Hepatic 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase

THE ROLE OF SURFACE LOOP BASIC RESIDUES IN SUBSTRATE BINDING TO THE FRUCTOSE-2,6-BISPHOSPHATASE DOMAIN*

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Lys-356 has been implicated as a critical residue for binding the C-6 phospho group of fructose 2,6-bisphosphate to the fructose-2,6-bisphosphatase domain of rat liver 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (Li, L., Lin, K., Correia, J., and Pilkis, S. J. (1992) J. Biol. Chem. 267, 16669-16675). To ascertain whether the three other basic residues (Arg-352, Arg-358, and Arg-360), which are located in a surface loop (residues 331-362) which contains Lys-356, are important in substrate binding, these arginyl residues were mutated to Ala, and each arginyl mutant was expressed in Escherichia coli and purified to homogeneity. The far UV circular dichroism spectra of the mutants were identical to that of the wild-type enzyme. The kinetic parameters of 6-phosphofructo-2-kinase of the mutants revealed only small changes. However, the $K_m$ for fructose 2,6-bisphosphate, $K_i$ for fructose 6-phosphate, and $K_i$ for inorganic phosphate of fructose-2,6-bisphosphatase for Arg352Ala were, respectively, 2,800-, 4,500-, and 1,500-fold higher than those for the wild-type enzyme, whereas there was no change in the maximal velocity or the $K_i$ for inorganic phosphate. The $K_m$ for fructose 2,6-bisphosphate and $K_i$ for inorganic phosphate of Arg360Ala were 10- and 12-fold higher, respectively, than those of the wild-type enzyme, whereas the maximal velocity and $K_i$ for fructose 6-phosphate were unchanged. In addition, substrate inhibition was not observed with Arg352Ala and greatly reduced with Arg360Ala. The properties of the Arg358Ala mutant were identical to those of the wild-type enzyme. The results demonstrate that in addition to Lys-356, Arg-352 is another critical residue in fructose-2,6-bisphosphatase for binding the C-6 phospho group of fructose 2,6-bisphosphate and that Arg-360 binds the C-2 phospho group of fructose 2,6-bisphosphate in the phosphoenzyme fructose 2,6-bisphosphate complex. The results also provide support for Arg-352, Lys-356, and Arg-360 constituting a specificity pocket for fructose-2,6-bisphosphatase.

* The bifunctional enzyme 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (6-PF-2-kinase/Fru-2,6-Pase, EC 2.7.1.105/3.1.3.46) catalyzes both the synthesis and degradation of Fru-2,6-P$_2$, an important regulatory metabolite whose steady-state concentration determines glycolytic/gluconeogenic flux in the hepatocyte and glycolytic flux in extrahepatic tissues (1-4). Rat liver 6-PF-2-kinase/Fru-2,6-Pase is a homodimeric protein, and each subunit is composed of 470 amino acids. The enzyme consists of two catalytic domains (5, 6). The NH$_2$-terminal kinase domain (residues 1-250) and the COOH-terminal bisphosphatase domain (residues 251-470) have been postulated to be structurally similar to the glycolytic enzymes 6-phosphofructo-1-kinase and phosphoglycerate mutase, respectively (7). This two-domain structure of the enzyme is found in all mammalian forms of the enzyme (8-14), even though there are a number of tissue-specific isoforms encoded by at least four different genes (8, 11, 12, 14).

Recent work has begun to elucidate the chemical basis for catalysis and substrate/product binding in the Fru-2,6-P$_2$ase reaction of the bifunctional enzyme. The Fru-2,6-P$_2$ase reaction is catalyzed via an E-P intermediate (15, 16). Site-directed mutagenesis studies have demonstrated that His-258, His-392, and Glu-327 comprise a putative catalytic triad in rat liver Fru-2,6-P$_2$ase (17, 18); His-258 is the phosphoacceptor domain during Fru-2,6-P$_2$ hydrolysis (15-17, 19); His-392 plays an important role in catalysis, probably by acting as a proton donor to the leaving group Fru-6-P (17); and Glu-327 plays an important role in E-P formation by influencing the ionization state of the active site histidine(s) and/or acts as a general base catalyst (18). Arg-257 and Arg-307 are responsible for the binding of the C-2 phospho group of Fru-2,6-P$_2$ and product P (20).

