Crystal structure of the neutral form of fructose-1,6-bisphosphatase complexed with the product fructose 6-phosphate at 2.1-Å resolution

(x-ray crystallography/allosteric enzyme/structure refinement/binding site)

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ABSTRACT The crystal structure of fructose-1,6-bisphosphatase (EC 3.1.3.11) complexed with the product fructose 6-phosphate (F6P) has been refined at 2.1-Å resolution to an *R* factor of 0.177 with root-mean-square deviations of 0.014 Å and 2.9° from the ideal geometries of bond lengths and bond angles, respectively. The secondary structures but not the trace of the unligated enzyme have been slightly revised in the F6P complex at this higher resolution. Helix H4 in the unligated structure has been refined to a helix-like coil, and two very short 3_{10} helices have been found, one in H4 and one in H5. F6P at 10 mM concentration in the absence of divalent metals in our study shows major binding at the active site and minor binding at the AMP site. The major site has almost equal full occupancy in the C1 and C2 chains of the crystallographic asymmetric unit, while the minor site shows occupancy only in the C1 chain at about 50%. The electron density in both ($2F_o - F_c$) and ($F_o - F_c$) maps calculated by omitting F6P slightly favors the β anomer of D-F6P over the α anomer. Possible functions of the active-site residues are discussed, and candidates are suggested for site-directed mutagenesis.

Fructose-1,6-bisphosphatase (Fru-1,6-Pase; EC 3.1.3.11), a key regulatory enzyme in gluconeogenesis, catalyzes the hydrolysis of fructose 1,6-bisphosphate (Fru-1,6- P_2) to fructose 6-phosphate (F6P) and inorganic phosphate. A divalent metal ion (e.g., Mg^{2+} or Mn^{2+}) is a necessary cofactor for the enzymatic reaction. The catalytic and regulatory properties of the enzyme from various sources have been extensively studied (1–4). Two inhibitors, AMP and fructose 2,6-bisphosphate (Fru-2,6- P_2), control the activity of Fru-1,6-Pase. The inhibition by AMP is allosteric (5). However, controversial views from kinetic experiments suggest that Fru-2,6- P_2 binds to the active site (4), to an allosteric site (3), or to both (6). Eight complete amino acid sequences have been reported (7–13). The sequence homology among the mammalian enzymes is >85% in comparison to about 45% homology among all Fru-1,6-Pases.

Fru-1,6-Pase is a tetrameric molecule with four identical polypeptide chains that aggregates into a flat hexagonal disk with D_2 symmetry (Fig. 1). Three-dimensional structures have been described for the crystals of the unligated Fru-1,6-Pase and an active-site complex (Fru-2,6- P_2 or a hydrolyzed product) (14, 15), the F6P-AMP- Mg^{2+} complex (16), and the AMP complex (17). The two T-form structures show AMP binding to the allosteric site, which is about 28 Å from the active site of the enzyme, and suggest possible allosteric mechanisms of AMP inhibition (17, 18).

The F6P complex has essentially the same secondary and quaternary structures as the unligated enzyme. Conformations of some loops that have random coil configurations have been repositioned during the refinement of the F6P

structure and are described in detail here.[†] On the basis of the structure, a partial list of amino acids that are important for the activity of the enzyme is suggested for site-directed mutagenesis experiments.

METHODS

The neutral form of Fru-1,6-Pase was purified from pig kidney (15). Crystals of the F6P complex were grown by dialyzing enzyme solution (10–15 mg/ml) against 20 mM Tris base/2 mM maleic acid/0.1 mM EDTA/5 mM NaN_3 /1 mM 2-mercaptoethanol/7.5% (wt/vol) polyethylene glycol (molecular weight = 3350)/1 mM F6P at pH 7.4. A 4-day dialysis produced crystals with a typical size of $0.6 \times 1.0 \times 1.0$ mm in a diamond shape. To saturate the binding sites, the F6P cocrystals were further soaked in 10 mM F6P for at least 3 days. The space group is $P3_221$ with unit cell dimensions of $a = b = 131.1$ Å and $c = 69.7$ Å. Two subunits, designated C1 and C2, exist in the crystallographic asymmetric unit. The F6P complex is isomorphous to the unligated enzyme except for changes of about 1–2.5% in the cell parameters ($a = b = 132.3$ Å and $c = 68.0$ Å for the unligated enzyme).

