# Chemical Mechanism of the Fructose-6-Phosphate,2-Kinase Reaction from the pH Dependence of Kinetic Parameters of Site-Directed Mutants of Active Site Basic Residues†

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ABSTRACT: A bifunctional enzyme, fructose-6-phosphate 2-kinase−fructose 2,6-bisphosphatase, catalyzes synthesis and degradation of fructose 2,6-bisphosphate. Mutants of basic residues, including Lys51, Arg78, Arg79, Arg136, Lys172, and Arg193, immediately around the active site of rat testis fructose 6-P,2-kinase were constructed, and their steady state kinetics, ATP binding, and the effect of pH on the kinetics were characterized. All mutants showed a several-fold increase in $K_{MgATP}$, much larger increases in $K_{Fru\ 6-P}$, and decreased $V$ compared to those of the wild type enzyme (WT). Replacement of Lys172 and Arg193 with Ala and Leu, respectively, also produced mutants with large $K_{Fru\ 6-P}$ values. Substitution of Lys51, which is located in a Walker-A motif (GXXGXGKT, amino acids 45−52), with Ala or His resulted in enzymes with increased $K_{MgATP}$ values and unable to bind Fru 6-P. The dissociation constants for 2′-(3′)-*O*-(*N*-methylanthraniloyl)-ATP (mantATP) and ATP of all these mutants except Lys51 were similar. Lys51 mutants were unable to bind mantATP. The pH dependence of $V$ and the $V/K$s for MgATP and Fru 6-P suggest a mechanism in which reactants and enzyme combine irrespective of the protonation state of groups required for binding and catalysis, but only the correctly protonated enzyme−substrate complex is catalytically active. A chemical mechanism is suggested in which a general base accepts a proton from the 2-hydroxyl of Fru 6-P concomitant with nucleophilic attack on the $\gamma$-phosphate of MgATP. Phosphoryl transfer is also facilitated by interaction of the $\gamma$-phosphate with a positively charged residue that neutralizes the remaining negative charge. The dianionic form of the 6-phosphate of fructose 6-P is required for binding, and it is likely anchored by a positively charged enzyme residue. A comparison of the pH dependence of kinetic parameters for Ala or His mutant proteins at Lys51, Lys172, and Arg79 suggests that Lys51 interacts with the $\gamma$-phosphate of MgATP and that several other arginines likely participate in transition state stabilization of the transferred phosphoryl. The active site general base has yet to be identified.

Fructose-6-phosphate 2-kinase−fructose 2,6-bisphosphatase is a bifunctional enzyme that catalyzes the synthesis and degradation of Fru 2,6-$P_2$,[1] the most potent activator of phosphofructokinase (PFK) known (*1*).

$$\beta\text{-D-fructose 6-phosphate} + \text{MgATP} \rightleftharpoons \beta\text{-D-fructose 2,6-bisphosphate} + \text{MgADP} \quad (1)$$

$$\beta\text{-D-fructose 2,6-bisphosphate} \rightarrow \beta\text{-D-fructose 6-phosphate} + HPO_4^{2-} \quad (2)$$

Thus far, several tissue-specific isozymes have been purified from mammalian sources, and cDNAs encoding these isozymes have been isolated (*2*−*8*). The primary structures of the tissue-specific isozymes indicate that the catalytic domains of both the kinase and the phosphatase are highly conserved. The high degree of conservation suggests that the active site and therefore the reaction mechanism are similar for all isozymes. The Fru 6-P,2-kinase domain resides in the N-terminal half of the protein, while the Fru 2,6-Pase domain is located at the C terminus.

The reaction catalyzed by Fru 6-P,2-kinase follows a sequential kinetic mechanism (*9*) with a proposed direct transfer of the $\gamma$-phosphate of MgATP to the 2-OH of $\beta$-D-Fru 6-P (*10*). The crystal structure of the rat testis Fru 6-P,2-kinase−Fru 2,6-Pase complexed with MgATP has been determined to 2.0 Å resolution (*11*, *12*). The resulting structure indicates that the Fru 6-P,2-kinase domain is structurally similar to nucleoside monophosphate (NMP) kinases, such as adenylate kinase (ADK) and uridylate kinase (UDK), and also to the catalytic core of the GTP-binding proteins, ras and $G_{i\alpha}$. However, the structure of the Fru 6-P,2-kinase domain differs from PFK, even though the two enzymes catalyze similar reactions. The similarity of the Fru 6-P,2-kinase domain to those of the NMP kinases and the catalytic core of G-proteins has led to speculation that all of these enzymes employ similar catalytic mechanisms.

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[1] Abbreviations: Fru 6-P,2-kinase, fructose-6-phosphate, 2-kinase; Fru 2,6-Pase, fructose 2,6-bisphosphatase; Glu 6-P, $\beta$-D-glucose 6-phosphate; Fru 6-P, $\beta$-D-fructose 6-phosphate; Fru 2,6-$P_2$, fructose 2,6-bisphosphate; mantATP, 2′(3′)-*O*-(*N*-methylanthraniloyl)-ATP; DTT, dithiothreitol.

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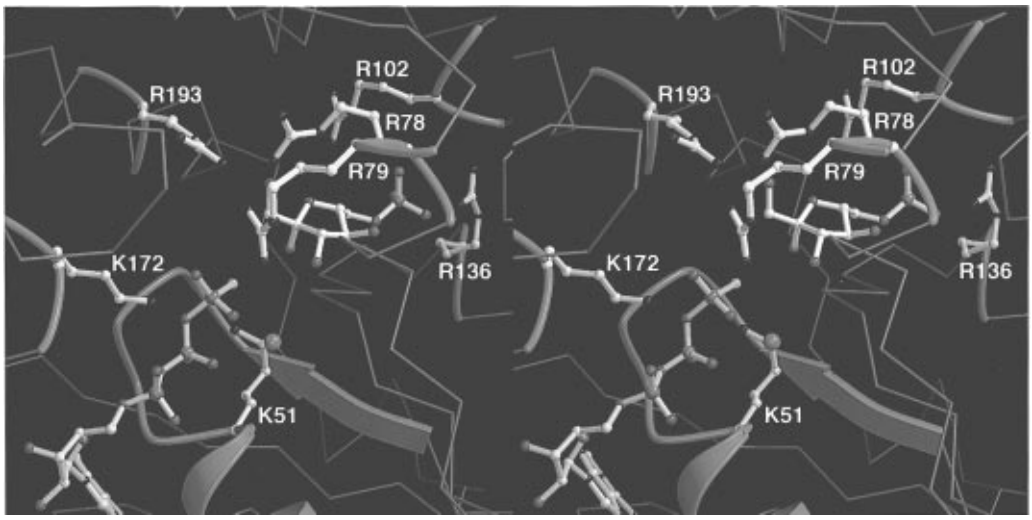



FIGURE 1: Stereodiagram of the substrate binding region of the Fru 6-P,2-kinase domain of Fru 6-P,2-kinase−Fru 2,6-Pase. The side chains mutated in this study are drawn as ball and stick models as is the MgATP. K51 and K172 are in contact distance to the $\gamma$-phosphate and the $\beta-\gamma$ bridge oxygen of ATP, respectively. Fru 6-P has been modeled into the proposed Fru 6-P binding site on the basis of (1) the coincidence of an empty cavity in the crystal structure with the substrate binding site of the homologous NMP kinases and (2) the convergence of residues shown to affect $K_{\text{Fru 6-P}}$ in this region. R193, R78, and R79 are in the proximity of the $\gamma$-phosphate of ATP and/or the 2-OH of the modeled Fru 6-P in a position to stabilize the transition state and/or product formation. The proximity of R136 and R102 to the 6-phosphate of Fru 6-P is apparent.

