Crystal Structure of the H256A Mutant of Rat Testis Fructose-6-phosphate,2-kinase/Fructose-2,6-bisphosphatase

FRUCTOSE 6-PHOSPHATE IN THE ACTIVE SITE LEADS TO MECHANISMS FOR BOTH MUTANT AND Wild TYPE BISPHOSPHATASE ACTIVITIES

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Fructose-6-phosphate,2-kinase/fructose-2,6-bisphosphatase (Fru-6-P,2-kinase/Fru-2,6-Pase) is a bifunctional enzyme, catalyzing the interconversion of β-D-fructose-6-phosphate (Fru-6-P) and fructose-2,6-bisphosphate (Fru-2,6-P2) at distinct active sites. A mutant rat testis isozyme with an alanine replacement for the catalytic histidine (H256A) in the Fru-2,6-Pase domain retains 17% of the wild type activity (Mizuguchi, H., Cook, P. F., Tai, C-H., Hasemann, C. A., and Uyeda, K. (1998) J. Biol. Chem. 274, 2166–2175). We have solved the crystal structure of H256A to a resolution of 2.4 Å by molecular replacement. Clear electron density for Fru-6-P is found at the Fru-2,6-Pase active site, revealing the important interactions in substrate/product binding. A superposition of the H256A structure with the RT2K-Wo structure reveals no significant readjustment of the active site resulting from the binding of Fru-6-P or the H256A mutation. Using this superposition, we have built a view of the Fru-2,6-P2-bound enzyme and identify the residues responsible for catalysis. This analysis yields distinct catalytic mechanisms for the wild type and mutant proteins. The wild type mechanism would lead to an inefficient transfer of a proton to the leaving group Fru-6-P, which is consistent with a view of this event being rate-limiting, explaining the extremely slow turnover (0.032 s−1) of the Fru-2,6-Pase in all Fru-6-P,2-kinase/Fru-2,6-Pase isozymes.

The bifunctional enzyme fructose-6-phosphate,2-kinase/fructose-2,6-bisphosphatase (Fru-6-P,2-kinase/Fru-2,6-Pase) participates in glucose homeostasis by regulating the intracellular concentration of fructose-2,6-bisphosphate (Fru-2,6-P2). Fru-2,6-P2 is both a potent physiological activator of 6-phosphofructokinase, and an in vitro inhibitor of fructose-1,6-bisphosphatase (reviewed in Refs. 2–4). There is significant sequence homology among the Fru-2,6-Pase domain, the phosphoglycerate mutases, and the acid phosphatases (5, 6). We previously determined the crystal structure of the rat testis Fru-6-P,2-kinase/Fru-2,6-Pase (RT2K-Wo), showing the enzyme to be a head-to-head homodimer of 55-kDa subunits, with each monomer consisting of independent kinase and phosphatase domains (7). The kinase domains are in close contact, forming an extended hydrophobic core between them, while the phosphatase domains are essentially independent of one another. The structure of the isolated rat liver Fru-6-P,2-kinase/Fru-2,6-Pase (RL2K) Fru-2,6-Pase domain has also been determined (8), and as would be predicted by the 85% identity between the Fru-2,6-Pase domains, the structures are nearly identical. Comparison of the RT2K-Wo Fru-2,6-Pase domain structure with those of yeast phosphoglycerate mutase (9) and rat acid phosphatase (10) confirmed that these enzymes also have very similar tertiary structures (7). These enzymes share a common catalytic mechanism involving a covalent phosphohistidine intermediate (11–14) located in a conserved sequence motif (ZXRHG/E/Q)XXN, where Z is a hydrophobic residue (15). The sequence motif LCHGESNL292 is found in the RT2K phosphatase domain, where His292 is the catalytic histidine. This conserved sequence motif, together with Arg255, Glu256, and His292, forms a larger conserved structural motif defining the catalytic center of all three enzymes (7).

