Isolation of a Human Liver Fructose-1,6-bisphosphatase cDNA and Expression of the Protein in Escherichia coli

ROLE OF ASP-118 AND ASP-121 IN CATALYSIS*

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The gluconeogenic enzyme fructose-1,6-bisphosphatase, Fru-1,6-Pase, catalyzes the conversion of fructose-1,6-bisphosphate to fructose-6-phosphate and D-glyceraldehyde-3-phosphate. The catalytic activity of this enzyme is governed primarily by changes in the concentration of the inhibitor Fru-2,6-P₂, which competes with the substrate at the active site and also potentiates inhibition by the allosteric effector AMP (1-3). The levels of Fru-2,6-P₂ are in turn regulated by, and inversely proportional to, the intracellular cAMP concentrations (3-5), such that high Fru-1,6-Pase activity, necessary for net hepatic gluconeogenesis, is manifest when cAMP levels are increased and Fru-2,6-P₂ is low (6). Fru-1,6-Pase gene expression is also regulated by cAMP and insulin in liver (7-9) and by vitamin D₃ in human leukocytes (10). Although it is unlikely that vitamin D₃ plays a significant role in the regulation of the gluconeogenic pathway, enhanced transcription rates of the gene in response to increased intracellular cAMP and to low plasma insulin provides an explanation for the induction of hepatic Fru-1,6-Pase mRNA in the diabetic state (11).

Fru-1,6-Pase from pig kidney (12), sheep liver (13), and rat liver (11) have been purified, and their complete amino acid sequences have been determined. Their primary sequence is highly homologous (approximately 90%), and there is complete identity of putative active and allosteric site residues as well as in the flanking sequences (14). This homology extends to enzyme forms from plants (15, 16), yeast (17), and Escherichia coli (18), which suggests that Fru-1,6-Pase has undergone minimal changes throughout evolution, even though regulation of the enzyme's activity differs in different cell types.

The human enzyme has, for obvious reasons, been studied the least (19, 20). Since increased rates of gluconeogenesis contribute significantly to hyperglycemia in type I and type II diabetes (21), a better understanding of the residues that contribute significantly to hyperglycemia is needed. For example, the conserved active site residue Lys-274 in rat liver Fru-1,6-Pase revealed that it is an important substrate/Fru-2,6-P₂ binding residue but is not involved in catalysis (22). Here, as a first step in elucidating the mechanism of catalysis of Fru-1,6-Pase, we describe the isolation of the cDNA for the human liver enzyme, the construction of a plasmid for its expression in E. coli, and purification and characterization of the recombinant enzyme. In addition, the catalytic role of 2 aspartate residues (Asp-118 and Asp-121), conserved in all known Fru-1,6-Pase sequences and predicted to participate in the binding of divalent metal cations (14), was studied by mutating each residue to alanine and by analyzing the kinetic properties of the mutants.

EXPERIMENTAL PROCEDURES

Materials—The pET3a expression vector containing the α1 promoter for T7 RNA polymerase and the bacterial host cell strains...
BL21(DE3) pLysS were kind gifts of Dr. William Studier (Brookhaven National Laboratories). Restriction enzymes were from New England Biolabs, and T4 DNA ligase and polyadenylate kinase were from Boehringer Mannheim as were yeast glucose-6-phosphate dehydrogenase and phosphoglucomerase isomerase. Iso-Propyl-β-D-thiogalactopyranoside was from New Jersey Vine and L-[35S]methionine (1061 Ci/mmol) was from Du Pont-New England Nuclear. Oligodeoxynucleotides were synthesized on an Applied Biosystems Model 380A synthesizer and purified on OPC™ cartridges. The human liver cDNA/Agt11 library was from Clontech, and the plasmid vector Bluescript was from Stratagene. Hot Tub DNA polymerase and 10X buffer were from Amersham, and the thermal cycler was a Model 550 TempCycler from Coy Laboratory Products Inc.

