Structures of Mammalian and Bacterial Fructose-1,6-bisphosphatase Reveal the Basis for Synergism in AMP/Fructose 2,6-Bisphosphate Inhibition*

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Fructose-1,6-bisphosphatase (FBPase) operates at a control point in mammalian gluconeogenesis, being inhibited synergistically by fructose-2,6-bisphosphate (Fru-2,6-P2) and AMP. AMP and Fru-2,6-P2 bind to allosteric and active sites, respectively, but the mechanism responsible for AMP/Fru-2,6-P2 synergy is unclear. Demonstrated here for the first time is a global conformational change in porcine FBPase induced by Fru-2,6-P2, in the absence of AMP. The Fru-2,6-P2 complex exhibits a subunit pair rotation of 13° from the R-state (compared with the 15° rotation of the T-state AMP complex) with active site loops in the disengaged conformation. A three-state thermodynamic model in which Fru-2,6-P2 drives a conformational change to a T-like intermediate state can account for AMP/Fru-2,6-P2 synergism in mammalian FBPases. AMP and Fru-2,6-P2 are not synergistic inhibitors of the Type I FBPase from Escherichia coli, and consistent with that model, the complex of E. coli FBPase with Fru-2,6-P2 remains in the R-state with dynamic loops in the engaged conformation. Evidently in porcine FBPase, the actions of AMP at the allosteric site and Fru-2,6-P2 at the active site displace engaged dynamic loops by distinct mechanisms, resulting in similar quaternary end-states. Conceivably, Type I FBPases from all eukaryotes may undergo similar global conformational changes in response to Fru-2,6-P2 ligation.

Fructose-1,6-bisphosphatase (α-fructose-1,6-bisphosphate 1-phosphohydrolase; EC 3.1.3.11; FBPase) is a regulatory enzyme in gluconeogenesis, cleaving the 1-phosphoryl group from fructose-1,6-bisphosphate (Fru-1,6-P2) to produce fructose 6-phosphate and P1 (1, 2). FBPase and fructose-6-phosphate-1-kinase (PFK) define a futile cycle that consumes ATP. In order to avoid unnecessary loss of ATP, enzymes associated with futile cycles are often subject to coordinate regulation. Mammalian FBPases have two primary physiological inhibitors, fructose-2,6-bisphosphate (Fru-2,6-P2), the levels of which are under hormonal control, and AMP, which remains at a relatively constant concentration under aerobic conditions (3–7). Fru-2,6-P2 binds to the active site and inhibits catalysis competitively with respect to Fru-1,6-P2 (6–9), whereas AMP binds to allosteric sites separated by no less than 28 Å from the nearest active site (10). Despite binding to well separated sites on the enzyme, Fru-2,6-P2 enhances AMP inhibition by 3–10-fold (6, 7). AMP and Fru-2,6-P2 also synergistically activate PFK in glycolysis (11, 12), and both activation of PFK and inhibition of FBPase occur at concentrations near the physiological range for Fru-2,6-P2 in the liver (6, 7, 13–15).

Although five nonhomologous FBPases may exist in living organisms, the most prevalent (Type I) is the sole form of FBPase in eukaryotes and the primary form in many bacteria (16–20). The mammalian and bacterial Type I enzymes are reportedly homotetramers (subunits labeled C1–C4 by convention) and exist in distinct quaternary states R and T. Physiological effectors influence the relative stability of the R- and T-states. AMP transforms the active mammalian R-state enzyme, observed in crystal structures in the absence of inhibitors (21–24), to an inactive T-state (10, 24–26). Transition to the T-state entails a rigid body rotation of 15° of the upper (C1–C2) subunit pair relative to the lower (C3–C4) subunit pair (10, 27). Mutant FBPases with destabilized T-states adopt intermediate quaternary states, I R (27) and I T,4 in the presence of AMP. In the porcine liver enzyme, the AMP-induced transition to the T-state displaces a dynamic loop (residues 52–72) from the active site, disrupting at least one of three binding sites for essential metal cofactors (23, 24, 27). The conformational change in the dynamic loop is consistent with competitive inhibition of catalysis by AMP with respect to Mg2+ (28).

Four molecules of Fru-2,6-P2 bind per tetramer (29, 30). Fru-2,6-P2 is a linear competitive inhibitor with respect to Fru-1,6-P2, lowers the concentration of AMP necessary for 50% inhibition of mammalian FBPases (AMP/Fru-2,6-P2 synergism), and

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The atomic coordinates and structure factors (code 2QVR, 2QVU, and 2QVV) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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3 The abbreviations used are: FBPase, fructose-1,6-bisphosphatase; Fru-1,6-P2, fructose-1,6-bisphosphate; Fru-2,6-P2, fructose-2,6-bisphosphate; PFK, fructose-6-phosphate-1-kinase; PEP, phosphoenolpyruvate; Glc-6-P, glucose-6-phosphate.

4 C. V. Iancu, S. Mukund, H. J. Fromm, and R. B. Honzatko, unpublished results.
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induces sigmoidicity in the binding of Fru-1,6-P₂ (7, 31), an
effect not caused by AMP (3). Binding studies are consistent
with kinetics; the presence of Fru-2,6-P₂ enhances AMP bind-
ing (30). Moreover, the binding of one equivalent of Fru-2,6-P₂
elicits change in UV difference spectra similar to that induced
by AMP (30). The conformational changes in FBPase that
underlie these observations are unclear. Specifically, how
FBPase responds to Fru-2,6-P₂ alone is not known. The Fru-
2,6-P₂:AMP complex is isomorphous to the AMP complex
(T-state) (26), and FBPase crystal structures with Fru-2,6-P₂,
in the absence of AMP, are in the R-state. These R-state struc-
tures, however, either lack density for the 2-phosphoryl group
(a likely consequence of degradation of Fru-2,6-P₂ to fructose
6-phosphate (8)) or are the result of soaking R-state crystals
with the inhibitor (9). The crystallization of FBPase with Fru-
2,6-P₂ from solution and clear evidence of an intact ligand
would go a long way to understanding the conformational
changes induced by Fru-2,6-P₂.

