The enteric bacterium *Escherichia coli* requires fructose-1,6-bisphosphatase (FBPase) for growth on gluconeogenic carbon sources. Constitutive expression of FBPase and fructose-6-phosphate-1-kinase coupled with the absence of futile cycling implies an undetermined mechanism of coordinate regulation involving both enzymes. Tricarboxylic acids and phosphorylated three-carbon carboxylic acids, all intermediates of glycolysis and the tricarboxylic acid cycle, are shown here to activate *E. coli* FBPase. The two most potent activators, phosphoenolpyruvate and citrate, bind to the sulfate anion site, revealed previously in the first crystal structure of the *E. coli* enzyme. Tetramers ligated with either phosphoenolpyruvate or citrate, in contrast to the sulfate-bound structure, are in the canonical R-state of porcine FBPase but nevertheless retain sterically blocked AMP pockets. At physiologically relevant concentrations, phosphoenolpyruvate and citrate stabilize an active tetramer over a less active enzyme form of mass comparable with that of a dimer. The above implies the conservation of the R-state through evolution. FBPases of heterotrophic organisms of distantly related phylogenetic groups retain residues of the allosteric activator site and in those instances where data are available exhibit activation by phosphoenolpyruvate. Findings here unify disparate observations regarding bacterial FBPases, implicating a mechanism of feed-forward activation in bacterial central metabolism.

Fructose-1,6-bisphosphatase (α-fructose-1,6-bisphosphate 1-phosphohydrolase, EC 3.1.3.11; FBPase) hydrolyzes fructose 1,6-bisphosphate (Fru-1,6-P₂) to fructose 6-phosphate (Fru-6-P) and inorganic phosphate (Pᵢ) (1, 2) and is a principle regulatory enzyme in gluconeogenesis (1, 2). With few exceptions, FBPase exists in nearly all organisms (2) and at some level in nearly all tissues (3). Mammalian FBPases are tetrameric and adopt distinct quaternary states. In the absence of AMP, the enzyme is in an active R-state conformation, 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 (4–7). In principle, hormonal control of levels of fructose 2,6-bisphosphate (Fru-2,6-P₂), a potent inhibitor of FBPase in mammals, determines flux through the gluconeogenic pathway (8, 9). Little is known, however, regarding the regulation of FBPases in prokaryotes, organisms that lack Fru-2,6-P₂.

Prokaryotic organisms express a diverse group of FBPases, classified by levels of sequence homology. The Type I enzyme, which is the subject of this investigation, is the most widely distributed among living organisms, being the primary FBPase in *Escherichia coli*, most bacteria, a few Archaea, and all eukaryotic organisms known to possess the gluconeogenic pathway (10–14). Many bacteria also express a second kind of FBPase (Type II), which in *E. coli* is encoded by the *glpX* gene (13). The physiological role of the Type II enzyme in *E. coli* is unclear, but it may be the principle FBPase in other organisms (13, 15, 16). Some organisms of the phyla Firmicutes and Fusobacteria, and several of the class Bacteroides, also possess a third kind of FBPase (Type III), whereas Type IV FBPase is prevalent in Archaea and Type V in thermophilic Archaea and at least one hyperthermophilic bacterium (17–19).

Type I FBPase in *E. coli* most likely generates anabolic precursors for nucleotide, polysaccharide, and aromatic amino acid biosynthesis from gluconeogenic substrates (10, 11). Fructose-6-phosphate-1-kinase (PFK) and FBPase define a futile cycle (20). Fraenkel and Horecker (10), who originally identified the enzyme in *E. coli*, reported the constitutive expression of FBPase under all growth conditions and suggested the need for a mechanism of metabolic control. Subsequent studies of FBPases in bacteria demonstrated only 2–3-fold variations in enzyme levels under different conditions of growth (10, 21, 22). F futile cycling, nonetheless, is minimal under both glycolytic (23) and gluconeogenic (24) growth conditions. AMP is the only known physiological inhibitor of *E. coli* FBPase (25); however, AMP levels remain relatively constant under all growth conditions (26). Additionally, an *E. coli* strain, deficient in wild-type FBPase but complemented with an AMP-insensitive mutant of FBPase, exhibits normal growth (25). Hence, AMP is
an unlikely dynamic regulator of *E. coli* FBPase *in vivo*. Phosphoenolpyruvate (PEP) on the other hand relieves AMP inhibition (27, 28) and could be a physiological activator of FBPase in *E. coli* (14, 27). PEP activates the Type I enzyme from thermophilic bacterium *Flavobacterium thermophilum* HB88 (29) and the non-homologous Type II and Type III enzymes from *E. coli* (13) and the genus *Bacillus* (30, 31). Before our previous investigation (14), the only proposed mechanism for PEP activation was the possible competition between PEP and AMP for a common site on the Type III enzyme from *Bacillus licheniformis* (30).