The mechanism of the Fru 6-P,2-kinase is presently unknown, but it is clear that there are differences from the mechanism of PFK, despite the similarity in reaction. Data suggest that, unlike the PFK reaction, acidic residues such as Asp or Glu likely do not serve as a general base (*13*). The NMP kinases contain two mobile segments for MgATP and substrate binding (*14*, *15*). The proposed function of the mobile segments is exclusion of water from the bound MgATP to prevent hydrolysis. The Fru 6-P,2-kinase contains analogous segments (residues 74−100 and 159−194) that sequester Fru 6-P and MgATP, respectively. In the crystal structure of rat testis enzyme, the ATP binding loop of the Fru 6-P,2-kinase domain is closed as predicted since MgATP is bound, while the Fru 6-P binding loop is only partially closed (*12*).

Figure 1 shows the substrate binding sites of the Fru 6-P,2-kinase domain of rat testis Fru 6-P,2-kinase−Fru 2,6-Pase (*12*). The nucleotide, MgATP, is bound to the active site of the kinase, primarily by interaction with the main chain nitrogens of the "Walker-A motif" (*16*), and by the side chains of K51 and K172, which are essential for neutralizing the negative charges of the polyphosphate backbone of ATP. The crystal structure has shown that Thr52 and Asp128 are involved in the binding of the $Mg^{2+}$ ion which is chelated to ATP (*12*). Although the structure of the enzyme complexed with Fru 6-P has not been determined, its binding site and the residues involved can be predicted by computer-docking experiments. Thr130, Asn73, and Tyr197 are located in the active site in close proximity to Fru 6-P (as it is modeled into the site). Arg78, Arg79, and Arg193 are proposed to be located near the phosphate to be transferred (the 2-phosphate of Fru 2,6-$P_2$, as modeled into the active site, or the $\gamma$-phosphate of MgATP), while Arg102 and Arg136 interact with the 6-phosphate (*12*). Some of the above-mentioned residues, including Arg102 and Arg193 of the rat testis bifunctional enzyme (corresponding to Arg104 and Arg195 of the liver isozyme), have been previously implicated in the binding of Fru 6-P by site-directed mutagenesis (*17*−*20*) and chemical modification (*21*, *22*).

In order to confirm our proposed binding site for Fru 6-P and also the ATP binding site as determined by X-ray crystallography (*12*), we have constructed mutants of the above-mentioned basic residues of the rat testis Fru 6-P,2-kinase and investigated their steady state kinetics and nucleotide binding characteristics compared to those of the native enzyme. The effect of pH on the kinetic parameters and the effect of mutation of the basic residues on the pH dependency have also been studied with a goal toward identifying the amino acids responsible for the pH−activity profile with an added hope that such studies may shed some light into a general base involved in the catalysis of Fru 6-P,2-kinase.

## MATERIALS AND METHODS

*Materials*. [$\alpha$-$^{35}$S]dATP (1000 Ci/mmol) was purchased from Amersham Corp. (Arlington Heights, IL). 2′(3′)-*O*-(*N*-Methylanthraniloyl)-ATP (mantATP) was synthesized by the method of Hiratsuka (*23*) and purified by HPLC (*24*). Rabbit muscle PFK was prepared as described previously (*25*). The cDNA encoding rat testis Fru 6-P,2-kinase−Fru 2,6-Pase (RT2K) was prepared as reported previously (*7*). The pT7-7 RNA polymerase/promoter plasmid (*26*) was a gift from S. Tabor (Harvard Medical School). Restriction enzymes and T4 polynucleotide kinase were purchased from New England Biolabs (Beverly, MA). The DNA ligation kit was from Takara Shuzo (Kyoto, Japan). The Muta-Gene M13 *in vitro* mutagenesis kit was from BioRad Laboratories (Hercules, CA). The sequenase version 2.0 sequencing kit was from U.S. Biochemicals (Cleveland, OH). All other chemicals were reagent grade and obtained from commercial sources.

*Site-Directed Mutagenesis*. Oligonucleotide-directed *in vitro* mutagenesis was performed as described by Kunkel (*27*), using the Muta-Gene M13 *in vitro* mutagenesis kit. Plasmid RT2K/pT7-7 containing the RT2K gene, cloned in a pT7-7 vector (*7*), was digested with *Xba*I and *Hin*dIII, and the isolated 1.7 kb fragment containing the RT2K gene was ligated into the *Xba*I−*Hin*dIII site of M13mp18 (M13/

RT2K). The phage harboring M13/RT2K was transfected into *Escherichia coli* CJ236 (*dut*$^-$*ung*$^-$). The recombinant M13/RT2K phage was purified and used to prepare uracil-containing single-stranded template. Synthetic oligonucleotide primers used for constructing various mutants are listed in Table 1. The synthesized double-stranded DNA was used to transform *E. coli* MV1190. Mutant derivatives were identified by DNA sequencing (*28*). The mutant DNAs were digested with *Nde*I and *Hin*dIII, and the DNA fragments containing a mutated RT2K gene were subcloned into the *Nde*I-*Hin*dIII site of pT7-7 (RT2K/pT7-7). The obtained expression plasmids were transformed into *E. coli* BL21 (DE3) cells, and recombinant mutant enzymes were purified as described below. Before assay, all enzymes were desalted by column centrifugation (*29*) in 50 mM Tris-phosphate (pH 7.5) containing 0.5 mM EDTA, 0.5 mM EGTA, 2 mM DTT, 1% polyethylene glycol ($M_r$ = 300), and 5% glycerol.

*Expression and Purification of Mutant Enzymes.* The mutant enzymes described here were purified using essentially the same procedure as that for the wild type enzyme (*30*). For those mutants that did not bind to the Yellow-3 column, a slight modification was required. For the purification of K172E and K51H mutants, a Blue-Sepharose column (2.5 cm × 8 cm) was used instead of the Yellow-3 column. The K172E enzyme was eluted from the column with 15 mM Tris-phosphate (pH 7.5), containing 1 M KCl, while the K51H enzyme was eluted from the column with a linear gradient of 1 to 2.4 M KCl in 15 mM Tris-phosphate (pH 7.5). All the enzymes were purified to apparent homogeneity as judged by SDS−polyacrylamide gel electrophoresis (SDS−PAGE).

*Assay Method for Fru 6-P,2-Kinase.* The assay was based on the determination of the amount of Fru 2,6-$P_2$ produced and is the same as that described previously (*31*) 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, and the indicated concentrations of Fru 6-P, $MgCl_2$, and ATP. The reaction was initiated by the addition of enzyme, and the reaction mixture was incubated at 30 °C for 10 min. After the incubation, the reaction was terminated by adding 50 $\mu$L of 0.1 N NaOH, and the mixture was incubated at 85 °C for 90 s. Suitable aliquots were assayed for Fru 2,6-$P_2$ as described by Uyeda *et al.* (*32*). One unit of enzyme activity is defined as the amount of enzyme that catalyzes the formation of 1 $\mu$mol of Fru 2,6-$P_2$ per minute under these conditions.

*Assay Method for Fru 2,6-Pase.* The assay continuously measures the formation of Fru 6-P fluorometrically coupling its production to the formation of NADPH via the phosphoglucose isomerase and Glu 6-P dehydrogenase reactions, as described previously (*30*). The reaction mixture, in a final volume of 1 mL, contained 100 mM Tris-HCl (pH 7.5), 0.2 mM EDTA, 0.1 mM NADP, 0.4 unit of Glu 6-P dehydrogenase, 1 unit of phosphoglucose isomerase, and varying amounts of Fru 2,6-$P_2$. The reaction was initiated by the addition of enzyme and followed at 25 °C by measuring the NADPH formation with excitation and emission wavelengths of 350 and 452 nm, respectively. One unit of enzyme activity is defined as the amount of enzyme that catalyzes the formation of 1 $\mu$mol of NADPH per minute under the above conditions.

*Binding of MantATP and ATP.* MantATP binding to enzymes was determined fluorometrically as described previously (*13*). The binding of MgATP was determined by measuring displacement of enzyme-bound mantATP. A saturating amount of mantATP, an amount that gave the maximum increase in fluorescence intensity, was added to enzyme (0.1 mg/mL in a final volume of 0.2 mL). Aliquots (1 $\mu$L each) of an ATP solution were sequentially added to the reaction mixture, and a decrease in fluorescence intensity was recorded. Correction for dilution was made by adding 1 $\mu$L each of $H_2O$ instead of ATP to the mantATP enzyme solution.

