Allosteric activation of fructose-1,6-bisphosphatase (FBPase) from *Escherichia coli* by phosphoenolpyruvate implies rapid feed-forward activation of gluconeogenesis in heterotrophic bacteria. But how do such bacteria rapidly down-regulate an activated FBPase in order to avoid futile cycling? Demonstrated here is the allosteric inhibition of *E. coli* FBPase by glucose 6-phosphate (Glc-6-P), the first metabolite produced upon glucose transport into the cell. FBPase undergoes a quaternary transition from the canonical R-state to a T-like state in response to Glc-6-P and AMP ligation. By displacing Phe15, AMP binds to an allosteric site comparable with that of mammalian FBPase. Relative movements in helices H1 and H2 perturb allosteric activator sites for phosphoenolpyruvate. Glc-6-P binds to allosteric sites heretofore not observed in previous structures, perturbing subunits that in pairs form complete active sites of FBPase. Glc-6-P and AMP are synergistic inhibitors of *E. coli* FBPase, placing AMP/Glc-6-P inhibition in a new intermediate state (IT), which lies 12–13° away from the R-state of the mammalian enzyme (8). Moreover, concentration of AMP alone, with no exceptions, signature residues of allosteric activators appear in bacterial sequences along with key residues of the Glc-6-P site. FBPases in such organisms may be components of metabolic switches that allow rapid changeover between gluconeogenesis and glycolysis in response to nutrient availability.

Fructose-1,6-bisphosphatase (α-fructose-1,6-bisphosphate 1-phosphohydrolase; EC 3.1.3.11; FBPase) catalyzes the hydrolysis of fructose 1,6-bisphosphate (Fru-1,6-P₂) to fructose 6-phosphate (Fru-6-P) and P, and is a principal regulatory enzyme in gluconeogenesis (1, 2). Primary sequence comparisons infer five nonhomologous FBPases in living organisms. All five types exist in various prokaryotes, but the Type I enzyme is the only form in eukaryotes (3–8). The enteric bacterium *Escherichia coli* requires Type I FBPase for growth on gluconeogenic substrates (3). That enzyme is subject to regulation by metabolites (4, 7–9).

Type I FBPases are tetramers with subunits labeled C1–C4 by convention. The porcine enzyme, the most studied of all mammalian FBPases, adopts distinct quaternary states called R, I₁, and T (10–14). The porcine liver enzyme takes on an active R-state conformation in the absence of the inhibitor AMP, whereas the AMP-bound form assumes an inactive T-state that differs from the R-state by a 15° rotation between “top” and “bottom” subunit pairs (10–13). The competitive inhibitor fructose 2,6-bisphosphate (Fru-2,6-P₂) converts the enzyme to an intermediate state (I₃), which lies 12–13° away from the R-state (14).

The constitutive expression of both fructose-6-phosphatase-1-kinase (PFK) and FBPase in bacteria demands a strategy of coordinate metabolite regulation (3, 8, 15). Expression levels of FBPase and PFK in *E. coli* vary only 2–3-fold between glycolytic and gluconeogenic conditions, yet futile cycling remains low under both conditions (16–19). Phosphoenolpyruvate (PEP) and citrate are probable *in vivo* allosteric effectors in the feed-forward activation of *E. coli* FBPase and gluconeogenesis (8). Activators of *E. coli* FBPase bind to allosteric sites between subunit pairs C1–C2 and C3–C4, stabilizing a quaternary state nearly identical to the R-state of the mammalian enzyme (8). Moreover, concentrations of PEP rise coordinately with the availability of nonglucose nutrients (20, 21). But then how does *E. coli* respond to a sudden influx of glucose? Eukaryotic organisms produce Fru-2,6-P₂ to activate PFK and inhibit FBPase. *Saccharomyces cerevisiae*, for instance, produces inhibitory levels of Fru-2,6-P₂ within 1 min of glucose exposure (22). Heterotrophic bacteria, such as *E. coli*, however, have no known mechanism for the rapid inhibition of FBPase in response to a sudden influx of glucose.

Glucose is immediately phosphorylated to glucose 6-phosphate (Glc-6-P) upon transport into the bacterial cell via the action of either the PEP phosphotransferase system or glucokinase (23). Glc-6-P levels vary as much as 10-fold...
between *E. coli* grown on different carbon sources and can rapidly fluctuate 5–7-fold in response to nutrient availability (21). Glc-6-P, being downstream of the FBPase reaction and subject to nutrient-induced variations in concentration, has the desired attributes of a dynamic regulator of FBPase. A mutant strain of *E. coli*, lacking phosphoglucone isomerase and glucose-6-phosphate dehydrogenase activities, accumulates large amounts of intracellular Glc-6-P (∼50 mM, normal range 0.2–2 mM); its failure to grow on glycerol suggests in vivo inhibition of gluconeogenesis by Glc-6-P (21, 24).