The first crystal structure of an FBPase from bacteria revealed several bound sulfate anions, one of which is at a putative site of allosteric activation (14). New conditions of crystal growth have resulted here in structure determinations of *E. coli* FBPase with bound PEP and with bound citrate. These structures confirm the site of allosteric activation implied by the ligation of sulfate. Unlike AMP, PEP concentrations in bacteria can fluctuate by more than 10-fold between glycolytic and gluconeogenic conditions of growth (32). Variations in PEP concentration could be the basis of coordinate regulation of FBPase and PFK, as PEP is also an inhibitor of the latter enzyme (33).

We report the direct activation of *E. coli* FBPase by PEP, citrate, and similar metabolites, providing strong evidence of feed-forward activation of gluconeogenesis in bacteria belonging to distinct phylogenetic groups. PEP and citrate may activate these bacterial FBPases directly by stabilizing the R-state tetramer and indirectly by blocking the AMP pocket via the insertion of tinct phylogenetic groups. PEP and citrate may activate these enzymes in bacteria belonging to different phylogenetic groups. PEP and citrate may activate these bacterial FBPases directly by stabilizing the R-state tetramer and indirectly by blocking the AMP pocket via the insertion of a conserved aromatic side chain (Phe$^{15}$). On the basis of sequence conservation, the anion activation site is present in FBPases from a variety of organisms, including distantly related Flavobacteria. FBPases from organisms within this class should be activated by PEP, a prediction supported by the literature (29). A consensus sequence for the allosteric activator site suggests a widespread mechanism of regulation in central metabolism in bacteria and other microbes.

**EXPERIMENTAL PROCEDURES**

**Materials**—Fru-1,6-P$_2$, Fru-2,6-P$_2$, NADP$^+$, oxaloacetate, cis-aconitate, and α-glycerophosphate came from Sigma. PEP, glyceraldehyde 3-phosphate, fumarate, and malate came from MP Biomedicals. Glucose-6-phosphate dehydrogenase and glyceraldehyde 3-phosphate, fumarate, and malate came from MP Biomedicals. Glucose-6-phosphate dehydrogenase and phosphogluco isomerase were from Roche Applied Sciences. All other chemicals were of reagent grade or equivalent.

**Crystallization of R-state Complexes**—Native and selenomethionine-substituted proteins were prepared as previously described (14). Crystals were grown by hanging drop in vapor diffusion VDX-plates (Hampton Research). PEP-bound crystals grew from droplets containing 2 μl of a protein solution (15 mg/ml selenomethionine-substituted FBPase, 20 mM dithiothreitol, 0.2 mM EDTA, 5 mM Fru-1,6-P$_2$, 5 mM MgCl$_2$, and 10 mM PEP) and 2 μl of a precipitant solution (50 mM sodium acetate, pH 5.6, 16% (w/v) polyethylene glycol 1500, and 20% (w/v) sucrose) and were equilibrated over 500 μl of precipitant solution. Citrate-bound crystals were grown similarly using the same protein solution (without PEP) and a precipitant solution containing 50 mM sodium citrate, pH 5.3, 20% (w/v) polyethylene glycol 3350, and 20% (w/v) sucrose. Equal-dimensional crystals (0.2–0.3 mm) grew within 3 days at 22 °C under each of the conditions.

**Data Collection**—Crystals were frozen directly in a cold stream of nitrogen without additional cryoprotectants. Data were collected at Iowa State University on a Rigaku R-AXIS IV++ rotating anode/image plate system using CuK$_{α_1}$ radiation from an Osmic confocal optics system at a temperature of 110 K. The program d’trek (34) was used to index, integrate, scale, and merge intensities, which were then converted to structure factors using the CCP4 (35) program TRUNCATE (36).