Based on computer-assisted modeling, residues 333-361 of Fru-2,6-P$_2$ase form a loosely packed loop structure which overhangs the Fru-2,6-P$_2$ase active site pocket (7). Previous work has implicated this loop structure in substrate/product binding in Fru-2,6-P$_2$ase (21). For example, Lys-356 has been shown to be a critical residue for binding the C-6 phospho group of Fru-2,6-P$_2$/Fru-6-P to the Fru-2,6-P$_2$ase domain (21). In addition to Lys-356, there are only 3 other basic residues (Arg-352, Arg-358, and Arg-360) in this loop structure. Furthermore, the alignment of the bifunctional enzyme sequences from several species reveal that these 3 arginyl residues are conserved in all liver forms of the enzymes whose sequences are known (6, 10, 13). The object of this report was to

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The abbreviations used are: 6-PF-2-kinase, 6-phosphofructo-2-kinase; Fru-2,6-P$_2$ase, fructose-2,6-bisphosphatase; Fru-2,6-P$_2$, fructose 2,6-bisphosphate; Fru-6-P, fructose 6-phosphate; E-P, phosphoenzyme; TES, 2-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]-aminoethanesulfonic acid.

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ascertained whether Arg-352, Arg-358, and Arg-360 play a role in Fru-2,6-P₂ase substrate binding and/or catalysis.

EXPERIMENTAL PROCEDURES

Materials—Restriction enzymes, bacteriophage T₄ DNA ligase, and T₄ DNA polymerase were obtained from New England BioLabs. Phosphocellulose (P₁₁) was from Whatman. Q-Sepharose Fast Flow was obtained from Pharmacia LKB Biotechnology Inc. Fru-6-P, Fru-2,6-P₂, and ATP were obtained from Sigma. [γ-³²P]ATP was from Du Pont-New England Nuclear.

Site-directed Mutagenesis—The mutagenic oligonucleotides for Arg352Ala, Arg358Ala, and Arg360Ala were synthesized by phosphoramidite chemistry on an Applied Biosystems model 381A synthesizer and purified on oligonucleotide purification cartridges according to the Applied Biosystems manual. As shown in Table I, the codon CGG, GCT, and GCC were, respectively, used to mutate Arg-352 (CGG), Arg-358 (GCT), and Arg-360 (GCC) to Ala. The three mutant cDNAs were engineered as described previously for mutagenesis of Arg-195 (22) and Lys-356 (21). The mutant cDNAs were identified by sequencing (23), and the nucleotide sequence of the entire coding region was determined on the alkali-denatured double-stranded DNA to ensure that there were no other point mutations (24).

Expression in Escherichia coli and Purification of Arg352Ala, Arg358Ala, and Arg360Ala Mutant and Wild-type 6-PF-2 kinase/Fru-2,6-P₂ase—The wild-type and mutant bifunctional enzymes were expressed in E. coli BL21 (DE3) using the phage T7 RNA polymerase-based system described by Studier and Moffatt (25) modified as described previously (26). The soluble proteins were extracted from cells as described previously (22). The wild-type and mutant enzymes were purified by polyethylene glycol fractionation and Fru-6-P elution from phosphocellulose as described previously (26).

Determination of Circular Dichroism Spectra—All far UV spectra were collected on a Jasco 500 A in a 0.1-mm cell at 20 °C. Scans were collected at 1 mm width with a time constant of 64 s, and all data are the average of three blank corrected samples. Samples were dialyzed against 20 mM Tris-HCl, pH 8.0, 0.2 mM EDTA, 1 mM dithiothreitol, 100 mM KCl, and 20% glycerol. Near UV spectra were collected in a 0.5-mm cell at the same temperature, scan rate, and time constant. Samples were centrifuged at 14,000 rpm for 10 min to remove any precipitated protein, and the A₅₃₂ was measured to scale the CD data to the same concentration. The CD data presented correspond to samples with an A₅₃₂ of 1.0 in a 1-cm cell. The data have not been scaled for path length in the CD cell.

