Central Cavity of Fructose-1,6-bisphosphatase and the Evolution of AMP/Fructose 2,6-bisphosphate Synergism in Eukaryotic Organisms

Yang Gao, Lu Shen, and Richard B. Honzatko

From the Roy J. Carver Department of Biochemistry, Biophysics and Molecular Biology, Iowa State University, Ames, Iowa 50011.

Background: AMP and fructose 2,6-bisphosphate (Fru-2,6-P$_2$) synergistically inhibit eukaryotic fructose-1,6-bisphosphatase (FBPase). How did this property evolve?

Results: Directed mutations, kinetics, and structure determinations link the central cavity of FBPase to AMP/Fru-2,6-P$_2$ synergism.

Conclusion: A central cavity in any FBPase implies AMP/Fru-2,6-P$_2$ and AMP/Fru-6-P synergism.

Significance: AMP/Fru-2,6-P$_2$ synergism of eukaryotic FBPases probably evolved from AMP/Fru-6-P synergism of an ancestral FBPase.

The effects of AMP and fructose 2,6-bisphosphate (Fru-2,6-P$_2$) on porcine fructose-1,6-bisphosphatase (pFBPase) and Escherichia coli FBPase (eFBPase) differ in three respects. AMP/Fru-2,6-P$_2$ synergism in pFBPase is absent in eFBPase. Fru-2,6-P$_2$ induces a 13° subunit pair rotation in pFBPase but no rotation in eFBPase. Hydrophilic side chains in eFBPase occupy what otherwise would be a central aqueous cavity observed in pFBPase. Explored here is the linkage of AMP/Fru-2,6-P$_2$ synergism to the central cavity and the evolution of synergism in FBPases. The single mutation Ser$_{45}$ → His substantially fills the central cavity of pFBPase, and the triple mutation Ser$_{45}$ → His, Thr$_{46}$ → Arg, and Leu$_{186}$ → Tyr replaces porcine with E. coli type side chains. Both single and triple mutations significantly reduce synergism while retaining other wild-type kinetic properties. Similar to the effect of Fru-2,6-P$_2$ on eFBPase, the triple mutant of pFBPase with bound Fru-2,6-P$_2$ exhibits only a 2° subunit pair rotation as opposed to the 13° rotation exhibited by the Fru-2,6-P$_2$ complex of wild-type pFBPase. The side chain at position 45 is small in all available eukaryotic FBPases but large and hydrophilic in bacterial FBPases, similar to eFBPase. Sequence information indicates the likelihood of synergism in the FBPase from Leptospira interrogans (iFBPase), and indeed recombinant iFBPase exhibits AMP/Fru-2,6-P$_2$ synergism. Unexpectedly, however, AMP also enhances Fru-6-P binding to iFBPase. Taken together, these observations suggest the evolution of AMP/Fru-2,6-P$_2$ synergism in eukaryotic FBPases from an ancestral FBPase having a central aqueous cavity and exhibiting synergistic feedback inhibition by AMP and Fru-6-P.

Fructose-1,6-bisphosphatase (α-fructose-1,6-bisphosphate 1-phosphohydrolase; EC 3.1.1; FBPase) catalyzes the penultimate step in gluconeogenesis, hydrolyzing fructose 1,6-bisphosphate (Fru-1,6-P$_2$) to fructose 6-phosphate (Fru-6-P) and phosphate (P$_i$) (1, 2). Fructose-6-phosphate-1-kinase (PFK-1) in glycolysis opposes the action of FBPase by the ATP-dependent phosphorylation of Fru-6-P (3). The coordinated regulation of FBPase and PFK-1 limits futile cycling in vivo. In mammals and presumably other eukaryotes, fructose 2,6-bisphosphate (Fru-2,6-P$_2$) and AMP inhibit FBPase while activating PFK-1 (3–10). In vivo AMP concentrations are nearly constant due to the action of adenylate kinase (11), whereas the level of Fru-2,6-P$_2$ varies in response to changing nutritional states (10). AMP/Fru-2,6-P$_2$ synergism in the inhibition of eukaryotic FBPases (5, 6, 12, 13), however, lowers the apparent $K_i$ for AMP as concentrations of Fru-2,6-P$_2$ increase, making both AMP and Fru-2,6-P$_2$ dynamic physiological regulators.

Porcine liver FBPase (pFBPase) is a model for eukaryotic FBPases (14). Four identical subunits (molecular mass of 37 kDa) form an approximate square-planar arrangement with D2 symmetry. AMP, which binds 28 Å away from the active site (15, 16), is a non-competitive inhibitor with respect to Fru-1,6-P$_2$ but a competitive inhibitor with respect to catalytically essential divalent cations (Mg$^{2+}$, Mn$^{2+}$, or Zn$^{2+}$) (17, 18). Binding of AMP induces a conformational transition from active R-state to inactive T-state (16, 19). The R- and T-states differ principally by a 15° rotation of the upper relative to the lower subunit pairs of the tetramer and by the displacement of loop 50–72 from an active site-engaged conformation to a disengaged conformation (16). At least two AMP molecules are required for the R- to T-state transition (20). The dimer-dimer interface is the origin of AMP cooperativity (20). Mutations that disrupt interactions between subunit pairs eliminate the cooperativity.

2 The abbreviations used are: FBPase, fructose-1,6-bisphosphatase; pFBPase, porcine liver FBPase; eFBPase, Escherichia coli FBPase; iFBPase, Leptospira interrogans FBPase; Fru-1,6-P$_2$, fructose 1,6-bisphosphate; Fru-2,6-P$_2$, fructose 2,6-bisphosphate; Fru-6-P, fructose 6-phosphate; PFK-1, fructose-6-phosphate-1-kinase; Glc-6-P, glucose 6-phosphate; PDB, Protein Data Bank.

This work was supported, in whole or in part, by National Institutes of Health Grant NS 10546.

The atomic coordinates and structure factors (codes 4GWS and 4GWU) have been deposited in the Protein Data Bank (http://wwpdb.org/).

This article contains supplemental Fig. S1.

1 To whom correspondence should be addressed: Iowa State University, 4206 Mol. Biol. Bldg., Ames, IA 50011. Fax: 515-294-0453; E-mail: honzatko@iastate.edu.

Received for publication, January 7, 2014 Published, JBC Papers in Press, January 16, 2014, DOI 10.1074/jbc.M114.548586
tive binding of AMP (21, 22). Two AMP-bound intermediate states (I<sub>K</sub>-state and I<sub>T</sub>-state) have been identified, both of which have engaged loop 50–72 and subunit pair rotations of 3 and 12°, respectively (23, 24). The I<sub>K</sub>- and I<sub>T</sub>-states exist in AMP complexes because mutations Ala<sup>54</sup> → Leu and Ile<sup>10</sup> → Asp, respectively, destabilize the disengaged conformation of loop 50–72.