*pH Titration of Fru 6-P,2-Kinase Activity.* In order to determine the pH dependence of the kinetic parameters of the rat testis Fru 6-P,2-kinase reaction, activity was measured as a function of pH from pH 6.5 to 9.5. The buffer system consisted of 0.1 M Mes, 0.051 M *N*-ethylmorpholine, and 0.051 M diethanolamine (MND buffer) (*33*). The Fru 6-P,2-kinase activity was assayed using the method described above with slight modification. The reaction mixture, in a final volume of 100 $\mu$L, contained MND buffer at various pH values, 0.1 mM EDTA, and the indicated concentrations of Fru 6-P, $MgCl_2$, and ATP. The reaction was initiated by adding enzyme, and the reaction mixture was incubated at 30 °C for 10 min. The reaction was terminated by adding 100 $\mu$L of 0.1 N NaOH, and the mixture was heated to 85 °C for 90 s. Suitable aliquots of the reaction mixture were assayed for Fru 2,6-$P_2$. The pH of the reaction mixture was measured before the addition of the enzyme. Since Fru 2,6-$P_2$ is labile in acid, loss of Fru 2,6-$P_2$ during the reaction was checked by incubating a known amount of Fru 2,6-$P_2$ at various pHs. There was no significant loss (less than 10%) of Fru 2,6-$P_2$ under these conditions.

*Data Analysis.* The following rate equations were fitted to the data using a program developed by Cleland (*34*) with the DeltaGraph software (DeltaPoint, Monterey, CA). All plots of reciprocal initial velocity *vs* reciprocal substrate concentration were linear.

$$v = VAB/(K_{ia}K_b + K_aB + K_bA + AB) \quad (3)$$

$$\log y = \log[C/(1+K_2/H)] \quad (4)$$

$$\log y = \log[C/[1+H/K_1)] \quad (5)$$

$$\log y = \log[C/(1+H/K_1+K_2/H+H^2/K_1K_3)] \quad (6)$$

For the determination of kinetic parameters, initial velocity data were fitted using eq 3, in which $v$ and $V$ are the initial and maximal velocities of the reaction, respectively, $A$ and $B$ are substrate concentrations, $K_a$ and $K_b$ are Michaelis constants for substrates A and B, respectively, and $K_{ia}$ is the inhibition constant for A. For the determination of $V/K$ values as a function of pH, the concentration of the fixed substrate was saturating at all pHs, and the resulting data were fitted using eqs 4−6. In eqs 4−6, $y$ is $V$ or $V/K$, $C$ is the pH-independent value of $y$, $H$ is the hydrogen ion concentration, and $K_1$, $K_2$, and $K_3$ are acid dissociation constants for groups on the enzyme or substrate.

*Other Methods.* The protein concentration was determined by the Bradford method (*35*), using bovine serum albumin as a standard. SDS−PAGE was carried out according to the procedure of Laemmli (*36*), using the Phast system (Pharmacia Biotech Inc., Uppsala, Sweden).

Table 1: Oligonucleotides Used for Mutagenesis

| mutants | oligonucleotide sequence[a] |
|---|---|
| K51A | 5′-GA AAT GTA GGT CGC GCC CCT G-3′ |
| K51H | 5′-GA AAT GTA GGT CTG GCC CCT G-3′ |
| R78L | 5′-CAT GTC CCG GAG ATA CTG ACC-3′ |
| R78H | 5′-CAT GTC CCG GTG ATA CTG ACC-3′ |
| R79L | 5′-GAC CAT GTC CAG GCG ATA CTG-3′ |
| R79H | 5′-GAC CAT GTC GTG GCG ATA CTG-3′ |
| R136K | 5′-AT CAT CGC TCT CTT TTC TCG GG-3′ |
| R136L | 5′-CAT CGC TCT CTG TTC TCG GGT-3′ |
| R136S | 5′-AT CAT CGC TCT GCT TTC TCG GG-3′ |
| K172A | 5′-AGG GCT GCC CAG CGC CAC TTG CA-3′ |
| K172E | 5′-GCT GCC CAG CTC CAC TTG CAC-3′ |
| K172H | 5′-GCT GCC CAG ATG CAC TTG CA-3′ |
| K172R | 5′-AGG GCT GCC CAG CCT CAC TTG CA-3′ |
| R193L | 5′-CA TTC AAT GAG CCT CAT GAA G-3′ |
| R193H | 5′-CA TTC AAT GTG CCT CAT GAA G-3′ |

[a] The mutated nucleotides are underlined. These oligonucleotides are the complementary DNA of the wild type RT2K at 181−201 (K51A and K51H), 259−280 (R78L and R78H), 263−283 (R79L and R79H), 435−456 (R136S and R136K), 434−454 (R136L), 543−565 (K172A and K172R), 542−562 (K172E and K172H), and 604−624 (R193L and R193H).

## RESULTS

*Steady State Kinetics of Fru 6-P,2-Kinase.* The kinetic constants of the Fru 6-P,2-kinase activity of wild type (WT) and mutant enzymes are summarized in Table 2. Data for the mutant enzymes fall into three categories that roughly reflect the function proposed for the residues changed. Changes made to either of the two lysine residues, K51 or K172, give the most substantive changes in all kinetic parameters, with decreases of about 2, 3, and 4 orders of magnitude observed for $V$, $V/K_{MgATP}$, and $V/K_{Fru\ 6-P}$, respectively. The lysine residues are the best candidates for catalytically important residues. Arginine 136, which is predicted to interact with the 6-phosphate of Fru 6-P, gives, as expected, substantial changes in $V/K_{Fru\ 6-P}$. The $V/K_{Fru\ 6-P}$ is decreased most (3 orders of magnitude) when replaced by the hydrophobic residue, leucine. The remaining three arginine residues, R78, R79, and R193, are thought to be in the vicinity of the transferred phosphate. The largest decreases in kinetic parameters are observed with changes to R193, and in all three cases, decreases in $V/K_{Fru\ 6-P}$ are more pronounced than changes in either $V$ or $V/K_{MgATP}$.

Interestingly, the R78H mutant gives a positively cooperative saturation curve for Fru 6-P (Figure 2). The reason for this change is unclear.

These changes in the kinetic properties of the mutant enzymes were not due to altered protein structure because all these enzymes except K51H and K172E were purified to homogeneity with the same procedure employed for the wild type enzyme. For K51H and K172E, we made a minor change in the elution conditions for affinity chromatography. Furthermore, the Fru 2,6-Pase activities of the mutant enzymes were unchanged (data not shown).

*Mg(MantATP) and MgATP Binding.* Binding of Mg(mantATP) by the WT enzyme and various mutant enzymes was determined in order to compare the affinity of free enzymes for MgATP. Results in Table 3 demonstrate that the dissociation constants for E−Mg(mantATP) are increased 2−3-fold for mutant enzymes compared to those of the WT enzyme, with the exception of K172E, K51A, and K51H. It is not surprising that K172E binds Mg(mantATP) poorly because of the negatively charged Glu residue in the vicinity of the $\beta$- and $\gamma$-phosphate(s) of ATP. The conclusion concerning K172 is supported by the fact that the other mutants of K172, including K172A and K172H, give $K_d$ values comparable to that of the WT enzyme. However, both K51 mutant enzymes give a large increase in the $K_d$ value, suggesting that K51 is essential for binding.