We demonstrate here the inhibition of purified *E. coli* FBPase (specific activity 1000-fold higher than that used in Ref. 24) at physiologically relevant concentrations of Glc-6-P. Moreover, Glc-6-P enhances AMP inhibition by as much as 10-fold, even in the presence of 1 mM PEP. The latter property might allow Glc-6-P to override PEP activation of gluconeogenesis. Glc-6-P binds to distinct allosteric sites never before observed in FBPase structures, shearing subunits that pair off in forming complete active sites. *E. coli* FBPase, ligated by Glc-6-P and AMP, is in a T-like state, similar to the T1-state of the porcine enzyme. AMP displaces the side chain of Phe15 and axially dislocates helices H1 and H2 in opposite directions. Helix displacement distorts the PEP activation site, destabilizes the R-state, and allows Arg80 to stabilize the T-like state through its interaction with Glu6 from a symmetry-related subunit. Arg80 and Glu6 are conserved in organisms possessing signature residues of the PEP and Glc-6-P sites. Similarities between Glc-6-P and Fru-2,6-P2 inhibition suggest an evolutionary linkage; both inhibitors are one-reaction products of Fru-6-P, and both inhibit their respective FBPases synergistically with AMP.

**EXPERIMENTAL PROCEDURES**

**Materials**—Fru-1,6-P2, Glc-6-P, Fru-6-P, AMP, NADP+, and ammonium molybdate came from Sigma; PEP was from MP Biomedicals; and zinc acetate, ascorbic acid, and sulfuric acid were from Fisher. 1,5-Anhydro-6-glucitol 6-phosphate (AnG6P) was synthesized from 1,5-anhydro-d-glucitol (Toronto Research Chemicals) and quantified as previously described (25, 26). Glucose-6-phosphate dehydrogenase, phosphoglucoisomerase, and alkaline phosphatase were from Roche Applied Science. All other chemicals were of reagent grade.

**Enzyme Isolation and Purity**—Native and selenomethionine-substituted *E. coli* FBPases, prepared as previously described (7), are structurally and kinetically indistinguishable. Preparations of selenomethionine-substituted enzyme were used for structure determinations and native enzyme for kinetics. Purified enzymes migrate as single bands on SDS-PAGE, have specific activities of 35–40 units/mg, and have a single residue type (methionine or selenomethionine) at the N terminus.

**Kinetic Experiments**—Assays of *E. coli* FBPase use coupling enzymes phosphoglucone isomerase and glucose-6-phosphate dehydrogenase and monitor the formation of NADPH at 22 °C by either fluorescence emission at 470 nm or absorbance at 340 nm (13). Assay mixtures (total volume, 2 ml) included 50 mM Hepes, pH 7.5, 100 mM EDTA, and 150 μM NADP+ with up to saturating levels of Fru-1,6-P2 (40 μM) and MgCl2 (10 mM). Assays were initiated either by the addition of 1.4 μg of enzyme (enzyme-initiated assays) or by incubating the enzyme in assay mixtures for 1 h at 22 °C without MgCl2 and then initiating the reaction by the addition of metal (enzyme incubation assays).

Glc-6-P is an intermediate in the coupled assay. Hence, studies of Glc-6-P inhibition monitor FBPase turnover by the evolution of P1 (26). Reducing reagent was prepared daily (26) with the addition of H2SO4 to a final concentration of 1 N (4). Assay mixtures (50 mM Hepes, pH 7.5, 0.1 mM EDTA, 0.5 mM Fru-1,6-P2, 0.75 μg of enzyme, and varying amounts of PEP, AMP, and Glc-6-P in a total volume of 60 μl) were incubated in 96-well microtiter plates (Evergreen) at 25 °C for 1 h prior to the initiation of the reaction by the addition of 5 mM MgCl2. The addition of 250 μl of reducing agent at
fixed intervals quenched reactions. Plates were sealed and incubated at 25 °C for at least 2 h to allow color development. Absorbances (λ = 655 nm) were measured on a Bio-Rad Benchmark microplate reader. Blank-corrected standard curves relating \( A_{655} \) to [P] (0–1.5 mM) were linear (\( R^2 > 0.998 \)).

Determinations of kinetic mechanism require low concentrations of substrate and result in levels of Pi undetectable by the phosphate release assay. Such determinations employed the coupled assay with AnG6P as the inhibitor. AnG6P does not interfere with enzymes of the coupled assay (25).

Initial rate data taken at saturating substrate, fixed effector, and systematically varied inhibitor concentrations were fit to a Hill equation,

\[
V = \frac{V_{\text{max}}}{1 + \left(\frac{I}{I_0}\right)^n} + V_s
\]

where \( I \) represents the inhibitor concentration; \( V, V_{\text{max}} \), and \( V_s \) are the velocity, maximum velocity (at \( I = 0 \)), and the limiting velocity (at \( I \) saturating); \( n \) is the Hill coefficient associated with the inhibitor; and \( I_{0.5} \) is the inhibitor concentration at 50% inhibition. Data taken from experiments in which inhibitor/substrate or inhibitor/metal concentrations were varied systematically were fit to rapid equilibrium models for competitive, uncompetitive, noncompetitive, and mixed inhibition, fixing the Hill coefficient of the ligand (\( n = 1 \) or \( n = 2 \)) as circumstances dictate. AMP behaves cooperatively (\( n = 2 \)) in enzyme-initiated assays or in the presence of PEP and noncooperatively (\( n = 1 \)) in enzyme incubation assays in the absence of PEP (8). Kinetic data were fit to models using the programs IGOR-PRO (WaveMetrics) or DYNAFIT (27). Estimates of in vivo metabolite concentrations in \( E. \ coli \) were determined as previously described (8).