**Structure Determination and Refinement**—A molecular replacement solution was obtained from the program AMORE (37) using a single subunit from sulfate-bound *E. coli* FBPase (PDB accession identifier 2GQ1), from which all ligand and water molecules had been removed. Manual adjustments in the conformation of specific residues employed the program XTALVIEW (38). The resulting model underwent simulated annealing from 3000 to 300 K in steps of 25 K followed by 100 cycles of energy minimization and thermal parameter refinement using CNS (39). Force constants and parameters of stereochemistry were from Engh and Huber (40). Restraints for thermal parameter refinement were as follows: 1.5 Å$^2$ for bonded main-chain atoms, 2.0 Å$^2$ for angle main-chain atoms and angle side-chain atoms, and 2.5 Å$^2$ for angle side-chain atoms. Ligands (fructose 6-phosphate and PEP or citrate) and water molecules were fit to omit electron density until no improvement in $R_{free}$ was evident. Water molecules with thermal parameters above 50 Å$^2$ or more than 3.2 Å from the nearest hydrogen bonding partner were removed from the final model.

**Structure and Sequence Comparisons of FBPases**—Dimer and tetramer models of FBPase were constructed from single subunits and used in pairwise least squares superpositions between *E. coli* and porcine liver enzymes using the CCP4 programs PDBSET (41) and LSQKAB (42). Individual alignments between Cα positions in structures were measured using XTALVIEW (38). The canonical R and T states of porcine FBPase used in superpositions have PDB identifiers 1CNQ and 1EYK, respectively, whereas the I$_T$ and I$_B$ states have identifiers 1Q9D and 1YYZ, respectively. Because the angle of rotation between subunit pairs in various quaternary states of FBPases is sensitive to the subset of residues employed in the least squares fit, the previously established residues used to compare *E. coli* and porcine FBPases were used for all alignments (14). Multiple sequence alignments of Type I FBPases employed the program ClustalW (43). Optimal global pairwise alignments, which served as the basis for calculating sequence identity, utilized the program ALIGN (44).

**Kinetic Experiments**—Assays for FBPase activity employed the coupling enzymes phosphogluco isomerase and glucose-6-phosphate dehydrogenase and monitored formation of NADPH by either absorbance at 340 nm or fluorescence emission at 470 nm (7). Coupling enzymes were dialyzed against a 1000-fold volume of 50 mM Hepes, pH 7.5, to remove ammonium sulfate that would otherwise be present at ~3 mM in all assays. Assays (total volume, 2 ml) were performed at 22 °C in 50 mM Hepes, pH 7.5, with 100 μM EDTA and 150 μM NADP$^+$. Saturating levels of Fru-1,6-P$_2$ (40 μM) and MgCl$_2$ (10 mM) were used to measure specific activity and anion activation effects.
Assays were 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 subsequently initiating the reaction by the addition of metal (enzyme incubation assays). Equations (legend of Fig. 2) were fit to kinetic data using the program IGORPRO (WaveMetrics).

**Dynamic Light Scattering**—Experiments were performed in the assay buffer without coupling enzymes and Mg²⁺. Samples of E. coli FBPase with subunit concentrations of 1, 2, 3, 5, 8, and 14 μM were incubated in the presence and absence of 5 mM PEP for 2 h before light scattering measurements at 22 °C. Data were collected in 1-s intervals subdivided into 10 s acquisitions on a Protein Solutions DynaPro dynamic light scattering instrument. Instrumentation software (DYNAMICS V6) facilitated the determination of diffusion coefficients from time correlation curves and the use of such data in the estimation of molecular masses.

**Estimation of Metabolite Concentrations in E. coli**—Amounts of metabolites are often reported in units of mol/g dry weight of bacteria and may be converted to concentrations subject to several assumptions; the cytoplasm is homogenous, the cytoplasmic volume is the free-solution volume of the metabolite, and the total concentration of metabolite is its free concentration. At an optimal osmolarity of 0.28 osmol/liter, Cayley et al. (45) determined a cytoplasmic volume of 2.08 ± 0.06 × 10⁻⁶ liter/mg of dry weight of bacteria. Division by this value converts mol/g dry weight of bacteria to molar concentration.

**RESULTS**

**Enzyme Purity**—Native and selenomethionine-substituted enzymes are kinetically and structurally indistinguishable (14). Preparations of enzyme here give specific activities of 35–40 units/mg, appear as single bands on SDS-polyacrylamide gel electrophoresis (purity level of at least 95%), and have a single residue type (methionine or selenomethionine) at the N terminus.