Isolation of a Human Liver cDNA and Construction of the Expression Plasmid—Four independent cDNA clones of human liver Fru-1,6-Pase were isolated by screening an Agt11 library with a random-prime 32P-labeled cDNA of the rat liver enzyme (11). The cDNA was ligated into the EcoRI site of a Bluescript plasmid vector to give pHLPFB-BS. Since there were no convenient restriction sites with which to subclone the cDNA into the pET3a expression vector, an oligonucleotide-directed polymerase chain reaction (PCR) was used to introduce an NdeI recognition site at the protein initiation codon: 5'GATCCCTTGCTGGATCTTCC-3'. The PCR product was then restriction-digested with SacI1 and ClaI, and used to replace the equivalent wild-type sequences in pHLFBP-ET1. Mutated expression sequences. The primer, 5'-GATCCCTTGCTGGATCTTCC-3', encoding the NdeI site, the initiation Met and flanking 25 base pairs, was used in conjunction with an opposing primer 5'-GGCGTATCAATCCCCTTGAT-3' and 5'-GATCCCTTGCTGGATCTTCC-3' that encode Asp-118 Ala and Asp-121 Ala of the human liver Fru-1,6-Pase. The latter "megaprimer" was then used with the M13 reverse primer in the PCR. This primer introduces an EcoRI recognition site immediately following the translation termination codon, and the resulting PCR product was also ligated into the pET3a vector to give pHLPFB-ET2. Both constructs were verified by double-stranded DNA sequencing of both strands by dideoxy-chain termination as previously described (8).

Preparation of Asp-118 → Ala and Asp-121 → Ala Mutants of Human Liver Fru-1,6-Pase—Asp-118 and Asp-121 residues of human liver Fru-1,6-Pase were each mutated to alanine by PCR as previously described for mutation of Lys-274 of the rat liver enzyme (22). Two synthetic oligonucleotide primers (5'-CTCGTTTTGCT-GCCCTTGTG-3' and 5'-GATCCCTTGCTGGATCTTCC-3') that encode Asp-118 → Ala and Asp-121 → Ala mutations, respectively, were synthesized. Each primer was combined with primer 5'-GCAGGCCC-3', derived from pET3a sequences (23), to generate the first PCR product. The latter "megaprimer" was then used with the T7 primer of pET3a to generate the second PCR product (24), which was restriction-digested with SacI and Clal, and used to replace the equivalent wild-type sequences in pHLPFB-ET1. Mutated expression plasmids were verified by sequencing.

Expression of Fru-1,6-Pase—Competent E. coli strains BL21(DE5) pLysS were transformed with pHLFPFB-ET1 and -2 and grown in LB medium at 37 °C with shaking at 250 rpm. After 18 h of growth, the cells were harvested by centrifugation at 30,000 x g for 30 min and the supernatant was adjusted to 10 mM EDTA and heated at 58 °C for 3 min. The denatured protein was removed by another centrifugation, and Fru-1,6-Pase was precipitated between 45 and 75% saturation of (NH4)2SO4. The precipitate was collected by centrifugation and dissolved in a minimum volume of buffer (5 mM sodium malonate, pH 7.2, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 0.1 mM EDTA) and leupeptin (2.5 μg/ml). The enzyme, 10 μg/ml, was centrifuged for 10 min at 30,000 x g, and the desalted sample was applied to a cyanogenmethyldextrin-C60 column equilibrated with the same buffer. The column was washed until the A280 of the eluate was ≤0.05, and the enzyme was eluted with 2 mM Fru-1,6-P2, 1 mM AMP. The enzyme was then precipitated at 75% saturation of (NH4)2SO4, and the precipitate was washed, dissolved in buffer (20 mM KPi, pH 7.5, 50% glycerol, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 0.1 mM EDTA, and leupeptin (2.5 μg/ml), dialyzed extensively against the same buffer, and stored at −70 °C.

Assay of Fru-1,6-Pase Activity—Fru-1,6-Pase activity was measured spectrophotometrically in a reaction that coupled the production of Fru-6-P to the reduction of NADP+, catalyzed by phosphoglucomerase isomerase and glucose-6-phosphate dehydrogenase (25). Cells from induction time courses (1 ml) were harvested by centrifugation, suspended in 100 μl of lysing buffer, and lysed as described above, and after DNase treatment, the clear supernatant was assayed for enzyme activity.

Other Methods—Total protein was determined by the method of Lowry et al. (26). Total Fru-1,6-Pase induction was monitored by suspending the cell pellets (100 μl) from induction time courses in SDS sample loading buffer and subjecting them to electrophoresis in SDS-polyacrylamide gels according to Laemmli (27). Protein synthetic rates were monitored by preincubating 100 μl of the cells with 1.5 μCi of [35S]Met at 37 °C for 3 min. The cells were then harvested and dissolved in SDS-PAGE sample buffer.