Assay of 6-PF-2 kinase and Fru-2,6-P₂ase Activities—6-PF-2 kinase activity was determined by following the production of Fru-2,6-P₂ from ATP and Fru-6-P with the potato pyrophosphate:Fru-2,6-P₂-phosphotransferase assay as described previously (27). All assays were performed in buffer containing 50 mM Tris-HCl, pH 8.0, 10 mM MgCl₂, 100 mM KCl, 0.5 mM EDTA, and 1 mM dithiothreitol. The concentrations of ATP, Fru-6-P, and Fru-2,6-P₂ were varied as indicated in the Table II legend under Results. The 2-6-P₂ase activity was determined by measuring the production of ³²P from [2-³²P]Fru-2,6-P₂ as described previously (28, 29). The buffer used for bisphosphatase assays was 20 mM Tris-HCl, pH 8.0, 0.5 mM EDTA. The concentrations of the Fru-2,6-P₂, Fru-6-P, and Pi were varied as indicated in the Table II legend under Results.

Preparation of [³²P]Fru-2,6-P₂ for Fru-2,6-P₂ase Assay—[³²P]Fru-2,6-P₂ was prepared by incubating 0.05 mM [γ-³²P]ATP (>400,000 cpm/pmol) and 10 mM Fru-6-P in the presence of 20 mM TES, pH 7.8, 100 mM KCl, 5 mM MgCl₂, 5 mM P₂O₅, 0.5 mM EDTA, and 1 mM dithiothreitol with 40 μg of the purified His×258Ala mutant of 6-PF-2-kinase/Fru-2,6-P₂ase for 2 h at 30 °C (21). The use of the His×258Ala mutant, which has no Fru-2,6-P₂ase activity, allows for the quantitative conversion of [γ-³²P]ATP to [²-³²P]Fru-2,6-P₂, with no net hydrolysis of ATP. [²-³²P]Fru-2,6-P₂ was purified by a DEAEdexadex A-55 column as described previously (28), and its concentration was determined by its activation of pyrophosphate:fructose-6-phosphate-1-phosphotransferase (27).

Assay of E-P Formation—The E-P steady-state level was assayed as acid-precipitable radioactivity on phosphocellulose paper as described previously (29). The time course of E-P formation was measured by mixing the enzyme (0.5–1 μM) and 10 μM [²-³²P]Fru-2,6-P₂, 50 mM Tris-HCl, pH 8.0, 10 mM MgCl₂, 150 mM KCl, and 20% glycerol. Near UV spectra were collected in a 0.5-cm cell at the same temperature, scan rate, and time constant. Samples were centrifuged at 14,000 rpm for 10 min to remove any precipitated protein, and the A₅₃₂ was measured to scale the CD data to the same concentration. The CD data presented correspond to samples with an A₅₃₂ of 1.0 in a 1-cm cell. The data have not been scaled for path length in the CD cell.

RESULTS

Purification of E. coli-expressed Wild-type and Mutant 6-PF-2-kinase/Fru-2,6-P₂ases—Wild-type bifunctional enzyme is routinely purified to homogeneity by Fru-6-P elution from a phosphocellulose column (26). 6-PF-2-kinase/Fru-2,6-P₂ase is believed to bind to phosphocellulose via Arg-195, the residue primarily responsible for Fru-6-P binding to the 6-PF-2-kinase domain (22). In support of this hypothesis, mutation of Arg-195 to alanine resulted in a form which did not bind to phosphocellulose (22). The Arg352Ala, Arg358Ala, and Arg360Ala mutant enzymes all bound to phosphocellulose and were eluted with 2 mM Fru-6-P, which suggests that these residues do not play a major role in binding Fru-6-P in the 6-PF-2-kinase domain. Wild-type and mutant enzymes were extracted from E. coli cells, subjected to polyethylene glycol fractionation (6–15%), followed by Fru-6-P elution from phosphocellulose columns, as described previously (18, 20–22). As shown in Fig. 1, the wild-type and Arg352Ala, Arg358Ala, and Arg360Ala mutant enzymes were purified to homogeneity by this purification scheme (as judged by the criteria of sodium dodecyl sulfate-polyacrylamide gel electrophoresis), and had the same molecular mass (55 kDa). The wild-type and mutant enzymes are homodimeric with native molecular masses of 110 kDa (data not shown), which indicates that mutation of

![FIG. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of purified mutant and wild-type enzymes. 2–5 μg of each of the enzymes was subjected to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the gel was stained with Coomassie Blue. The protein standards were a mixture of 4 μg each of netropsin (24 kDa), soybean trypsin inhibitor (20 kDa), and bovine milk a-lactalbumin (14 kDa).](image-url)
solvent exposure of only the aromatics. It is concluded that analysis of CD spectra demonstrates that the mutation of the surface loop basic residues does not change the core secondary structure of the enzyme but does alter the solvent exposure of some of the tyrosyl residues interposed between Lys-356, Arg-358, and Arg-360.