Reported here for the first time are co-crystallizations of porc-
ine FBPase with Fru-2,6-P₂. Ligation of the active site by Fru-
2,6-P₂, in the presence of Mg²⁺ or Zn²⁺ causes a quaternary
transition to the I₁-state, similar to that induced by AMP.
Hence, we propose that AMP/Fru-2,6-P₂ synergism arises from
the presence of AMP/Fru-2,6-P₂,2m M ZnCl₂ and 2 m of a precipitant solution (50 mM sodium citrate, pH 5.3, 25% (w/v) polyethylene
glycol 1500, and 20% (w/v) sucrose). Crystals of the Mg²⁺-Fru-
2,6-P₂ complex of porcine FBPase grew from droplets contain-
ing 2 m of a protein solution (10 mg/ml enzyme, 0.2 mM EDTA,
5 mM Fru-2,6-P₂,5 mM MgCl₂) and 2 m of a precipitant solution
(8.5% (w/v) polyethylene glycol 3350, 5% (v/v) t-butanol, 27%
(v/v) glycerol, and 100 mM Hepes-NaOH, pH 7.0). Crystals of the
Zn²⁺-Fru-2,6-P₂ complex of porcine FBPase grew from drop-
lets containing 2 m of a protein solution (10 mg/ml enzyme, 5
mM Fru-2,6-P₂, 2 mM ZnCl₂) and 2 m of a precipitant solution
(10% (w/v) polyethylene glycol 3350, 5% (v/v) t-butanol, 27%
(v/v) glycerol, and 100 mM Hepes-NaOH, pH 7.0). In all cases,
the droplets were equilibrated over 500 l of precipitant solu-
tion. Equal dimensional crystals (0.2–0.3 mm) grew within 3
days at 22 °C. Crystals were frozen directly in a cold stream of
nitrogen without additional cryoprotectants.

Data Collection—Crystals were screened, and data were col-
clected for the bacterial enzyme at Iowa State University on a
Rigaku R-AXIS IV++ rotating anode/image plate system using
CuKα radiation from an Osmic confocal optics system at a tem-
perature of 110 K. Data were collected from the mammalian
enzyme crystals at 100 K on Beamline 4.2.2 of the Advanced
Light Source, Lawrence Berkeley Laboratory. The program
dₜrek (40) was used to index, integrate, scale, and merge en-
terprises, which were then converted to structure factors using
the CCP4 (41) program TRUNCATE (42).

Structure Determination and Refinement—The subunit from
the canonical R-state of porcine FBPase (Protein Data Bank
accession identifier 1CNQ), less ligands and water molecules,
was transformed by CCP4 programs PDBSET (43) and
LSQKAB (44) into a C₁–C₂ dimer. That dimer and the pro-
gram AMORE (45) were used in a molecular replacement solu-
tion of the porcine Mg²⁺-Fru-2,6-P₂ complex. The C₁–C₂
dimer of the refined Mg²⁺-Fru-2,6-P₂ complex (less ligands and
water molecules) was used in turn as the initial model for the
Zn²⁺-Fru-2,6-P₂ complex. The subunit of R-state E. coli FBPase
(Protein Data Bank identifier 2OWZ), less ligands and water
molecules, enabled a molecular replacement solution of the
E. coli Mg²⁺-citrate-Fru-2,6-P₂ complex.

Structural models underwent energy minimization followed
by individual thermal parameter refinement using CNS (46).
Force constants and parameters of stereochemistry were from
Engh and Huber (47). Restraints for thermal parameter refine-
ment were as follows: 1.5 Å² for bonded main-chain atoms, 2.0
Å² for angle main-chain atoms and angle side-chain atoms, and
2.5 Å² for angle side-chain atoms. Noncrystallographic restrai-
nts were not used in the refinement. Manual adjustments in
the conformation of specific residues employed the program
XTALVIEW (48). Ligands (Mg²⁺, Zn²⁺, Fru-2,6-P₂, HPO₄²⁻,
**Fru-2,6-P₂ Inhibition of Fructose-1,6-bisphosphatase**

Dehydrogenase and monitor the formation of NADPH by either absorbance at 340 nm or fluorescence emission at 470 nm (27). Assays (total volume, 2 ml) were conducted at 22 °C in 50 mM Hapes, pH 7.5, with 100 μM EDTA and 150 μM NADP⁺. 150 mM KCl was present in porcine FBPase assays only. Saturating levels of Fru-1,6-P₂ (40 or 20 μM) and MgCl₂ (10 or 5 mM) for E. coli and porcine enzymes, respectively, were used to measure specific activity. E. coli FBPase (but not porcine FBPase) is sensitive to the method of assay, so two assays were employed (32). Assays were either initiated by the addition of 1.4 μg of enzyme (enzyme-initiated assays) or by incubating the enzyme in assay mixtures for 1–2 h at 22 °C without MgCl₂ and then initiating the reaction by the addition of metal (metal-initiated assays). Porcine FBPase assays were initiated by the addition of metal.