**Time-dependent Loss of E. coli FBPase Activity**—Activity of E. coli FBPase diminishes upon dilution into assay buffers (Fig. 1). Relative activity falls to ~70% after 2 min and reaches a threshold of ~35% after 1 h. Similar results occur in the presence or absence of substrate (40 μM Fru-1,6-P₂), reducing agents (5 mM dithiothreitol or β-mercaptoethanol), glycerol, salts (20 mM NaCl, KCl, NH₄Cl, or NaC₂H₃O₂), and several buffers (50 mM Tris-malonate, Hepes-NaOH, Tris-HCl, or MES-NaOH). Inorganic sulfates (20 mM Na₂S₂O₃, K₂S₂O₃, or (NH₄)₂S₂O₃) and to a lesser extent inorganic phosphates (KH₂PO₄ or NaH₂PO₄) reduce activity loss. 5 mM PEP or citrate prevents time-dependent loss of activity while decreasing relative activity by ~20% (Fig. 1). On the other hand, 5 mM AMP increases the rate of activity loss, reaching a threshold value of ~15% relative activity. By way of comparison, relative activity is ~70% in the presence of 5 mM AMP in enzyme-initiated assays. 5 mM PEP (Fig. 1) or citrate (not shown) blocks the effects of 5 μM AMP. In contrast to the E. coli enzyme, dilution and PEP have no discernable effect on the activity of porcine FBPase (Fig. 1). Even after incubation in the assay buffer for 4 h at 22 °C, porcine FBPase retains full activity.

**Variation of Kinetic Parameters with Assay Protocol**—Kinetic parameters for E. coli FBPase, determined in the absence of PEP, differ for assays initiated by the addition of enzyme (no incubation) and initiated by the addition of Mg²⁺ after incubation of the enzyme under conditions of assay (Table 1). Incubation of enzyme for 1 h before initiation increases the Kₘ for Fru-1,6-P₂ and the Kᵣ for Mg²⁺ and decreases kₘₐₜ AMP inhibition, cooperative in enzyme-initiated assays (Hill coefficient, ~1.7), increases dramatically in incubation assays and is non-cooperative (Hill coefficient, ~0.8). Kinetic parameters from incubation assays that include 1 mM PEP, however, are similar to those of enzyme-initiated assays (Table 1).

**Metabolic Activators of E. coli FBPase**—Activation by citrate, PEP, and other effectors is evident only in incubation assays. In
enzyme-initiated assays, 1 mM PEP has little effect. PEP (or citrate) concentrations of 1–5 mM weakly inhibit enzyme activity, but inhibition is small relative to the magnitude of activation attributed to these ligands in incubation assays.

Incubation of E. coli FBPase for 2 h (see “Experimental Procedures” for specific conditions) individually with intermediates of the tricarboxylic acid cycle or 3-carbon intermediates of glycolysis increase relative activity by as much as 500% (Fig. 2). PEP, citrate, 3-phosphoglycerate, cis-aconitate, isocitrate, oxaloacetate, and α-ketoglutarate enhance enzyme activity at concentrations up to 5 mM. PEP is the most potent activator, achieving nearly full activation at ~50 μM. Citrate exhibits maximal activation comparable with PEP at a concentration of ~1 mM. Relative velocity increases hyperbolically with the concentration of PEP and citrate (Fig. 2), with 50% of maximum activation (A_{0.5}) coming at 27 ± 2 and 210 ± 10 μM, respectively. Concentrations of 10 mM malate or fumarate exhibit weak activation (data not shown).

PEP and citrate are antagonists of AMP inhibition (Table 1). The concentration of AMP causing 50% inhibition (I_{0.5}) in incubation assays is 2.2 μM, but in the presence of 40 μM, 1 mM, and 2 mM PEP increases to 6.1, 20, and 44 μM, respectively. The nonlinear variation of I_{0.5} for AMP with the concentration of PEP probably stems from cooperativity in AMP binding and/or changes in the quaternary state of the enzyme. Citrate (2 mM) increases the I_{0.5} for AMP to 10 μM. As illustrated in Fig. 1, 5 μM AMP causes no measurable inhibition in the presence of 5 mM PEP (or citrate, not shown). Alternatively, the A_{0.5} for PEP is 27 ± 2 μM in the absence of AMP and increases to 1.4 ± 0.2 mM in the presence of 40 μM AMP. In contrast to the E. coli enzyme, PEP (2 mM) has no effect on AMP inhibition in porcine FBPase.