Effect of Mutation of Arg-352, Arg-358, and Arg-360 on the Rate of E-P Formation and the Steady-state E-P Level—Fru-2,6-P_2ase catalyzes its reaction through the formation of an E-P intermediate (15-17). E-P formation and breakdown have been characterized in wild-type (15) and several mutant enzymes (17, 18, 20, 21). For the wild-type enzyme, the rate of E-P formation determined by measuring ^32P incorporation into the enzyme in the presence of [2-^32P]Fru-2,6-P_2 is 500-1000-fold faster than the rate of E-P breakdown, which is believed to be the step which determines the overall rate of the reaction (15). Determination of presteady-state kinetics of E-P formation of the wild-type enzyme requires a stop-quench apparatus since steady-state values are reached by 80 ms (15). However, with mutants whose rates of E-P formation are decreased, the rate of E-P formation can be measured manually (17, 18, 20). The steady-state E-P level in the wild-type enzyme is 1 mol of phosphorus/mol of dimer (17, 18, 20, 21). Mutants which have differential effects on E-P formation and breakdown rates would be expected to exhibit changes in the steady-state E-P level (18, 20, 21). When the time course of E-P formation was measured manually as described under "Experimental Procedures," the steady-state E-P levels of the Arg352Ala, Arg358Ala, and Arg360Ala mutant and wild-type enzymes were reached in less than 2 s at 0 °C (data not shown). Furthermore, no changes in the rate of E-P formation of the mutant enzymes were detected when compared with that for the wild-type enzyme in preliminary experiments using the quench-flow apparatus (data not shown).

However, as shown in Fig. 3, in contrast to the wild-type enzyme and the Arg358Ala mutant whose steady-state E-P values remained constant for 15 min, the steady-state E-P value for the Arg360Ala mutant decreased linearly from 0.8 mol of ^32P/mol of enzyme to 0.1 mol of ^32P/mol of enzyme in 15 min, while the value for the Arg352Ala mutant remained constant at 10% that of the wild-type value. The low steady-state E-P value for the Arg352Ala mutant may reflect either an effect on catalysis per se, e.g. the chemical C-2 phospho group transfer step, and/or an effect on the substrate concent-

![Figure 2](image-url)

**Fig. 2.** Circular dichroism spectra of wild-type, Arg352Ala, Arg358Ala, and Arg360Ala 6-PF-2-kinase/Fru-2,6-P_2ase. Panel A, far UV-CD spectra; panel B, near UV-CD spectra. Millidegrees of CD signal are plotted versus wavelength for the wild-type (solid line) and the Arg352Ala (open circle), Arg358Ala (open triangle), and Arg360Ala (open square) mutant enzymes.

Arg-352, Arg-358, and Arg-360 had no effect on the quaternary structure of the enzyme.