Fru-2,6-P<sub>2</sub> is a competitive inhibitor with respect to Fru-1,6-P<sub>2</sub> (5, 6). Chemical modification, NMR, mutagenesis, and crystallographic studies consistently indicate Fru-2,6-P<sub>2</sub>-binding to the active site (14, 25–28). In the presence of Fru-2,6-P<sub>2</sub>, the <i>K<sub>i</sub></i> for AMP falls 10-fold (AMP/Fru-2,6-P<sub>2</sub> synergism) (5, 6, 29). Hines et al. (14) suggest that AMP/Fru-2,6-P<sub>2</sub> synergism arises from a similar end state induced independently by AMP and Fru-2,6-P<sub>2</sub>, and indeed, Fru-2,6-P<sub>2</sub> and AMP cause comparable subunit pair rotations in FBPase. Moreover, the crystallographic work is in harmony with similar UV spectra of FBPase in complexes with Fru-2,6-P<sub>2</sub> and AMP (14, 29).

<em>Escherichia coli</em> FBPase (eFBPase) has 41% sequence identity with pFBPase but a fundamentally different mechanism of regulation (30–32). eFBPase is subject to feed-forward activation by phosphoenolpyruvate, the binding of which favors an active tetramer over an inactive or less active dimer (30–31). AMP and glucose 6-phosphate (Glc-6-P) are synergistic inhibitors that stabilize subunit pair rotations in phosphoenolpyruvate, the binding of which favors an active conformation (30–32).

Expression and Purification of Wild-type and Mutant pFBPases—<em>E. coli</em> strain DF 657 was used for expression of pFBPases. The cell culture of <em>E. coli</em> DF657, transformed with plasmid, grew to <i>A<sub>600</sub></i> of 1.0, at which time transciption was induced by the addition of isopropyl-<i>β</i>-d-thiogalactopyranoside (final concentration 1 mM). The culture was maintained (with shaking, 37 °C) for an additional 16 h before harvesting. The supernatant solution of a cell-free extract was loaded onto a Cibracon Blue-Sepharose column previously equilibrated with 20 mM Tris-HCl, pH 7.5, and 5 mM MgCl<sub>2</sub>. The column was washed with 20 mM Tris-HCl, pH 7.5. Enzyme was eluted with 5 mM AMP and 20 mM Tris-HCl, pH 7.5. The pH of eluted protein solution was adjusted to 8.5 before loading onto a DEAE-Sepharose column equilibrated with 20 mM Tris-HCl, pH 8.3. Purified enzyme was eluted with a NaCl gradient (0–0.5 M) in 10 mM Tris-HCl, pH 8.3, and then dialyzed extensively against 50 mM Hepes, pH 7.4, for kinetic investigations and for crystallization experiments. Purity and protein concentrations of FBPase preparations were confirmed by SDS-PAGE (34) and by the Bradford assay (35), respectively.

Cloning, Expression, and Purification of IFBPs—The gene for IFBPs (UniProt ID Q8F421) was synthesized and cloned into a pET24b plasmid from Genscript. The integrity of the construct was confirmed by sequencing the promoter region and the entire open reading frame as above for mutant constructs of pFBPase. Expression of IFBPs followed the procedure outlined above for pFBPase. The supernatant component of the cell-free extract was subject to ammonium sulfate fractionation. The 30–50% ammonium sulfate fraction was collected and dialyzed in 20 mM Tris, pH 8.0, before loading onto a DEAE-Sepharose column. Purified enzyme was eluted with a NaCl gradient (0–0.5 M) in 20 mM Tris-HCl, pH 8.0. Enzyme was dialyzed extensively against 50 mM Hepes, pH 7.4, for kinetics studies. Purity and protein concentrations of IFBPs preparations were confirmed by SDS-PAGE (34) and by Bradford assay (35), respectively.

Kinetics—Phosphoglucone isomerase and glucose-6-phosphate dehydrogenase were used as coupling enzymes in assays for FBPase (1). For specific activity measurements, reduction of NADP<sup>+</sup> to NADPH was monitored by absorbance at 340 nm. Other assays used the same coupling enzymes but monitored the formation of NADPH by its fluorescence emission at 470 nm using an excitation wavelength of 340 nm. Assays were performed at 22 °C in 50 mM Hepes, pH 7.5. Assay solutions for wild-type and mutant pFBPases contained EDTA and KCl at concentrations of 10 μM and 150 mM, respectively, whereas KCl was absent in the assay solution for IFBPs. Data for AMP inhibition with respect to Mg<sup>2+</sup> and Fru-
AMP/Fru-2,6-P₂ Synergy in Fructose-1,6-bisphosphatase

2,6-P₂ inhibition with respect to Fru-1,6-P₂ were fit to several models using Grafit (36), with best fits of data to models reported here.

Tryptophan Fluorescence—Fluorescence measurements were made at room temperature using a 1-cm² quartz cuvette on an SLM Amico 8000 fluorometer. The excitation wavelength was 295 nm, and emission scans were integrated from 310 to 370 nm. Fluorescence scans (repeat of three scans for each data point) were performed after the additions of small volumes of ligand to 2 ml of a 2 μM FBPase solution. The total volume of added titrant did not exceed 1% of the initial volume. Data fitting was carried out using protocols in the literature (37).

Crystallization of Cav-pFBPase—The method of hanging drops combined 2 μl of protein solution with 2 μl of precipitant solution. Wells contained 500 μl of the precipitant solution. Crystals of the Fru-2,6-P₂ complex grew from a protein solution (10 mg/ml Cav-pFBPase, 25 mM Heps, pH 7.4, 5 mM MgCl₂, and 5 mM Fru-2,6-P₂) combined with a precipitant solution (100 mM Heps, pH 7.4, 8% (w/v) polyethylene glycol 3350, 27% (v/v) glycerol, and 5% (v/v) 2-buty alcohol). Crystals of the AMP complex grew from a protein solution (10 mg/ml Cav-pFBPase, 25 mM Heps, pH 7.4, 5 mM MgCl₂, and 5 mM AMP) combined with a precipitant solution (100 mM Heps, pH 7.4, 12% (w/v) polyethylene glycol 3350, 23% (v/v) glycerol, and 5% (v/v) t-butyl alcohol). Crystals were of equal dimensions (0.2–0.4 mm), growing in ~3 days at 20 °C, and were transferred directly from droplet to liquid nitrogen as cryoprotectants were included in the conditions of crystallization.

X-ray Data Collection, Structure Determination, and Refinement—Data were collected at Iowa State University from single crystals on a Rigaku R-AXIS IV++ rotating anode/image plate system using CuKα radiation from an Osmic confocal optics system and a temperature of 110 K. The program d*trek (38) was used in reducing data.