Mutagenesis and kinetic analyses on the rat liver isozyme (16–20) led to a model of Fru-2,6-P2 binding and a potential phosphatase reaction mechanism. Thus, the catalytic histidine (His256 in RL2K, His256 in RT2K) was proposed to make a nucleophilic attack on the 2-phosphate of Fru2,6-P2, while positively charged side chains (Arg257 and Arg257 in RL2K, Arg255 and Arg265 in RT2K) orient the 2-phosphate and stabilize the transition state. His392 (His390 in RT2K) was proposed to be coupled to Glu327 (Glu325 in RT2K), making His392 doubly protonated, and capable of donating a proton to the leaving group oxygen of Fru-6-P. A water, activated by Glu327, would then attack the phosphoenzyme intermediate, regenerating the enzyme and producing a free PO4 ion (8, 14). This interpretation of the mutagenesis and the proposed reaction mechanism are largely in agreement with the RT2K-Wo and RL2K Fru-2,6-Pase domain structures, with the exception of the role of H256A, a mutant form of RT2K-Wo with an additional histidine 256 to alanine mutation.

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†† The atomic coordinates and structure factors (code 2bif) have been deposited in the Protein Data Bank, Brookhaven National Laboratory, Upton, NY.

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1 The abbreviations used are: Fru-6-P,2-kinase, fructose-6-phosphate,2-kinase; Fru-6-P, fructose-6-phosphate; Fru-6-P2, fructose-2,6-bisphosphate; Fru-6-P, β-D-fructose-6-phosphate; AMP-PNP, 5′-adenosyl imidodiphosphate; RL2K, rat liver isozyme of fructose-6-phosphate,2-kinase/fructose-2,6-bisphosphatase; RT2K, rat testis isozyme of fructose-6-phosphate,2-kinase/fructose-2,6-bisphosphatase; RT2K-Wo, RT2K with all four tryptophans mutated to phenylala-
Bisphosphatase Mechanism from Structure with Bound Fru-6-P

His\textsuperscript{390}, which is neither coupled to Glu\textsuperscript{325}, nor in a position to donate a proton to Fru-6-P. It is important to note, however, that neither the RT2K-Wo nor the original RL2K Fru-2,6-P\textsubscript{ase} domain structures contained bound ligands (Fru-6-P or Fru-2,6-P\textsubscript{2}), which has hampered a thorough characterization of the Fru-2,6-P\textsubscript{ase} mechanism. The report of a trapped RL2K phosphoenzyme intermediate (21) and the work presented here combine to remedy this deficiency.

To facilitate our own crystallographic studies of the RT2K kinase reaction, we generated a mutant protein, H256A, that as reported for the RL2K H258A mutant (16), would be devoid of phosphatase activity (and allow us to soak Fru-2,6-P\textsubscript{2} into crystals without consumption by the Fru-2,6-P\textsubscript{ase} reaction). This enzyme unexpectedly retains 17% of the RT2K-Wo Fru-2,6-P\textsubscript{ase} crystals without consumption by the Fru-2,6-P\textsubscript{ase} reaction). This enzyme unexpectedly retains 17% of the RT2K-Wo Fru-2,6-P\textsubscript{ase} crystals without consumption by the Fru-2,6-P\textsubscript{ase} reaction).

**EXPERIMENTAL PROCEDURES**

**Protein Preparation and Crystallization**—The preparation and purification of the Wo form of RT2K was described previously (22, 23). A single point mutation of the Wo enzyme, converting His\textsuperscript{356} to Ala was generated and purified by the same procedure (1). Crystallization was performed by the hanging drop vapor diffusion method. The conditions for crystallization were a modified version of those reported previously (24). Briefly, 10 mg/ml of protein (in 50 mM Tris-P\textsubscript{O}4, pH 7.5, 5% glycerol, 40 mM EDTA, 40 mM EGTA, 800 mM dithiothreitol, 0.4% PEG300, 1.7 mM AMP-PNP, 1% β-octyl glucoside) was mixed with 1:1 with a well solution of 17% polyethylene glycol 4000, 60–90 mM sucinate, pH 6.0, 10% glycerol, and 10 mM MgCl\textsubscript{2}. Crystals were grown and stored at 4 °C.