RESULTS AND DISCUSSION

Isolation of Human Liver Fru-1,6-Pase cDNA—Four independent clones were isolated from a human liver cDNA library by screening with the cDNA to the rat liver Fru-1,6-Pase. Three of the clones were too short to contain the entire protein coding sequence. The fourth and longest clone (1421 base pairs) contained a 385-amino acid open reading frame and 260 base pairs of the 3′-untranslated region including a polyadenylation signal sequence and polyadenylation tail (Fig. 1). The deduced amino acid sequence, starting after the Met, contains 336 residues and is highly homologous (about 90%) with those of the pig kidney (12), rat liver (11), and that of chicken liver Fru-1,6-Pase, deduced from a recently isolated cDNA (Fig. 1). The conserved residues include those predicted to interact with the 6-phosphate group of Fru-1,6-P2 (Tyr-215, Arg-243, Tyr-244, Tyr-264, and Asn-212), the 2-phosphate group of Fru-2,6-P2 (Gly-122, Ser-124, Lys-274, Arg-275, Phe-312, and Arg-314). The furanose ring (Lys-274, Met-248), the predicted to bind metal ions (Glu-97, Glu-98, and Asp-118), and those predicted to interact with the allostERIC inhibitor, AMP (Leu-30, Ala-24, Met-177, Thr-31, Tyr-113, and Arg-140) (14). These findings attest to the identity of the isolated human cDNA, as well as provide additional support for a very high conservation in the primary sequence of all known eukaryotic Fru-1,6-Pases. Based on the sequence, the estimated subunit molecular weight (36,697) of human liver Fru-1,6-Pase is very similar to those of the pig kidney and sheep liver enzymes (Fig. 1). Although the rat liver Fru-1,6-Pase sequence ends in a unique carboxy-terminal 25- amino acid extension, which yields a subunit of higher molecular weight (~40,000) (8), the remainder of the sequence is homologous to other eukaryotic sequences.

The nucleotide sequence of the human liver cDNA, with the exception of a single conservative mutation (A1a-216 for Gly-216), two other mutations in the noncoding regions (an additional T at position 1188 and a G for A substitution at
Fig. 1. cDNA and deduced amino acid sequence of human liver Fru-1,6-P$_2$ase and comparison with amino acid sequences of pig kidney, rat liver, and chicken liver Fru-1,6-P$_2$ase. Pig kidney (PK) (12), rat liver (RL) (11), and chicken liver (CL).$^2$ Lowercase
Expression of Human Liver Fru-1,6-P$_2$ase in E. coli—The human liver Fru-1,6-P$_2$ase cDNA was subcloned into a T7 RNA polymerase-transcribed expression vector, pET3a (23, 28), by mutation of the translation initiation region to create a unique NdeI recognition site, as described under “Experimental Procedures.” Fig. 2 shows a Coomassie Blue-stained polyacrylamide gel of the time course of expression of total (Fig. 2, lanes 2–8) and soluble (Fig. 2, lanes 9–15) protein in E. coli BL21(DE3) at 37°C in LB medium. There was no expression of the human form in the absence of IPTG. After the addition of IPTG, a band corresponding to the predicted size of the human Fru-1,6-P$_2$ase subunit accumulated, reaching a maximum level after 4–6 h (Fig. 2, lanes 6–8). Analysis of protein synthesis by [35S]methionine incorporation showed that 70–80% of newly synthesized protein during the first 2 h was Fru-1,6-P$_2$ase (data not shown). Approximately half of this protein was in the soluble fraction (Fig. 2, lanes 13–15) and was coincident with the appearance of Fru-1,6-P$_2$ase activity (Fig. 3). The induction of Fru-1,6-P$_2$ase activity also reached a maximal value after 6 h, remaining unchanged for up to 22 h (Fig. 3). Based on the amount of enzyme in the soluble fraction (Fig. 2) and the specific activity of the purified rat liver enzyme (20 units/mg), the yield of the expressed human enzyme was calculated to be approximately 20 mg/liter of cells.

A second construct, lacking the 3′-untranslated region and polyadenylation tail, showed essentially identical induction profiles, indicating that this region of the gene had no effect on Fru-1,6-P$_2$ase expression in E. coli (data not shown). On the other hand, expression of human Fru-1,6-P$_2$ase in a BL21(DE3) strain carrying the pLysS plasmid, whose inclusion has been reported to result in slower rates of T7 RNA polymerase activity (28), yielded consistently lower total, as well as enzymatically active, Fru-1,6-P$_2$ase protein (data not shown). These findings are similar to those reported for the expression of the rat liver enzyme in E. coli (25). Substituting an even richer medium, 2 × YT containing 0.4% glyceral, for the LB medium doubled the yield of Fru-1,6-P$_2$ase and was, therefore, used for the large-scale purification of the enzyme.