R-state Structures of E. coli FBPase—R-state crystals of E. coli FBPase belong to the space group I222 for both PEP-bound (a = 44.7, b = 83.1, c = 174.4 Å) and citrate-bound (a = 45.3, b = 83.3, c = 173.4 Å) structures and are isomorphous to crystals of the E. coli enzyme grown in the presence of high concentrations of sulfate (Ref. 14; PDB identifier 2GGQ). Unit cell parameters here and for crystals of the sulfate-ligated enzyme differ by less than 3%. The models for the PEP and citrate complexes (Table 2) are complete except for some residues of the dynamic loop (residues 46–62) for which there is no interpretable electron density. Electron density is present for a molecule of Fru-6-P bound to the active site, but no density appears for inorganic phosphate or Mg^{2+}. Disorder in the dynamic loop may stem from the absence of bound metal and phosphate. The absence of bound phosphate and Mg^{2+} could be a consequence of low pH and/or non-saturating concentrations of metal ion and/or phosphate. One molecule of PEP or citrate binds per monomer to the same allosteric activator site (Fig. 3). PEP and
citrate bind to FBPase stoichiometrically with one water molecule and form extensive networks of hydrogen bonds between top (C1–C2) and bottom (C3–C4) dimers of E. coli FBPase (Fig. 4). Interactions between ligands and protein appear in Table 3.

The PEP- and citrate-bound structures exhibit no differences beyond coordinate error, differing principally by the bound ligand. The quaternary states of the citrate and PEP complexes of the E. coli enzyme match the canonical R-state of porcine FBPase on the basis of tetramer superpositions (the rotation angle between subunits pairs C1–C2 and C3–C4 differs by no more than 0.1° in any superposition). By way of comparison, the rotation angle between subunit pairs C1–C2 and C3–C4 of the sulfate complex of the E. coli FBPase is 6.0° away from the canonical R-state and toward the T state. The side chain of Phe15 occupies the AMP pocket in the PEP and citrate complexes as it does in the sulfate complex (14). The tertiary structures of mammalian and E. coli enzymes are nearly identical on the basis of Cα traces (Fig. 5), differing mainly in loop regions connecting secondary structural elements.

**Dynamic Light Scattering**—Native mass determinations using E. coli FBPase subunit concentrations of 1–14 μM and 5 mM PEP were substantially larger (122 ± 4 kDa) than corresponding determinations made in the absence of PEP (70 ± 15 kDa). Mass determinations varied little with protein concentration, suggesting the existence of homogenous states in the presence and absence of PEP that differ in subunit association, such as a tetramer (calculated mass ~147 kDa) in the presence of PEP and a dimer (calculated mass ~74 kDa) in its absence. The dissociation constant for a tetramer to dimer equilibrium must be no higher than 10⁻⁷ M in the presence of PEP and no lower than 10⁻⁵ M in the absence of PEP.

**Identification of Organisms Possessing Anion-activated FBPase**—Five residues signify the probable existence of anion activation in FBPase. Gly5, Lys30, and Arg80 bind directly to the activator, Phe15 blocks the AMP pocket, and Val186 (in place of glutamate in FBPases from eukaryotic organisms) makes room for the anion to bind at the C1–C4 interface (See Fig. 3 for definition of subunit interfaces). The five signature residues are present in FBPase from known heterotrophic bacteria. With little exception, these residues appear in an all-or-none fashion, defining a consensus sequence (Table 4) linked to allosteric activation of gluconeogenesis. Alignments of sequences (>100) in previous work (14), expanded here to include >250 sequences, identifies 44 species having a Type I FBPase with the potential for allosteric activation. Excluding the sole eukaryote, the protozoan parasite Toxoplasma gondii, these organisms are all members of the domain bacteria and separate into two phyla. The majority belong to the phylum Proteobacteria, class Gammaproteobacteria, and occupy the orders Enterobacteriales, Alteromonadales, Vibrionales, Pasteurellales, Reinekeae, and Oceanospirillales. A second group occupies the phylum Bacteroidetes and the class Flavobacteria.

**DISCUSSION**

Results here unify many disparate observations reported by investigators of bacterial FBPases. E. coli FBPase undergoes a time-dependent change upon dilution (11, 27), a phenomenon originally attributed to an effect of the substrate in assay buffers (11). Past investigators initiated assays with enzyme (11, 27, 28, 46) but in so doing minimized the influence of anionic ligands on enzyme activity. Differences in assay protocols and the uncontrolled presence
of activating anions, such as sulfate, can account for significant variations in $k_{cat}$, $K_m$, and cooperativity in AMP inhibition (Table 1). For instance, Hill coefficients for AMP inhibition range from 1.1 to 3.2 in the literature (28, 46) and from 0.8 to 2.6 in the present study.

