Fructose-1,6-bisphosphatase (FBPase) governs a key step in gluconeogenesis, the conversion of fructose 1,6-bisphosphate into fructose 6-phosphate. In mammals, the enzyme is subject to metabolic regulation, but regulatory mechanisms of bacterial FBPases are not well understood. Presented here is the crystal structure (resolution, 1.45 Å) of recombinant FBPase from *Escherichia coli*, the first structure of a prokaryotic Type I FBPase. The *E. coli* enzyme is a homotetramer, but in a quaternary state between the canonical R- and T-states of porcine FBPase. Phe residues and residues at the C-terminal side of the first α-helix (helix H1) occupy the AMP binding pocket. Residues at the N-terminal side of helix H1 hydrogen bond with sulfate ions buried at a subunit interface, which in porcine FBPase undergoes significant conformational change in response to allosteric effectors. Phosphoenolpyruvate and sulfate activate *E. coli* FBPase by at least 300%. Key residues that bind sulfate anions are conserved among many heterotrophic bacteria, but are absent in FBPases of organisms that employ fructose 2,6-bisphosphate as a regulator. These observations suggest a new mechanism of regulation in the FBPase enzyme family: anionic ligands, most likely phosphoenolpyruvate, bind to allosteric activator sites, which in turn stabilize a tetramer and a polypeptide fold that obstructs AMP binding.

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Fructose-1,6-bisphosphatase (Fructose-1,6-bisphosphatase 1-phosphohydrolase, EC 3.1.3.11; FBPase) is a key enzyme of the gluconeogenic pathway, hydrolyzing fructose 1,6-bisphosphate (Fru-1,6-P₂) to fructose 6-phosphate (Fru-6-P) and inorganic phosphate (Pi) (1, 2). In *Escherichia coli*, the most likely role of FBPase is the generation of five-carbon precursors for nucleotide and polysaccharide production (3, 4). Fructose-2,6-bisphosphate (Fru-2,6-P₂), however, does inhibit *E. coli* FBPase in assays (15, 16). Dynamic regulation by AMP alone is unlikely, because AMP concentrations in vivo remain relatively constant under gluconeogenic and glycolytic conditions (17).

Donahue et al. (18) was the first to classify prokaryotic FBPases by sequence homology. Investigators now suggest five FBPase types (19). Types I-III are primarily in Bacteria, Type IV in Archaea, and Type V in thermophilic prokaryotes from both domains. Eukaryotic FBPases are homologous to prokaryotic Type I enzymes. Crystal structures are available for Types IV and V FBPases and for mammalian and chloroplast FBPases, but no structure of an FBPase from Bacteria is available.

FBPase is a target for potential drugs in the treatment of Type II diabetes (20–22). Novel inhibitors have been developed (20, 21), and in one case, knowledge of structure and mechanism contributed to the rational design of a new inhibitor (22). An important property of mammalian FBPase, synergism in Fru-2,6-P₂/AMP inhibition, however, is not understood in terms of a specific molecular mechanism. Moreover, no example is known of a mutant or wild-type mammalian FBPase that lacks synergism without significant disruption of AMP or Fru-2,6-P₂ inhibition. Fru-2,6-P₂ and AMP individually are potent inhibitors of *E. coli* FBPase, but exhibit no synergism (15). The sequence identity (41%) of the *E. coli* and porcine enzymes suggest similar folds. Other studies indicate the existence of an allosteric AMP binding site and a tetrameric subunit organization for the *E. coli* enzyme (14, 16). Hence, differences in the conformational responses of *E. coli* and porcine FBPases to AMP and/or Fru-2,6-P₂ could reveal the molecular basis of AMP/Fru-2,6-P₂ synergism.

Reported here is the high resolution structure of Type I FBPase from *E. coli*. The structure reveals a homotetramer between the canonical R- and T-states of porcine FBPase (11). Sulfate anions appear at the interface between top and bottom subunit pairs of the tetramer. Sulfate and phosphoenolpyruvate (PEP) enhance activity of the *E. coli* enzyme by at least 300%. Anionic ligands likely mimic a physiological effector that stabilizes *E. coli* FBPase as an active tetramer, a hypothesis consistent with previous reports of dilution-linked inactivation (4, 14). The true physiological activator is probably PEP, an inhibitor of *E. coli* fructose-6-phosphate 1-kinase (23), and an activator of FBPases in other prokaryotic organisms (18, 24–26). Sequence alignments reveal a number of organisms with Type I FBPases that probably possess the anion binding site of the *E. coli* enzyme. The anion binding site, however, is not present in organisms that employ Fru-2,6-P₂ in the regulation of FBPase activity.