**RESULTS**

**Enzyme Purity**—Preparations of native and selenomethionine-substituted E. coli FBPase, used for kinetic and structural investigations, respectively, have specific activities of 35–40 units/mg and appear as single bands on SDS-PAGE. Analysis of the N-terminal residue reveals a single type (methionine or selenomethionine), indicating no N-terminal proteolysis. Purified porcine FBPase used here has a specific activity of 30 units/mg, migrates as a single band on SDS-PAGE, and has a pH 7.5/9.6 activity ratio of 3.3, characteristic of a nonproteolyzed mammalian FBPase.

**Kinetics of AMP and Fru-2,6-P₂ Inhibition**—Enzymes isolated here have kinetic properties as previously reported for porcine (50) and E. coli (32) FBPases, including the presence or absence of AMP/Fru-2,6-P₂ synergy reported in previous investigations (6, 7, 37). Enzyme- or metal-initiated assays of E. coli FBPase result in different $I_{0.5}$ values and Hill coefficients for AMP (32). Regardless, Fru-2,6-P₂ has no effect on AMP inhibition of E. coli FBPase using either assay method (Fig. 1). Lines in $A–C$ of Fig. 1 are from fits of the Hill equation,

\[
V = \frac{V_{\text{max}}}{(1/\left[I_{0.5}\right]^n + 1)} \tag{1}
\]

where $V$ and $V_{\text{max}}$ represent the observed and maximum velocities, $I$ is the concentration of AMP, $I_{0.5}$ is the concentration of AMP that gives 50% inhibition, and $n$ is the Hill coefficient. Values of $n$ are 1.0 ± 0.2 and 1.7 ± 0.2 for data of $A$ and $B$, respectively, as is consistent for enzyme- and metal-initiated assays (32). In Fig. 1C, the Hill coefficient for AMP inhibition of porcine FBPase declines from 1.9 ± 0.2 to 1.3 ± 0.1 as the

and citrate) and water molecules were fit to omit electron density until no improvement in $R_{\text{free}}$ was observed. Water molecules with thermal parameters above 55 Å² or more than 3.2 Å from the nearest hydrogen bonding partner were removed from the final model. Protein geometry was analyzed using the program PROCHECK (49).

**Superposition of Structures**—CCP4 programs PDBSET (43) and LSQKAB (44) and models of dimers and tetramers of FBPase were used in pairwise superpositions. Displacements between corresponding Cα positions in superimposed structures were measured using XTALVIEW (48). Superpositions employed porcine FBPase structures with Protein Data Bank identifiers 1CNQ, 1EYK, 1Q9D, and 1YYZ, as canonical R-, T-, $I_R$-, and $I_T$-states, respectively. Citrate-bound E. coli FBPase (Protein Data Bank identifier 2OWZ) represents the R-state of the E. coli enzyme. The angle of rotation between subunit pairs in various quaternary states of FBPases is sensitive to the subset of residues used in superpositions. Established residue subsets were the basis of comparison of porcine structures with each other (27) and with E. coli structures (20). Determination of the subunit pair rotation first superimposes subunits C3–C4 of a tetramer onto the porcine R-state. The angle through which subunit pair C1–C2 must then rotate in order to be superimposed onto the subunits C1–C2 of the R-state determines the subunit pair rotation.

**Kinetic Experiments**—Activity assays employ the coupling enzymes phosphoglucone isomerase and glucose-6-phosphate dehydrogenase and monitor the formation of NADPH by either absorbance at 340 nm or fluorescence emission at 470 nm (27). Assays (total volume, 2 ml) were conducted at 22 °C in 50 mM Hapes, pH 7.5, with 100 μM EDTA and 150 μM NADP⁺. 150 mM KCl was present in porcine FBPase assays only. Saturating levels of Fru-1,6-P₂ (40 or 20 μM) and MgCl₂ (10 or 5 mM) for E. coli and porcine enzymes, respectively, were used to measure specific activity. E. coli FBPase (but not porcine FBPase) is sensitive to the method of assay, so two assays were employed (32). Assays were either initiated by the addition of 1.4 μg of enzyme (enzyme-initiated assays) or by incubating the enzyme in assay mixtures for 1–2 h at 22 °C without MgCl₂ and then initiating the reaction by the addition of metal (metal-initiated assays). Porcine FBPase assays were initiated by the addition of metal.

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Table 1

Statistics of data collection and refinement

|                          | Porcine (Mg²⁺-Fru-2,6-P₂) | Porcine (Zn²⁺-Fru-2,6-P₂) | E. coli (Mg²⁺-citrate-Fru-2,6-P₂) |
|--------------------------|-----------------------------|----------------------------|----------------------------------|
| Resolution (Å)           | 48.15-1.50 (1.55-1.50)      | 47.33-2.03 (2.13-2.03)     | 47.44-2.18 (2.32-2.18)           |
| Total/Unique reflections | 719,450/118,335              | 290,285/51,027             | 114,732/16,881                   |
| Average redundancy       | 6.1 (2.5)                   | 5.7 (5.7)                  | 6.8 (5.6)                        |
| Completeness (%)         | 93.9 (65.4)                 | 99.9 (100)                 | 99.3 (95.9)                      |
| Rmerge                  | 0.056 (0.213)               | 0.080 (0.321)              | 0.042 (0.203)                    |
| I/σ(I)                  | 21.5 (4.0)                  | 12.0 (3.9)                 | 17.6 (5.6)                       |
| No. of atoms            | 5513                        | 5343                       | 2755                             |
| Solvent sites           | 437                         | 269                        | 128                              |
| Rfree                   | 19.6                        | 20.0                       | 19.6                             |
| Rwork                   | 21.4                        | 23.4                       | 24.2                             |