**Crystallization of the AMP-Glc-6-P Complex**—Crystals were grown by hanging drop in vapor diffusion VDX plates (Hampton Research). Solutions of sucrose and polyethylene glycol had 0.05% (w/v) NaN3 to reduce microbial growth. All other solutions were sterile filtered prior to use. AMP/Glc-6-P-bound FBPase crystals grew from droplets containing 2 μl of a protein solution (15 mg/ml enzyme, 20 mM dithiothreitol, 0.1 mM EDTA, 5 mM Fru-1,6-P₂, 5 mM MgCl₂, and 5 mM AMP) and 2 μl of a precipitant solution (50 mM MES-NaOH, pH 6.5, 13% (w/v) polyethylene glycol 10,000, and 20% (w/v) sucrose) and were equilibrated over 500 μl of precipitant solution. All components of the protein solution were combined, except AMP, and incubated at room temperature for 30 min, allowing for the enzyme-catalyzed equilibration of Fru-1,6-P₂, Fru-6-P, and Glc-6-P prior to the addition of AMP. (FBPase isolated as described here has endogenous phosphoglucose isomerase activ-

---

### Table 1

| [PEP] (μM) | \( I_{0.5} \) AMP (μM) | \( I_{0.5} \) AMP with 500 μM Glc-6-P (μM) | \( I_{0.5} \) Glc-6-P (μM) |
|------------|------------------------|----------------------------------------|------------------------|
| 0          | 8 ± 2                  | 6 ± 1                                  | 38 ± 6                 |
| 50         | 12 ± 2                 | 4 ± 1                                  | 100 ± 20               |
| 1000       | 31 ± 7                 | 3 ± 2                                  | 340 ± 40               |

---

**FIGURE 2. Overview of the AMP-Glc-6-P complex.** Four subunits of the tetramer, labeled C1–C4 by convention, bind Fru-1,6-P₂ and two Mg²⁺ ions at the active site. AMP binds at an allosteric site corresponding to that of porcine FBPase. Helices H1 and H2 (dark lines and shading) contribute residues to the anion activator sites that lie between the top and bottom halves of the tetramer. Chloride ions probably occupy the sites in the present structure. Glc-6-P binds at a novel allosteric site at the interface between the Fru-1,6-P₂ domains of subunits C1 and C2 (as well as C3 and C4). Electron density is from \( F_o - F_c \); omit maps contoured at 3σ, using a cut-off radius of 2.0 Å. This drawing was prepared with MOLSCRIPT (44).
Allosteric Inhibition of E. coli FBPase

ity that produces Glc-6-P from Fru-6-P. Purifying a C-terminal polyhistidyl-tagged FBPase on nickel-nitrotriacetic acid-agarose (Novagen) removes the trace impurity, but the tagged FBPase has undetermined properties of crystallization. Rodlike crystals (0.2 × 0.2 × 0.8 mm) grew within 3 days at 22 °C. Crystals were cryoprotected by immersion for 60 s in a solution of 18% (w/v) polyethylene glycol 20,000, 20% (v/v) 2-methyl-2,4-pentanediol, and 50 mM MES-NaOH, pH 6.5, supplemented with dithiothreitol, EDTA, and ligands immediately prior to freezing in a cold stream of nitrogen.

Data Collection—Crystals were screened 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 temperature of 110 K. Data were collected from a single crystal at 100 K on Beamline 4.2.2 of the Advanced Light Source, Lawrence Berkeley Laboratory. The program d'trek (28) was used to index, integrate, scale, and merge intensities, which were then converted to structure factors using the CCP4 (29) program TRUNCATE (30).

Structure Determination and Refinement—The program AMORE (31) and a single subunit (less ligands) from the sulfate-bound E. coli FBPase structure (Protein Data Bank accession code 2GQ1) were used in constructing the asymmetric unit (32). Multiple sequence alignments of Type I FBPases (7) 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. Ligands (Fru-1,6-P₂, Glc-6-P, AMP, Mg²⁺, HPO₄²⁻, and Cl⁻) and water molecules were fit to omit electron density until no improvement in R_free was evident. Water molecules with thermal parameters above 60 Å² or more than 3.2 Å from the nearest hydrogen bonding partner were removed from the final model.

Structure and Sequence Comparisons of FBPases—Tetramer models of E. coli and porcine FBPases were constructed from crystallographic asymmetric units and used in pairwise superpositions using the CCP4 programs PDBSET (35) and LSQKAB (36). Displacements between Cα positions were measured using XTALVIEW (32). The canonical R- and T-states of porcine FBPase have Protein Data Bank identifiers 1CNQ and 1EYK, respectively, whereas the I₁- and I₂₆-states have identifiers 1Q9D and 1YYY. The measured angle of rotation between subunit pairs is sensitive to the set of residues used in the superposition. Hence, previously established residues employed in the comparison of E. coli and porcine FBPases were used for alignments (7). Multiple sequence alignments of Type I FBPases employed the program ClustalW (37).

RESULTS

Inhibition of E. coli FBPase by Glc-6-P and AMP—AMP is a noncompetitive inhibitor of E. coli FBPase with respect to the substrate Fru-1,6-P₂ under all methods of assay and in the presence or absence of PEP. Kᵢ values range from 2 to 100 μM, being sensitive to the conditions of assay and PEP concentration. At saturating concentrations of Fru-1,6-P₂ (40 μM) and in the presence of 1 mM PEP, AMP inhibition is mixed with respect to Mg²⁺. The constant of dissociation for AMP from the enzyme-Fru-1,6-P₂ complex (Kᵢ (Kₗ₋ is 13 ± 3 μM)) is 7-fold less than that for the dissociation of AMP from the enzyme-Fru-1,6-P₂-Mg²⁺ complex (Kᵢ (Kₗ₋ is 94 ± 10 μM)). AMP is a competitive inhibitor with respect to Mg²⁺ for porcine FBPase (38).