Cav-pFBPase with Fru-2,6-P₂ and AMP are isomorphous to R- and T-state FBPase complexes, respectively. R-state FBPase (PDB code 1CNQ) and T-state FBPase (PDB code 1EYJ) were used as initial models for molecular replacement. The resulting models underwent refinement using CNS (39) with force constants and parameters of stereochemistry from Engh and Huber (40). A cycle of refinement consisted of slow cooling from 1000 to 300 K in steps of 25 K followed by 100 cycles of conjugate gradient minimization and concluded by the refinement of individual thermal parameters. Restraints of 1.5 Å² on nearest neighbor and next-to-nearest neighbor main chain atoms, 2.0 Å² on nearest neighbor side chain atoms, and 2.5 Å² on next-to-nearest neighbor side chain atoms were employed in thermal parameter refinement. Water molecules were added to difference electron density of 2.5σ or better until no significant decrease was evident in the R_free value. Water molecules in the final models were within acceptable donor-acceptor distances to each other and/or a polar group of the protein. Stereochemistry of the models was analyzed by use of MolProbity (41).

RESULTS

Rationale for Directed Mutations—Directed mutations have identified residues essential for AMP binding (20, 42), regulation (43–46), cooperativity (21–22, 43), and Fru-2,6-P₂ binding (47); however, no mutation eliminates AMP/Fru-2,6-P₂ synergy while retaining potent AMP and Fru-2,6-P₂ inhibition. Because eFBPase shares 41% identity with pFBPase but lacks AMP/Fru-2,6-P₂ synergy, some subset of the remaining 59% of residues must be responsible.

An overview of FBPase showing the relationship of ligand binding sites and loop 50–72 to the central cavity appears in Fig. 1. The number of water molecules in the central cavities of pFBPases, varying in quaternary state and loop conformation (PDB codes 1CNQ, 1NUY, 1YYZ, 2F3D, 2QVQ, 2QVU, 1EYJ, and 1FRP), averages to 31, and that of a similar ensemble of eFBPases (PDB codes 2OX3, 2QVR, 2GQ1, and 2Q8M) averages to 14 (supplemental Fig. S1). The difference in water structures of the central cavities of pFBPase and eFBPase is not solely due to the resolution of crystal structures because cavities with the highest numbers of discrete sites for water molecules in eFBPase (2QG1; 22 water molecules, 1.5 Å resolution) and pFBPase (1YYZ; 48 water molecules, 1.9 Å resolution) follow the trend established by the ensemble averages (Fig. 1). Order/disorder transitions of water molecules in the cavity could be a factor in AMP/inhibitor synergy. Only three positions in or near the central cavity differ in residue type between eFBPase and pFBPase. Ser⁴⁵⁻, Thr⁴⁶⁻, and Leu¹⁸⁶⁻ in the porcine enzyme are His³⁷⁻, Arg³⁸⁻, and Tyr¹⁸⁶⁻, respectively, in eFBPase (Fig. 1). Side chains of Ser⁴⁵⁻/His³⁷⁻ point into the central cavity, whereas side chains of Thr⁴⁶⁻/Arg³⁸⁻ and Leu¹⁸⁶⁻/Tyr¹⁸⁶⁻ are at the edge. If the central cavity is essential to synergy, then mutations that replace residues of pFBPase with those of eFBPase should significantly reduce AMP/Fru-2,6-P₂ synergy.

Kinetics of Wild-type and Mutant pFBPases—His⁴⁵⁻, Arg⁴⁶⁻, and Cav⁻ FBPase have substantial activity relative to wild-type enzyme (Table 1). The Kᵣ for substrate for Tyr¹⁸⁶⁻ pFBPase is 2-fold greater than that for wild-type enzyme. Mutations cause only minor changes in the Kᵣ for Mg²⁺. AMP is a competitive inhibitor with respect to Mg²⁺ for all mutants. Hill coefficients of ~2 indicate positive cooperativity in AMP binding. The Kᵣ of AMP for Arg⁴⁶⁻ pFBPase decreases 6-fold, whereas for other mutants, the Kᵣ for AMP inhibition is comparable with that of wild-type enzyme. His⁴⁵⁻, Arg⁴⁶⁻, and Cav⁻ FBPases exhibit residual activity (10–20% of V_max) at saturating levels of AMP. This phenomenon has been attributed to active AMP complexes in intermediate quaternary states of the tetramer (24, 45). Fru-2,6-P₂ is a potent inhibitor, competitive with respect to Fru-1,6-P₂ for all FBPases here. In general, cavity mutations have relatively minor effects on activity and ligand binding affinities.

Data in Fig. 2 represent the linear increase in fluorescence with time due to the production of NADPH. In previous work (14), each curve was fit by an empirical relationship with adjustable parameters for maximal and minimum velocities and a Hill coefficient and then replotted as relative velocity versus [AMP]. An alternative model-based definition of synergy (Scheme 1) provides an analytical expression valid at all concentrations of Fru-2,6-P₂, including an infinite concentration.
FIGURE 1. Overview of FBPases. A, alignment of R-state (cyan, product complex, PDB code 1CNQ) and T-state (magenta, AMP complex, PDB code 1EYJ) pFBPases map the locations of AMP binding, active sites, and the central cavity. B, an expanded view of A, showing subunit C2 with loop 50–72 in engaged (cyan) and disengaged (magenta) conformations. C, the central cavity of Leu54pFBPase (left, R-state, AMP complex, 1.9 Å, PDB code 1YYZ) and the central region of eFBPase (right, ammonium sulfate complex, 1.5 Å, PDB code 2GQ1). Comparable regions exhibit significant differences in hydration levels. The surface renderings are of subunits C1 and C4 of the tetramer, with ball-and-stick models representing selected residues from subunits C2 and C3 and water molecules (red spheres). The icon with associated arrow indicates the region viewed and viewing direction, respectively. Image generated with PyMOL (57).

| TABLE 1 |
| Parameters from the kinetics of wild-type and mutant forms of pFBPases and wild type lFBPase |

Values in parenthesis are standard deviations in the last significant digit.

| Specific activity | $K_{m}$ Fr-1,6-P$_{2}$ | Hill coefficient Mg$^{2+}$ | $K_{m}$ Mg$^{2+}$ | Hill coefficient AMP | $K_{i}$ AMP | $K_{i}$ Fr-2,6-P$_{2}$ |
|------------------|-----------------------|------------------------|-----------------|----------------------|----------|-----------------------|
| WT pFBPase       | 25 (1)                | 1.47 (7)               | 1.7 (1)         | 0.70 (5)             | 2.2 (1)  | 0.76 (8)              | 0.14 (1)                          |
| S45H pFBPase     | 11.9 (7)              | 1.07 (7)               | 1.6 (1)         | 1.0 (1)              | 1.8 (1)  | 0.35 (5)              | 0.053 (9)                         |
| T46R pFBPase     | 12.3 (9)              | 0.68 (8)               | 1.8 (2)         | 0.93 (9)             | 2.4 (2)  | 4.8 (2)               | 0.27 (5)                          |
| L186Y pFBPase    | 21.0 (7)              | 1.1 (1)                | 1.6 (1)         | 0.68 (4)             | 1.7 (2)  | 0.58 (9)              | 0.16 (1)                          |
| Cav pFBPase      | 15.2 (9)              | 1.1 (2)                | 1.8 (1)         | 0.82 (6)             | 2.2 (2)  | 0.56 (4)              | 0.14 (1)                          |
| pFBPase          | 15 (1)                | 1.0 (1)                | 0.77 (8)        | 1.3 (1)              | 2.2 (2)  | 0.58 (5)              | 0.052 (1)                         |

* Determined at saturating Fru-1,6-P$_{2}$ (20 μM) and saturating Mg$^{2+}$ (5 mM for wild-type pFBPase and lFBPase and 10 mM for mutant pFBPases). Reported values come from five repetitions.