**Data Collection and Processing**—Crystals were serially transferred into solutions of surrogate mother liquor (19% PEG 4000, 10 mM MgCl\textsubscript{2}, 90 mM succinate, pH 6.0, 50 mM Tris-HCl, pH 7.5, 1% β-octyl glucoside, 10 mM Fru-6-P, and 1 mM AMP-PNP) with increasing concentrations of glycerol (10–20% in 5% steps). These cryoprotected crystals were flash frozen in liquid propane and subsequently maintained at 120 K in a dry nitrogen stream using an X-Stream crystal cooler (Molecular Structure Corp.). Data were collected on a DIP-2020 image plate detector (MacScience) mounted on a Rigaku rotating anode generator operated at 50 mA, 100 kV, with double mirror focusing (MacScience). All data were collected on a DIP-2020 image plate detector (MacScience) mounted on a Rigaku rotating anode generator operated at 50 mA, 100 kV, with double mirror focusing (MacScience). Data were collected on a DIP-2020 image plate detector (MacScience) mounted on a Rigaku rotating anode generator operated at 50 mA, 100 kV, with double mirror focusing (MacScience). Diffraction intensities were integrated using the program Denzo (25). All data were merged and scaled in Scalepack (25) and formatted for subsequent use in XPLOR (26). Automatic indexing in Denzo indicated that the crystals belong to space group P1, and subsequent refinement in Scalepack led to unit cell dimensions a = 61.7 Å, b = 73.5 Å, c = 76.7 Å; α = 116.9°, β = 99.31°, and γ = 105.2°. Assuming two monomers per asymmetric unit, the calculated Matthews coefficient (27) is 2.54 Å\textsuperscript{3}/Da.

**Phasing by Molecular Replacement, Model Building, and Refinement**—The rotation search and PC refinement were carried out in XPLOR, using a polyalanine model derived from the RT2K-Wo crystal structure (7). Because of the extensive interaction between monomers in the original P3121 crystal form, we anticipated that the global dimer structure would be unchanged in this new P1 crystal packing and thus used the entire dimer as a search model, rather than separate searches for the two monomers. The molecular replacement was unambiguous, with a rotation function peak 7.4° above the mean. Rigid body minimization of this rotation solution using data from 20- to 2.8-Å resolution led to an R\textsubscript{cryst} of 0.51 for the polyalanine model and an R\textsubscript{work} of 0.42 with all side chains included. To minimize model bias, initial electron density maps were calculated with the polyalanine molecular replacement phases and data from 30 to 2.4 Å, using SIGMAA (28) weighting as implemented in XPLOR. Model rebuilding was accomplished using the program O (version 6.1 (29)). The major changes in the structure are related to changes in the ligand-binding state of both catalytic domains and changes due to crystal packing. Refinement in XPLOR included the use of noncrystallographic restraints. Four groups were defined (kinase-tight, kinase-loose, phosphatase-tight, and phosphatase-loose), using a weight of 100 for the tighter and 50 for the looser restraints. Rebuilding, positional, and B-factor refinement yielded the final model reported here with indicators of model quality as reported in Table I. Coordinates have been deposited with the Protein Data Bank (30), with accession code 2bif.

**RESULTS**

**Protein Crystallization and Structure Solution**—Crystals of the RT2K H256A mutant protein were grown by the hanging drop vapor diffusion method as described under “Experimental Procedures.” Crystals grew with a rod-shaped morphology and were found to belong to space group P1. The unit cell dimensions are a = 61.7 Å, b = 73.5 Å, c = 76.7 Å; α = 116.9°, β = 99.31°, and γ = 105.2°, and there are two monomers (the functional dimer) in the asymmetric unit. These crystals diffract to 2.4 Å, and data were collected from specimens frozen at cryogenic (120 K) temperatures. The structure was solved by molecular replacement using the reported RT2K-Wo structure as a search model. A summary of the overall data and model qualities is presented in Table I. The overall structure of the RT2K dimer is similar to that reported earlier (7), i.e. a close interaction between kinase domains, with essentially independent phosphatase domains tethered to the kinase dimer. Because there are two monomers in the asymmetric unit, we have determined two independent structures of the monomer. For the kinase domains, the two copies are different, with subtle conformational changes, and different ligands bound. The two phosphatase domains are quite similar in conformations, and different ligands bound. The two phosphatase domains are quite similar in conformations, and different ligands bound.