### TABLE 3

| Ligand group and atom | Bonding partner | Distance $\AA$ |
|-----------------------|-----------------|---------------|
| Citrate/Wat$^{233}$   | Lys$^{30}$ NZ  | 2.71          |
| 1-Carboxyl O1         | Gly$^{36}$ N   | 2.88          |
| 1-Carboxyl O2         | Phe$^{37}$ N   | 2.79          |
| 2-Carboxyl O5         | Thr$^{3}$ OG   | 2.65          |
| 2-Carboxyl O6         | Arg$^{46}$ NH$^*$ | 2.55       |
| 3-Carboxyl O3         | Lys$^{34}$ NZ$^*$ | 2.65       |
| 3-Carboxyl O4         | Wat$^{733}$    | 2.85          |
| 2-Hydroxyl O7         | Lys$^{36}$ NZ  | 2.97          |
| Wat$^{731}$ O         | Lys$^{36}$ NZ  | 2.60          |

**PEP/Wat$^{541}$**

| Ligand group and atom | Bonding partner | Distance $\AA$ |
|-----------------------|-----------------|---------------|
| 2-Phosphoryl O1P      | Arg$^{46}$ NH$^*$ | 2.60         |
| 2-Phosphoryl O2P      | Thr$^{3}$ OG   | 2.56          |
| 2-Phosphoester O3     | Lys$^{30}$ NZ  | 2.99          |
| 1-Carboxyl O1         | Lys$^{34}$ NZ$^*$ | 2.69       |
| 1-Carboxyl O2         | Lys$^{34}$ NZ$^*$ | 2.79       |
| Wat$^{541}$ O         | Lys$^{36}$ NZ  | 2.81          |

**Fru-6-P**

| Ligand group and atom | Bonding partner | Distance $\AA$ |
|-----------------------|-----------------|---------------|
| 1-Hydroxyl O1         | Glu$^{273}$ OE1 | 2.63          |
| 3-Hydroxyl O3         | Asp$^{113}$ OD2 | 2.61          |
| 4-Hydroxyl O4         | Lys$^{269}$ NZ  | 2.89          |
| 2,5-Ether O5          | Wat$^{542}$    | 2.74          |
| 6-Phosphoester O6     | Lys$^{269}$ NZ  | 3.15          |
| 6-Phosphoryl O61      | Arg$^{230}$ NH$^*$ | 2.86      |
| 6-Phosphoryl O62      | Tyr$^{235}$ OH  | 2.56          |
| Wat$^{546}$           | Lys$^{269}$ NZ  | 2.63          |
| Wat$^{542}$           | Lys$^{269}$ NZ  | 2.73          |
| 6-Phosphoryl O63      | Arg$^{230}$ NH$^*$ | 3.14     |
| Asp$^{112}$ ND2       | Lys$^{269}$ NZ  | 2.74          |
| Tyr$^{235}$ OH        | 2.82           |

**FIGURE 5.** *Superposition of R-state subunits from E. coli and mammalian FBPases.* C$_\alpha$ atoms from the E. coli subunit are connected sequentially by solid lines, and those of the porcine subunit connected sequentially by dashed lines. Fru-6-P and PEP (bold lines) and residue numbers are from E. coli FBPase. Subunit orientation corresponds to that of subunit C2 of Fig. 3. Structural differences occur at N and C termini and in some loops. This drawing was prepared with XTALVIEW (38).

**PEP Activation of E. coli Fructose-1,6-bisphosphatase**

Dilution-linked loss of FBPase activity stems from a conformational change in the tetramer or from the dissociation of the tetramer into subunit monomers and/or dimers. Variation in the native mass of E. coli FBPase in the presence and absence of PEP would provide direct evidence in favor of the latter; however, the lowest reachable subunit concentration in dynamic light scattering measurements (~1 m$\mu$m) is 50-fold higher than the subunit concentration of FBPase in assays (~20 nM). Nonetheless in the absence of an activator, light scattering data indicate a native mass for E. coli FBPase in the range of a subunit dimer (observed, 70 ± 15 kDa, expected, 74 kDa), whereas the native mass of the enzyme in the presence of 2 m$\mu$M PEP is near that of the tetramer.

The subunits of the putative dimer could be related by the C1–C4 interface or the C1–C2 interface, but the latter is more likely. Ligands perturb the C1–C4 interface as evidenced by the rotation angles for C1–C2 subunit pairs in the PEP and citrate complexes (rotation angle of 0°) and the sulfate complex (rotation angle of 6°). In the absence of anionic ligands, the C1–C4 subunit interface would have a net positive charge (14). Finally, porcine FBPase requires functional AMP sites in the top (C1–C2) and bottom (C3–C4) halves of the tetramer for cooperativity in AMP inhibition (47), and mutations to residues that facilitate interactions across the C1–C4 interface eliminate AMP cooperativity (6, 48). By analogy, a C1–C2 dimer of E. coli FBPase should exhibit noncooperative inhibition by AMP, and in fact, AMP inhibition in enzyme-incubated assays in the absence of PEP is without cooperativity (Table 1). All data then are consistent with the complete dissociation of the E. coli tetramer into less active C1–C2 subunit dimers under conditions of low protein concentration and in the absence of anion activators.