The diffraction data were collected at 4°C with the MARK III system at the Resource for Crystallography at the University of California, San Diego (19, 20). A total of 200,580 diffraction maxima were measured, resulting in 38,643 symmetry-independent reflections to a resolution of 2.05 Å with an R_{merge} factor of 0.072. ($R_{\text{merge}} = \sum_{hkl} \sum_i |I_i - \langle I \rangle| / \sum_{hkl} \langle I \rangle$.) The data were about 97% complete to 2.1-Å resolution and about 87% of the reflections were stronger than twice the average background.

The coordinates of the active-site complex (14, 15) were used as the initial model of the F6P structure and were rebuilt using the program FRODO (21) for some loops that had high *B* values. By a five-stage procedure of the simulated annealing refinement (22), the structure was refined using the program XPLOR on either the Cray YMP at Pittsburgh Supercomputing Center or a DECstation 3100 in Gibbs laboratory at Harvard University.

STRUCTURE DESCRIPTION

Quality of the Structure. The final refinement yielded an *R* factor of 0.177 for those 35,069 reflections between 10- and 2.1-Å resolution that were at least three times stronger than the averaged background. Root-mean-square deviations are 0.014 Å and 2.89° from the ideal geometries of bond lengths

Abbreviations: Fru-1,6-Pase, fructose-1,6-bisphosphatase; Fru-1,6- P_2 , fructose 1,6-bisphosphate; Fru-2,6- P_2 , fructose 2,6-bisphosphate; F6P, fructose 6-phosphate.

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[†]The atomic coordinates and structure factors have been deposited in the Protein Data Bank, Chemistry Department, Brookhaven National Laboratory, Upton, NY 11973 (reference 5FBP).

FIG. 1. Schematic diagram of Fru-1,6-Pase viewed down a molecular twofold axis, q (labeled as the tiny black oval in the center). The other two molecular twofold axes are labeled p (vertical) and r (horizontal). The three twofold axes are perpendicular to one another, forming the D_2 symmetry of the molecule. The axis r is coaxial with the crystallographic twofold axis. Subunits C1 and C2 make up the crystallographic asymmetric unit in the space group $P3_221$. The major binding of F6P (O), the divalent metal site (★), and the AMP binding site (X) are marked. The active site of the enzyme is composed of the F6P and metal sites. F6P at 10 mM in the absence of AMP also binds to the AMP site, only in the C1 chain at about half occupancy.

and bond angles, respectively. A total of 343 water molecules have been included in the refinement. Three pairs of ϕ and ψ angles in a dimer fall into disallowed regions of the Ramachandran plot (23). However, these three residues are located at the surface of the molecule and have weak electron density.

The electron density for the loops around residues 146 and 217, which was poor in the structures of the unligated enzyme, has been dramatically improved in the F6P complex. However, the loop of residues 268–271 is still poorly defined in the F6P complex. In addition to the loop of 54–67 and the first five residues at the N terminus, which were omitted in the unligated structure, residues 55–71 were omitted in the F6P structure because of lack of electron density.

Kinetic experiments have identified the loop of residues 54–67 as a proteolytically sensitive region for all Fru-1,6-Pases (24–27). To test whether this loop is disordered or removed by proteolytic cleavage, a microsequencing technique was performed at the Chiron Corporation on a sample prepared by redissolving the F6P crystals. The cleavage between Asn-236 and Ser-237 was established at <10%. Therefore, we conclude that the missing loop of residues 54–71 is disordered in this structure. This lack of cleavage is consistent with the neutral form of the enzyme.

The overall B factor was 25.5 Å² for all atoms. Higher B values for the loops around residues 235 and 269 are consistent with their weaker density in the $(2F_o - F_c)$ map and probably indicate conformational flexibility of those loops.