Purification of Recombinant Human Liver Fru-1,6-P$_2$ase—The protocol used to purify human liver Fru-1,6-P$_2$ase from E. coli extracts was a modification of that used to purify the rat liver enzyme (25), which involved heat treatment, ammonium sulfate fractionation, and a substrate/effector elution of the homogeneous enzyme from a cation exchange resin (see “Experimental Procedures”). Human liver Fru-1,6-P$_2$ase was stable to heat treatment, but precipitated at a higher saturation of (NH$_4$)$_2$SO$_4$ (50 to 70%) than that which precipitated the rat liver enzyme (25). It was also necessary to raise the pH of the cation exchange chromatographic step from 5.8 to 7.2, in order to bind and subsequently to elute the human liver Fru-1,6-P$_2$ase. This may be a consequence of a higher calculated pI value (6.96) for the human form, in comparison to the rat form (pI 5.96), due to the presence of 2 additional basic residues and 5 fewer acidic residues in the human liver enzyme (Fig. 1).

Fig. 4 shows a Coomassie Blue-stained SDS-polyacrylamide gel of human liver Fru-1,6-P$_2$ase of fractions at various stages of purification. The final preparation appeared homogeneous (Fig. 4, lane 5), migrating as a 37,000-dalton peptide, slightly faster than the rat liver enzyme subunit (M$_r$ = 40,000, Fig. 4, lane 6). Table I contains a summary of enzyme recovery during purification from 4 liters of cells in 2 × YT medium. Approximately 800 units of Fru-1,6-P$_2$ase activity was present in the bacterial extract, 40% of which was recovered in the final elution from the CM-Sephadex column. The overall yield of recombinant Fru-1,6-P$_2$ase was 25% of that of the rat liver enzyme.
Recombinant Human Fructose-1,6-bisphosphatase Catalysis

Fig. 3. Time course of induction of Fru-1,6-P2ase activity by IPTG. Aliquots taken during the time course shown in Fig. 2 were assayed for Fru-1,6-P2ase activity as described under "Experimental Procedures" with 5 μM substrate.

Fig. 4. SDS-polyacrylamide gel electrophoresis of human liver Fru-1,6-P2ase during purification. Aliquots of fractions taken during purification of the enzyme from 4 liters of bacterial extract. Lane 1, marker standards with sizes shown on left in kilodaltons; lane 2, bacterial extract; lane 3, heat treatment supernatant; lane 4, Sephadex G-50 pool; lane 5, CM-Sephadex pool; lane 6, 1 μg of purified rat liver Fru-1,6-P2ase.

was 32 mg of purified enzyme/4 liters of cells (Table I).

Characterization of Expressed Human Liver Fru-1,6-P2ase—In order to confirm that the purified protein was Fru-

1,6-P2ase, it was subjected to 10 cycles of automated sequencing by Edman degradation, and the following amino acid sequence was obtained: Ala-Asp-Gln-Ala-Pro-Phe-Asp-Thr-

Asp-Val-. This sequence is consistent with that deduced from the cDNA and predicted from the expression construct.

The kinetic properties of the expressed human liver enzyme are summarized in Table II and compared to those of rat liver Fru-1,6-P2ase. The specific activity of the homogeneous enzyme was 9.8 units/mg of protein or approximately half that of the enzyme purified from rat liver (18 units/mg)(25), although both had identical \( K_a \) values for Fru-1,6-P2 (2 μM). The human liver enzyme appeared to be more sensitive to inhibition by Fru-2,6-P2 and AMP, having \( K_i \) values of 0.3 μM and 12 μM, respectively. Under the same assay conditions,

### Table I

| Fraction          | Volume (ml) | Units (μM) | Protein (mg) | Yield (%) |
|-------------------|-------------|------------|--------------|-----------|
| Lysate supernatant| 200         | 810        | 950          | 0.8       | 100       |
| Heat supernatant  | 180         | 820        | 320          | 2.7       | 101       |
| Sephadex G-50     | 33          | 670        | 170          | 3.9       | 83        |
| CM-Sephadex       | 22          | 314        | 32           | 9.8       | 39        |