The determination of the kinetic mechanism of Glc-6-P inhibition employed the coupled assay and the analog AnG6P (which inhibits with 5–10-fold lower affinity than Glc-6-P). In assays with Fru-1,6-P₂ or Mg²⁺ at saturating concentrations and 1 mM PEP, AnG6P inhibited E. coli FBPase noncompetitively with respect to both Fru-1,6-P₂ (Kᵢ (Kₗ₋ is 660 ± 70 μM)) and Mg²⁺ (Kᵢ (Kₗ₋ is 850 ± 70 μM)). Glc-6-P inhibition, as measured by the phosphate release assay, varied with fixed concentrations of PEP and AMP (Fig. 1 and Table 1). PEP antagonizes AMP and Glc-6-P inhibition. I₃₅ values for Glc-6-P and AMP inhibition increase 10- and 4-fold, respectively, over a range of 0–1 mM PEP. 500 μM Glc-6-P enhances AMP inhibition by 10-fold.

Structure of the AMP-Glc-6-P Complex of E. coli FBPase (Protein Data Bank Accession Code 2Q8M)—Crystals belong to the space group P4₁2₁2₁ (a = b = 124.6, c = 132.3 Å) with a C₁–C₄ dimer pair in the asymmetric unit (Fig. 2 and Table 2). Additional subunits related by crystallographic symmetry complete the biological tetramer. Four AMP and two Glc-6-P molecules bind in full occupancy to the tetramer. AMP molecules are at allosteric sites corresponding to those of the porcine tetramer (10), whereas Glc-6-P molecules bind at allosteric sites that incorporate a 2-fold axis of molecular/crystallographic symme-

### Table 1

| Glc-6-P AMP E. coli FBPase |  |
|----------------------------|--|
| Resolution (Å) | 37.8-2.05 (2.12-2.05) |
| Total reflections/unique reflections | 154,807/63,536 |
| Average redundancy | 2.4 (2.5) |
| Completeness (%) | 96.5 (95.7) |
| Mean Rmerge | 0.068 (0.255) |
| I/σ(I) | 9.2 (5.5) |
| No. of atoms | 5305 |
| No. of solvent sites | 312 |
| Rfree (%) | 20.9 |
| Rfree* | 23.5 |

### Table 2

Statistics of data collection and refinement of the AMP-Glc-6-P complex

Space group and unit cell parameters are described under “Results.” Parenthetical values pertain to the data shell of highest resolution. Ramachandran plots from PROCHECK (45) indicate that >90% of residues are in most favored regions and no residues are in disallowed conformations.

---

4 J. K. Hines and R. B. Honzatko, unpublished observations.
try. Hence, Glc-6-P in the model adopts mutually exclusive modes of binding related by symmetry (Fig. 2). Chloride ions (a total of six per tetramer) and water molecules occupy conformationally altered sites associated with PEP activation in the R-state (8). Fru-1,6-P₂ and two Mg²⁺ are in each active site. The electron density for the 1-phosphoryl groups and Mg²⁺, however, is less robust than that for the rest of the ligand. A mixture of Fru-1,6-P₂ and Fru-6-P probably binds to the active site. To reflect this circumstance, occupancy factors for 1-phosphoryl groups and Mg²⁺ are set to 0.5.

Magnesium ions occupy loci analogous to metal sites 1 and 2 of porcine FBPase (39) and have five and six coordinating oxygen atoms, respectively. A complete list of protein ligand interactions appears in Table 3. The model does not include residues 46–67 (most of the dynamic loop and the N-terminal end of helix H3) due to the absence of interpretable electron density; however, residues 42–46 at the N-terminal end of the loop occupy a position consistent with the disengaged conformation of the dynamic loop in T-state models of porcine FBPase (40).

The angle of rotation of subunit pair C1–C2 relative to C3–C4 is 9° (Fig. 3). (By convention, the R-state of porcine FBPase defines as subunit pair rotation angle of 0°, equivalent to that of the E. coli R-state). The subunit pair rotation of the AMP-Glc-6-P tetramer is nearest to the I₁-state of the porcine enzyme (12–13°) (14). In addition, the subunit pair C2–C3 rotates relative to subunit pair C1–C4 about the molecular 2-fold axis that relates subunits C1 and C4 (Fig. 3). The rotation is modest (3°) but causes a significant shear between the Fru-1,6-P₂ domains (residues 200–337) of subunits C1 and C2 (as well as the Fru-1,6-P₂ domains of subunits C3 and C4). Finally, each individual subunit undergoes a rotation of 3° about an axis that passes through its center of mass (Fig. 3).

**Glc-6-P Site of Binding and Mechanism of Inhibition—Glc-6-P binds as the β-anomer to an allosteric site that incorporates a 2-fold axis of symmetry. Glc-6-P, itself lacking 2-fold symmetry, binds then with equal weight in two mutually exclusive orientations. Acceptable interactions are possible for the two orientations due in part to the chair conformation of the pyranose ring and the equatorial positions of the four hydroxyl groups. Rotation of bound Glc-6-P about the symmetry axis superimposes all hydroxyl groups and atoms of the pyranose ring onto nearly equivalent atom types and positions. Omit electron density (Fig. 2) clearly defines the chirality of each of four hydroxyl-bearing carbons of the pyranose ring. Bound in either orientation, Glc-6-P mediates interactions across the C1–C2 interface involving Glu²²⁵, Lys²²², and Tyr²¹⁰ (Fig. 4). The hydroxyl group of Tyr²¹⁰ hydrogen bonds with two water molecules that in turn hydrogen-bond with the 1-hydroxyl and 6-phosphoryl groups of Glc-6-P. In the 2-fold-related orientation, Tyr²¹⁰ and its two water molecules interact with the 4-hydroxyl and 6-phosphoryl groups of Glc-6-P (Table 3).