**Circular Dichroism Spectra of the Wild-type and Mutant 6-PF-2-kinase/Fru-2,6-P_2ase—**To determine whether mutation of Arg-352, Arg-358, and Arg-360 to alanine changed the secondary structure of the enzyme, the circular dichroism spectra of the three mutant and wild-type enzymes were determined. As shown in Fig. 2A, the far UV circular dichroism spectra for the three mutant and the wild-type enzymes were similar, consistent with no significant change in the secondary structure of the enzyme. The Arg352Ala, Arg358Ala, and Arg360Ala mutants. The near UV CD spectra, Fig. 2B, were identical for the wild-type and mutant Arg360Ala, which suggests identical aromatic environments and thus identical tertiary structures. However, the spectra for the Arg352Ala and the Arg358Ala mutants deviate from the wild-type enzyme in the 270-290 nm region, consistent with small changes in solvent exposure of both tyrosine and tryptophan residues. The positive peak at 295 nm is probably caused by tryptophan (38). The major increases occur at the peak centered at 288.5 nm and at the broad minima centered at 277 nm. These bands could be caused by tyrosine and/or tryptophan. The small changes observed in the near UV CD spectra probably reflect changes in solvent exposure of only a few residues, resulting from changes in the surface charge of the enzyme (+2 from one arginine to alanine substitution on each subunit) and thus to conformational rearrangements of the surface loop (residues 351-362) to stabilize the protein. There are 5 tyrosine residues in the surface loop region, and 3 of these tyrosines (Tyr-356, Tyr-359, Tyr-361) are interposed between Lys-356, Arg-358, and Arg-360 (5). Similar changes in the near UV CD spectra extending to 260 nm were also observed with the Lys356Ala mutant (21) and are thus consistent with mutations in this surface loop resulting in specific changes in the surface charge. It is unlikely that these mutations caused any change in core protein structure, in which case we would expect changes in the far CD spectra, not just an increase in

![Figure 3](image-url)

**Fig. 3.** Time course of E-P levels for the wild-type and Arg352Ala, Arg358Ala, and Arg360Ala mutant enzymes. A 1 μM concentration of each of the enzymes was mixed with 20 μM [2-^32P]Fru-2,6-P_2 and incubated at 30 °C. The E-P levels were assayed as acid-precipitable radioactivity at various time as described previously (29). Open circles, wild-type; filled circles, Arg360Ala; open squares, Arg352Ala; filled squares, Arg358Ala.
tation dependence of E-P formation. The decline in the steady-state E-P value of the Arg352Ala mutant was caused by altered substrate dependence, the substrate dependence of the steady-state E-P value was determined, and the results are shown in Fig. 4.

To determine whether the lower steady-state value for the Arg352Ala mutant was caused by altered substrate dependence, the substrate dependence of the steady-state E-P value was determined, and the results are shown in Fig. 4. The maximal steady-state E-P values for the wild-type, Arg352Ala, Arg358Ala, and Arg360Ala mutant enzymes were 0.98, 0.49, 1.13, and 0.89 mol/mmol of dimer, respectively. The steady-state E-P values for the wild-type and Arg358Ala mutant reached saturation at the lowest concentration of Fru-2,6-P$_2$ (4 nM) used, whereas that for the Arg352Ala mutant was reached at concentrations of Fru-2,6-P$_2$ between 80 and 100 nM. The steady-state E-P value for the Arg360Ala mutant was 80% of the saturated value at 4 nM Fru-2,6-P$_2$. The results suggest that mutations at Arg-352 and Arg-360 affect the affinity of the enzyme for Fru-2,6-P$_2$.

Effect of Mutation of Arg-352, Arg-358, and Arg-360 on the $K_m$ for Fru-2,6-P$_2$ and Maximal Velocity of Fru-2,6-P$_2$ase—As shown in Table II, the maximal velocity for the Arg352Ala mutant was slightly higher than that for the wild-type enzyme. The maximal velocities for Arg358Ala and Arg360Ala were identical to that for the wild-type enzyme. However, the $K_m$ values for Fru-2,6-P$_2$ of Arg352Ala and Arg360Ala were, respectively, 16.6 nM and 63 nM, which are 2,800-fold and 10-fold higher, respectively, than that of the wild-type enzyme (6 nM). The $K_m$ for Fru-2,6-P$_2$ of the Arg358Ala was 9 nM and was not significantly different from that of the wild-type enzyme. The results are consistent with the results shown in Fig. 4 on the substrate dependence of steady-state labeling and indicate that Arg352 is an important Fru-2,6-P$_2$ binding site residue, whereas Arg360 also contributes, but to a lesser extent, to the binding of Fru-2,6-P$_2$. The results also demonstrate that Arg358 has no important role in the binding and catalysis in Fru-2,6-P$_2$ase.