Secondary and Quaternary Structure. A total of 317 out of 335 residues in the monomer have been built in the trans configuration, including all 15 prolines. The cis configuration for Pro-147 in our previous study is thus revised to trans. Also, in our earlier study some ω angles of proline peptide bonds deviated significantly from the ideal cis or trans configurations (15). Here, all ω angles of proline have values of $180 \pm 5^\circ$ except for 170° for Pro-271 in C1. These differences reflect improvement in the resolution limit from 2.8 Å in the earlier studies (14, 15) to 2.1 Å in the present study.

The F6P complex has D_2 symmetry (Fig. 1). Two domains in each monomer, the AMP domain of residues 1–200 and the FBP domain of residues 201–335, are linked by a covalent bond between Val-200 and Lys-201 and are tightly associated by numerous side-chain interactions.

The secondary structures of the unligated enzyme have been slightly revised in the F6P complex (Fig. 2 and Table 1). Although some of these changes might be subtle differences between the unligated form and the F6P complex, most represent the more accurate positioning of atoms in the F6P structure. The α -helix H4 (residues 214–219) in the unligated structure was adjusted to an α -helix-like coil in the F6P structure. Two new short 3_{10} α -helices were identified in the F6P structure: Ser-123 to Asp-127 (new H4) and Glu-149 to Leu-153 (new H5). Each monomer of the F6P structure consists of 9 α -helices (35%), 13 β -strands (21%), and 5 β -turns (6%).

Asymmetry of the Structure. The asymmetric binding of divalent metals found when Mg^{2+} , Mn^{2+} , and Zn^{2+} bind with higher occupancy in C1 than in C2 (H.K. and W.N.L., unpublished results) implies structural asymmetry possibly intrinsic or possibly due to crystal packing or to negative cooperativity at less than full ligand occupancy. Superposition of C1 over C2 shows an average displacement of 0.53 Å for all atoms and of 0.28 Å for C^α atoms. These differences between the two chains include the coordinate errors from the structural determination and intrinsic structural differences. However, the displacement of about 1.1 Å for C^α positions of residues 18–27 marginally suggests conformational differences of those residues in the two chains. The electron density in the $(2F_o - F_c)$ map is strong for residues 18–27 in the C1 chain, but poor for those in the C2 chain. Moreover, the temperature (B) factors for the backbone atoms of residues 18–27 in the C1 chain are about 20 Å², whereas some of those in the C2 chain are as much as 100 Å².

FIG. 2. Secondary structure of one subunit (C1) of Fru-1,6-Pase. The letters N and C represent the N and C termini, respectively. The α -helices are shown as cylinders, and the β -strands as arrows. The dotted line between H2 and H3 represents the missing loop of Ala-55 to Gly-71. Two circles with labels of FBP and AMP indicate the locations of the active site and the AMP site, respectively.

Table 1. Correspondence between the secondary structure and the sequence of Fru-1,6-Pase

Secondary element*	Residue range
H1	Thr-12 to Ala-24
H2	Gly-28 to Lys-50
H3	Lys-72 to Ser-88
B1	Cys-92 to Val-95
T1	Thr-96 to Asp-99
B2	Ile-103 to Glu-106
B3	Tyr-113 to Asp-118
H4	Ser-123 to Asp-127
B4	Ile-132 to Tyr-139
H5	Glu-149 to Leu-153
B5	Val-160 to Tyr-167
T2	Gly-168 to Ala-170
B6	Thr-171 to Ala-176
T3	Met-177 to Gly-180
B7	Val-181 to Asp-187
T4	Pro-188 to Gly-191
B8	Glu-192 to Asn-199
B9	Ser-208 to Ser-210
H6	Pro-221 to Phe-232
B10	Tyr-240 to Arg-243
H7	Ser-247 to Tyr-258
B11	Gly-260 to Tyr-264
H8	Arg-276 to Ala-291
B12	Leu-294 to Thr-296
T5	Thr-297 to Glu-300
B13	Ile-316 to Gly-319
H9	Ser-320 to Lys-333

*H, α -helix; B, β -strand; T, reverse turn.

These results suggest disorder or multiple conformations of residues 18–27 in the C2 chain, but comparative order in the C1 chain. Residues 18–24 in the α -helix H1 (Table 1) are located at the AMP binding site and may have an important role in the allosteric inhibition by AMP.