If one compares the $K_d$ of Mg(mantATP) (Table 3) to values of $K_{MgATP}$ (Table 2) for all mutant enzymes and the WT enzyme, the $K_d/K_m$ ratio is 0.01−0.001. Data suggest that binding of the fluorophore to the protein is dominated by the hydrophobic interaction of the mant group with the active site and that the hydrophobic interaction is not greatly affected by mutation of the basic amino acid residues. The dissociation constants for E−MgATP were determined by measuring the decrease in fluorescence accompanying displacement of the bound mantATP by ATP. As shown in Table 3, the $K_{d,ATP}$ values of the mutant enzyme increased from 0.8−3.3-fold compared to that of the WT enzyme. However, if one compares the $K_{d,ATP}$ and $K_{MgATP}$ (Table 2), the $K_m$ values are 1−54-fold higher in the mutant enzymes, while those of the WT enzyme are essentially the same. (mantFru 6-P does not bind to enzyme.)

*pH Dependence of the Kinetic Parameters of Wild Type Testis Fru 6-P,2-Kinase.* The pH dependence of kinetic parameters for the wild type Fru 6-P,2-kinase is shown in Figure 3. The $V/E_t$ for the kinase is relatively constant at approximately $0.071 \pm 0.01\ s^{-1}$ (Figure 3A) between pH 6.5 and 9. A decrease in $V/E_t$ is observed at the extremes of pH, giving approximate p$K$ values of 6.2 and 9.5. The value of $V/K_{MgATP}E_t$ is also constant from pH 6.1 to 8.6 (Figure 3B) but decreases above pH 8.6 with a limiting slope of −1. A p$K$ of 8.9 ± 0.08 and a pH-independent value of $(1.2 \pm 0.1) \times 10^3\ M^{-1}\ s^{-1}$ are estimated from the data. A plot of the pH dependence of $V/K_{Fru\ 6-P}E_t$ (Figure 3C) exhibits a pH optimum between pH 7.3 and 8.4. The $V/K$ decreases at low pH with a limiting slope of +2 and at high pH with a limiting slope of −1. Estimated p$K$ values of 6.7 ± 0.1 and 7.5 ± 0.1 are obtained for the two ionizable groups on the acid side, and a p$K$ of 8.7 ± 0.1 is obtained for the basic group. The pH-independent value of $V/K_{Fru\ 6-P}E_t$ is $(5.9 \pm 0.3) \times 10^3\ M^{-1}\ s^{-1}$. All p$K$ values are summarized in Table 4.

*Attempts To Identify the Residues Involved in the pH−Activity Profile.* In order to identify amino acid residues that contribute to the pH dependence of kinetic parameters of the Fru 6-P,2-kinase reaction, amino acid residues situated at the active site and potentially implicated in the overall reaction mechanism (see above) were altered by site-directed mutagenesis, and the pH dependence of the mutant kinases was investigated.

A measurement of Fru 6-P,2-kinase activity *vs* pH indicated that the mutant proteins fell into distinct classes with respect to their behavior. The descending limb above pH 9 in the pH−rate profiles for the K51A, K172E, and R79L mutants was eliminated (data not shown). The R79H and K172H mutants showed a shift in the pH optimum to lower pH (pH 9 to below 8), as did the K51H mutant (data not shown). Preparation of other mutant enzymes, including R78H, R78L, R193H, R193L, and R136S, did not exhibit any significant changes in their pH activity profiles compared to that of the wild type enzyme (data not shown). Data suggest that Lys51, Lys172, and Arg79 are involved in the

Table 2: Summary of Kinetic Data for Wild Type and Mutant Enzymes Measured at pH 7.5

| | $K_{Fru\ 6-P}$ (mM) | $K_{MgATP}$ (mM) | $V/E_t$ ($s^{-1}$) | $V$ (fold decrease) | $V/K_{Fru\ 6P}E_t$ ($M^{-1}\ s^{-1}$) | $V/K$ (fold decrease) | $V/K_{MgATP}E_t$ ($M^{-1}\ s^{-1}$) | $V/K$ (fold decrease) |
|---|---|---|---|---|---|---|---|---|
| wild type | 0.04 | 0.1 | 0.092 | | 2300 | | 920 | |
| K51A | >20 | 1.3 | 0.0010[a] | 90 | ≤0.05 | ≥46000 | 0.77 | 1200 |
| K51H | >20 | 4.9 | 0.0010[a] | 90 | ≤0.05 | ≥46000 | 0.20 | 4600 |
| R78H | 8 | 2.8 | 0.010 | 9 | 1.3 | 1800 | 3.6 | 250 |
| R78L | 9 | 2.7 | 0.050 | 2 | 5.60 | 400 | 19 | 50 |
| R79H | 0.17 | 0.053 | 0.012 | 8 | 71 | 30 | 230 | 4 |
| R79L | 0.8 | 0.63 | 0.0073 | 13 | 9.2 | 250 | 12 | 80 |
| R136K | 0.64 | 3.9 | 0.10 | 9 | 160 | 15 | 26 | 35 |
| R136L | 58 | 0.73 | 0.053 | 2 | 0.92 | 2500 | 73 | 13 |
| R136S | 10 | 1.1 | 0.11 | – | 11 | 200 | 100 | 9 |
| K172A | 24 | 0.44 | 0.00039 | 240 | 0.016 | 14000 | 0.90 | 1000 |
| K172E | >20 | 3.2 | 0.0011[a] | 85 | ≤0.055 | ≥42000 | 0.34 | 2700 |
| K172H | 7.2 | 4.5 | 0.0058 | 16 | 0.80 | 2900 | 1.3 | 700 |
| K172R | 26 | 0.063 | 0.00039 | 240 | 0.015 | 150000 | 6.3 | 150 |
| R193H | >20 | 6.8 | 0.0018[a] | 50 | ≤0.09 | ≥26000 | 0.26 | 3500 |
| R193L | 25 | 0.43 | 0.010 | 9 | 0.40 | 5800 | 23 | 40 |

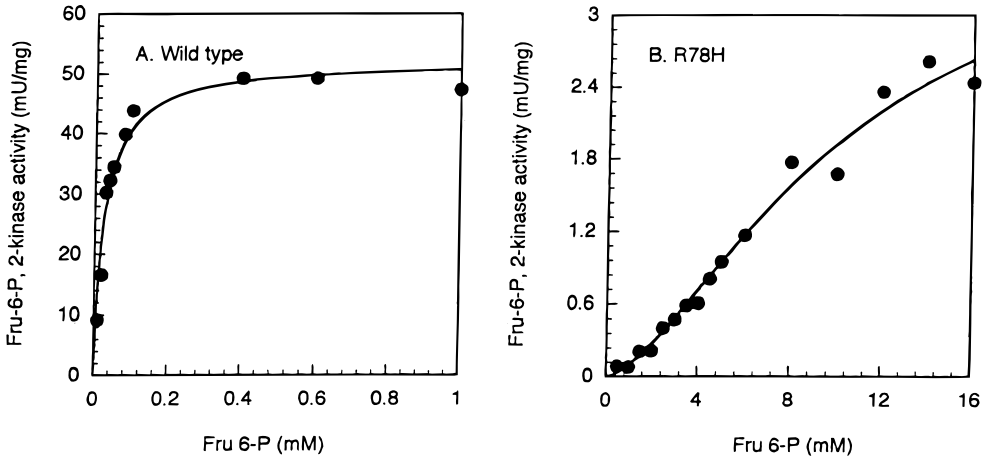



FIGURE 2: Fru 6-P saturation curves for wild type and R78H Fru 6-P,2-kinase. Fru 6-P,2-kinase activity was assayed as described in Materials and Methods, with the exception that [ATP] = 20 mM for R78H and 3 mM for the wild type enzyme. The concentration of Fru 6-P was varied as indicated: (A) wild type enzyme and (B) R78H mutant enzyme.

Table 3: MantATP Binding and Displacement by ATP[a]

| enzyme | $K_d^{mantATP}$ ($\mu$M) | $K_d^{ATP}$ ($\mu$M) | enzyme | $K_d^{mantATP}$ ($\mu$M) | $K_d^{ATP}$ ($\mu$M) |
|---|---|---|---|---|---|
| wild type | 0.9 | 51 | K172A | 1.1 | 42 |
| K51A | – | – | K172H | 4.6 | 170 |
| K51H | – | – | K172E | – | – |
| R78H | 5.2 | 102 | K172R | 1.7 | 64 |
| R78L | 4.1 | 85 | R193H | 7.2 | 124 |
| R79H | 2.5 | 68 | R193L | 2.9 | 113 |
| R79L | 3.3 | 70 | | | |

[a] MantATP binding to these enzymes was determined as described in Materials and Methods. Binding constant $K_d^{mantATP}$ was calculated from a double-reciprocal plot of fluorescence intensity *vs* mantATP concentration. Standard deviations were less than ±7%.

mechanism, and thus, the R79H, K172H, and K51H mutants were further characterized.