* Determined at saturating Mg$^{2+}$ (5 mM for wild-type pFBPase and lFBPase and 10 mM for mutant pFBPases). The concentrations of Fru-1,6-P$_{2}$ vary from 0.3 to 20 μM.

* Hill coefficient for Mg$^{2+}$ employed 0.2–20 mM Mg$^{2+}$ and saturating Fru-1,6-P$_{2}$ (20 μM).

* Determined by using concentrations in Mg$^{2+}$ from 0.5 to 5 mM and Fru-1,6-P$_{2}$ from 0.5 to 10 μM.

* Determined by using concentrations in AMP from 0.1 to 100 μM and saturating Fru-1,6-P$_{2}$ (20 μM) and Mg$^{2+}$ (10 mM).

* Determined by using concentrations in Mg$^{2+}$ from 0.5 to 5 mM with saturating Fru-1,6-P$_{2}$ (20 μM). AMP concentrations range from 0 to 5 μM for wild-type pFBPase and lFBPase, from 0 to 3 μM for His$_{55}$ pFBPase, from 0 to 20 μM for Arg$_{66}$ pFBPase, and from 0 to 5 μM for Tyr$_{186}$ and Cav$^{-}$ pFBPases.

* Determined by using Mg$^{2+}$ (5 mM for wild-type pFBPase and lFBPase and 10 mM for mutant pFBPases) and Fru-1,6-P$_{2}$ concentrations from 0.5 to 10 μM. Concentrations of Fru-2,6-P$_{2}$ range from 0 to 1 μM for wild-type pFBPase, from 0 to 0.5 μM for His$_{55}$ pFBPase and lFBPase, from 0 to 1 μM for Arg$_{66}$ pFBPase, and from 0 to 1 μM for Tyr$_{186}$ pFBPase and Cav$^{-}$ pFBPases.

AM/Fru-2,6-P$_{2}$ Synergism in Fructose-1,6-bisphosphatase
AMP/Fru-2,6-P₂ Synergism in Fructose-1,6-bisphosphatase

In Scheme 1, substrate $S$ binds to FBPase subunits independently ($k_{cat}$), resulting in an ES complex that contributes to activity ($k_{cat}$). Two molecules of AMP (represented by $A$) bind with a Hill coefficient of 2 and affect an R- to T-state transition (20). The $ESA_2$ complexes for some mutant $p$FBPases retain residual activity ($k_{cat}''$) is nonzero). Fru-2,6-P₂ (represented by $B$) and substrate are mutually exclusive in binding to the enzyme (5, 6); however, the binding of AMP and substrate are not mutually exclusive (43). AMP and Fru-2,6-P₂ bind to distinct sites (EBA and $EBA_2$). The Hill coefficient for AMP becomes unity in the presence of Fru-2,6-P₂. Hence, the equilibrium constant ($K_{iba}$) for the binding of $A$ to EBA equals $4K_{iba}$, as required for the independent binding of ligands to two identical sites.

Equation 1 gives the velocity $v$ based on Scheme 1.

$$v = \frac{(SV_{max}/K_s)(1 + (k_{cat}'/(K_{cat}K_{iba})))A^2}{(1 + (S/K_s) + (1/K_{iba} + S/(K_{iba}K_{iba})))A^2 + B/K_{iba} + BA/(K_{iba}K_{iba}) + BA/(4K_{iba}K_{iba}^2)}$$

(Eq. 1)

In fitting Equation 1, $V_{max}$, $k_{cat}'/(K_{cat}K_{iba})$, $(1/K_{iba} + S/(K_{iba}K_{iba}))$, and $K_{iba}$ are adjustable parameters. $K_s$ and $K_{iba}$ are determined by separate experiments for each enzyme ($K_m$ and $K_i$ Fru-2,6-P₂ of Table 1, respectively), and $S$ is 20 μM for all determinations. Values for parameters for each FBPase are shown in Table 2 with fits of Equation 1 in Fig. 2.

Rewriting Equation 1 with $A$ as the independent variable leads to Equation 2,

$$aA_{0.5}^2 + bA_{0.5} + c = 0$$

(Eq. 2)

where $a = (v_{0.5})(1/K_{iba} + S/K_{iba}K_{iba} + B/(K_{iba}K_{iba}^2)) - SV_{max}K_{cat}/K_sK_{iba}K_{cat}''$, $b = (v_{0.5})(B/(K_{iba}K_{iba})); c = (v_{0.5})(1 + S/K_s + B/K_{iba}) - SV_{max}/K_s$; and $v_{0.5}$ is one-half of the sum of the maximum (at $A = 0$) and minimum (at $A = \infty$) velocities for a given concentration of $B$. The quadratic formula provides $A_{0.5}$ at any value of $B$ including $B = \infty$ (Table 2). Synergism is defined here.
as \( A_{R}^{\text{WT}} / A_{R}^{\text{Cav}} \). For wild-type, Arg46, and Tyr186 pFBPases, \( A_{R}^{\text{WT}} \) decreases significantly as Fru-2,6-P2 concentration increases. On the other hand, His45 and Cav pFBPases show a two-thirds loss of AMP/Fru-2,6-P2 synergism (Table 2).

**AMP Complex of Cav pFBPase (Protein Data Bank Code 4GW5)**—Crystals of the AMP complex of Cav pFBPase (space group \( P2_12_12_1 \), \( a = 60.24 \, \text{Å}, b = 164.29 \, \text{Å}, c = 79.14 \, \text{Å} \)) belong to the same space group and lattice as crystals of canonical T-state pFBPase. A C1-C2 dimer exists in the asymmetric unit. Both monomers in the model start from residue 9. Electron density for residues 60–72 in the dynamic loop is weak and is associated with high B-values. Statistics for data collection and refinement are shown in Table 3.

The AMP complex of Cav pFBPase is in the T-state, with a 15° subunit pair rotation and a disengaged loop 50–72. One molecule each of Fru-6-P, Mg2+, \( P_i \), and AMP binds to each subunit. The root mean square deviation in Ca carbons between AMP complexes of Cav and wild-type pFBPase (both in the T-state) is 0.3 Å. Further details regarding the structure of the AMP complex of pFBPase can be found in the literature (16).