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2 The abbreviations used are: FBPase, fructose-1,6-bisphosphatase; Fru-1,6-P₂, fructose 1,6-bisphosphate; Fru-2,6-P₂, fructose 2,6-bisphosphate; PEP, phosphoenolpyruvate; DTT, dithiothreitol.

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This work was supported in part by National Institutes of Health Research Grant NS 10546 and by a graduate research fellowship award from the American Foundation for Aging Research. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact. The atomic coordinates and structure factors (code 2GQ1) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

1 To whom correspondence should be addressed: Dept. of Biochemistry, Biophysics, and Molecular Biology, 4206 Molecular Biology Bldg., Iowa State University, Ames, IA 50011-3260. Tel.: 515-294-6116; Fax: 515-294-0453; E-mail: honzatko@iastate.edu.

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E. coli in reactions (PCR). Forward (5′-GATCAGGAAACGGT-3′) and reverse (5′-TACGCGTCCGGGAACTCACGG-3′) deoxyoligonucleotides and DNA sequences. All other chemicals were of reagent grade or equivalent.

**EXPERIMENTAL PROCEDURES**

**Materials**—Fru-1,6-P₃, NADP⁺, PEP, leupeptin, ampicillin, phenylmethylsulfonyl fluoride (PMSF), DEAE-Sepharose and phenyl-Sepharose were from Roche Applied Sciences. The Iowa State University DNA Sequencing and Synthesis Facility prepared deoxyoligonucleotides and DNA sequences. All other chemicals were of reagent grade or equivalent.

**Cloning E. coli FBPase**—The fbp gene that encodes Type I FBPase in *E. coli* (27) was cloned from genomic DNA isolated from strain XL1-Blue (Stratagene) by primer extension using polymerase chain reaction (PCR). Forward (′-GTATCGACCATATGAAAACGGTAGGTGAATTATTGTG-3′) and reverse (′-CAGAATTCTTACGGGTCGGGAACCTACCCGGG-3′) primers introduced Ndel and EcoRI sites, respectively (sites underlined). The initial round of PCR employed VENT DNA polymerase. The amplified product was the template for a second PCR, using the same primers and the BIO-X-ACT Short DNA polymerase (Midwest Scientific), which leaves a 3′-A overhang. The resulting oligonucleotide was ligated into the pGem-T Easy Vector (Promega) and the resulting plasmid used to transform electrocompetent XL1-Blue cells. Transformants were selected by growth on LB-agarose supplemented with 50 μg/ml ampicillin, whereas recombinants were selected by blue/white screening. Plasmid DNA was isolated from cultures grown from single colonies. The presence of the insert was verified by digestion with EcoRI and gel electrophoresis.

The internal Ndel site within the fbp gene was eliminated by PCR using the mutagenic primer 5′-TTTGTGGCAACGCACGGATCGTTGAAATGTCGAACGCACTG-3′ and its reverse complement to introduce a silent mutation (base change underlined). Parental methylated DNA was digested with DpnI, and DH10B cells were transformed with the resulting nicked plasmid. Plasmids from single colonies were isolated, digested with Ndel, and separated by gel electrophoresis, using non-mutated plasmid as a control. The desired mutation was confirmed by DNA sequencing. The resulting construct was amplified by PCR, and then digested with Ndel and EcoRI. Simultaneously the bacterial protein expression vector pET-24b (Novagen), carrying the kan* selectable marker, was digested with Ndel and EcoRI and the cut plasmid isolated by gel electrophoresis. The excised oligonucleotide and vector DNA were purified, ligated, and transformed as previously described, using the cut and uncut vectors as negative and positive controls, respectively. The final plasmid (pECFBP) was verified by DNA sequencing.