It was not possible to obtain the $V/K_{MgATP}$−pH profile for the K51H and K172H mutants, because of the high measured $K_{Fru\ 6-P}$. The $V/K_{Fru\ 6-P}E_t$ profiles for the K51H and K172H mutants are shown in Figure 4. The approximate pH-independent values of $V/K_{Fru\ 6-P}E_t$ for the two mutants are 0.23 ± 0.023 $M^{-1}\ s^{-1}$ (K51H) and 0.61 ± 0.05 $M^{-1}\ s^{-1}$ (K172H), respectively, 0.004 and 0.01% of the value obtained for the wild type enzyme. In both cases, p$K$ values are altered. For the K172H mutant, both of the acid side p$K$ values are observed and have values similar to those measured for wild type enzyme, 6.6 ± 0.1, and 7.7 ± 0.1 while if there is a base side p$K$, it is >9 (∼9.5) compared to the value of 8.7 observed for the wild type enzyme. In the case of the K51H mutant, a single acid side p$K$ is observed with a value of about 6.9, and the base side p$K$ is not observed. The $V/K_{Fru\ 6-P}E_t$−pH profile of the R79H mutant gives p$K$ values identical within error to those of the wild type enzyme (Figure 5A), but the pH-independent value of 83 ± 3 is 71-fold lower than that obtained for the wild type enzyme. The biggest change noted for R79H is in the $V/K_{MgATP}$ profile, which is essentially pH-independent from 6 to 9.4 (Figure 5B), in contrast to that of the wild type enzyme. All p$K$ values are summarized in Table 4.

## DISCUSSION

*Effect of Mutagenesis on MgATP Binding.* The observation that the dissociation constants for mantATP and ATP were not greatly affected by mutation, with the exception of the K51 mutants, suggests that the mutations did not significantly affect ATP binding to the enzyme. However, since $K_{MgATP}$ was increased much more than $K_{d,ATP}$ for the mutant enzymes, but not for the WT enzyme, it is likely that the mutation altered the interaction of Fru 6-P with the E−MgATP complex to form a ternary complex. The altered interaction, as a result of the mutations, may involve changes in substrate-induced conformational changes. The NMP

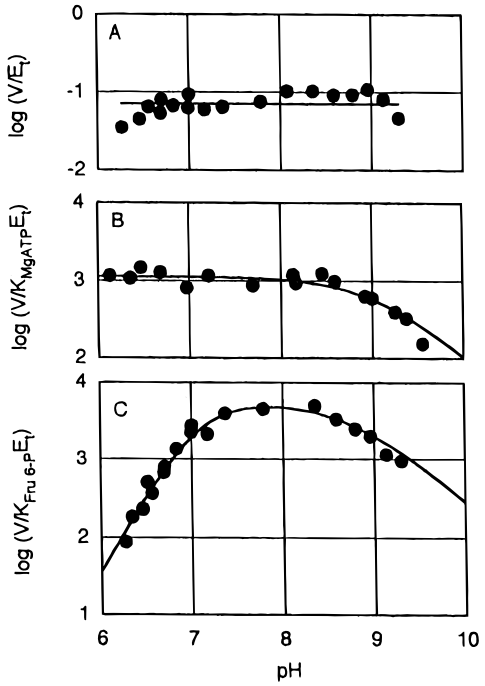



FIGURE 3: pH dependence of $V/E_t$ (A), $V/K_{MgATP}E_t$ (B), and $V/K_{Fru\ 6-P}E_t$ (C) values of the rat testis Fru 6-P,2-kinase. Fru 6-P,2-kinase activity was assayed as described in Materials and Methods except for the following changes: (A and C) 2 mM ATP, 10 mM $MgCl_2$, and varying concentrations of Fru 6-P; and (B) 2 mM Fru 6-P, 10 mM $MgCl_2$, and varying concentrations of ATP.

Table 4: Summary of p$K$ Values Estimated from the pH Dependence of Kinetic Parameters

| | p$K_a \pm$ SE | p$K_b \pm$ SE |
|---|---|---|
| wild type | | |
| $V/E_t$ | 6.2[a] | 9.5[a] |
| $V/K_{MgATP}E_t$ | − | $8.9 \pm 0.08$ |
| $V/K_{Fru\ 6-P}E_t$ | $6.7 \pm 0.05$ | $8.7 \pm 0.08$ |
| | $7.5 \pm 0.06$ | |
| K51H | | |
| $V/K_{Fru\ 6-P}E_t$ | 6.9[a] | − |
| K172H | | |
| $V/K_{Fru\ 6-P}E_t$ | $6.6 \pm 0.10$ | 9.5[a] |
| | $7.7 \pm 0.10$ | |
| R79H | | |
| $V/K_{Fru\ 6-P}E_t$ | $6.5 \pm 0.04$ | $8.6 \pm 0.04$ |
| | $7.4 \pm 0.05$ | |

[a] p$K$ values estimated graphically.

kinases have two mobile loops which close upon binding substrates (*14*, *15*), and the proposed function of these segments is to exclude solvent to prevent MgATP hydrolysis. Fru 6-P,2-kinase is proposed to contain similar flexible segments, *viz*. amino acids 74−100 and 159−194 for MgATP and Fru 6-P binding sites, respectively (*12*). Conformational changes induced by binding both substrates involve closing the MgATP and Fru 6-P binding loops. It is possible that closure of the Fru 6-P loop could be affected by changes in the MgATP binding loop. The large increase in $K_{Fru\ 6-P}$ observed for the Lys51 and Lys172 mutants may be due in part to altered closure of one or both of the substrate-binding loops.

*Interpretation of the Effect of the Mutation.* The crystal structure of Fru 6-P,2-kinase−Fru 2,6-Pase (*12*) has demonstrated that the binding of MgATP to the Fru 6-P,2-kinase

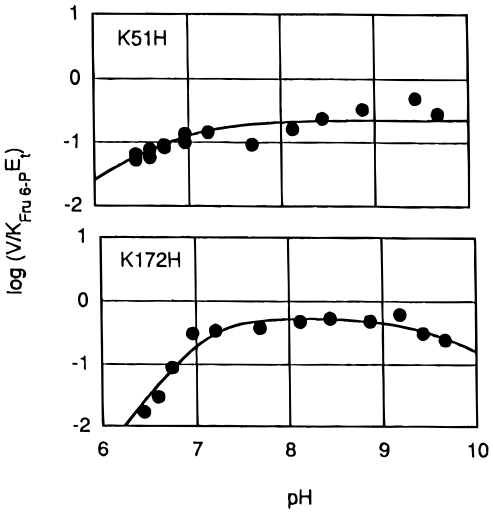



FIGURE 4: pH dependence of $V/K_{Fru\ 6-P}E_t$ values for the K51H and K172H mutants of the rat testis Fru 6-P,2-kinase. Fru 6-P,2-kinase activity was assayed as described in Materials and Methods except for using 10 mM ATP and 20 mM $MgCl_2$ (for K51H) and 20 mM ATP and 20 mM $MgCl_2$ (for K172H).