Mean thermal parameters (Å²):

|                          | Protein          | Fru-2,6-P₂ | Mg²⁺ or Zn²⁺ | HPO₄²⁻ | Citrate |
|--------------------------|------------------|------------|---------------|--------|---------|
| Mean                     | 21               | 30         | 36            | 31     | 33      |

Root mean square deviations

|                          | Bond lengths (Å) | Bond angles | Dihedral angles | Improper angles | Improper angles |
|--------------------------|------------------|-------------|-----------------|-----------------|-----------------|
| Bond lengths (Å)         | 0.004            | 1.3         | 22.8            | 0.8              | 1.0              |
| Bond angles              | 0.005            | 1.3         | 22.8            | 0.8              | 1.0              |
| Dihedral angles          | 22.8             | 22.8        | 22.8            | 22.8             | 22.8             |
| Improper angles          | 0.8              | 0.8         | 1.0             | 1.0              | 1.0              |

In the above, I₀.₅ is the concentration of AMP causing 50% inhibition, I₀.₅max is the observed value for 50% inhibition by AMP in the absence of Fru-2,6-P₂, and α₀.₅ is the concentration of Fru-2,6-P₂ that produces 50% of the synergistic effect. The parameter α₀.₅ represents an apparent dissociation constant for Fru-2,6-P₂. Its value from the data of D is 0.36 ± 0.04 μM, within range of observed dissociation constants (0.1–2 μM) reported for Fru-2,6-P₂ from kinetics and binding experiments (6, 7, 29, 31, 51). Enhancement of AMP inhibition by Fru-2,6-P₂ is simply I₀.₅max/I₀.₅min ~ 4.5-fold based on the data of Fig. 1. I₀.₅ for Fru-2,6-P₂ inhibition also decreases as a function of AMP concentration (7) (data not shown).

Fru-2,6-P₂-bound Structures of Porcine FBPase

Concentration of Fru-2,6-P₂ increases. Moreover, Fru-2,6-P₂ clearly enhances the inhibition of porcine FBPase by AMP. I₀.₅ values decline as a function of increasing concentration of Fru-2,6-P₂ (Fig. 1D). The decrease in I₀.₅ of AMP follows Equation 2, which is of the same form as the Hill equation with n set to unity.

\[ I₀.₅ = I₀.₅min + (I₀.₅max - I₀.₅min)/(I(Fru-2,6-P₂)/α₀.₅ + 1) \]  

(Eq. 2)

In the above, I₀.₅ is the concentration of AMP causing 50% inhibition, I₀.₅min is the limiting I₀.₅ value for AMP inhibition in the presence of an infinite concentration of Fru-2,6-P₂, I₀.₅max is the observed value for 50% inhibition by AMP in the absence of Fru-2,6-P₂, and α₀.₅ is the concentration of Fru-2,6-P₂ that produces 50% of the synergistic effect. The parameter α₀.₅ represents an apparent dissociation constant for Fru-2,6-P₂. Its value from the data of D is 0.36 ± 0.04 μM, within range of observed dissociation constants (0.1–2 μM) reported for Fru-2,6-P₂ from kinetics and binding experiments (6, 7, 29, 31, 51). Enhancement of AMP inhibition by Fru-2,6-P₂ is simply I₀.₅max/I₀.₅min ~ 4.5-fold based on the data of Fig. 1. I₀.₅ for Fru-2,6-P₂ inhibition also decreases as a function of AMP concentration (7) (data not shown).

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(Eq. 2)

In the above, I₀.₅ is the concentration of AMP causing 50% inhibition, I₀.₅min is the limiting I₀.₅ value for AMP inhibition in the presence of an infinite concentration of Fru-2,6-P₂, I₀.₅max is the observed value for 50% inhibition by AMP in the absence of Fru-2,6-P₂, and α₀.₅ is the concentration of Fru-2,6-P₂ that produces 50% of the synergistic effect. The parameter α₀.₅ represents an apparent dissociation constant for Fru-2,6-P₂. Its value from the data of D is 0.36 ± 0.04 μM, within range of observed dissociation constants (0.1–2 μM) reported for Fru-2,6-P₂ from kinetics and binding experiments (6, 7, 29, 31, 51). Enhancement of AMP inhibition by Fru-2,6-P₂ is simply I₀.₅max/I₀.₅min ~ 4.5-fold based on the data of Fig. 1. I₀.₅ for Fru-2,6-P₂ inhibition also decreases as a function of AMP concentration (7) (data not shown).

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\[ I₀.₅ = I₀.₅min + (I₀.₅max - I₀.₅min)/(I(Fru-2,6-P₂)/α₀.₅ + 1) \]  

(Eq. 2)
FIGURE 2. Structures of Fru-2,6-P$_2$ complexes of porcine and E. coli FBPases. A, overview of the Fru-2,6-P$_2$–Mg$^{2+}$ complex of porcine FBPase (left) in its I$_r$-state. Dynamic loops (residues 52–72, heavy lines) are in the disengaged conformation, previously observed in AMP-bound structures. Shown is a stereoview of ligands and selected residues at the active site (right) covered in electron density ($2F_o - F_c$) contoured at 2σ with a cut-off radius of 2.0 Å. B, omit density ($F_o - F_c$) contoured at 4σ with a cut-off radius of 2.0 Å for Fru-2,6-P$_2$ and Zn$^{2+}$ from the Fru-2,6-P$_2$–Zn$^{2+}$ complex of the porcine enzyme. C, overview of the Fru-2,6-P$_2$–Mg$^{2+}$–citrate complex of E. coli FBPase in the R-state. Fru-2,6-P$_2$ and Mg$^{2+}$ are in each active site, and citrate is in each anion activation site. Ligands are covered in omit density ($F_o - F_c$) contoured at 4σ with a cut-off radius of 2.0 Å. The dynamic loop (residues 42–63, heavy lines) adopts an engaged conformation. Parts of this drawing were prepared with XTALVIEW (48) and MOLSCRIPT (78).
of the dynamic loop. The rotation angle between subunit pairs C1–C2 and C3–C4 of the Fru-2,6-P2 complexes differs by 13° from the canonical R-state and 2° from the T-state.