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2 M. H. Yuen and C. A. Hasemann, manuscript in preparation.
Fru-6-P Binding in the Active Site—In our previous structure of RT2K-Wo, there was not a Fru-6-P molecule bound to the Fru-2,6-Pase domain, despite its presence in the crystallization mixture. Instead, there were phosphate ions bound in the presumed binding sites for the 2- and 6-phosphates of Fru-6-P. In the structure of the RT2K H256A mutant reported here, there is clear electron density for Fru-6-P in both monomers (Fig. 1). The refined coordinates of the Fru-6-P molecule in the binding site (Fig. 2) reveal that the Fru-6-P traverses a crevice composed of Ile267, Glu325, Tyr336, Arg350, Lys354, Tyr365, Gln391, and Arg395. The polar residues form hydrogen bonds with the Fru-6-P hydroxyls and 6-phosphate (Table II and Fig. 2), while Ile267 forms a Van der Waals interaction by stacking with the fructose ring (Fig. 3). Tyr336, Arg350, Lys354, and Tyr365 interact with the 6-phosphate in exactly the same way that the free phosphate was bound in the RT2K-Wo structure. Eight of the nine oxygens of Fru-6-P are involved in direct hydrogen bonds to the protein or to well ordered waters, which are in turn bound by protein. Fru-6-P-O-1 interacts with two water molecules, which are in turn bound to main chain carbonyls and side chains. Fru-6-P-O-2, which is of course the site of bond cleavage from the 2-phosphate during the Fru-2,6-Pase reaction, forms a hydrogen bond with Glu325, making Glu325 a prime candidate for a catalytic residue, as will be discussed below. The main chain nitrogen of Gly268 interacts with the Fru-6-P-O-3 hydroxyl, while the nitrogen of Gln391 forms two hydrogen bonds to fructose oxygens (O-5 and O-6). Only Fru-6-P-O-4 is not involved in a hydrogen bond interaction, and that is because the stacking of Ile267 with the fructose ring includes an interaction with the C-4-O-4 bond, preventing a close approach of any other protein groups that might interact with Fru-6-P-O-4. This mode of Fru-6-P binding (a combination of many hydrogen bonds and hydrophobic stacking) is typical for carbohydrate binding proteins that bind their substrate in an internal pocket (type I proteins, as defined by Quiocho (31) and Vyas (32)) and promotes both tight binding and substrate specificity.

Phosphate Binding at the 2-Phosphate Pocket—A phosphate ion is bound in the 2-phosphate pocket of both monomers in the crystal. The phosphate is in hydrogen bonding distance to the side chains of Arg255, Glu325, Arg305, His390, Asn262, and the main chain nitrogen of Gln391, as well as being in contact with Fru-6-P-O-2. A superposition of the H256A and RT2K-Wo structures shows that the phosphate occupies a different position in the two enzymes. In the H256A structure, the ion shifts (by an average distance of 1.46 Å for the two monomers) to occupy the space created by the loss of His2356. While the phosphate still contacts the same set of protein atoms, most of the hydrogen bonds are longer in the H256A mutant (Table III). The phosphate in the H256A structure has, of course, lost its interaction with His2356 but has gained an interaction with Fru-6-P.