Another region of conformational difference between the two chains occurs around residues 142–145. Here, the average C α displacement of 0.73 Å is about 3 times the average C α displacement in the whole chain. This loop is located at the surface of the enzyme, where extra amino acids are inserted in the *Escherichia coli* enzyme (see the sequence alignment in ref. 15). Furthermore, this loop has insertions of 12 or 16 amino acids in the enzymes from wheat chloroplast and spinach chloroplast (13). Conformational differences in this

loop may reflect flexibility to accommodate the insertions. It is unclear whether this loop affects AMP binding, although it is close to the AMP binding site. These two chloroplast enzymes are insensitive to inhibition by AMP (13).

DISCUSSION

Catalytic Function of Active-Site Residues. At 10 mM concentration, F6P binds to two different sites of the enzyme: a major binding at the active site and a minor binding at the AMP site. The major site of F6P has almost equally strong electron density in the chains C1 and C2. However, the minor site of F6P shows recognizable electron density in C1 but not in C2. This asymmetry is comparable with that noted when AMP binds to the C1 chain much more strongly than to the C2 chain in an active quaternary structure in the space group P3₂21 (14).

In the major binding site of F6P, the 6-phosphate interacts with side-chain atoms of residues Asn-212, Tyr-215, Tyr-244, and Tyr-264 in the same chain and with side-chain atoms of Arg-243 from the neighboring chain (Fig. 3). The furanose ring contacts side-chain atoms of Lys-274 and Asp-121 and backbone atoms of Met-248. When the C1 chain is superimposed over the C2 chain, the F6P-binding residues show no significant positional shifts. A small difference between the F6P sites of C1 and C2 is that the O1 atom of F6P forms a hydrogen bond with Glu-280 in C1 but not in C2. Another difference is that a water molecule is close to the divalent metal binding site and interacts with Asp-118 and Asp-121 in C1, but no water is found near the metal site in C2.

Arg-243 is located at the C1/C2 interface and interacts with the 6-phosphate of F6P from the neighboring chain, presumably to compensate partly the negative charge of the 6-phosphate. We predict that mutation of Arg-243 to Ala-243 will destabilize the 6-phosphate binding and reduce the affinity of the substrate for the enzyme. This or a similar mutation can be predicted to reduce the activity of the enzyme and affect the cooperativity of the substrate or product binding by reducing interaction and communication between the monomers. Also, Tyr-244, Tyr-264, and Asn-212 interact with the 6-phosphate of F6P. If the 6-phosphate group of substrate has the same binding as the product F6P, mutation of each of these three residues can be expected to decrease binding of substrate and of the product F6P. Lys-274 interacts with the O5 and O6 oxygens of F6P and is a candidate for site-directed mutagenesis. It is known that chemical modification of Lys-274 by pyridoxal 5'-phosphate, a bulky group, is associated with loss of activity (7, 28, 29).

FIG. 3. Stereo plot of the major F6P binding at the active site of the enzyme. Residues Ala-241 and Arg-243 (top left corner) are from the neighboring chain C2, while all other residues are from the C1 chain. A336 in the center denotes β -D-F6P. Solvent molecules are marked as crosses, and hydrogen bonds between F6P and the protein are shown by dotted lines.

FIG. 4. Schematic drawing of the equilibrium of α -D-F6P (left, about 20%) and β -D-F6P (right, about 80%) in a solution.

Residues at the negatively charged pocket include Glu-280, Asp-121, Asp-118, Glu-97, and Glu-98. The first four of these residues form four- to five-coordination with Mg^{2+} and Mn^{2+} , and five- to six-coordination with Zn^{2+} (H.K. and W.N.L., unpublished results). Mutation of each of these negatively charged residues can be expected to decrease the binding of the divalent metals to the enzyme. The loop consisting of Asp-121, Gly-122, Ser-123, Ser-124, and Asn-125 is fully conserved in the published sequences. In particular, Ser-123 and Ser-124 may be near the 1-phosphate of Fru-1,6- P_2 , assuming that the α anomer is the true substrate (1, 30). All of these residues should be subjected to site-specific mutagenesis, and studies are needed of complexes with analogues of both α and β anomers of Fru-1,6- P_2 .