The quaternary state of the porcine tetramer in its Fru-2,6-P2 complexes is close to that of the I-like state (subunit pair rotation angle of 12°), recognized first in an enzyme complex with the pseudotetrapeptide OC252 (38). A survey of FBPase quaternary states and crystal forms (Table 3) reveals the R-state and the I1-state in two space groups (I222 and P21212). Hence, the quaternary state is not a likely consequence of crystal "packing forces." Subunit pair rotations of individual R-state structures vary by ~0.5°, and the Mg2+ and Zn2+ Fru-2,6-P2 complexes reported here differ by ~0.75°. Moreover, the OC252 complex, AMP-bound Ile10 → Asp FBPase, and Fru-2,6-P2-ligated struc-

### Table 3

Quaternary states of Type I FBPase

| Quaternary state | Subunit pair rotation | Ligands                     | Space group | Resolution | Protein Data Bank code | Reference |
|------------------|-----------------------|-----------------------------|-------------|------------|-------------------------|-----------|
| Porcine FBPase   |                       |                             |             |            |                         |           |
| R-state          | 0°                    | None                        | P3,21       | 2.80       | 2FBP                   | 21        |
| R-state          | 0°                    | Fru-6-P, P, P, Zn2+         | I222        | 2.27       | 1CNQ                   | 24        |
| R-state          | 0°                    | Fru-6-P, F, P, Mg2+         | I222        | 2.32       | 1EYI                   | 24        |
| I1-state         | 3°                    | AMP, Fru-6-P, P, P, Mg2+    | I222        | 1.85       | 1YYZ                   | 27        |
| I-like state     | 12°                   | AMP, Fru-6-P, P, Zn2+       | P2,2,2,     | 2.35       | 1Q9D                   | 39        |
| L-like state     | 13°                   | Fru-2,6-P, P, Zn2+          | P2,2,2,     | 1.83       | 2FSD                   | 28        |
| L-state          | 13°                   | Fru-2,6-P, P, Zn2+          | P2,2,2,     | 1.50       | 2OUV                   | 28        |
| T-state          | 15°                   | AMP, Fru-6-P, P, Zn2+       | P2,2,2,     | 2.28       | 1EYI                   | 24        |
| T-like state     | 15°                   | AMP, Fru-6-P, P, Zn2+       | P2,2,2,     | 2.22       | 1EYK                   | 24        |
| T-like state     | 9°                    | AMP, Glc-6-P, Fru-1,6-P, P, | P4,22       | 2.00       | 1FRP                   | 26        |

| E. coli FBPase   |                       |                             |             |            |                         |           |
| R-state          | 0°                    | Fru-6-P, PEP                | I222        | 2.18       | 20X3                   | 33        |
| R-state          | 0°                    | Fru-6-P, citrate            | I222        | 2.18       | 20WZ                   | 33        |
| R-state          | 0°                    | Fru-6-P, citrate, Mg2+      | I222        | 2.18       | 2QVR                   | 20        |
| T-like state     | 6°                    | SO4                      | I222        | 1.45       | 2QG1                   | 20        |
| T-like state     | 9°                    | AMP, Glc-6-P, Fru-1,6-P, P, | P4,22       | 2.05       | 2Q8M                   | 34        |

a A1a110 → Leu FBPase.

b Le10 → Asp FBPase.
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FIGURE 3. Stereoview of the active site in the vicinity of bound Mg²⁺ and Fru-2,6-P₂. Porcine FBPase (top) with heavy lines representing the Fru-2,6-P₂-bound enzyme and light lines representing the superimposed R-state enzyme (products and metals are omitted to improve clarity). The superposition aligns residues that bind the 6-phosphoryl group of the fructophosphoryl sugars in each structure. Residues 97–99 move toward the 6-phosphoryl group relative to those of Tyr279 (porcine enzyme) (boxed area). This drawing was prepared with XTALVIEW (48).

Conformational differences between R-state (product complex) and I₇-state (Fru-2,6-P₂ complex) subunits indicate factors contributing to global conformational change in the porcine tetramer. In the presence of Fru-2,6-P₂, movements in residues 97–99 and 123–126 block Asp⁷⁴ from its R-state, loop-engaged conformation (Fig. 3). Moreover, metal activators do not recognize coordination site 2 and have difficulty recognizing site 3. Asp⁷⁴ hydrogen bonds with the backbone amide of Lys⁷¹ in the loop-engaged conformation, and its mutation to alanine dramatically reduces Kₐcat (52). Metal coordination is significant to the stability of the engaged loop, since mutations that disrupt the integrity of the loop also cause significant increases in the Kₐ for metal activation (53). Hence, after a catalytic turnover, Fru-2,6-P₂ probably binds to an open active site containing a metal ion only at site 1. The position of the 2-phosphoryl group relative to that of the 1-phosphoryl group destabilizes metal coordination at sites 2 and 3, and conformational relaxation of residues 123–126 and 97–99 sterically precludes the engaged conformation of the loop. The Fru-2,6-P₂-bound R-state subunit has high energy, and a transition to the I₇-state occurs when the requisite number of molecules of Fru-2,6-P₂ bind to the R-state tetramer.