### Table II

| Enzyme          | Units/mg | \( K_a \) (μM) | \( K_i \) (μM) | \( K_i \) (μM) |
|-----------------|----------|----------------|----------------|---------------|
| Human liver     | 9.8 ± 0.8| 2 ± 0.2        | 0.3 ± 0.02     | 12 ± 1.1      |
| Rat liver       | 17.8 ± 1.5| 2 ± 0.1        | 4.9 ± 0.3      | 40 ± 4.5      |
that are postulated to interact with Fru-2,6-P2 and AMP (13). AMP inhibition of the human enzyme is not clear. All residues in binding the 2-phospho group of Fru-2,6-P2 to rat liver Fru-1,6-P2ase (22), is conserved in the human sequence. One possible explanation for the differences in specific activity and sensitivity between human and rat liver enzymes is the presence of the 25-amino acid carboxyl-terminal extension in rat liver Fru-1,6-P2ase (Fig. 1), which may have a positive effect on catalysis, or decrease binding of Fru-2,6-P2, but not Fru-1,6-P2, to the active site. Based on this argument, the specific activity and Fru-2,6-P2 inhibition of the pig kidney and sheep liver enzymes should be similar to that of the human liver Fru-1,6-P2ase and to a form of the rat liver enzyme from which the carboxyl-terminal extension has been deleted. Because of differences in isolation procedures and assay conditions, a comparison of the published kinetic parameters of pig, sheep, and rat enzymes is not possible. Analysis of the effect of deleting the carboxyl-terminal extension of the rat liver enzyme, however, is currently under investigation.

Mutation of Human Liver Fru-1,6-P2ase Asp-118 → Ala and Asp-121 → Ala and Expression and Characterization of the Mutant Enzyme Forms—The initial crystallographic analysis of pig kidney Fru-1,6-P2ase suggested that a group of residues including Glu-97, Asp-118, and Asp-121 form a negatively charged pocket in the active site of the enzyme, at which Mg\(^{2+}\) or Mn\(^{2+}\) is predicted to bind (14). Zhang et al. (29) recently reported refined structures of pig kidney Fru-1,6-P2ase complexed with Fru-1,6-P2 and of various ternary complexes of Fru-1,6-P2ase with divalent metal ions and nonhydrolyzable substrate analogs. Two metal ion binding sites were located at the enzyme active site complexed with substrate analog. Metal site 1 is coordinated by the carboxylate groups of Glu-97, Asp-118, Glu-280, and the 1-phosphate group of the substrate analog, while metal site 2 is coordinated by the carboxylate groups of Glu-97, Asp-118, the phosphate group of the substrate analog, and the carboxyl oxygen of Leu-120. Based on these findings, Zhang et al. (29) postulate a reaction mechanism in which a metal-activated water molecule attacks the 1-phosphate of the α-analog of the substrate, while a proton is transferred from the carboxyl group of Asp-121 to the substrate ester oxygen either directly or with the help of the C2 hydroxyl group of the substrate. This hypothesis predicts that mutation of either Asp-118 or Asp-121 would greatly decrease Fru-1,6-P2ase catalysis.

In order to determine whether these residues play a role in catalysis, they were mutated to alanine, the mutant enzyme forms expressed as described under "Experimental Procedures," and purified as described above for the wild-type enzyme. Fig. 5 shows a polyacrylamide gel of the two purified mutant enzymes, which were homogeneous and migrated as 37,000-dalton peptides. Both the Asp-118 → Ala and the Asp-121 → Ala mutants bound to CM-Sephadex and were eluted by substrate in exactly the same manner as the wild-type enzyme, suggesting that it is unlikely that either mutation altered the gross secondary structure of the enzyme. In addition, the circular dichroism spectra of the mutant enzymes were indistinguishable from the wild-type enzyme (data not shown). As shown in Table III, the Asp-118 → Ala mutant had a specific activity of 2 milliunits/mg, while that of the Asp-121 → Ala mutant was 0.1 milliunit/mg, or about 1/5000 and 1/20000, respectively, of the activity of the wild-type human liver Fru-1,6-P2ase (10 units/mg). No effect on the $K_m$ for Fru-1,6-P2 was observed, which suggests that these residues are not involved in substrate binding per se, but rather mediate catalysis. The specific activities of the mutants did not increase when they were assayed in the presence of 10- to 50-fold higher concentrations of Mg\(^{2+}\) than that routinely used in the assay (2 mM). These results suggest that Asp-118 may play a more important role in catalysis than in Mg\(^{2+}\) binding, and that other residues such as Glu-97 and Glu-280 are more important for the latter. The results are consistent, however, with the proposed essential role of Asp-121 in catalysis.

