264*, 6344−6348.
Kunkel, T. A. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 488−492.
Li, L., Liu, K., Kurland, I. J., Correia, J. J., & Pilkis, S. J. (1992) *J. Biol. Chem. 267*, 4386−4393.
Lively, M. O., El-Maghrabi, M. R., Pilkis, J., D'Angelo, G., Colosia, A. D., Ciavola, J. A., Fraser, B. A., & Pilkis, S. J. (1988) *J. Biol. Chem. 263*, 839−849.
Penefsky, H. S. (1977) *J. Biol. Chem. 252*, 2891−2899.
Rider, M. H., & Hue, L. (1992) *Eur. J. Biochem. 207*, 967−972.
Rider, M. H., Marchand, M. J., Hue, L., & Rousseau, G. G. (1987) *FEBS Lett. 2243*, 317−321.
Sakakibara, R., Kitajima, S., Hartman, F. C., & Uyeda, K. (1984) *J. Biol. Chem. 259*, 14023−14028.
Sakata, J., & Uyeda, K. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 4951−4955.
Sakata, J., Abe, Y., & Uyeda, K. (1991) *J. Biol. Chem. 266*, 15764−15770.
Sanger, F., Nicklen, S., & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463−5467.
Shirakihara, V., & Evans, P. R. (1988) *J. Mol. Biol. 204*, 973−994.
Tabor, S., & Richardson, C. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 1074−1078.
Tominaga, N., Minami, Y.; Sakakibara, R., & Uyeda, K. (1993) *J. Biol. Chem. 268*, 15951−15957.
Uyeda, K., Miyatake, A., Luby, L. J., & Richards, E. G. (1978) *J. Biol. Chem. 253*, 8319−8327.
Uyeda, K., Furuya, E., & Luby, L. J. (1981) *J. Biol. Chem. 256*, 8394−8399.

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