Fru-2,6-P₂-bound Structure of E. coli FBPase (Protein Data Bank Code 2QVR) — Crystals of E. coli FBPase with bound Fru-2,6-P₂, Mg²⁺, and citrate belong to the space group I222 (a = 44.0, b = 82.3, c = 174.2 Å) and are isomorphous with citrate or PEP complexes (32). One monomer is present in the asymmetric unit. The tetramer (reproduced from crystallographic symmetry) is in the canonical R-state, although Fru-2,6-P₂ is in the active site, covered by strong electron density (Fig. 2). Mg²⁺ is at metal site 1, and citrate is at the allosteric activation site. The model has all 332 residues of E. coli FBPase in good or strong electron density.

The dynamic loop (residues 42–63) in association with Fru-2,6-P₂, appears ordered for the first time in a crystal structure of a bacterial Type I FBPase (Fig. 4), adopting a conformation similar to that of the engaged loop of porcine FBPase (23). The C-terminal half of the dynamic loops for the E. coli and porcine enzymes exhibit similarities in sequence and interactions (Table 4). Glu⁶⁰ (corresponding to Asp⁶⁸ in porcine FBPase) interacts with the 2-phosphoryl group of the inhibitor through water molecules and interacts directly with Arg⁷¹. Additionally, Wat⁶², which interacts with Glu⁶⁰ and Glu⁸⁹, occupies the position corresponding to metal site 3 in the mammalian enzyme. Electron density levels and donor acceptor distances of ~2.8 Å are not consistent with the assignment of Mg²⁺ to this locus.

The engaged dynamic loop for the E. coli complex stands in contrast to the porcine Fru-2,6-P₂ complexes that have disengaged loops. Interactions of a disengaged loop for E. coli FBPase (if it were to exist) would differ significantly from those of the porcine enzyme (Table 4). Ala⁸⁵ of porcine FBPase, for instance, packs with Ile⁸⁸, Ile⁹⁸, and Ile¹⁹⁴, but all corresponding residues differ for the E. coli enzyme. Replacements of Thr⁴⁶ and Leu⁸⁶ in porcine FBPase with Arg⁴⁸ and Lys⁷², respectively, would introduce electrostatic charge in predomi-
nantly hydrophobic regions of the loop-disengaged conformer. Hence, a stabilized disengaged loop in the porcine system could be the driving force for an R- to T-state transition. The probable absence of a stabilized disengaged loop in the *E. coli* system may explain in part the lack of a global conformational change in that system in response to Fru-2,6-P$_2$.

**DISCUSSION**

Initial models for AMP/Fru-2,6-P$_2$ synergy have Fru-2,6-P$_2$ binding to the AMP site or an unknown allosteric site (9, 54–59). In conflict with these models is the binding of Fru-2,6-P$_2$ to the active site in crystal structures (9) (present study). Moreover, Fru-2,6-P$_2$ (but not AMP) protects the active site from acetylation (30). Kinetic and binding studies support Fru-2,6-P$_2$ binding only to the active site (6, 60–64). Lys$_{274}$ interacts directly with oxygen atoms of fructose 6-phosphate and Fru-2,6-P$_2$ (8, 9, 22), and the mutation of Lys$_{274}$ to alanine increases the $K_m$ for Fru-1,6-P$_2$ and $K_i$ for Fru-2,6-P$_2$ with no effect on AMP inhibition (54). Lys$_{274}$ Ala FBPase still exhibits AMP/Fru-2,6-P$_2$ synergy at concentrations of Fru-2,6-P$_2$ that correlate quantitatively with the mutation-linked increase in $K_i$. Fru-2,6-P$_2$ may indeed bind to the AMP site at sufficiently high concentrations (9), but whether such binding has function is doubtful. Mutant enzymes with reduced AMP affinity retain AMP/Fru-2,6-P$_2$ synergism and Fru-2,6-P$_2$ inhibition (65). A successful model for AMP/Fru-2,6-P$_2$ synergism then must connect the action of AMP at its allosteric site to that of Fru-2,6-P$_2$ at the active site. AMP and Fru-2,6-P$_2$ complexes of mammalian FBPases are deficient in essential metal cofactors (63), and hence AMP/Fru-2,6-P$_2$ synergy could arise from the expulsion of metal ions from the active site by independent actions of AMP and Fru-2,6-P$_2$. AMP expels metal ions by promoting an R- to T-state transition and a disengaged dynamic loop as confirmed by investigations in directed mutation, crystallography, and kinetics (23, 24, 50). Inorganic phosphate in product complexes coordinates metal activators at sites 2 and 3 (24), but Fru-2,6-P$_2$ (by virtue of a different location for its 2-phosphoryl group) destabilizes metal coordi-

**FIGURE 4.** Stereoview of the engaged dynamic loop of the Fru-2,6-P$_2$ complex of *E. coli* FBPase. Electron density comes from a 2$\sigma$ = $F_c$ map contoured at 2$\sigma$ with a cut-off radius of 2.0 Å.