**Fru-2,6-P2 Complex of Cav pFBPase (PDB Code 4GWU)**—Crystals of the Fru-2,6-P2 complex of Cav pFBPase (space group \( P2_12_12_1 \), \( a = 54.00 \, \text{Å}, b = 81.22 \, \text{Å}, c = 165.06 \, \text{Å} \)) belong to the same space group and lattice as crystals of wild-type pFBPase in its R-state (PDB code 1CNQ). The asymmetric unit is a monomer. Electron density for residues 1–9 and 52–70 is absent. Electron density for residues 22–27 at the AMP binding site is weak, consistent with the previously reported R-state structure (49). Side chains of His25 and Tyr186 are clear in electron density maps, whereas the side chain of Arg46 is partially disordered (Fig. 3). Electron density confirms the presence of bound Fru-2,6-P2, Mg2+ at metal site-1 in contact with the 1-hydroxyl group and 2-phosphoryl group of Fru-2,6-P2.

Superposition of the entire tetramer of the Fru-2,6-P2 complex of Cav pFBPase onto the wild-type R-state, wild-type Fru-2,6-P2 complex, and wild-type T-state yields root mean square deviations in Ca coordinates of 0.75, 2.3, and 2.5 Å, respectively. The Fru-2,6-P2 complex of Cav pFBPase is near the canonical R-state, exhibiting a 3° subunit pair rotation as opposed to the 13° rotation of the Fru-2,6-P2 complex of wild-type pFBPase (14) (Fig. 3). The 2° rotation in Fru-2,6-P2 complex of Cav pFBPase is similar to the 3° rotation of the I-state induced by AMP in Leu54 pFBPase (23). The shear of helices H1 and H2 observed in the AMP complex of Leu54 pFBPase is evident in the Fru-2,6-P2 complex of Cav pFBPase, as is the loss of the hydrogen bond between Asn35 and Thr14 and the movement of Ile10 from its hydrophobic pocket (Fig. 3); however, unlike the AMP complex of Leu54 pFBPase, disruption of the Glu192-Thr29 hydrogen bond across the C1-C4 subunit interface does not occur.

Fru-2,6-P2 induces local conformational change in Cav pFBPase similar to that induced in wild-type pFBPase (Fig. 3) (14). Loop 121–126 moves toward Fru-2,6-P2 probably in response to hydrogen bonds formed between backbone amide groups of Ser123 and Ser124 and the 2-phosphoryl group of Fru-2,6-P2, \( \alpha \)-Carbons of Glu97 and Glu98 move toward the Mg2+ at site-1, most likely a consequence of altered metal coordination due to the absence of metal at site-2. Movements in residues 97–98 and 121–126 may block Asp74 from forming a functionally essential hydrogen bond with the C-terminal end of loop 50–72. Loop 50–72 itself is disordered, and metal cations are absent from sites 2 and 3. The conformation of loop 264–274 in the Fru-2,6-P2 complex of Cav pFBPase is nearly identical to that of the product complex of wild-type pFBPase (R-state), having not undergone the conformational change observed in the R- to T-state transition.

### Table 2

Fru-2,6-P2/AMP synerigism of FBPases

| WT pFBPase | S4H pFBPase | T46R pFBPase | L186Y pFBPase | Cav- pFBPase | WT pFBPase |
|-----------|-------------|-------------|---------------|--------------|-----------|
| \( V_{\text{max}} \) (arbitrary units min\(^{-1}\)) | 0.194 (4) | 0.88 (3) | 0.434 (8) | 0.495 (9) | 0.47 (1) | 0.48 (1) |
| \( K_m^{\text{pF}} \) (mM) | 0* | 0.0018 (4) | 0.0006 (1) | 0.0017 (2) | 0.0024 (3) | 0* |
| \( K_m^{\text{ATP}} \) (mM) | 0.51 (4) | 0.30 (2) | 4.1 (5) | 0.28 (3) | 0.20 (2) | 0.21 (1) |
| \( 1/(K_m^p + S/(K_m^p)) \) (mM) | 0.78 (6) | 0.85 (6) | 1.7 (1) | 0.35 (3) | 0.72 (4) | 0.17 (2) |
| \( A_{R}^{\text{WT}} \) (μM) | 0.42 (4) | 0.71 (6) | 1.4 (1) | 0.29 (3) | 0.66 (4) | 0.14 (2) |
| \( A_{R}^{\text{Cav}} \) (μM) | 3.9 (3) | 2.4 (2) | 11 (1) | 2.3 (2) | 2.0 (2) | 2.1 (2) |
| \( A_{R}^{\text{Cav}} / A_{R}^{\text{WT}} \) | 9 (1) | 3.4 (9) | 8 (1) | 8 (2) | 3.3 (6) | 15 (4) |

* Value fixed to zero, reflecting full inhibition at saturating concentrations of AMP.

### Table 3

Statistics of data collection and refinement for Cav- pFBPase

| Fru-2,6-P2 complex | AMP complex |
|-------------------|-------------|
| Space group | \( P2_12_12_1 \) |
| Unit cell lengths (Å) | 54.00, 81.22, 165.06 |
| Unit cell angles (degrees) | 90, 90, 90 |
| Resolution limit (Å) | 3.00 |
| No. of measurements | 26,045 |
| No. of unique reflections | 7478 |
| Completeness of data (%) | Overall 98.4 |
| | Last shell/resolution range (Å) 99.9/3.11–3.00 |
| | Last shell/resolution range (Å) 99.9/3.11–3.00 |
| | No. of reflections in refinement 7104 |
| | Number of atoms 2489 |
| | Number of solvent sites 88 |
| | Root mean square deviations Bond lengths (Å) 0.026 |
| | Bond angles (degrees) 1.8 |
| | Ramachandran statistics Preferred (%) 98.6 |
| | Outlier (%) 0 |

* \( R_{\text{sym}} = \Sigma \sum |I_i - \langle I_j \rangle| / \Sigma \sum |I_i| \), where \( i \) runs over multiple observations of the same intensity, and \( j \) runs over all crystallographically unique intensities.

* \( R_{\text{free}} = 2 \sum |I_i - \langle I_j \rangle| / |\sum |I_i|\rangle \), where \( I_{\text{obs}} \) > 0.

* \( R_{\text{sym}} \) based upon 10% of the data randomly culled and not used in the refinement.
Sequence Analysis of Type I FBPases—The alignment and hierarchical clustering of 307 Type I FBPase sequences from the Uniprot database (50) employed the ClustalW Web server on the GenomeNet database. Comparisons of sequence positions occupied by signature residues responsible for AMP inhibition, tetramer stability, Glc-6-P inhibition, phosphoenolpyruvate activation, and cavity formation segregate FBPases into groups with mutually exclusive regulatory mechanisms. Position 45, for instance, is a small side-chain residue in eukaryotic FBPases but large and hydrophilic in *E. coli*-like FBPases (Fig. 4). Residue 112 is critical for AMP inhibition and the salt link between Lys 42 and Glu 192 is essential for AMP inhibition, tetramer stability, and central cavity. A block with 64 sequences in the middle of the similarity matrix includes *eFBPase*. Additional blocks, such as those for *E. coli*-like FBPases, have signature residues for AMP inhibition, tetramer formation, and AMP/Fru-2,6-P2 synergism. More than half of all similarity blocks have scant or no experimental data. FBPases in the large block at the top right of the similarity matrix have no AMP/Fru-2,6-P2 synergism. A bacterial FBPase with properties of a eukaryotic FBPase fall into blocks of relatively high similarity corresponding to fungi, mammalian muscle, mammalian liver, and plants (Fig. 5). The eukaryotic FBPases should be tetramers subject to synergistic inhibition by AMP and Fru-2,6-P2 (signature residues are present for the AMP site, tetramer stability, and central cavity). A block with 64 sequences in the middle of the similarity matrix includes eFBPase. *E. coli*-like FBPases have residue types associated with phosphoenolpyruvate activation, a dynamic equilibrium between dimer and tetramer forms, AMP inhibition, no central cavity, and, hence, no AMP/Fru-2,6-P2 synergism.