An initial study revealed sulfate and PEP as activators of E. coli FBPase (14); however, anion activation is widely shared by metabolites of central metabolism. In fact, a high formal negative charge is the principle attribute common to all activators of E. coli FBPase. PEP and citrate, for instance, are structurally dissimilar but have a formal charge at or near −3 at neutral pH. Other potent activators are either phosphorylated carboxylic acids (PEP-like) or tricarboxylic acids (citrate-like). 3-Phosphoglycerate, cis-aconitate, and isocitrate, exhibiting maximal activation at concentrations of 1 m$m$M, probably form interactions with the protein similar to those of PEP or citrate. 2-Phosphoglycerate (not tested) is probably an activator, being similar to PEP and 3-phosphoglycerate in charge and structure. Oxaloacetate, α-ketoglutarate, malate, fumarate, sulfate, and phosphate with a formal charge at or near −2 at neutral pH act only at elevated concentrations. Indeed at pH 7.5, sulfate is a dianion, whereas phosphate is an equilibrium mixture of di- and mono-an-
ions, which may explain the superior activating effect of sulfate relative to phosphate (14). Activation by oxaloacetate and α-ketoglutarate at concentrations of 5 mM and by malate and fumarate at concentrations of 10 mM are probably unimportant physiologically. For the latter pair, observed activation could arise from as little as 0.5% contamination by citrate.

Structures of the PEP- and citrate-bound FBPases reveal the mechanism by which the same effector site can recognize different ligands. In one sense the activator site recognizes neither PEP nor citrate but, rather, hydrated forms of each ligand (Fig. 4). In the PEP complex a water molecule occupies the position of an oxygen atom of the 1-carboxyl group of citrate, and in the citrate complex a water molecule is near the position of an oxygen atom of the 1-carboxyl group of citrate. The PEP/citrate hydrogen bond to Lys34 spans the C1–C4 interface and may rotate subunit pairs into their eclipsed relationship of the canonical R-state.

Alternatively, the different quaternary state of the sulfate-ligated complex may stem from the presence of an additional sulfate anion. Superpositions of single subunits of the sulfate, PEP, and citrate complexes localize the largest change in tertiary conformation (displacements of ~1 Å) to the C-terminal end of helix H2 near the center of the tetramer (Fig. 7). A sulfate anion (sulfate341 in reference 14) on the C1–C2 molecular 2-fold axis interacts with the side chains of Arg38 and His37, and conformational changes in these side chains correlate with rigid body movements of helix H2. The quaternary state of the sulfate complex then may be due to sulfate341, which may bind to the enzyme by virtue of its elevated concentration (~1 mM) in previous crystallization experiments. Synthetic inhibitors that bind to the center of porcine FBPase also stabilize an intermediate quaternary state (50). Whether these synthetic inhibitors of the mammalian enzyme and sulfate341 of the E. coli enzyme mimic the interaction of a physiological effector at the center of the tetramer is unclear.

The interaction of PEP/citrate with Lys34 in E. coli FBPase is structurally equivalent to the interactions of Glu192 with Lys42 of R-state porcine FBPase. Hence, the porcine enzyme may represent evolution toward a permanently activated state of FBPase. Basic residues (Lys30, Lys34, and Arg80) of the anion binding site of E. coli FBPase balance the electrostatic charge of the most potent anion activators (Fig. 4). The formal positive charge here is +1 for porcine FBPase, with Cys38 and Ser38 replacing Lys30 and Arg80, respectively. In fact, Glu192 occupies the “anion site” in porcine FBPase, balancing the positive charge and making hydrogen bonds that bridge the C1–C4 interface. Despite being vastly different from the porcine enzyme at the C1–C4 interface and differing in residue type at 195 positions, E. coli and porcine FBPases adopt virtually identical R states. Evidently, the R-state tetramer of FBPase has been

### TABLE 4

| Sequence position | Consensus residue type | Exceptions |
|-------------------|-----------------------|------------|
| 5                 | Gly                   | H. influenzae (Ser) |
| 15                | Phe/Tyr               | M. succiniciproducens (Asp) |
| 30                | Lys/Arg               | T. gondii (Leu) |
| 80                | Arg                   | None |
| 186               | Val/Thr/Ser           | A. pleurophoeniae (Leu) |