Effect of Mutation of Arg-352, Arg-358, and Arg-360 on $K_m$ Values for Fru-6-P and $P_i$ of Fru-2,6-P$_2$ase—Three basic residues, Arg-257 and Arg-307, Lys-356, have been previously shown to bind the substrate, Fru-2,6-P$_2$. Examination of the pattern of product inhibition revealed that Lys-356 binds the C-6 phospho group (21) and Arg-257 and Arg-307 bind the C-2 phospho group of Fru-2,6-P$_2$ (31). The $K_i$ values for Fru-2,6-P$_2$ for the Arg352Ala and Arg360Ala mutants were increased as shown in Table II, which indicates that these 2 arginyl residues are also important for binding Fru-2,6-P$_2$, in addition to Arg-257, Arg-307, and Lys-356. As demonstrated previously (15, 21, 31-37), the product, Fru-6-P, is a noncompetitive inhibitor, and the other product, $P_i$, is a competitive inhibitor (15, 31, 32) with respect to the substrate, Fru-2,6-P$_2$. To ascertain which phospho group of Fru-2,6-P$_2$ contacts Arg-352 and Arg-360, the $K_i$ values for Fru-2,6-P$_2$ and $P_i$ for both mutants were obtained from the plot of 1/v versus Fru-6-P concentration and the plot of $K_i$ for Fru-2,6-P$_2$ versus $P_i$ concentration, respectively. As shown in Table II, the $K_i$ for Fru-2,6-P$_2$ of the Arg352Ala mutant was 810 nM, which is 4,500-fold higher than that of the wild-type enzyme (0.18 nM), whereas the $K_i$ for $P_i$ of the Arg352Ala mutant was similar to that of the wild-type enzyme. The result indicates that, like Lys-356 (21), Arg-352 is a critical residue for binding the C-6, but not C-2, phospho group of Fru-2,6-P$_2$ in bisphosphatase domain. The $K_i$ for the Arg360Ala mutant was 21.11 nM, which is 12-fold higher than that of the wild-type enzyme, but the $K_i$ for Fru-6-P was increased by only a factor of 2 compared with that for wild-type, which suggests that Arg-360 contacts the C-2, but not C-6, phospho group of Fru-2,6-P$_2$.

Effect of Mutation of Arg-352, Arg-358, and Arg-360 on Substrate Inhibition of Fru-2,6-P$_2$ase—Previous work has demonstrated that substrate inhibition of Fru-2,6-P$_2$ can be explained by Fru-2,6-P$_2$ binding to the same site as the noncompetitive product inhibitor, Fru-6-P (21). The major findings in support of this hypothesis were that mutation of Lys-356 to Ala abolished substrate inhibition and decreased the affinity of the enzyme for Fru-6-P by a factor of 2,200-fold. In addition, as described previously (21), P$_i$ activates Fru-2,6-P$_2$ase in the presence of saturating substrate concentration by competing with Fru-6-P and Fru-2,6-P$_2$ for the same site; that is to say, the activator, $P_i$, the product, Fru-6-P, and the inhibitor Fru-2,6-P$_2$, bind to the same site. Since the results shown in Table II show that Arg-352 is another critical residue for binding the C-6 phospho group of Fru-6-P/Fru-2,6-P$_2$, it was of interest to determine whether mutation of Arg-352 also affected substrate inhibition and P$_i$ activation, to provide additional support for the hypothesis that the inhibitors, Fru-2,6-P$_2$ and Fru-6-P, and activator $P_i$ bind to the same site. As shown in Fig. 5 and Table II, there was no inhibition by substrate of the Arg352Ala mutant, whereas the $K_i$ for $P_i$ of this mutant was 1,500-fold higher than that of the wild-type enzyme. The results support the hypothesis that the Fru-6-P binding site is responsible for Fru-2,6-P$_2$ inhibition and $P_i$ activation of Fru-2,6-P$_2$ase.