**Kinetics of FBPase**—FBPases from *Leptospira* are between *eFBPase* and *E. coli*-like FBPases in the similarity matrix but resemble eukaryotic FBPases, having signature residues for AMP inhibition, tetramer formation, and AMP/Fru-2,6-P2 synergism. A bacterial FBPase with properties of a eukaryotic
AMP/Fru-2,6-P₂ Synergism in Fructose-1,6-bisphosphatase

AMP inhibition of FFBPase is comparable with that of pFBPase. The Hill coefficient for AMP inhibition is close to 2. AMP is a competitive inhibitor with respect to Mg²⁺ ($K_i = 0.58 \pm 0.05 \mu M$) and a non-competitive inhibitor with respect to Fru-1,6-P₂ ($K_i = K_d = 0.83 \pm 0.05 \mu M$).

Although the genome does not reveal an obvious candidate for a fructose-6-phosphate-2-kinase in L. interrogans, Fru-2,6-P₂ is a potent inhibitor of FFBPase (Table 1), competitive with substrate. In the presence of saturating substrate (20 μM) and Mg²⁺ (5 mM), the concentration of Fru-2,6-P₂ causing 50% inhibition is 0.33 ± 0.02 μM, with a Hill coefficient 1.05 ± 0.08.

Fru-2,6-P₂ enhances AMP inhibition of FFBPase by 15-fold (Table 2), confirming the prediction from sequence analysis. The Hill coefficient for AMP inhibition drops from 2.2 to unity upon the addition of Fru-2,6-P₂. AMP at a concentration that causes 50% inhibition (1.8 μM) enhances Fru-2,6-P₂ inhibition by 2-fold (Fig. 6).

Anion activators, such as PEP or citrate, activate eFBPase by promoting tetramer formation (31). To test the effect of PEP on FFBPase, Mg²⁺-initialized assays were used (31). PEP activation is not present for FFBPase. Instead, millimolar levels of PEP cause moderate inhibition. Assays of Glc-6-P inhibition followed the published protocol for eFBPase (32). Glc-6-P inhibition becomes evident only at millimolar levels, ~50-fold weaker than Glc-6-P inhibition of eFBPase. Glc-6-P inhibits pFBPase and FFBPase at comparable concentrations.

Sedimentation Equilibrium—FFBPase and pFBPase at three protein concentrations ($A_{280} = 0.3, 0.5$, and 0.7) were sedimented to equilibrium at angular speeds of 15,000, 18,000, and 21,000 rpm. Sedimentation equilibrium data were fit to single component and dimer-tetramer equilibrium models with UltraScan (53). Regardless of model, pFBPase and FFBPase give similar apparent molecular masses, 111.9 and 112.1 kDa, respectively. The determined molecular masses are less than the calculated mass of the tetramer (140 kDa).

Tryptophan Fluorescence—FFBPase has but one tryptophan (Trp²¹⁹), which on the basis of structural similarity is near the common binding sites for the 6-phosphoryl groups of substrate, product, and Fru-2,6-P₂. Steady-state fluorescence emission from Trp²¹⁹ increases as levels of substrate, product, or Fru-2,6-P₂ increase, whereas increasing concentrations of AMP cause little change. Substrate, product, Fru-2,6-P₂, and AMP have no effect on fluorescence emission from 100 μM tryptophan in the absence of FFBPase. The fluorescence change caused by Fru-6-P, Fru-1,6-P₂, or Fru-2,6-P₂ is arguably due to a change in the local environment of Trp²¹⁹. Indeed, at saturating concentrations, the three active site ligands induce nearly identical limiting changes in fluorescence emission.

Data from titrations of 6-phosphoryl ligands in the presence and absence of AMP are fit to Equation 3,

$$\frac{\Delta F}{F_0} = \left( \frac{\Delta F_{\text{max}}}{F_0} \right) \cdot \frac{L}{K_d + L}$$

(Eq 3)

where $\Delta F$ is the change in fluorescence upon the addition of ligand $L$, $F_0$ is the fluorescence in the absence of ligand, and $K_d$ is the dissociation constant. AMP does not affect the binding of Fru-1,6-P₂ (Fig. 6 and Table 4); however, AMP at a concentr-
AMP/Fru-2,6-P₂ Synergism in Fructose-1,6-bisphosphatase

FIGURE 5. Similarity matrix of Type I FBPase sequences and the prediction of structural and regulatory properties. The fraction of identical residues in sequence pairs are represented by color according to the scale to the right of the similarity matrix. The positions of eFBPase, pFBPase, and iFBPase are marked by vertical and horizontal dotted lines. FBPases from eukaryotic systems and E. coli-like FBPases are indicated by brackets. Regulatory properties are predicted (gray bar) according to residue types associated with key positions in the amino acid sequence. AMP inhibition requires serine or threonine for residue 31, lysine or arginine for residue 112, and tyrosine for residue 113. Tetramer stability requires lysine or arginine for residue 42 and glutamate for residue 192. Glc-6-P inhibition requires tyrosine for residue 216, lysine or arginine for residue 228, and glutamine for residue 231. Anion activation requires glycine for residue 14, lysine or arginine for residue 38, lysine or arginine for residue 88, and not glutamate for residue 192. The presence of a central cavity (AMP/Fru-2,6-P₂ synergism) requires small side chains (glycine, alanine, cysteine, serine, or gap) for residue 45.