**TABLE 4**

| Corresponding residue types and interactions involving the dynamic loops of porcine and *E. coli* FBPases |
|---------------------------------------------------------------|
| **Porcine FBPase**                                           | **E. coli FBPase** |
| Loop sequences                                               | Lys$_{54}$–Ser$_{183}$ |
| KAGIAHLYGIA$_{50}$                                           | NP$_a$ |
| GSTNVTGDOVKK$_{72}$                                          | Asn$_{56}$–Asp$_{90}$ |
| Donor-acceptor interactions with engaged loops               | Glu$_{58}$–Arg$_{271}$ |
| Lys$_{56}$–Asp$_{187}$                                        | Glu$_{58}$–Arg$_{271}$ |
| Tyr$_{57}$–Water–Ser$_{189}$                                 | Glu$_{58}$–Asp$_{68}$ |
| Asn$_{58}$–Glu$_{98}$                                         | Asp$_{58}$–Metal, Site 3-P$_a$ |
| Asn$_{58}$–Glu$_{97}$                                         | Asp$_{58}$–Arg$_{76}$ |
| Thr$_{66}$–Arg$_{276}$                                        | Thr$_{66}$–Arg$_{276}$ |
| Tyr$_{57}$–Water–Ser$_{189}$                                 | Lys$_{71}$–Asp$_{74}$ |
| Asp$_{68}$–Metal, Site 3-P$_a$                                | Asp$_{68}$–Arg$_{276}$ |
| Asp$_{68}$–Metal, Site 3-P$_a$                                | Lys$_{71}$–Ser$_{123}$ |
| Lys$_{72}$–Asp$_{74}$                                         | Lys$_{72}$–Asp$_{74}$ |
| Hydrophobic interactions of the disengaged loop$^d$           | Lys$_{72}$–(Leu$_{65}$, Ser$_{176}$, Thr$_{176}$) |
| Lys$_{50}$–(Ile$_{53}$, Ala$_{170}$, Met$_{176}$)            | Ala$_{51}$–(Arg$_{196}$, Ser$_{197}$) |
| Ala$_{54}$–(Thr$_{39}$, Ala$_{197}$)                         | Leu$_{57}$–(Ile$_{186}$, Ala$_{204}$, Ala$_{204}$, Leu$_{204}$, Val$_{204}$, Ile$_{204}$) |
| Leu$_{57}$–(Ile$_{186}$, Ala$_{204}$, Ala$_{204}$, Leu$_{204}$, Val$_{204}$, Ile$_{204}$) | Ala$_{51}$–(Leu$_{65}$, Ser$_{176}$) |
| Ala$_{51}$–(Leu$_{65}$, Ser$_{176}$)                         | Lys$_{54}$–(Ile$_{10}$, Val$_{196}$) |
| Ile$_{59}$–(Thr$_{39}$, Ala$_{197}$, Val$_{204}$, Ile$_{204}$) | Tyr$_{57}$–(Ile$_{186}$, Ile$_{204}$, Leu$_{204}$, Ile$_{204}$) |
| Leu$_{57}$–(Ile$_{186}$, Ala$_{204}$, Ala$_{204}$, Leu$_{204}$, Val$_{204}$, Ile$_{204}$) | Leu$_{57}$–(Ile$_{186}$, Ile$_{204}$, Val$_{204}$, Ala$_{204}$, Leu$_{204}$) |
| Ala$_{60}$–(Leu$_{76}$, Leu$_{80}$)                          | Ser$_{172}$–(Phe$_{187}$, Lys$_{176}$) |

$^a$ Residue from a symmetry-related subunit.  
$^b$ Corresponding interaction not possible due to sequence difference.  
$^c$ NO, corresponding interactions possible but not observed.  
$^d$ Disengaged loop observed only for porcine FBPase. Residues listed for the *E. coli* enzyme correspond to those of porcine FBPase.
nation and the engaged conformation of the dynamic loop, evidently promoting an R- to I1-state transition. UV difference spectroscopy (30) and fluorescence emission from a tryptophan reporter group (50) support similar conformational responses to AMP and Fru-2,6-P2 by mammalian FBPases. AMP/Fru-2,6-P2 synergism correlates with the $I_{0.5}$ values for Fru-2,6-P2 and AMP, and these in turn depend on the type of metal cofactor (31). Mn$^{2+}$ (followed by Zn$^{2+}$ and then Mg$^{2+}$) is most effective in stabilizing the engaged conformation of the dynamic loop against AMP- and Fru-2,6-P2-induced conformational change (50), and Mn$^{2+}$-activated FBPase also requires the highest concentrations of AMP and Fru-2,6-P2 in order to observe inhibition and synergism (31).

In binding experiments without metal activators and substrate, one bound molecule of Fru-2,6-P2, generates a UV difference spectrum indicative of conformational change (30). However, in kinetics, where metal and substrate are present, Fru-2,6-P2 inhibits without cooperativity. Hyperbolic inhibition of FBPase would result if the R- to I1-state transition occurs only in response to the binding of the fourth molecule of Fru-2,6-P2. If so, then a single engaged dynamic loop will maintain the R-state. Indeed, substrate saturation curves for FBPase, hyperbolic in the absence of Fru-2,6-P2, become sigmoidal in its presence (7, 31). The binding of the first molecule of substrate to an I1-state tetramer saturated with Fru-2,6-P2 would cause a transition to the R-state and enhance catalysis for the remaining subunits. Although speculative, this model is consistent with the observed data. In principle, the number of bound molecules of Fru-2,6-P2 that causes an R- to I1-state transition could be determined using hybrid tetramers of FBPase.

A thermodynamic model for AMP/Fru-2,6-P2 synergism in porcine FBPase assumes three quaternary states, R, T, and I1 (Fig. 5). In the presence of substrate and metal cofactors and in the absence of AMP or Fru-2,6-P2, the porcine enzyme is in the R-state, defined by a subunit pair rotation angle of 0°. Transition to the T-state (subunit pair rotation of 15°) by a sequential allosteric mechanism requires a minimum of two bound AMP molecules (66). By binding to an R-state subunit, AMP drives a shearing motion of helices H1 and H2, disrupting intra- and intersubunit hydrogen bonds, the end result of which is an R-state AMP complex of high energy (27). A significant drop in free energy favors the transition from R(AMP)$_2$ to T(AMP)$_2$. The equilibrium constant governing AMP inhibition is $\Delta G_0^\circ$ in Fig. 5.