Modeling Fru-2,6-P₂ Binding in the Active Site—Superposition of the H256A crystal structure with the RT2K-Wo structure allows us to generate a model of Fru-2,6-P₂ in the phosphatase active site. This is possible because the positions of the 6-phosphate and protein atoms in the active site have not changed (root mean square coordinate change for 143 atoms = 0.20 Å) beyond the intrinsic coordinate error of the H256A model (0.35 Å). Thus, by combining the protein and 2-phosphate analogue from the RT2K-Wo structure with the Fru-6-P from the H256A structure, we achieve an excellent approximation of the Fru-2,6-P₂ complex (Fig. 3). The arrangement of protein/Fru-6-P hydrogen bonds and Van der Waals interactions described above holds the Fru-2,6-P₂ molecule in the ideal orientation for an in-line associative transfer of the 2-phosphate to His2356. Thus, the crystallographically determined location of Fru-6-P in the active site (and the model of Fru-2,6-P₂) allow us to re-evaluate the proposed catalytic mechanism in more detail.

**DISCUSSION**

Fru-6-P and Fru-2,6-P₂ Binding—The structure presented here represents the first structure of a Fru-6-P-bound form of an intact Fru-2,6-Pase domain in a dimeric Fru-6-P,2-kinase/Fru-2,6-Pase. Fru-6-P is a potent inhibitor of Fru-2,6-Pase activity at physiologic concentrations of Fru-6-P ($K_i = 51$ nM, hepatocyte concentration of 20–50 μM). In the case of the RT2K isozone, this should result in an inhibited Fru-2,6-Pase activity and a net production of Fru-2,6-P₂ and consequent driving force for glycolysis in tissues that express this isozone. Thus, the elucidation of the mode of binding of this potent inhibitor is itself significant. Recently, a structure of the truncated form of...
an isolated RL2K Fru-2,6-Pase domain was determined with a covalent phosphohistidine intermediate in the presence of Fru-6-P (21). The position of the Fru-6-P molecule is notably different in the two structures. While it is impossible to say that one conformation is correct and the other incorrect, there are several points that argue that the conformation of Fru-6-P reported here is more likely to be the native conformation. First, the RL2K structures are of a truncated protein that is missing 30 C-terminal amino acids. The analogous stretch of polypeptide in our RT2K structure passes within 3.5 Å of the Fru-6-P molecule, at Thr443. The carbonyl oxygen of Thr443 is hydrogen-bonded to a water, which in turn coordinates the 1-hydroxyl of Fru-6-P (see Fig. 2). In addition, the region of RL2K structure that should have been occupied by the truncated protein is instead involved in a crystal contact, such that amino acids from a neighboring molecule are within 5.8 Å of the Fru-6-P. One of these amino acids (Gln391) actually interacts with Gln391 (the analogue of Gln391 in RT2K). Remember that Gln391 makes two hydrogen bonds with Fru-6-P that are important in maintaining its position in the active site (see Fig. 2). Last, the Fru-6-P in the RL2K structure is in an unusual conformation compared with two Fru-6-P and two Fru-1,6-P2 structures from the Cambridge small molecule database. In contrast, the Fru-6-P conformation reported here closely resembles the extended, low energy conformations determined by small molecule crystallography. Finally, recall that Ile267 forms a typical stacking interaction with the fructose ring in the structure reported here. This stacking interaction is absent in the RL2K structure.

The determination of a Fru-6-P-bound form of the RT2K H256A Fru-2,6-Pase domain has allowed us to build a reliable model of the Fru-2,6-P2-bound enzyme. As seen in Fig. 3, the active site of the Fru-2,6-Pase domain is perfectly tailored to accommodate Fru-2,6-P2. The active site serves as a molecular ruler, measuring the length of the bound substrate along an axis defined by the line between the Fru-6-P 2-hydroxyl and His256 (5.5 Å in the RT2K-Wo structure; see Fig. 4). In this space, there is room for one phosphate, one covalent bond to phosphate (1.6–1.8 Å), and one noncovalent interaction (3.2 Å). Thus, Fru-2,6-P2 is accommodated, with a covalent Fru-6-P-2-phosphate bond and the 2-phosphate in Van der Waals contact with His256. After the catalytic transfer of the 2-phosphate to His256, the ruler is still satisfied, with a covalent...
phosphohistidine and a noncovalent interaction between the transferred 2-phosphate and Fru-6-P. However, there is not enough room for either the combination of phosphohistidine, hydrolytic water, and Fru-6-P or for a noncovalently bound phosphate ion with a Fru-6-P molecule. In those cases, the ruler would be measuring two noncovalent interactions, a distance that cannot be accommodated. The arrangement of molecules in the RL2K trapped phosphohistidine structure described above (E-P-H2O-Fru-6-P) is an arrangement that violates this molecular ruler concept. This violation is only allowed because of the unnatural position of Fru-6-P in the binding pocket, which is a consequence of the C-terminal truncation of the RL2K Fru-2,6-Pase domain.