The mechanism postulated by Zhang et al. (29) implicates Asp-118 in metal ion binding and activation of water. In the crystal structure, only one Mg\(^{2+}\) ion is found near the metal site 1 of the active site in the complex of the α-analog of substrate and enzyme even with concentrations of 50 mM Mg\(^{2+}\) in the crystallization buffer. It has been postulated that metal ions act to position the 1-phosphate group of the substrate in the correct conformation so that the ester oxygen can receive hydrogen bonds from the C-2 hydroxyl group of the substrate, from the main chain NH group of Gly-122, and perhaps from the carboxyl group of Asp-121 (29). Mutation of Asp-118 to alanine probably results in looser metal binding and decreased activation of water. However, some water activation still occurs in the Asp-118 → Ala mutant, judging from a reduction in catalytic rate of only 3 orders of magnitude. Other residues (Glu-97, Glu-280) can coordinate metal ions to block the ester oxygen from receiving hydrogen bonds from the substrate.

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**Table III**

Kinetic properties of the wild-type and Asp-118 → Ala and Asp-121 → Ala mutants

| Enzyme          | Specific activity | $k_{cat}$ | $K_m$ Fru-1,6-P2 | $V_{max}$ |
|-----------------|------------------|----------|-----------------|-----------|
|                 | units/mg         | $M^{-1}$ | $\mu$M          | $\mu$M    |
| Wild-type       | 1.00 ± 0.1       | 3.0 × 10$^9$ | 2.1 ± 0.2       |           |
| Asp-118 → Ala   | 0.0020 ± 0.0001  | 6.3 × 10$^9$ | 2.5 ± 0.4       |           |
| Asp-121 → Ala   | 0.0005 ± 0.00009 | 1.5 × 10$^9$ | 2.9 ± 0.3       |           |
ion, and, therefore, Mg\textsuperscript{2+} binding and water activation would not be expected to be completely abolished by mutation of Asp-118. The greater decrease in catalytic rate of the Asp-121 → Ala mutant of more than 4 orders of magnitude is consistent with the loss of the proton donor function of the carboxyl group of Asp-121 and, therefore, supports its postulated role in catalysis. However, in the α-substrate analog complex with Mg\textsuperscript{2+}, the side chain carboxyl group of Asp-121 is also coordinated to Mg\textsuperscript{2+} (29), which suggests that part of the reduction in the catalytic rate of the Asp-121 → Ala mutant may be the result of decreased metal binding as well. Under the assay conditions employed in this study, i.e. pH 7.4 at 2 mM Mg\textsuperscript{2+} and even at 20 mM Mg\textsuperscript{2+}, only 1 Mg\textsuperscript{2+} ion is present in the active site, and it is unclear whether a second Mg\textsuperscript{2+} ion is required to activate the enzyme and/or whether Mg\textsuperscript{2+} ion may activate the enzyme by a mechanism different from other divalent cations, i.e. Mn\textsuperscript{2+} and Zn\textsuperscript{2+} (29). Additional characterization of the metal ion binding properties of these mutants and their crystallization are in progress. Crystals of the recombinant wild-type human liver Fru-1,6-P\textsubscript{2}ase have already been obtained, and the structure solved at 2.1 Å resolution. Examination of this structure revealed that the same active site constellation of the transferred phosphate group is present in the human liver enzyme, including the negatively charged pocket containing Glu-97, Glu-98, Asp-118, and Asp-121. Therefore, conclusions with regard to reaction mechanism based on the pig kidney x-ray structure can be confidently applied to the human liver enzyme. Also in progress are site-directed mutagenesis experiments of Glu-97 and Glu-98, and their crystallization are in progress. Crystals of the recombinant enzyme family (31, 32) and the Fru-2,6-P\textsubscript{2}ase/cofactor-independent phosphoglycerate mutase enzyme family (33). Catalysis in the former family utilizes two zinc ions, and even at 20 mM Mg\textsuperscript{2+}, only 1 M\textsuperscript{2+} ion is present in the active site, and thus will be invaluable in determining the molecular basis for several inherited Fru-1,6-P\textsubscript{2}ase deficiencies reported in human patients (34).

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