**FIGURE 6.** Superposition of bound PEP and citrate molecules. The superposition of PEP (bold lines) and citrate is based on the Cα atoms of the PEP- and citrate-bound tetramers. Single water molecules (octahedral crosses) combine with PEP and citrate to complement the interactions of each effector. This drawing was prepared with MOLSCRIPT (59).
PEP Activation of E. coli Fructose-1,6-bisphosphatase

Figure 7. Tertiary conformational changes of helix H2 correlated with the presence of sulfate. The area near the sulfate anion at the C1–C2 interface and the C terminus of helix H2 is enlarged. The superposition of single subunits of the PEP- and sulfate-bound structures reveals an 8-Å movement in the side chain of Arg38 (illustrated by the arrow), hydrogen bonding internally with sulfate3^41 (bold lines) near the center of the tetramer but moving to a surface-exposed conformation in the PEP-bound structure. Movement of Arg38 and the C-terminal end of helix H2 are correlated. This drawing was prepared with MOLSCRIPT (59).

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In principal, a decrease in PFK activity and a concomitant increase in FBPase activity would transform glycolytic into gluconeogenic flux. The Fru regulon controls flux through PEP carboxykinase and PEP synthase by adjusting the expression levels of these enzymes (21, 51, 52); however, levels of FBPase and PFK change by only 2–3-fold on growth media of glucose and acetate (21, 22). Metabolite regulation of FBPase and PFK-1 in vivo then seems the only option, but such a mechanism requires coordinated changes in the concentrations of free AMP, PEP, and citrate. Estimates of AMP concentrations in vivo range from 20 to 60 μM (26) to as high as 150 μM (27). Although these estimates may be erroneously high due to the uncontrolled degradation of ADP and ATP, AMP levels are certainly 5-fold higher than the I_0.5 value (~2 μM) for E. coli FBPase in the absence of an activating anion. Hence, FBPase should be inactive if AMP were the only effector. PEP concentrations, however, change from 50 to 1000 μM in E. coli between glycolytic and gluconeogenic conditions of growth, respectively. The A_0.5 values for PEP in the presence of either 0 or 40 μM AMP determined here are 27 ± 2 μM and 1.4 ± 0.2 mM, spanning the range of PEP concentrations found in E. coli. Moreover, PEP also inhibits PFK in E. coli (33), and the in vitro behavior of PFK at physiological concentrations of substrates and effectors (including PEP) is consistent with a metabolite mechanism of regulation in vivo (53). As a final consideration, changes in the concentration of PEP respond rapidly to changes in nutrients; PEP levels fall 70% within 3 min after the addition of glucose to a succinate growth medium (32). All of these observations support the hypothesis of metabolite regulation of glycolysis and gluconeogenesis by the coupling of PFK and FBPase activities to the in vivo concentration of PEP.

Organisms that have the potential for PEP regulation of gluconeogenesis via the bacterial anion activation site (see Table 4) exhibit several trends worth noting. Many are free-living, opportunistic protozoan parasites, and all are obligate heterotrophs, with the ability to use nutrients that enter glycolysis/glucconeogenesis above and below the FBPase/PFK step. The requirement for metabolite regulation of FBPase may stem from a need to adapt quickly to changes in nutrient availability, utilizing a variety of carbon sources for both energy and biochemical synthesis. Absent from Table 4 are lithotrophs, phototrophs, and autotrophs; these organisms likely run their glycolytic/glucconeogenic pathways unidirectionally (predominantly anabolic).

In some instances the anion activation site of bacterial FBPases may be a potential target for anti-microbial agents. The requirement for a functional FBPase in pathogenic organisms varies dramatically. At one extreme the protozoan parasite Plasmodium falciparum, the cause of malaria, no longer possesses the FBPase gene (among other gluconeogenic genes). P. falciparum presumably uses glucose from the host for energy production and biochemical synthesis (54, 55). In sharp contrast, FBPase activity in another protozoan, the parasite Leishmania major, which causes leishmaniasis, is vital to pathogenesis (56). The pathogenic protozoan, T. gondii, the cause of toxoplasmosis, possesses a bacteria-like FBPase, but the relationship between FBPase activity and pathogenicity (if any) is unclear. The accurate prediction of FBPase dependence of seemingly related microorganisms is problematic, necessitating for the present, an experimental evaluation of each target.

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