Binding of F6P at the Active Site. F6P exists in an equilibrium of about 20% α and 80% β anomers (Fig. 4), as well as less than 2% keto form in a solution at room temperature (31–35). The structure in the crystal of β -D-F6P (36) was used for the β anomer and modified for the α anomer. The electron density map, using $(2F_o - F_c)$ as its coefficients and phases from the structure with β -D-Fru-2,6- P_2 , showed density for the cyclic form of F6P. From this electron density we favor slightly the β anomer over the α anomer. Also, both $(2F_o - F_c)$ and $(F_o - F_c)$ maps calculated by omitting F6P from the refined structure fit the β anomer slightly better than the α anomer (Fig. 5).

In the absence of enzyme, β -F6P is the major component in solution as measured by NMR. Crystallization yields only the β anomer of either F6P (36) or Fru-1,6- P_2 (37, 38). Finally, kinetic experiments indicate that analogues of the β anomer bind more tightly to the enzyme than do those of the α anomer (39, 40). However, there are kinetic indications that a slow conversion occurs between a form (R') that preferentially binds Fru-2,6- P_2 and a form (R) that preferentially binds the substrate Fru-1,6- P_2 (41, 42).

Binding of F6P at the AMP Site. Besides binding to the active sites, F6P at 10 mM in our experiment also binds to the AMP site in C1, but not in C2 (Fig. 6). The occupancy of F6P at this minor site in C1 is estimated as about half of the occupancy at the active site. The 6-phosphate group of F6P at the minor site in C1 shows interactions with the protein that are similar to those of the phosphate group of AMP. The asymmetric binding of AMP to the R' form of the enzyme may be due to crystal packing in the space group $P3_221$ (14), may be intrinsic to the molecule (17), or may be induced by negative cooperativity at partial occupancy. Similarly, intermolecular interactions of Asn-142 with Tyr-140 and with Ala-24 are observed in the C1 chain of this F6P structure, but not in the C2 chain. In addition, superposition of C2 over C1 shows a shift of >1 Å in the position of the helix H1 (residues 12–24) (Fig. 6), a shift that may reflect a difference in affinity of the two chains for F6P.

The minor site is occupied by AMP in the presence of both 1 mM AMP and 1 mM F6P (16). However, in the absence of AMP binding, this minor site may serve as a regulatory site at high concentration of F6P. For example, negative cooperativity is associated with binding of F6P (1, 2). The activity of rabbit liver Fru-1,6-Pase is about 50% inhibited by ≈ 0.2 mM F6P, and this inhibition is independent of the concentration of substrate (43). Competitive inhibition of rabbit liver Fru-1,6-Pase has been reported (44), in which the K_i is about 70 μM at pH 8.5. Thus, F6P may show competitive inhibition and/or negative cooperativity.

Model Building. When the results for F6P are extended by model building to the α or β anomer of Fru-1,6- P_2 , we find that Asp-121 and Glu-280 are the closest of potential metal-binding residues to the 1-phosphate, and they are closer to the β anomer. These residues, and possibly the 1-phosphate, may enhance binding of a second metal ion, which occurs for Mn^{2+} in the rabbit liver enzyme (45). AMP decreases binding of a catalytic Mg^{2+} (46, 47), probably an additional metal site.

FIG. 5. Model of F6P in the electron density of the $(F_o - F_c)$ map that was calculated by omitting F6P from the structure. The one contour level shown is about 5 times the average background. The thick lines inside the density represent β -D-F6P and the dashed lines represent α -D-F6P. The density on the right side of the sugar (the position labeled as O1 A339) is higher than that on the left, an indication that β -D-F6P binds to the enzyme in preference to α -D-F6P. In order to fit O1 of α -D-F6P into the density, it was necessary to place C2 slightly off the density. Higher resolution would be desirable to confirm these results.

FIG. 6. Stereoview of the minor F6P binding at the AMP site in the C1 chain. The thin lines represent residues from the C1 chain. The thick lines are the residues from the C2 chain, which are generated from the superposition of the C2 chain over the C1 chain. Conformational differences of the α -helix H1 (bottom right) are obvious and probably determine the differing affinity for F6P in the two chains.

Structures of the enzyme complexed with substrate analogues can be expected to provide a basis for active-site and allosteric mechanisms in this enzyme.

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