**Correlation of Structures with Kinetics**—The molecular ruler concept predicts an ordered reaction mechanism (Scheme 1), where Fru-6-P release must precede phosphohistidine hydrolysis. After Fru-6-P has dissociated, a water molecule would be accommodated in the active site for hydrolysis of the E-P intermediate. This model is consistent with previous kinetic experiments where it has been reported that the rate of phosphoenzyme formation is greater than the overall reaction rate (33), that Fru-6-P inhibits the Fru-2,6-Pase reaction (34), that Fru-6-P release is the rate-limiting step in the overall reaction (35), and that Pi accelerates E-P hydrolysis by competing for Fru-6-P binding (presumably at the 6-phosphate binding site) (33). In our own studies, we showed that Fru-6-P release and phosphohistidine breakdown occur in parallel in the wild type enzyme (1). Together, the structural and kinetic data indicate that E-P hydrolysis does not occur until after Fru-6-P has dissociated from the enzyme, and that E-P hydrolysis is fast (Scheme 1).

There have been several models of Fru-2,6-Pase catalysis proposed, based first on mutagenesis directed by the homology of the Fru-2,6-Pase domain to the phosphoglycerate mutase family (16, 17) and later based on crystal structures (1, 7, 8, 21). These studies have all concluded that all of the amino acids that define the 2-phosphate binding pocket (His256, His390, Arg255, Arg305, Asn262, and Glu325) are influential in catalysis. Specifically, His256 has conclusively been demonstrated to act as a nucleophile that attacks the 2-phosphate, resulting in a His256 phosphohistidine intermediate (1, 13, 14, 21). His256 is perfectly placed for an in-line attack that would break the O-2-P-2 bond of Fru-2,6-P2 with an inversion of phosphate geometry (Fig. 3). Our own data on the retention of significant catalytic activity in the H256A mutant (1) are contradictory to previous findings in the RL2K enzyme (16) and indicate that this phosphoenzyme intermediate is not an obligatory intermediate for the enzyme. Arg255 and Arg305 have been proposed to neutralize the charge of the 2-phosphate and/or to stabilize the transition state (1, 18), consistent with their location as equatorial ligands to the 2-phosphate. Glu325 has been proposed to act both to promote the protonation of His390 and to polarize a water molecule as a nucleophile for attack on the phosphohistidine intermediate (8, 17, 21). The capacity of Glu325 to alter
the pKₐ of His₃⁹⁰ is doubtful, since the ionizable groups of these amino acids are not near each other (see Figs. 2 and 3). The role for Glu₃²⁵ as a catalytic base in phosphohistidine hydrolysis is consistent with both its location in the structure and the available kinetic data on Glu₃²⁵ mutants (1, 17). Finally, His₃⁹⁰ has been proposed to act as a catalytic acid, donating a proton to the leaving group O-2 of Fru-6-P (8, 16). This, however, is inconsistent with the structure, since His₃⁹⁰ is not in the vicinity of the leaving group (Fig. 3), nor does it exist in an environment that would promote its protonation. Our kinetic data from the H₃⁹⁰A mutant protein are also inconsistent with its role as a proton donor to the leaving group, indicating that His₃⁹⁰ is involved in phosphohistidine hydrolysis. Thus, the role of H₃⁹⁰, the mechanism of catalysis in the H256A mutant, and the identity of the proton donor for the leaving group O-2 of Fru-6-P remain unresolved from previous findings. We believe that the Fru-2,6-Pase kinetic data (as cited above) and the structural findings reported here lead to a complete catalytic mechanism for the wild type enzyme and a distinct mechanism for the H256A mutant enzyme.