In the absence of structures with only one bound ligand, we cannot ascribe conformational changes with certainty to any specific ligand. Glc-6-P, however, seems responsible for the shear between Fru-1,6-P₂ domains caused by the rotation of subunit pair C2–C3 relative to subunit pair C1–C4.

### Table 3

| Ligand, group, and/or atom | Binding partner | Distance (Å) |
|----------------------------|-----------------|--------------|
| Fru-1,6-P₂                 |                 |              |
| 1-Phosphoryl O11           | Glu¹⁴⁴ N        | 2.6          |
| 1-Phosphoryl O13           | Mg²⁺ (Site 2)   | 2.0          |
| 1-Phosphoester O1          | Mg²⁺ (Site 1)   | 2.5          |
| 3-Hydroxyl                 | Asp¹⁰¹ OD1      | 2.5          |
| 4-Hydroxyl                 | Asp¹⁰¹ OD2      | 3.1          |
| Endocyclic O5              | Leu²⁴⁵ N        | 2.9          |
| 6-Phosphoester O6          | Wat⁴⁵          | 2.7          |
| 6-Phosphoester O61         | Tyr²⁷⁷ OH       | 2.7          |
| 6-Phosphoryl O62           | Lys²⁶⁸ NZ       | 3.0          |
| 6-Phosphoryl O63           | Lys²⁶⁸ NZ       | 2.9          |
| AMP                        |                 |              |
| N1                         | Wat³⁷ (Tyr²⁹⁸)  | 3.2          |
| N6                         | Thr¹⁴¹ OG¹      | 3.1          |
| N7                         | Ile¹² O         | 3.0          |
| 2'-Hydroxyl                | Wat³⁶³ (Thr²³⁵ N) | 2.9       |
| 3'-Hydroxyl                | Wat³⁶³          | 3.0          |
| 5'-Phosphoryl O1A          | Tyr²⁰⁸ OH       | 2.7          |
| 5'-Phosphoryl O2A          | Thr¹⁹ OG¹      | 2.8          |
| 5'-Phosphoryl O3A          | Thr¹⁹ N        | 2.7          |
| Glc-6-P                    |                 |              |
| 1-Hydroxyl                 | Glu²²² OE¹      | 2.6          |
| 2-Hydroxyl                 | Glu²²² NE²      | 2.9          |
| 3-Hydroxyl                 | Glu²²² NE²*     | 2.8          |
| 4-Hydroxyl                 | Glu²²² OE¹*     | 2.8          |
| 6-Phosphoester O6          | Lys²²² NZ*      | 2.8          |
| 6-Phosphoester O1P         | Lys²²² NZ       | 2.7          |
| 6-Phosphoryl O2P           | Lys²²² (Tyr²⁰⁸*) | 3.0       |
| Mg²⁺ (Site 1)              |                 |              |
| Fru-1,6-P₂, O1             | Fru-1,6-P₂, O1  | 2.1          |
| Fru-1,6-P₂, O13            | Fru-1,6-P₂, O13 | 2.5          |
| Glu²⁷⁷ OE¹                 | 2.2          |
| Asp¹⁰¹ OD1                 | 1.9          |
| Asp¹⁰¹ OD2                 | 2.3          |
| Mg²⁺ (Site 2)              |                 |              |
| Fru-1,6-P₂, O13            | 2.0          |
| Asp¹⁰¹ OD2                 | 2.2          |
| Glu¹⁴⁴ OE¹                  | 2.4          |
| Leu²⁴⁵ O                   | 2.5          |
| Wat⁴⁵                      | 2.2          |
| Glu⁶⁹ (Glu⁴⁶)              | 2.2          |
Allosteric Inhibition of E. coli FBPase

AMP Site of Binding and Mechanism of Inhibition—AMP binds to E. coli FBPase at an allosteric site corresponding to that of the porcine enzyme (10) (Figs. 2 and 5). The backbone carboxyl of Ile8 and the side chain of Thr22 hydrogen-bond with the 6-amino group of the adenine base, and Tyr169 and Thr171 hydrogen-bond with the adenine base through water molecules (Table 3). Residues 19–23 (part of the loop connecting helices H1 and H2), Lys104, and Tyr105 interact with the 5′-phosphoryl group. Lys11, Glu12, Phe15, Ile18, Leu22, Leu26, Val154, and Thr171 are in contact with the adenine base and/or ribose sugar. Backbone atoms and aliphatic side chain atoms of Lys11, Glu12, Phe15, and Leu22 sandwich the adenine base in stacking interactions (Fig. 5).