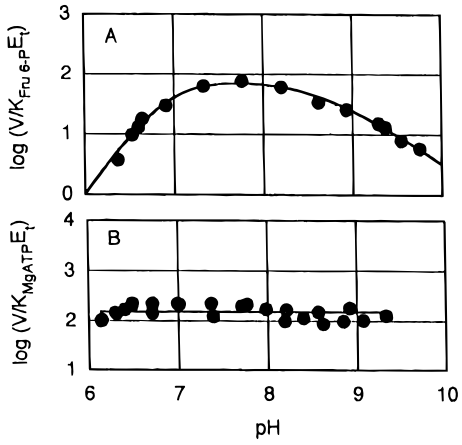



FIGURE 5: pH dependence of $V/K_{Fru\ 6-P}E_t$ (A) and $V/K_{MgATP}E_t$ (B) values for the K79H mutant of the rat testis Fru 6-P,2-kinase. Fru 6-P,2-kinase activity was assayed as described in Materials and Methods except for using 3 mM Fru 6-P (for the ATP saturation curve) and 5 mM ATP and 10 mM $MgCl_2$ (for the Fru 6-P saturation curve).

domain is very similar to that observed in the NMP kinases, which possess a Walker-A motif (GXXGXGKT) represented by amino acid residues 45−52 ($G^{45}$LPARGK$T^{52}$) of the Fru 6-P,2-kinase. The binding pocket for the adenosine moiety of ATP has very few specific interactions (*12*), and all are van der Waals interactions between the adenine moiety and nonpolar side chains such as Cys158, Val163, Val220, and Val246 and between the ribose ring and Thr130. The binding pocket for the phosphates of ATP is an anion hole generated by the α1 helix dipole and the main chain nitrogens of residues 48−53. The highly conserved Lys51 in the Walker-A motif interacts with both the $\beta$- and $\gamma$-phosphates of ATP, while the conserved Thr52 along with Asp128 of the Walker-B motif are ligands to the octahedrally coordinated $Mg^{2+}$, which also coordinates with the $\beta$- and $\gamma$-phosphates of ATP (*12*). (Thr52 is also directly bound to the $\beta$-phosphate of ATP.)

Mutation of basic residues, including Lys51 and Lys172 which are in the vicinity of the $\beta$- and $\gamma$-phosphates of ATP, affects all three kinetic parameters, $V$, $V/K_{MgATP}$, and

$V/K_{Fru\ 6-P}$. A likely explanation for the apparent lack of greater effect on the *V/K* for ATP (and mantATP) in these mutants is the fact that the side chains of these basic residues play only a minor role in the binding of the nucleotide. The major determinant in the binding of ATP is the Walker-A motif (P-loop) which wraps around the phosphates. In a study of a similar Walker-A motif of the p21 ras oncoprotein (*37*), it was demonstrated that the primary determinant for GTP/GDP binding is the anion hole formed by the helix dipole and main chain dipoles in the P-loop. The crystal structure of Fru 6-P,2-kinase demonstrates that the glycine-rich motif also wraps around the $\beta$-phosphate of the nucleotide and chelates the $Mg^{2+}$ ion through Thr52 in the loop (*12*).

*K51 and K172*. Replacement of K51 with either A or H gives similar decreases in all kinetic parameters, while results differ in the case of K172, dependent on the nature of the replacement amino acid. Interestingly, at position 172, it is not sufficient to conserve the charge as shown by K172R and K172H (although His may not be ionized, see below). For example, the $V/K_{MgATP}$ is decreased by 150-fold in the case of the K172R mutant even though the positive charge is conserved, compared to a decrease of 3000-fold with opposite charge (K172E), or a decrease of 1000-fold with a hydrophobic replacement (K172A). $V/K_{Fru\ 6-P}$ is 1 order of magnitude lower in K172R than in the hydrophobic K172A mutant and about 4-fold lower than in the negatively charged K172E mutant. The observation that $V/K_{Fru\ 6-P}E_t$ values of the lysine mutant enzymes are dramatically decreased (less than 0.03% of WT enzyme) suggests that these residues play an important role in binding and/or catalysis.

The above results are in contrast to those of the rat liver bifunctional enzyme (*38*). Mutation of K54 (analogous to K51 in the testis enzyme) to Met resulted in a large increase in the $K_m$ values for both substrates such that they were immeasurable, while the $V_{max}$ decreased to less than 0.01 milliunit/mg. Differences between the liver and testis enzymes could be due to the assay conditions employed or the thoroughness of the study. In the present studies, a complete initial velocity study was carried out.

*R136.* As suggested in the Results, the R136 mutant enzymes corroborate the suspected role of R136 as a binding group of the 6-phosphate of Fru 6-P. Very little change is observed in *V*, while changes in $V/K_{MgATP}$ are also small, and parallel those observed for *V*. On the other hand, conservation of charge, as in the R136K mutation, gives only a small change in $V/K_{Fru\ 6-P}$, while elimination of charge (K136S or K136L) gives a decrease in the $V/K_{Fru\ 6-P}$ of 2−3 order of magnitude. It has been reported that R102 is also involved in the binding of Fru 6-P (*18*). However, in contrast to R102 mutants, which show no remarkable change in $K_{MgATP}$, R136 mutations affect $K_{MgATP}$.

*R78, R79, and R193*. The most substantial changes observed with mutation of these arginines are decreases in $V/K_{Fru\ 6-P}$. In all cases, with the possible exception of the R193H mutant, decreases are $\leq$3 orders of magnitude, suggesting that none of these residues alone are absolutely required for catalysis and/or binding of MgATP or Fru 6-P. Similar results have been reported by Li *et al.* (*17*) for R195 in the rat liver enzyme (corresponding to R193 in the testis enzyme). Perhaps the most interesting result of the arginine mutations is the development of a positively cooperative saturation isotherm for Fru 6-P. A similar saturation curve has been observed for the WT enzyme at pHs below 6.8.[2] Results suggest that a positive charge density is provided by the three arginines, essential for the binding of both substrates.

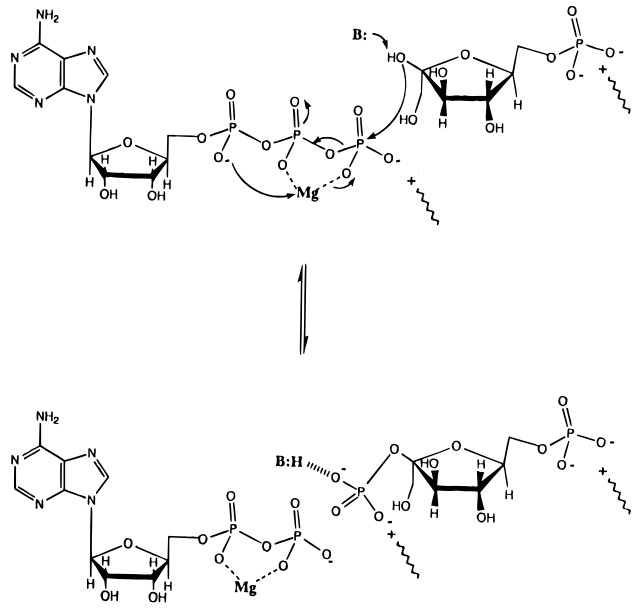
*Interpretation of pH Profiles for Wild Type Fru 6-P,2-Kinase.* The kinetic mechanism is random sequential with either MgATP or Fru 6-P adding to the kinase first (*9*). The *V* profile will reflect groups in the enzyme−substrate complex that build up in the steady state. If the binding of reactants locks all enzyme and reactant groups in their correct protonation state, the *V* profile will be pH-independent (*39*). In the latter case, all p*K* values observed in the *V/K* profiles will be intrinsic values (that is, p*K* values that are unperturbed as a result of rate limitation by noncatalytic steps) for the appropriate enzyme−reactant binary complex, E−Fru 6-P for $V/K_{MgATP}$, E−MgATP for $V/K_{Fru\ 6-P}$ and free reactant, MgATP for $V/K_{MgATP}$, and Fru 6-P for $V/K_{Fru\ 6-P}$. If reactant binding does not lock the protonation state of enzyme and reactant groups, *V* will be pH-dependent, and the p*K*s observed will be perturbed to lower (acid side) and higher (base side) pH unless the phosphoryl transfer step is solely rate-limiting. The *V* profile for wild type Fru 6-P,2-kinase is pH-dependent and decreases at low and high pH, indicating that one group must be unprotonated and another protonated for activity. The p*K* values observed in the *V* profile (6.2 and 9.5) are lower and higher, respectively, than those observed in the *V/K* profiles (7.4 and 8.7; the 6-phosphate p*K* will not be observed since Fru 6-P is already bound).