**Wild Type Mechanism**—Fig. 5 shows a Fru-2,6-Pase catalytic cycle where Glu₃²⁵ acts as the sole amino acid involved in protonation exchanges. At the beginning of the cycle (E’Fru-2,6-P₂ from Scheme I), His²⁵⁶ attacks the phosphate, leading to a pentacoordinate transition state, with the excess negative charge of the transition state being stabilized by Arg²⁵⁷, Asn³⁰², Arg³⁰⁵, and His³⁹⁰. A protonated Glu³²⁵ then donates a proton to the leaving group O-2 of Fru-6-P. As discussed above, Fru-6-P dissociation must precede the next step, where the ionized Glu³²⁵ polarizes a water molecule for attack on the phosphohistidine intermediate, regenerating the ground state of the enzyme. The attractive features of this model are its simplicity and its consistency with both the kinetic and structural data. The proximity of Glu³²⁵ to the 2-oxygen of Fru-6-P and/or the model of Fru-
2,6-P₂ and the kinetic data from Glu 325 mutants that show a drastic loss of Fru-2,6-Pase activity (1, 17) make Glu325 a prime candidate for a major role in catalysis. Glu325 is well positioned for proton transfer to the 2-oxygen of Fru-6-P (Fig. 3, and Table II) and is well positioned to polarize water for attack on the phosphohistidine intermediate (21). The role of the amino acids that are equatorial ligands in transition state stabilization is consistent with the decreases in $k_{cat}/K_m$ that are observed when they are mutated to amino acids that cannot fill this role. Where it has been studied, mutation of these equatorial ligands clearly affects both transition states (i.e. phosphohistidine formation and phosphohistidine hydrolysis). Note that His390, 

**Fig. 5. Fru-2,6-Pase catalytic cycle.** The proposed mechanism begins with formation of the E-Fru-2,6-P₂ complex shown in the upper left panel. His256 attacks the 2-phosphate, resulting in a tetrahedral intermediate that is stabilized by its interactions with Arg305, Arg255, His390, and Asn262 as shown in the upper right panel. A proton derived from Glu325 then joins the leaving group Fru-6-P as the O-2–P bond is broken. The resulting E-P-Fru-6-P complex is stable, and Fru-6-P release from the active site is rate-limiting. After Fru-6-P diffuses out of the active site, a water molecule will diffuse in. Glu325 then activates the water as a nucleophile to attack the E-P intermediate as shown in the lower right panel. The E-P hydrolysis requires the formation of another tetrahedral intermediate, again stabilized by Arg305, Arg255, His390, and Asn262 (not shown). Finally, the free phosphate remains bound in the 2-phosphate binding site (lower left panel) until it is displaced by another molecule of Fru-2,6-P₂, restarting the cycle.
which had previously been designated as a donor proton, is now simply another equatorial ligand. This is consistent with the kinetic data, where His390 mutations have effects similar to the mutation of other equatorial ligands. Most important, though, is the observation that the H390A mutation causes a significant lag in P2 release after Fru-6-P release (1). This lag in P2 release is consistent with the role of His390 in transition state stabilization but not consistent with His390 as a proton donor to Fru-6-P.