Conformational changes in the immediate neighborhood of AMP include a 3-Å movement of helix H2 relative to helix H1 (Fig. 6). Helix H2, slightly bent in the R-state complex, is straight in the AMP-Glc-6-P complex. The movement of helix H2 and the rotation of subunit pair C1–C2 relative to C3–C4 reduce the volume of symmetry-paired PEP sites at the C1–C4 interface. The distance between Cα atoms of Thr22 in subunits C1 and C4, for instance, is 18.5 Å in the R-state and 16 Å in the AMP-Glc-6-P complex. In the R-state, each activation site divides into binding loci for the phosphoryl and carboxyl groups of PEP, a total of four loci for the symmetry-paired sites. In the AMP-Glc-6-P complex, only three loci remain; the two binding loci for the carboxyl groups of PEP merge into a single locus. A spheroid of OMIT electron density appears in each of the three loci. Attempts to model water molecules, metal ions, cryoprotectants, or MES did not account for the electron density and/or provide plausible interactions with the protein. Chloride ions, present in crystallization experiments at a concentration of ~5 mM, provided the best fit to the electron density, a reasonable balance of formal electrostatic charge, and appropriate donor-acceptor distances in hydrogen bonding interactions. Additionally, ordered water molecules fill the remaining space between subunits C1 and C4.

Arg80 undergoes a significant change in conformation, linked directly to perturbations at the PEP pocket. Arg80, which in the R-state interacts with PEP, moves out of the PEP pocket into an intersubunit salt link with Glu6 (Fig. 7). The new interactions of Arg80 may be less effective in stabilizing the position of helix H3 than its former interactions in the R-state. Destabilization of helix H3 could perturb residues 46–67 at the N-terminal side of helix H3. If residues 46–67 are a dynamic loop involved in the binding of essential metal cofactors, then disorder in helix (Fig. 3). At the C1–C2 subunit interface of the Fru-1,6-P2 domains, symmetry equivalent residues exhibit displacements of ~1 Å. For instance, distances between symmetry-related Cα atoms of Glu225 and Lys222 increase by 1.5 and 0.8 Å, respectively, relative to the R-state complex. Active sites of FBPase combine residues from juxtaposed Fru-1,6-P2 domains. Hence, the observed shear may account for Glc-6-P inhibition.
H3 probably impairs catalysis by destabilizing the active conformation of that loop.

A second mechanism of inhibition associates disorder in helix H3 with conformational change in Glu89 and its chelation of metal cofactors at metal site 2. Traces of the two polypeptide chains in the asymmetric unit of the crystal are everywhere identical (within experimental uncertainty) except for residues 89-100. In chain A, Glu89, Gly90, and Tyr210 participate in lattice contacts, and residues 97–100 form a Type II reverse turn similar in conformation to that of the R-state subunit. In the absence of lattice contacts in chain B, residues 97–100 adopt an extended conformation. Conformational changes in residues 89 and 90 compensate for those of 97–100, limiting differences to residues 89–100. DespitedifferencesintheCαpositionsofGlu89inearachsubunit, the side chains remain coordinated to Mg2+ (Table 3). Nonetheless, destabilization of helix H3 by AMP displacement of PEP could perturb turn 97–100, Glu89, and the binding of Mg2+ at site 2.

Conservation of Residues Involved in Allosteric Regulation—Sequence alignments of Type I FBPases from more than 250 organisms demonstrate the probable conservation of the Glc-6-P binding site in FBPases that have an anion activation site (8). The only exception is the FBPase from the eukaryotic organism Toxoplasma gondii, which has only an activation site. The Glc-6-P binding site is absent in eukaryotic FBPases. Three signature residues (Tyr210, Lys222, and Gln225) infer Glc-6-P binding, and with the exception of a single lysine to arginine substitution, these three residues are invariant among these heterotrophic organisms (Table 4). Residues of the Glc-6-P pocket are conserved largely due to the lack of suitable alternatives. For example, a phenylalanine replacement for Tyr210 eliminates significant hydrogen bonds involving the aromatic hydroxyl group. A glutamate substitution for Gln225 introduces unbalanced charge, and nonisosteric replacements will disrupt hydrogen bonds. Lys222 could be replaced by arginine (and this may be the case in Flavobacterium sp. MED217), but the occurrence of this replacement in nature is rare. The Glc-6-P pocket seems to be an all-or-nothing proposition.

Other sequence-conserved residues are involved in conformational change associated with effector interactions. Phe15 occupies the AMP site when the enzyme is in the R-state and then packs against the bound AMP molecule in the inhibited state. Conformational changes in Phe15 and AMP ligation arguably are linked phenomena. Gly20 is conserved in most eukaryotes and some prokaryotes. It may be a hinge that allows the connecting element between helices H1 and H2 to undergo conformational change in response to AMP ligation. The ϕ/ψ angles of Gly20 change from ϕ/ψ = −55/−40° in the R-state to ϕ/ψ = 105°/−1° in the T-like state. Hence, the residue goes through a disallowed region of the Ramachandran plot as it passes between conformational states. The reason for sequence conservation of Glu6 along with residues of the PEP site is now clear. In the R-state, Glu6 hydrogen-bonds with solvent and with the backbone amide of Thr13, whereas in the AMP–Glc-6-P complex, Arg80 interacts directly with Glu6 from a symmetry-related subunit (Fig. 7). Arg80 and Glu6 are absent in FBPases from organisms that produce Fru-2,6-P2.
Allosteric Inhibition of E. coli FBPase

![Diagram of E. coli FBPase]  

**FIGURE 7. Interactions of Arg\(^{80}\) in the R-state PEP complex and the AMP Glc-6-P complex.** Electron density is from a 2F\(_{\alpha}\) – F\(_{\alpha}\) map contoured at 1σ and using a cut-off radius of 2 Å. Helices H1, H2, and H3 are shown by thick lines with dark shading. Arg\(^{80}\) from subunit C4 interacts with subunit C1 through a bridging molecule of PEP (left). Arg\(^{80}\) from subunit C4 interacts with Glu\(^{6}\) of subunit C1 in the AMP-Glc-6-P complex (right). The tetramer icon illustrates the viewing direction and region. Parts of this drawing were prepared with MOLSCRIPT (44) and XTALVIEW (32).