Fru-2,6-P2 promotes a transition from the R- to the I1-state. Since AMP inhibition retains a Hill coefficient above unity in the presence of Fru-2,6-P2, the I1- to T-state transition may yet require two molecules of bound AMP. The equilibrium constant for the binding of AMP in the presence of Fru-2,6-P2, however, is now determined by $\Delta G_0^\circ$ in Fig. 5. AMP/Fru-2,6-P2 synergism appears if $\Delta G_0^\circ$ is $>\Delta G_1^\circ$.

If a three-state system as described exhibits synergism, then single mutations may have little impact on the phenomenon. For instance, mutations that destabilize interactions of AMP will raise the energy of all AMP-associated forms of the enzyme by roughly similar amounts. As a consequence, $\Delta G_1^\circ$ and $\Delta G_0^\circ$ will become smaller by equal amounts, but $\Delta G_0^\circ > \Delta G_1^\circ$ still holds. Similarly, mutations at the active site destabilize all the complexes with Fru-2,6-P2, but have no effect on $\Delta G_1^\circ$ and $\Delta G_0^\circ$. In fact, no mutation of a mammalian FBPase has eliminated AMP/Fru-2,6-P2 synergism without severely reducing or eliminating inhibition by either ligand.

In one sense, E. coli FBPase is an extensively altered porcine FBPase (>40% sequence identity with comparable folds of the polypeptide chain). Unlike porcine FBPase, the E. coli enzyme exhibits no AMP/Fru-2,6-P2 synergism (Fig. 1) despite being potently inhibited by both ligands individually (37). The absence of AMP/Fru-2,6-P2 synergism implies the absence of an intermediate state stabilized by Fru-2,6-P2. (Glc-6-P, which inhibits E. coli FBPase synergistically with AMP (33), however, might stabilize an intermediate state.) Indeed, AMP (along with Glc-6-P) causes a quaternary transition out of the R-state (33), but Fru-2,6-P2-bound E. coli FBPase remains in the R-state with dynamic loops approximating the engaged conformation of porcine FBPase. Moreover, allosteric activators (PEP and citrate) that stabilize the R-state of the E. coli enzyme, enhance Fru-2,6-P2 inhibition (32). Evidently, Fru-2,6-P2 lowers the energy of the R- and T-states of E. coli FBPase by equal amounts, and hence, $\Delta G_0^\circ = \Delta G_1^\circ$ (Fig. 6). Fru-2,6-P2 neither enhances nor antagonizes inhibition by AMP.

The unicellular eukaryote Saccharomyces cerevisiae produces Fru-2,6-P2 within 1 min of its exposure to exogenous glucose (67), whereas no investigation has reported Fru-2,6-P2
Fru-2,6-P$_2$ Inhibition of Fructose-1,6-bisphosphatase

In bacteria, FBPase from *S. cerevisiae* exhibits Fru-2,6-P$_2$/AMP synergy (68) but lacks both the PEP/citrate activator site and the glucose 6-phosphate inhibition site of the bacterial enzyme (20, 32, 33). Moreover, Fru-2,6-P$_2$ allosterically activates PFK of *S. cerevisiae*, whereas *E. coli* PFK lacks the allosteric site for Fru-2,6-P$_2$ (69). From an evolutionary perspective, eukaryotes and bacteria differ significantly in mechanisms of regulation of glycolysis and gluconeogenesis, yet Fru-2,6-P$_2$, regardless of whether it exists in these organisms, potently inhibits their FBPases. Others suggest that Fru-2,6-P$_2$ probably existed in evolution as an inhibitor of eukaryotic FBPase before it became an allosteric activator of PFK (36, 54). Observations reported here and elsewhere (32, 33) regarding the bacterial enzyme support this hypothesis and are consistent with the evolution of AMP/Fru-2,6-P$_2$ synergy after Fru-2,6-P$_2$ inhibition in eukaryotic FBPases.

Fru-2,6-P$_2$ inhibition of liver FBPase *in vivo* may depend on levels of stored glycogen. The glucose gavage of fasted animals (liver glycogen-depleted) results in elevated levels of serum glucose and the rapid biosynthesis of glycogen; however, about half of the glucose in such newly synthesized glycogen comes from gluconeogenesis (70). Hence, gluconeogenesis could run unabated when glycogen reserves are low, and indeed levels of total Fru-2,6-P$_2$ rise sufficiently to inhibit FBPase only after the restoration of liver glycogen reserves (15, 70–72).

To the extent that excessive hepatic gluconeogenesis contributes to diabetic hyperglycemia (70, 73), mammalian FBPase is a target for the development of anti-diabetic drugs (38, 74, 75). In principle, the reduction of gluconeogenic flux via the inhibition of FBPase could reduce serum glucose levels. Several novel inhibitors have been developed (38, 74), and detailed knowledge of the structural mechanism of AMP inhibition has resulted in the rational design of a potential drug that targets the AMP site (75). This FBPase-specific inhibitor alleviates diabetic hyperglycemia in rats, providing a proof-of-principle for the use of FBPase inhibitors in diabetes therapy (76, 77). On the basis of work presented here, inhibitors that destabilize the engaged conformation of the dynamic loop of FBPase or stabilize the disengaged conformation of that loop, will act synergistically with AMP. Indeed, the inhibitor OC252, which stabilizes the 1$^\circ$-state of porcine FBPase with a disengaged dynamic loop, exhibits such synergistic inhibition (38).

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