However, an unexpected finding was the greatly diminished substrate inhibition observed with Arg360Ala mutant (Fig. 5). For example, at 20 nM Fru-2,6-P$_2$, the wild-type enzyme and the Arg360Ala mutant were inhibited by more than 90%, whereas the Arg360Ala mutant exhibited less than 20% inhibition (Fig. 5B). When the Fru-2,6-P$_2$ concentration was raised to 100 nM, Arg360Ala still retained more than 50% of its maximal activity (Fig. 5B), whereas the wild-type enzyme and the Arg358Ala mutant had activities below detectable levels (data not shown). This observation provides an explanation for the finding that the E-P steady-state value of the Arg360Ala mutant decreased much more quickly than that for the wild-type enzyme as shown in Fig. 3. Since the maxi-

![Fig. 4. Fru-2,6-P$_2$ dependence of E-P steady-state level of wild-type, Arg352Ala, Arg358Ala, and Arg360Ala enzymes.](Image)
Therefore it is reasonable to argue that Arg-360 is responsible for binding the C-2 phospho group of Fru-2,6-P₂ to the E-P form of the enzyme (E-P:Fru-2,6-P₂). However, the $K_m$ for Fru-2,6-P₂ and the $K_i$ for P, of the Arg360Ala mutant were 10- and 12-fold higher than those of the wild-type enzyme, respectively, which also suggests that this arginyl residue provides a weak contact to the C-2 phospho group of Fru-2,6-P₂ to form the enzyme substrate complex (E-Fru-2,6-P₂).

**Effect of Mutation of Arg-352, Arg-358, and Arg-360 to Ala on 6-PF-2-kinase Activity**—It has been shown previously that Arg-195 and Lys-356 are critical residues for binding Fru-6-P to the kinase domain (22) and bisphosphatase domain (21), respectively. Mutation of Arg-195 to alanine results in reduced affinity of Fru-6-P to the kinase domain by a factor of 3,000-fold but had no effect on the affinity of Fru-6-P for the bisphosphatase domain (22). The mutation of Lys-356 also had no effect on the affinity of Fru-6-P for the kinase domain but greatly changed the affinity of Fru-6-P for the bisphosphatase domain (21). These findings support the hypothesis that the bifunctional enzyme has two distinct binding sites of substrate/product. To ascertain whether Arg-352, Arg-358, and Arg-360, which are located in bisphosphatase domain, have important roles in the kinase reaction, the kinetic parameters of the 6-PF-2-kinase of the three mutant enzymes were analyzed and compared with that of the wild-type enzyme. As shown in Table II, the kinetic parameters for the three mutant enzymes i.e. $K_m$ for Fru-6-P, $K_m$ for ATP, $K_i$ for P, and $V_{max}$ were little changed from those for the wild-type enzyme. The results suggest that Arg-352, Arg-358, and Arg-360 have no important role in the binding and catalysis of 6-PF-2-kinase. In addition, the small changes observed by near UV CD (Fig. 25) for the Arg352Ala and the Arg358Ala mutants are not global changes in structure that affect enzyme activity in the 6-PF-2-kinase domain.

**DISCUSSION**

The results of this study provide solid support for the hypothesis that the surface loop encompassing residues 333-361 of Fru-2,6-Pase not only provides a connection between $\beta$-strand C and $\alpha$-helix 4 (7) but also participates in forming the Fru-2,6-P₂/Fru-6-P binding site in the Fru-2,6-Pase active site. Like many loop regions in proteins, this structure is rich in charged and polar hydrophilic residues. This 29-amino acid chain contains 8 glutamic acids, 2 aspartic acids, and 4 basic residues. The basic residues (Arg-352, Lys-356, Arg-358, and Arg-360) are clustered at the COOH-terminal end of the
Substrate Binding Site of Fructose-2,6-bisphosphatase

Comparison of 6-phosphofructo-2-kinase activities of wild-type and Arg352Ala, Arg358Ala, and Arg360Ala mutant enzymes

6PF-2-kinase activity of wild-type and mutant enzymes was measured as described under "Experimental Procedures." The \( K_v \) values for Fru-6-P were obtained in the absence and presence of 5 mM KPi from 1/\( v \) versus 1/s plots at 5 mM ATP-Mg\(^{2+}\). Fru-6-P concentrations ranging from 20 to 100 \( \mu M \) were used in the presence of 5 mM Pi, and Fru-6-P concentrations from 0.2 to 5 mM were used in the absence of 5 mM Pi. \( V_{max} \) and \( K_v \) values for ATP were obtained in the presence of 5 mM KPi, 5 mM Fru-6-P, 5 mM Mg\(^{2+}\), and ATP concentrations ranging from 50 to 500 \( \mu M \) from the 1/\( v \) versus 1/s plots. \( K_v \) values were obtained by determining the concentration of Pi giving half-maximal activation in the presence of 1 mM Fru-6-P and 5 mM ATP-Mg\(^{2+}\), and the Pi concentration range used was from 5 to 200 \( \mu M \). All of the values represent average of three determinations.