The *V/K*−pH profiles will reflect titratable groups on enzyme or substrate required in a given protonation state for optimum binding and/or catalysis (*39*). The *V/K* for MgATP reflects predominately the E−Fru 6-P binary complex, at saturating Fru 6-P, and free MgATP. The p*K* value for the $\gamma$-phosphate of MgATP is about 4.9 (*40*), outside the pH range investigated in these studies. The p*K* of 8.9 observed in the $V/K_{MgATP}$ profile thus reflects an enzyme group that must be protonated for optimum binding of MgATP and/or catalysis. In order to carry out a nucleophilic attack on the $\gamma$-phosphate of MgATP, the negative charge on the $\gamma$-phosphate must be neutralized by an ionic or hydrogen bonding interaction with an enzyme residue, and this is likely the function of the enzyme group with a p*K* of 8.9. There are a number of residues in the active site of Fru 6-P,2-kinase that could serve in this capacity, but K51 is the most likely candidate, as will be discussed below.

The *V/K* for Fru 6-P reflects predominately the E−MgATP binary complex, at saturating MgATP, and free Fru 6-P. Three ionizable groups are reflected in the $V/K_{Fru\ 6-P}$−pH profile, two that must be unprotonated and one protonated for optimum binding of Fru 6-P and/or catalysis. The p*K* value of 6-phosphate of Fru 6-P is about 6.4 (*40*) and may reflect the p*K* of 6.7 observed on the acid side of the $V/K_{Fru\ 6-P}$−pH profile, indicating that the 6-phosphate must be unprotonated for optimum binding of Fru 6-P to enzyme. A second p*K* of 7.4 is also observed on the acid side of the $V/K_{Fru\ 6-P}$−pH profile, suggesting that a group on enzyme must be unprotonated for binding and/or catalysis. The latter p*K* probably is derived from an enzymic general base that is required to accept a proton from the 2-OH of Fru 6-P to

[2] Unpublished results of K. Uyeda.

Scheme 1: Proposed Chemical Mechanism for the Rat Testis Fru 6-P,2-Kinase




facilitate nucleophilic attack on the $\gamma$-phosphate of MgATP. There are only two neutral acid residues, that is Asp or Glu, located close to the catalytic center as indicated by the crystal structure of the kinase (*12*). One of these is Asp128, which is shown to be chelated to the $Mg^{2+}$ ion of MgATP. The D128S and D128A mutants decrease $V_{max}$ 8−20-fold and the $V/K_{Fru\ 6-P}$ 240−4000-fold but do not affect the p*K* values observed in the $V/K_{Fru\ 6-P}$−pH profile (*13*). A second Asp residue, Asp177, is located within a purported mobile loop (*12*) and could approach the 2-OH close enough to serve as a base if a conformational change was induced by Fru 6-P binding. However, the $V/K_{Fru\ 6-P}$−pH profile of the D177N mutant (data not shown) is identical to that of the wild type enzyme. Another side that could serve as a general base is the imidazole of histidine. However, there is no histidine positioned so that it could serve as a general base (*12*).

The remaining p*K* of 8.7, observed in the $V/K_{Fru\ 6-P}$−pH profile must be protonated for maximum binding of Fru 6-P and/or catalysis. There are two likely possible functions for the group with a p*K* of 8.7. (1) It could be the same group observed in the $V/K_{MgATP}$−pH profile, needed to reduce the electron density around $\gamma$-phosphate of MgATP, or (2) it could be required for the binding of the 6-phosphate of Fru 6-P. As discussed below, it is likely neither of these possibilities.

A proposed chemical mechanism is provided in Scheme 1. Fructose 6-phosphate is shown bound via a positively charged residue (probably R136 and or R102, Figure 1) at its 6-phosphate (p$K \sim 6.4$). In addition, a general base (p$K \sim 7.4$) is positioned to accept the 2-hydroxyl proton. It is not unreasonable to believe that the base will be hydrogen-bonded or at least close to the 2-hydroxyl when Fru 6-P is bound. Another positively charged enzyme residue (p$K \sim 8.9$) is shown interacting with the $\gamma$-phosphate of MgATP, either K51 or K172 according to Figure 1. The phosphoryl transfer from MgATP to the 2-hydroxyl of Fru 6-P proceeds by nucleophilic attack of the 2-hydroxyl on the $\gamma$-phosphate aided by the general base that accepts the hydroxyl proton, and the positively charged enzyme group that interacts with the $\gamma$-phosphate to neutralize its negative charge. Phosphoryl

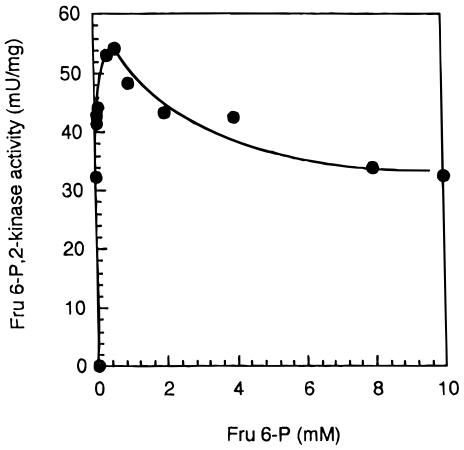



FIGURE 6: Effect of Fru 6-P concentration on rat testis Fru 6-P,2-kinase activity. Fru 6-P,2-kinase activity was assayed as described in Materials and Methods using 2 mM ATP, 5 mM $MgCl_2$, and varying concentrations of Fru 6-P.

transfer is followed by a ligand displacement reaction in which the 2-phosphate of the Fru 2,6-$P_2$ product is displaced from $Mg^{2+}$ by the α-phosphate of the MgADP product.

Kurland *et al.* (*20*) reported that the $V_{max}$−pH plot of rat liver enzyme exhibits two pH optima, 6.9 and 8.8, and a minimum around pH 7.5. A similar observation was made with the rat testis enzyme in the present study, depending on the concentration of Fru 6-P used. As the Fru 6-P concentration is increased above 0.7 mM, inhibition of the Fru 6-P,2-kinase activity of the bifunctional enzyme is observed (Figure 6). However, $K_{Fru\ 6-P}$ for the rat testis enzyme at pH values around 7.5 is ∼40 $\mu$M, and maintaining the Fru 6-P concentration at ≤0.2 mM alleviates the substrate inhibition. The decrease in $V_{max}$ at pH 7.5 noted by Kurland *et al.* can likely be attributed to substrate inhibition (or perhaps ionic strength effects) resulting from the high concentrations of Fru 6-P used in the reported studies. The inhibition may also be due to the presence of a contaminant in the Fru 6-P solution.

Rider *et al.* (*19*) showed that the activity of the rat muscle Fru 6-P,2-kinase increases continuously with increasing pH from 5.5 to 9.5. These authors further showed that the R104S mutant enzyme has an altered pH profile with a plateau between 8 and 8.5, suggesting that the Arg104 side chain may contribute to the alkaline part of the pH profile in the wild type enzyme. Kurland *et al.* (*20*) reported that the pH profile of the rat liver Fru 6-P,2-kinase is complicated and shows two sharp pH optima at 7 and 10 and a minimum at pH 8.5. However, these authors also showed that the pH profile of the R104A mutant was bell-shaped with a pH optimum at 8, similar to that of the muscle enzyme.

*Interpretation of Mutant pH Profiles and Attempts To Identify Groups Reflected in the Wild Type pH Profile.* The crystal structure of Fru 6-P,2-kinase indicates that Lys51 and Lys172 are in the vicinity of the $\beta$- and $\gamma$-phosphates of ATP (*12*). Mutation of Lys51 and Lys172 to His give a decrease of ≥46000-fold in $V/K_{Fru\ 6-P}E_t$ values and an about 100-fold decrease in $V/E_t$ (Table 2), implicating both residues in catalysis and/or binding. The mutation of Arg79 gave decreases of only about 30-fold and 4-fold in the $V/KE_t$ values for Fru 6-P and MgATP, respectively, suggesting that it not essential for catalysis but has some secondary function.