**TABLE 4**  
Consensus residue types indicative of allosteric regulation in bacterial FBPases  

| Sequence position in E. coli FBPase | Consensus residue type | Exceptions |
|------------------------------------|------------------------|------------|
| Glc-6-P site                       |                        |            |
| 210                                | Tyr                    | None       |
| 222                                | Lys                    | Flavobacterium sp. MED217 (Arg) |
| 225                                | Gln                    | None       |
| PEP/citrate activation site        |                        |            |
| 5                                  | Gly                    | Haemophilus influenzae (Ser) |
| 30                                 | Lys/Arg                | None       |
| 80                                 | Arg                    | None       |
| 186                                | Val/Thr/Ser            | Actinobacillus pleuropneumoniae (Lys) | Reinekeia sp. MED297 (Ile) |
| PEP/AMP allosteric mechanism       |                        |            |
| 6                                  | Glu                    | Pasteurella multocida (Gln) | Haemophilus somnus (Gln) |
| 15                                 | Phe/Tyr                | None       |
| 20                                 | Gly                    | None       |

**DISCUSSION**

The interactions and sequence conservation of Glu\(^{6}\) and Arg\(^{80}\) support a tetrameric structure for the inhibited state of E. coli FBPase. Hydrogen bonds between Arg\(^{80}\) and PEP (R-state) and Arg\(^{80}\) and Glu\(^{6}\) (AMP-Glc-6-P complex) are analogous to C1–C4 subunit interactions that are broken and reform when porcine FBPase goes from its R- to T-state (10, 13). A subunit dimer of E. coli FBPase is an alternative model for the inhibited state, but evidence exists for a dimer only in the absence of PEP and anionic ligands. The dimer could exist transiently in vivo during the transition between active and inhibited states of the tetramer, but at present we have no evidence for a dimer as a major form of FBPase under physiological conditions. Moreover, the “chloride” binding sites of the AMP-Glc-6-P complex could recognize a ligand of physiological significance that confers stability to an inhibited tetramer. The AMP-Glc-6-P tetramer observed here may indeed be the T-state of E. coli FBPase, but the C1–C2 subunit pair rotation angle falls 6° short of that for the T-state of porcine FBPase. We cannot exclude the possibility of yet another ligand binding (perhaps to the chloride sites) along with AMP and Glc-6-P and causing additional subunit movements.

An unambiguous determination of the individual effects of Glc-6-P and AMP on the structure of E. coli FBPase requires structures with only a single ligand. Unfortunately, the enzyme has yet to crystallize in the presence of Glc-6-P or AMP alone. Lack of success here may be due in part to the synergy of Glc-6-P and AMP inhibition. E. coli FBPase with only one bound ligand may not be stable as a tetramer or may exist as a tetramer in a manifold of quaternary states. If we associate conformational change with the proximal ligand, then AMP would induce the C1–C2 subunit pair rotation, and Glc-6-P would induce the C2–C3 subunit pair rotation. Synergism in AMP and Glc-6-P inhibition may be due to the rotations of monomers (Fig. 3), which perturb both the C1–C2 and C1–C4 interfaces of the tetramer. Monomer rotations, caused independently by AMP and Glc-6-P, would result in thermodynamic linkage and the manifestation of binding synergism. Allosteric inhibition comes about presumably by the disruption of metal cofactors through the destabilization of helix H3 and by the perturbation of the 6-phosphoryl pocket due to the shear dislocation at the C1–C2 interface. The mechanism differs from porcine FBPase, since no displacement is evident between subunits C1 and C2 in the R- to T-state transition (39).

Glc-6-P and AMP at saturating levels do not cause complete inhibition of E. coli FBPase (Fig. 1), in contrast to the complete inhibition of porcine FBPase by AMP. The most obvious structural difference between the two FBPases is the absence of the disengaged conformation of the dynamic loop in the E. coli system. The porcine system stabilizes a conformation in which the dynamic loop packs with hydrophobic residues well removed from the active site. Stabilization of a disengaged loop is not evident in AMP-Glc-6-P complex. Interestingly, maximal AMP inhibition of the Ile\(^{10}\) → Asp mutant of porcine FBPase is ~50% (41). The dynamic loop in Ile\(^{10}\) → Asp porcine FBPase

---

24704 JOURNAL OF BIOLOGICAL CHEMISTRY

VOLUME 282 • NUMBER 34 • AUGUST 24, 2007
Alterations in the functional state of E. coli FBPase, something (AMP or Phe\textsuperscript{5}) always occupies the AMP pocket, unlike porcine FBPase, which has an accessible AMP pocket in its R-state.