FIGURE 6. Synergism between active site ligands and AMP in iFBPase. A, Fru-2,6-P₂ inhibition in the absence (○) and presence (●) of AMP ([AMP] = 1.8 μM). All assays are carried out with saturating Fru-1,6-P₂ (20 μM) and Mg²⁺ (10 mM). B–D, titrations for Fru-1,6-P₂ (B), Fru-2,6-P₂ (C), and Fru-6-P (D) performed in the absence (open symbols) and presence (filled symbols) of AMP ([AMP] = 1.8 μM) reveal binding synergism between AMP and only Fru-6-P and Fru-2,6-P₂. Each titration data point represents integration of the Trp²¹⁹ emission spectrum from 310 to 370 nm.
TABLE 4
Effect of AMP on the binding of ligands to the active site
S.D. in the last significant digit is given parenthetically.

| Ligand     | $K_d$ (µM) | $K_d$ (µM) |
|------------|------------|------------|
| Fru-1,6-P$_2$ | 0.77 (9)   | 0.73 (5)   |
| Fru-2,6-P$_2$ | 0.32 (7)   | 0.17 (2)   |
| Fru-6-P     | 105 (9)    | 50 (7)     |

DISCUSSION

Ser$^{45} \rightarrow$ His and Cav$^{-}$ pFBPases represent first instances of modified pFBPases that retain potent AMP and Fru-2,6-P$_2$ inhibition with greatly reduced AMP/Fru-2,6-P$_2$ synergism. Kinetic parameters for catalysis, Mg$^{2+}$ activation, AMP inhibition, and Fru-2,6-P$_2$, inhibition of mutant enzymes investigated here are similar to those of wild-type pFBPase. Reduced Fru-2,6-P$_2$/AMP synergism in Cav$^{-}$ pFBPase is not due to the disruption of the active or allosteric binding sites but rather to an impaired quaternary response; Cav$^{-}$ pFBPase exhibits only a 2° subunit pair rotation in contrast to the 13° subunit pair rotation for the wild-type enzyme. Conceivably, Cav$^{-}$ mutations could stabilize the R-state (or destabilize the T-state), making the quaternary transition unfavorable; however, previously reported mutations that destabilize quaternary states (Ala$^{54} \rightarrow$ Leu and Ile$^{10} \rightarrow$ Asp, for instance) broadly impact the kinetics of pFBPase. Moreover, impairment of the R- to T-state transition is specific to Fru-2,6-P$_2$. AMP drives Cav$^{-}$ pFBPase to a canonical T-state, which is structurally indistinguishable from the T-state of the wild-type enzyme.

The effect of Fru-2,6-P$_2$ must be more than the displacement of loop 50–72 from its engaged conformation. Loop 50–72 is disordered in the Fru-2,6-P$_2$ complex of Cav$^{-}$ pFBPase, but the enzyme remains in an R-like state. In contrast, the loop 50–72 acquires its disengaged conformation in the Fru-2,6-P$_2$ complex of wild-type pFBPase, and the enzyme is in a near-T quaternary state. Structural evidence here indicates the subunit pair rotation must go beyond 2° to allow the disengaged conformer.

The solvated central cavity, absent in Cav$^{-}$ pFBPase, evidently enables the requisite subunit pair rotation in response to the binding of Fru-2,6-P$_2$. The binding of AMP to Leu$^{54}$ pFBPase causes a 3° subunit pair rotation, retaining the engaged conformation of loop 50–72 (23) and an organized water structure in the central cavity (Fig. 1). In contrast, in the T-state of Leu$^{54}$ pFBPase, loop 50–72 and the water structure in the cavity are disordered. Arguably, an order-to-disorder transition of water molecules within the central cavity, caused by the movement of loop 50–72 from its engaged conformation, provides the requisite motive force for subunit pair rotation. In Cav$^{-}$ pFBPase, the order-to-disorder transition of water structure is inoperative, and consequently movement of loop 50–72 from its engaged conformation results in virtually no subunit pair rotation. The mutation of Ser$^{45} \rightarrow$ His alone is largely responsible for this effect, suggesting a critical role for Ser$^{45}$ in maintaining an organized water structure when loop 50–72 is engaged. The volume of the histidyl side chain (137 Å$^3$) relative to that of serine (89 Å$^3$) is probably a factor; however, the side chain of serine in this specific context may offer the capacity, not shared by other amino acid types, to “seed” a network of hydrogen-bonded water molecules.

Fru-2,6-P$_2$/AMP synergism, although reduced, is still present in His$^{45}$ and Cav$^{-}$ pFBPase. The 2° subunit pair rotation caused by Fru-2,6-P$_2$ in Cav$^{-}$ pFBPase is similar to changes induced by AMP in Leu$^{54}$ pFBPase (I$_R$-state, 3° subunit pair rotation). Structural changes at the C1-C4 interface due to small subunit pair rotations could be the structural basis for the weak synergism that remains in Cav$^{-}$ pFBPase. Indeed, wild-type and mutant pFBPases here exhibit reduced AMP cooperativity in the presence of Fru-2,6-P$_2$, which may come from conformational changes at the C1-C4 interface (such as the weakening of the hydrogen bond between Glu$^{192}$ and Thr$^{39}$) within the first few degrees of subunit pair rotation. The model above suggests that an engaged dynamic loop in the presence of bound Fru-2,6-P$_2$ would eliminate Fru-2,6-P$_2$/AMP synergism altogether, and in fact, such is the case for eFBPase; there is no Fru-2,6-P$_2$/AMP synergism, and the Fru-2,6-P$_2$ complex of wild-type eFBPase reveals a dynamic loop in its engaged conformation (14).

The failure of the loop 50–72 to achieve a disengaged conformation in its Fru-2,6-P$_2$ complex of Cav$^{-}$ pFBPase is also consistent with the results of targeted molecular dynamics simulations, which indicate that the transition of loop 50–72 from an engaged to disengaged conformation comes late in the R- to T-state transition (24). Moreover, previous work ties the conformational change in loop 264–274 to subunit pair rotation from the I$_R$- to I$_T$-state (from 3 to 12°, respectively) (23, 24). The Fru-2,6-P$_2$ complexes of Cav$^{-}$ and wild-type pFBPases reveal a conformational change in loop 264–274 only for the latter complex.

The foregoing analysis supports the following hypothesis. Any tetrameric FBPase with signature residues of a central cavity should exhibit AMP/Fru-2,6-P$_2$ synergism. Hence, all eukaryotic organisms for which sequences of FBPases are known should exhibit AMP/Fru-2,6-P$_2$ synergism.

Fru-2,6-P$_2$ is not present in bacteria (54), so the evolution of AMP/Fru-2,6-P$_2$ synergism from a bacterial ancestor is problematic. E. coli FBPase employs Glc-6-P as a synergistic inhibitor with respect to AMP (32). Unlike Fru-2,6-P$_2$, Glc-6-P is an allosteric non-competitive inhibitor with respect to substrate. Vastly different kinetic properties and a distinct allosteric binding site for Glc-6-P in eFBPase make Glc-6-P an unlikely precursor of Fru-2,6-P$_2$ through evolution. The analysis of Type I FBPase sequences, however, indicates a central cavity in I FBPase. Moreover, AMP/Fru-6-P binding synergism in I FBPase suggests an evolutionary pathway from AMP/Fru-6-P synergism in bacteria to AMP/Fru-2,6-P$_2$ synergism in eukaryotic organisms.