There is one significant problem with this mechanism. The crystal structure clearly shows that Glu325 is capping the N terminus of an α-helix (α14, amino acids 391–400, Fig. 3). Such a protein environment would shift the pKa of Glu325 such that the existence of a protonated Glu325 is unlikely, making it a poor choice for the proton donor in Fru-6-P release. On the other hand, this is in fact an enzyme with a low turnover (i.e. the wild type RT2K Fru-2,6-Pase reaction has a kcat of 0.032 s−1). Because the biological role of the enzyme is regulatory and is not a step in a biosynthetic pathway, it makes some teleological sense that the enzyme would be slow. As such, a rare protonation of Glu325 and subsequent proton donation to Fru-6-P could be tolerated. This idea of a slow Glu325 protonation is not in agreement with the kinetic evidence, however, since phosphohistidine formation has been reported to be 2 orders of magnitude faster than the overall rate of catalysis, with the Fru-6-P release rate-limiting (33, 34). It is possible that Fru-2,6-P2 binding to the active site shifts the Glu325 pKa via the interaction of the 2-phosphate with the N terminus of helix α14 (Fig. 3).

One approach to address this apparent Glu325 paradox would be pH titration kinetics of phosphohistidine formation in the wild-type and active site mutant enzymes to determine the pKa values for Glu325 and other active site residues. Pilkis and co-workers reported the pH titration of kcat/Km for wild type, H392A, and E327Q mutant forms of the RL2K enzyme (36). Their results with the wild type enzyme indicated two ionizable groups with pKa values of 6.1 and 8.4, which would we interpret as the pKa of a Fru-2,6-P2 phosphate and an unidentified residue of the protein, respectively. The titration profiles of the H392A and E327Q mutants clearly showed a partial change in the low end pK values with an increase in the pK values. The curves were complex however, suggesting the possibility of compensating charge interactions. It is evident that further experiments in this area will be necessary to clarify the role of ionizable groups in the Fru-2,6-P2ase active site.

H256A Catalysis: Hydrolysis Versus Phosphohistidine 390—

The discovery that the H256A mutant of RT2K has 17% of the wild type bisphosphatase activity has led to two alternative explanations of the mechanism (1). Either the enzyme is able to utilize His390 as an alternate to His256 as a nucleophile to form a phosphohistidine intermediate, or another as yet undetermined mechanism exists for the H256A mutant. Examination of the active site structure (Fig. 3) leads to the conclusion that the transfer of a phosphate to His390 is quite unlikely. As noted above, His256 is ideally positioned for the in-line transfer of phosphate from Fru-2,6-P2. His390 on the other hand, is nearly 90° off this transfer axis, ideal positioning for an equatorial ligand to the transition state intermediate, but horribly suited for an in-line phosphotransfer. In order to accomplish a phosphotransfer to His390, either by direct in-line attack or by an attack from the side followed by pseudorotation (37, 38), the substrate and/or His390 would have to be repositioned in the active site. The position of His390 is of course constrained by the fold of the protein and thus could not move from its equatorial location to an axial position. Manual remodeling of Fru-6-P in the active site to a position that would lead to an axial His390 led only to eteric clashes of Fru-6-P with protein. From this evidence, combined with the inability to detect a phosphohistidine 390 intermediate pathway is not at all likely.

An alternative hypothesis is that the H256A mechanism involves the direct hydrolysis of Fru-2,6-P2 by water. Due to the deletion of His256, there is a hole in the enzyme where the imidazole ring used to be. In the crystal structure, this hole is occupied by a free phosphate as described above (Fig. 2 and Table III). When we model Fru-2,6-P2 bound to the enzyme by superimposing the H256A and RT2K-Wo structures, this hole would be empty in the H256A substrate complex. Since vacuums do not exist in proteins, this space will be filled, and most likely by a water molecule. Like the imidazole of His256, this water molecule would be in the perfect position for an in-line transfer of the phosphate. The equatorial ligands to the phosphate remain in the same position in both the RT2K-Wo and H256A structures, so they would be competent to stabilize this hydrolytic transition state in the mutant enzyme. The best experiment to verify this hypothesis would be to utilize a synthetic Fru-2,6-P2 that has been triply labeled at the 2-phosphate with 18O, 17O, and 16O, generating a chiral phosphate. In the event of a direct hydrolysis, there will be an inversion of the phosphate stereochemistry, while for a two-step process involving a phosphohistidine intermediate, there will be retention of stereochemistry. Such an experiment was used to verify the phosphohistidine intermediate in phosphoglycerate mutase (39).

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