E. coli FBPase can exist in distinct functional states, acting as a regulatory switch in response to natural effectors (Fig. 8). ADP activates, but PEP and citrate inhibit, PFK (23, 42, 43). PEP and citrate, on the other hand, activate FBPase (8), whereas AMP is inhibitory (4, 8, 9). Unlike AMP, the concentration of which is relatively constant \textit{in vivo}, Glc-6-P is a dynamic inhibitor of FBPase. Its concentration rises in concert with the availability of glucose. Levels of Glc-6-P that cause inhibition of \textit{E. coli} FBPase \textit{in vitro} match the range of Glc-6-P concentrations \textit{in vivo} (21). Although activation of FBPase by PEP is potent (8), physiologic combinations of Glc-6-P and AMP override activation even at PEP concentrations of 1 mM (Table 1). Hence, PEP and citrate under physiologic conditions become activators of FBPase only if Glc-6-P levels are low.

As with the anion activation site (8), the Glc-6-P binding site may be a possible target for antibiotic development. Structure and sequence information demonstrates the presence of the site in pathogenic bacteria and its absence in eukaryotic FBPases. The impact of a FBPase-targeted drug on a pathogen, however, depends on its local environment in the host. FBPase is critical for growth of \textit{E. coli} in the absence of glucose. Nonetheless, the presence of at least three distinct allosteric effector sites on \textit{E. coli} FBPase infers a requirement for rapid and precise regulation of the FBPase/PFK control point of glycolysis and gluconeogenesis. Loss of coordinate regulation will probably diminish the vigor of the organism.

Acknowledgments—We thank Dr. Xiaoming Chen, who synthesized and purified the AmbG6P; Dr. Jay Nix, who assisted with data acquisition and processing at Beamline 4.2.2 of the Advanced Light Source (Lawrence Berkeley Laboratory); and Professor S. Ramaswamy (Department of Biochemistry, University of Iowa) for providing synchrotron resources of the Molecular Biology Consortium.

## REFERENCES

1. Benkovic, S. T., and de Maine, M. M. (1982) \textit{Adv. Enzymol. Relat. Areas Mol. Biol.} 53, 45–82
2. Tejwani, G. A. (1983) \textit{Adv. Enzymol. Relat. Areas Mol. Biol.} 54, 121–194
3. Fraenkel, D. G., and Horecker, B. L. (1965) \textit{J. Bacteriol.} 90, 837–842
4. Fraenkel, D. G., Pontremoli, S., and Horecker, B. L. (1966) \textit{Arch. Biochem. Biophys.} 114, 4–12
5. Sato, T., Imanaka, H., Rashid, N., Fukui, T., Atomi, H., and Imanaka, T. (2004) \textit{J. Bacteriol.} 186, 5799–5807
6. Donahue, J. L., Bowman, J. L., Niehaus, W. G., and Larson, T. J. (2000) \textit{J. Bacteriol.} 182, 5624–5627
7. Hines, J. H., Fromm, H. J., and Honzatko, R. B. (2006) \textit{J. Biol. Chem.} 281, 18386–18393
8. Hines, J. H., Fromm, H. J., and Honzatko, R. B. (2007) \textit{J. Biol. Chem.} 282, 11696–11704
9. Babul, J., and Guixe, V. (1983) \textit{Arch. Biochem. Biophys.} 225, 944–949
10. Zhang, Y., Liang, J.-Y., Huang, S., and Lipscomb, W. N. (1994) \textit{J. Mol. Biol.} 244, 609–624
11. Ke, H., Zhang, Y., and Lipscomb, W. N. (1990) \textit{Proc. Natl. Acad. Sci. U. S. A.} 87, 5243–5247
12. Shyur, L. F., Aleshin, A. E., Honzatko, R. B., and Fromm, H. J. (1996) \textit{J. Biol. Chem.} 271, 33301–33307
13. Iancu, C. V., Mukund, S., Fromm, H. J., and Honzatko, R. B. (2005) \textit{J. Biol. Chem.} 280, 15498–15505
Allosteric Inhibition of E. coli FBPase

14. Choe, J.-Y., Nelson, S. W., Arienti, K. L., Axe, F. U., Collins, T. L., Jones, T. K., Kimmich, R. D., Newman, M. J., Norvell, K., Ripka, W. C., Romano, S. I., Short, K. M., Slee, D. H., Fromm, H. J., and Honzatko, R. B. (2003) J. Biol. Chem. 278, 51176–51183
15. Pilkis, S. J., El-Maghrabi, M. R., and Claus, T. H. (1988) Annu. Rev. Biochem. 57, 755–783
16. Chin, A. M., Feldheim, D. A., and Saier, M. H., Jr. (1989) J. Bacteriol. 171, 2424–2434
17. Oh, M.-K., Rohlin, L., Kao, K. C., and Liao, J. C. (2002) J. Biol. Chem. 277, 13175–13183
18. Chambost, J.-P., and Fraenkel, D. G. (1980) J. Biol. Chem. 255, 2867–2869
19. Daldal, F., and Fraenkel, D. G. (1983) J. Bacteriol. 153, 390–394
20. Franzen, J. S., and Binkley, S. B. (1960) J. Biol. Chem. 246, 6511–6521
21. Lowry, O. H., Carter, J., Ward, J. B., and Glaser, L. (1971) J. Biol. Chem. 246, 6511–6521
22. Drueckes, P., Schinzel, R., and Palm, D. (1995) Anal. Biochem. 230, 173–177
23. Kraulis, J. (1991) J. Appl. Crystallogr. 24, 946–950
24. Laskowski, R. A., MacArthur, M. W., Moss, D. S., and Thornton, J. M. (1993) J. Appl. Crystallogr. 26, 283–291