| Enzyme forms | \( V_{max} \) (milliunits/mg) | \( K_v \) (Fru-6-P) (mM) | \( K_v \) (ATP) (mM) | \( K_v \) (Pi) (\( \mu M \)) |
|--------------|-------------------------------|------------------------|-------------------|----------------------|
| Wild-type    | 59 ± 18                        | 1.42 ± 0.24            | 0.022 ± 0.009     | 0.11 ± 0.06          | 36 ± 14               |
| Arg352Ala    | 36 ± 9                         | 4.0 ± 1.1              | 0.052 ± 0.021     | 0.20 ± 0.09          | 52 ± 10               |
| Arg358Ala    | 53 ± 3                         | 0.54 ± 0.20            | 0.015 ± 0.005     | 0.16 ± 0.03          | 28 ± 18               |
| Arg360Ala    | 77 ± 23                        | 3.6 ± 0.4              | 0.041 ± 0.010     | 0.22 ± 0.11          | 47 ± 12               |

The results of this report and those of Li et al. (21) and Lin et al. (20) have implicated 5 basic residues, 3 of which are in the surface loop overhanging the active site, in substrate binding in the Fru-2,6-P\(_2\)-ase domain. Fig. 6 is a schematic representation of the residues responsible for the substrate binding to the free enzyme (E-Fru-2,6-P\(_2\)) and the E-P intermediate (E-P-Fru-2,6-P\(_2\)).

The presence of basic residues that bind the C-6 phospho group (Arg352 and Lys356) and the C-2 phospho group (Arg360) of the substrate in the putative surface loop region suggests that this part of the active site may serve as a "specificity pocket." Comparison of the sequence alignments of the related phosphoglycerate mutase and acid phosphatase enzyme families with a number of mammalian Fru-2,6-P\(_2\)-ase domains reveals that all of these enzymes possess such a surface loop between \( \beta \)-strand C and \( \alpha \)-helix 4 (7). However, the loop is highly variable between these families, and only in Fru-2,6-P\(_2\)-ases is the clustering of basic residues conserved in the COOH-terminal end of the loop. Interestingly, rat liver Arg358, which is not involved in substrate/product binding in either domain, is not conserved in the bovine heart Fru-2,6-P\(_2\)-ase domain but is conserved in all enzyme forms (Table IV).

The high degree of substrate inhibition (greater than 90%) observed for the rat liver Fru-2,6-P\(_2\)-ase with physiological

The work of Li et al. (21) clearly implicated Lys-356 as an important substrate/product binding site residue and provided the major impetus to mutate the remaining closely spaced basic residues to ascertain their role in binding and/or catalysis. The results clearly show that in addition to Lys-356, Arg-358, Arg-352, Arg-257, and Arg-307 bind the C-6 phospho group of Fru-6-P. Arg-257 and Arg-307 (20) contribute to the spatial orientation of the side chains of these residues is critical for efficient contacts to either the C2 of C6 phospho group of the substrate.
Concentrations of Fru-2,6-P_2 (20 μM) suggests that in vivo the enzyme is always inhibited by excess substrate. The residues responsible for substrate inhibition (Arg-352, Lys-356, and Arg-360) are all conserved in all known sequences of mammalian Fru-2,6-P_2ases, which suggests that this inhibition represents an important general regulatory feature of the Fru-2,6-P_2ase family. However, substrate inhibition has only been studied with the rat liver enzyme. It will be of interest to determine whether it is equally significant in other tissue-specific isoforms of the enzyme and/or whether it is modulated by Pi and/or by the presence of different NH_2- and COOH-terminal sequences in these different isoforms. It is conceivable that different degrees of substrate inhibition of tissue-specific isoforms of the bifunctional enzyme may have evolved to meet the metabolic exigencies of the particular tissue. Work is in progress to evaluate substrate inhibition and its regulation in these different enzyme forms including the bifunctional enzymes from skeletal muscle, testis, and brain.

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