At some point in evolution, a primordial FBPase may have existed as an equilibrium mixture of dimers and tetramers (Fig. 7). This primordial FBPase may have been subject to AMP inhibition and feedback inhibition by Fru-6-P. One pathway of evolution stabilizes the tetramer through the creation of an anion activation site. Over time, this form of FBPase acquires a site for Glc-6-P inhibition that acts synergistically with AMP, becoming the present day eFBPase.
**AMP/Fru-2,6-P₂ Synergism in Fructose-1,6-bisphosphatase**

An alternative pathway of evolution also leads to a stable FBPase tetramer through the formation of Glu₁⁹²–Lys₄² salt links. Because this form of the tetramer is at all times catalytically competent, a robust mechanism of inhibition would presumably co-evolve. Mutation of position 45 from histidine to serine would create a nascent central cavity in the primordial enzyme with no effect on activity. The introduction of the Glu₁⁹²–Lys₄² salt link in a Ser⁴⁵ ancestral FBPase would create a stable tetramer with a central cavity. The central cavity would enable AMP/Fru-6-P synergism as a counterpart to the enhanced activity of a stable tetramer. Over time, Fru-2,6-P₂ replaces Fru-6-P as a dynamic regulator. Crystal structures of Fru-2,6-P₂ bound to eFBPase clearly show that all atoms, excepting the 2-phosphoryl group, occupy positions corresponding to those of Fru-6-P (14).

*L. interrogans* is a pathogenic bacterium with a metabolism strikingly different from that of mammals (55). *L. interrogans* lacks hexokinase (as well as other enzymes of glycolysis) and cannot capture and directly use glucose from exogenous sources (55, 56). Instead, lipid is the principal energy source for *L. interrogans*.

The apparent role of gluconeogenesis in *L. interrogans* is the generation of Fru-6-P as a precursor for sugar-nucleotide biosynthesis (56). Given that a gene coding for glucose 6-phosphate dehydrogenase is absent in *L. interrogans* (56), Fru-6-P must enter the non-oxidative pentose phosphate pathway in order to produce ribose 5-phosphate. The conversion of Fru-1,6-P₂ into Fru-6-P in *L. interrogans* then represents the first committed step in ribose 5-phosphate biosynthesis and *de novo* nucleotide biosynthesis. In this scheme, Fru-6-P is a direct feedback inhibitor, and AMP is an allosteric feedback inhibitor. High levels of Fru-6-P should affect only FBPase, due to the absence of a glycolytic pathway. When nucleotides are in demand, the concentrations of AMP and Fru-6-P (the latter being in equilibrium with the pool of ribose 5-phosphate) should be low; however, rising concentrations of AMP and Fru-6-P may synergistically support each other in the feedback inhibition of FBPase.

*Leptospira* is the causative agent of leptospirosis, a disease of global concern, causing renal and hepatic damage and, in some instances, sudden and fatal hemoptysis (55). Infections in livestock and domestic animals can expose humans to *Leptospira* infections through sanitary waste or contaminated drinking water. Little information is known about the metabolism of *L. interrogans* by direct experimentation. Given the current status of our knowledge, however, one could regard FBPase from *Leptospira* as a potential target in the development of agents of anti-microbial growth.

**REFERENCES**

1. Benkovic, S. J., and deMaine, M. M. (1982) Mechanism of action of fructose 1,6-bisphosphatase. *Adv. Enzymol. Relat. Areas Mol. Biol.* 53, 45–82
2. Tejwani, G. A. (1983) Regulation of fructose-bisphosphatase activity. *Adv. Enzymol. Relat. Areas Mol. Biol.* 54, 121–194
3. Blangy, D., Buc, H., and Monod, J. (1968) Kinetics of the allosteric interactions of phosphofructokinase from *Escherichia coli*. *J. Mol. Biol.* 31, 13–35
4. Taketa, K., and Pogell, B. M. (1965) Allosteric inhibition of rat liver fructose-1,6-bisphosphatase by adenosine 5'-monophosphate. *J. Biol. Chem.* 240, 651–662
5. Pilkis, S. J., El-Maghrabi, M. R., Pilkis, J., and Claus, T. (1981) Inhibition of fructose-1,6-bisphosphatase by fructose 2,6-bisphosphate. *J. Biol. Chem.* 256, 3619–3622
6. Van Schaftingen, E., and Hers, H.-G. (1981) Inhibition of fructose-1,6-bisphosphatase by fructose 2,6-bisphosphate. *Proc. Natl. Acad. Sci. U.S.A.* 78, 2861–2863
7. Pilkis S. J., El-Maghrabi, M. R., Pilkis, J., Claus, T. H., and Cumming D. A. (1981) Fructose 2,6-bisphosphate. A new activator of phosphofructokinase. *J. Biol. Chem.* 256, 3171–3174
8. Van Schaftingen, E., Jett, M.-F., Hue, L., and Hers, H.-G. (1981) Control of liver 6-phosphofructokinase by fructose 2,6-bisphosphate and other effectors. *Proc. Natl. Acad. Sci. U.S.A.* 78, 3483–3486
9. Uyeda, K., Furuya, E., and Luby, L. J. (1981) The effect of natural and synthetic D-fructose 2,6-bisphosphate on the regulatory kinetic properties of liver and muscle phosphofructokinase. *J. Biol. Chem.* 256, 8394–8399
10. Okar, D. A., and Lange, A. I. (1999) Fructose-2,6-bisphosphate and control of carbohydrate metabolism in eukaryotes. *Biofactors* 10, 1–14
11. Derr, R. F., and Zieve, L. (1972) Adenylate energy charge. Relation to guanulate energy charge and the adenylate kinase equilibrium constant. *Biochem. Biophys. Res. Commun.* 49, 1385–1390
12. Han, P. F., Han, G. Y., Hayes, R. L., Moore, C. L., and Johnson, J. (1983) Synergistic effect of AMP and fructose 2,6-bisphosphate on the protection of fructose 1,6-bisphosphatase against inactivation by trypsin. *Experientia* 39, 1305–1307
13. Kruger, N. J., and Beevers, H. (1984) Effect of fructose 2,6-bisphosphate on the kinetic properties of cytoplasmic fructose 1,6-bisphosphatase from germinating castor bean endosperm. *Plant Physiol.* 76, 49–54
14. Hines, J. K., Chen, X., Nix, J. C., Fromm, H. J., and Honzatko, R. B. (2007) Structures of mammalian and bacterial fructose 1,6-bisphosphatase—reveal the basis for synergism in AMP/fructose 2,6-bisphosphate inhibition. *J. Biol. Chem.* 282, 36121–36131
15. Ke, H. M., Zhang, Y. P., and Lipscomb, W. N. (1990) Crystal structure of fructose-1,6-bisphosphatase complexed with fructose 6-phosphate, AMP and magnesium. *Proc. Natl. Acad. Sci. U.S.A.* 87, 5243–5247
16. Choe, J. Y., Fromm, H. J., and Honzatko, R. B. (2000) Crystal structures of fructose-1,6-bisphosphatase. Mechanism of catalysis and allosteric inhibition revealed in product complexes. *Biochemistry* 39, 8565–8574
17. Scheffer, J. E., and Fromm, H. J. (1986) Regulation of rabbit liver fructose-1,6-bisphosphatase by metals, nucleotides, and fructose 2,6-bisphosphate as determined from fluorescence studies. *Biochemistry* 25, 6659–6665
18. Liu, F., and Fromm, H. J. (1988) Purification and characterization of fructose-1,6-bisphosphatase from bovine brain. *Arch. Biochem. Biophys.* 260,