To explain the K51H/K172H mutant data one must consider the kinase active site structure (Figure 1). The

active site has an abundance of basic residues, including R193, R136, R102, R79, and R78, as well as K51 and K172. As a result, there will be substantial positive charge in the site, and this will certainly have an effect on the p*K* values of all of the groups listed, causing them to decrease significantly compared to their normal values in aqueous solution (that is about 10.5 for the $\epsilon$-amino of lysine and 12.5 for the guanidinium of arginine). Effects are, of course, distance-dependent, but because of the positive charge density, there will likely be some effect of positive charge neutralization on the p*K* values of remaining positively charged residues.

In each of the mutants studied in detail, the lysine was changed to a histidine, which normally has a p*K* of about 6. However, an imidazole in the kinase active site will likely have a lower p*K* given the high positive charge density in the site, will probably be un-ionized over the pH range studied, and will effectively eliminate a positive charge in the unliganded enzyme. The loss of the positive charge would cause an increase in the observed p*K* of at least the positively charged groups in the vicinity of the group that loses its positive charge.

Finally, the R79H mutant gives a $V/K_{\mathrm{Fru\ 6-P}}$−pH profile that is qualitatively identical to that of wild type. $V/K_{\mathrm{MgATP}}$ is pH-independent, while that for the wild type enzyme decreases at high pH, giving a p*K* of about 8.9. Since the group with a p*K* of 8.6−8.7 is still observed in the $V/K_{\mathrm{Fru\ 6-P}}$−pH profile, the groups responsible for the observed p*K*s of 8.9 in the $V/K_{\mathrm{MgATP}}$ profile and 8.7 in the $V/K_{\mathrm{Fru\ 6-P}}$ profile must be different. The group responsible for the base side p*K* in the $V/K_{\mathrm{MgATP}}$ profile is K51 (Figure 1). The location of K51, in hydrogen-bonding distance (3.13 ± 0.29 Å) to the $\gamma$-phosphate of MgATP, suggests it is the group that neutralizes the negative charge on the $\gamma$-phosphate, facilitating attack by the 2-hydroxyl of Fru 6-P. The absence of the base side p*K* is likely due to an increase in the p*K* of K51 as a result of the loss of positive charge for R79 upon its mutation to H. The latter would also explain the small change in the magnitude of the kinetic parameters of the R79H mutant compared to wild type, since R79 is nonessential, with the exception of contributing charge to the site. There is a reasonable expectation that, on the basis of the placement of some of the positively charged groups in the active site, *e.g.* R78, R79, and R193, they are involved in stabilization of the phosphoryl transfer transition state (Figure 1).

The K172H mutant shows an increase in the base side p*K* in the $V/K_{\mathrm{Fru\ 6-P}}$−pH profile by about 0.9 pH unit. However, all three of the p*K*s observed in the wild type $V/K_{\mathrm{Fru\ 6-P}}$−pH profile are present, suggesting that none of the groups responsible for the ionizations observed is K172. However, the dramatic loss in $V/K_{\mathrm{Fru\ 6-P}}E_{\mathrm{t}}$ (10000-fold) suggests that a catalytic advantage has been lost in the K172H mutant. On the basis of the placement of K172 with respect to the $\beta$- and $\gamma$-phosphates of MgATP (Figure 1) it is suggested that K172 could facilitate catalysis in two ways. The structure of the E−MgATP complex places K172 in the vicinity of the $\beta,\gamma$-bridge oxygen (2.78 ± 0.29 Å) and one of the nonbridge oxygens of the $\gamma$-phosphate (2.51 ± 0.29 Å). Its proximity to the $\gamma$-phosphate of MgATP might neutralize its remaining negative charge, facilitating the nucleophilic attack of the 2-hydroxyl of Fru 6-P on the $\gamma$-phosphate of MgATP. Its proximity to the $\beta,\gamma$-bridge oxygen suggests it could neutralize the transient negative charge which would accumulate on this oxygen in the transition state. On the basis of studies of GTP hydrolysis and the structure of the G-protein Gi$\alpha$ (*41*) wherein an arginine occupies a similar position near the $\beta,\gamma$-bridge oxygen, it has been proposed (*42*) that enzymatic GTP hyrolysis is a "dissociative-like" reaction. The placement of K172 and its critical role in catalysis would be consistent with such a theory which predicts a large negative charge accumulation on the $\beta,\gamma$-bridge oxygen in the transisiton state. The group responsible for the base side p*K* in the $V/K_{\mathrm{Fru\ 6-P}}$−pH profile for K172H is probably one of the arginines, R78 or R193, in the vicinity of the lysine.

The pH profiles for the K51H mutant exhibit very low activity (25000-fold lower than that of the wild type), and the data exhibit substantial error. It is thus difficult to determine an accurate shape for the $V/K_{\mathrm{Fru\ 6-P}}$ profile. However, there is little pH dependence on the basic side of the pH profile, and since elimination of a positive charge will result in an increase in the p*K* of other positively charged groups in the vicinity, it is most likely that the presence of K51 results in a decrease in the p*K* of R79 or R193, as observed for K172 (Figure 1). The identity of the general base in the kinase reaction will have to await future studies.

It has been reported that mutations of Arg104 of the rat liver Fru 6-P,2-kinase (the corresponding residue is Arg102 in the rat testis enzyme) result in an altered pH profile, suggesting that this residue contributes to the pH profile (*19*, *20*). However, the pH−activity profile of the R102L mutant in the testis enzyme is identical to that of the wild type enzyme (data not shown), suggesting that ionization of Arg102 does not affect the pH−activity profile of the enzyme. Closer examination of Kurland's data (*20*) revealed that the wild type enzyme showed two pH optima and one minimum around pH 7.5−8, similar to that of the testis enzyme (data not shown), while the R104A mutant showed only one broad pH optimum. These differences could be explained by the difference in the sensitivity to Fru 6-P inhibition, as demonstrated in the data of Figure 6. It seems reasonable that, since the $K_{\mathrm{Fru\ 6-P}}$ value of the mutant is much higher than that of the wild type enzyme, the inhibition by Fru 6-P (or its contaminant) at pH 7.5 is also decreased by the mutation. Thus, we believe that the pH curves of R104A and the wild type enzyme are essentially the same. The pH curve of R104S reported by Rider *et al.* (*19*) also appears to be similar to that of R104A of the liver isozyme and R102L of the testis isozyme. These authors reported that the pH−activity profile of the wild type muscle isozyme shows no pH optimum, differing from the profiles of the liver (*20*) and testis isozymes (data not shown). Since the catalytic domains of all of the above isozymes are essentially the same, it is difficult to explain the differences in the pH dependency of the kinase activity, but it could be attributed to experimental conditions.

*Conclusions.* In Scheme 1, according to the above studies and the avaliable structure of the kinase (*12*), the phoshate of Fru 6-P is likely to be ionized for optimum binding, anchored by R102 and or R136. Interaction of the $\gamma$-phosphate of MgATP with K51 is critical for neutralization of the charge and facilitation of nucleophilic attack by the 2-hydroxyl of Fru 6-P. The protonation state of K51 is locked upon binding of MgATP, since its p*K* is not observed in the $V/K_{\mathrm{Fru\ 6-P}}$−pH profile. The ionization state of R78

(and/or R193) is also important, and it is its p*K* that is likely to be observed on the basic side of the $V/K_{Fru\ 6-P}$−pH profile. As suggested above, R78, R79, and R193 are likely to be involved in transition state stabilization as the $\gamma$-phosphate is transferred to the 2-hydroxyl, building up additional negative charge on the phosphate oxygens. Unfortunately, the identity of the general base that accepts a proton from the 2-hydroxyl has not been elucidated.

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