**Expression and Purification of Native and Selenomethionine-substituted E. coli FBPase**—Separate preparations of native and selenomethionine-substituted enzyme were used for the kinetic and structural investigations, respectively. FBPase-deficient *E. coli* strain DF657 (tonA22, ompF627(T 2R), relA1, pit10, spoT1, without L-methionine-substituted enzyme were used for the kinetic and structural investigations, respectively. FBPase-deficient *E. coli* strain DF657 (tonA22, ompF627(T 2R), relA1, pit10, spoT1, without L-methionine-substituted enzyme were used for the kinetic and structural investigations, respectively. FBPase-deficient *E. coli* strain DF657 (tonA22, ompF627(T 2R), relA1, pit10, spoT1, without L-methionine-substituted enzyme were used for the kinetic and structural investigations, respectively. FBPase-deficient *E. coli* strain DF657 (tonA22, ompF627(T 2R), relA1, pit10, spoT1, without L-methionine-substituted enzyme were used for the kinetic and structural investigations, respectively. FBPase-deficient *E. coli* strain DF657 (tonA22, ompF627(T 2R), relA1, pit10, spoT1, without L-methionine-substituted enzyme were used for the kinetic and structural investigations, respectively. FBPase-deficient *E. coli* strain DF657 (tonA22, ompF627(T 2R), relA1, pit10, spoT1, without L-methionine-substituted enzyme were used for the kinetic and structural investigations, respectively. FBPase-deficient *E. coli* strain DF657 (tonA22, ompF627(T 2R), relA1, pit10, spoT1, without L-methionine-substituted enzyme were used for the kinetic and structural investigations, respectively. FBPase-deficient *E. coli* strain DF657 (tonA22, ompF627(T 2R), relA1, pit10, spoT1, without L-methionine-substituted enzyme were used for the kinetic and structural investigations, respectively. FBPase-deficient *E. coli* strain DF657 (tonA22, ompF627(T 2R), relA1, pit10, spoT1, without L-methionine-substituted enzyme were used for the kinetic and structural investigations, respectively. FBPase-deficient *E. coli* strain DF657 (tonA22, ompF627(T 2R), relA1, pit10, spoT1, without L-methionine-substituted enzyme were used for the kinetic and structural investigations, respectively. FBPase-deficient *E. coli* strain DF657 (tonA22, ompF627(T 2R), relA1, pit10, spoT1, without L-methionine-substituted enzyme were used for the kinetic and structural investigations, respectively. FBPase-deficient *E. coli* strain DF657 (tonA22, ompF627(T 2R), relA1, pit10, spoT1, without L-methionine-substituted enzyme were used for the kinetic and structural investigations, respectively. FBPase-deficient *E. coli* strain DF657 (tonA22,ompF627(T 2R), relA1, pit10, spoT1, Δ(fbp)287; Genetic Stock Center at Yale University, New Haven, CT) was transformed by the pECFBP plasmid and plated onto LB-agarose supplemented with 30 μg/ml kanamycin sulfate. A single colony inoculant (100 ml LB-kanamycin) grew (with shaking, 37 °C) for 24 h. Two percent inoculum was added to 4 liters of LB-kanamycin. The culture grew (with shaking, 37 °C), to an *A*₀.₆₀₀ of 1.0, at which time transcription was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG, final concentration, 0.85 mM). The culture was maintained (with shaking, 37 °C) for an additional 12 h. Sel enomethionine-substituted protein was produced as described previously with all growth steps at 37 °C.³

Modifications to the protocol of Kelley-Loughnane et al. (16) led to purified protein in a single day. All buffers employed after cell lysis were degassed (stirring under vacuum) and contained 0.2 M EDTA and 5 mM dithiothreitol (DTT). Cells were collected by centrifugation (3,000 × g) and resuspended in 75 ml of lysis buffer (40 mM KH₂PO₄/K₂HPO₄, pH 7.0, 0.1 mM PMSF, 1 mM EDTA, 5 μg/ml leupeptin, and 1 mM β-mercaptoethanol) for disruption by French press. Insoluble debris was removed by centrifugation (30 min, 33,000 × g) prior to ammonium sulfate fractionation. Precipitated protein was collected by

### TABLE 1

| Statistics of data collection and refinement for E. coli FBPase |
| --- |
| Space group and unit cell parameters are described under “Results.” |

| Resolution (Å) | 44.04–1.45 (1.50–1.45)² |
| --- |
| Number of reflections | 392,636 |
| Unique reflections/reflections used in refinement | 56,471 |
| Average redundancy | 6.95 (6.29)² |
| % Completeness | 99.9 (99.99)² |
| Rfree | 0.069 (0.293)² |
| I∽(I) | 13.3 (5.2)² |
| Number of atoms | 2807 |
| Number of solvent sites | 324 |
| R₁ | 21.9 |
| RF | 23.2 |
| Mean B for protein (Å²) | 18 |
| Mean B for SO₄²⁻ (Å²) | 46 |

Root mean-squared deviations

| Bond lengths (Å) | 0.004 |
| --- |
| Bond angles | 1.2 |
| Dihedral angles | 22.2 |
| Improper angles | 0.70 |

² Values in parentheses are for the last shell of data collection.

³ Ginder, N. D., Binkowski, D. J., Fromm, H. J., and Honzatko, R. B. (May 2, 2006) J. Biol. Chem. 10.1074/jbcM602109200.
**Escherichia coli Fructose-1,6-bisphosphatase**

**FIGURE 2. Stereoview of the anion activation site.** Electron density is from an omit map (contour level 1σ, cutoff radius 1.5 Å) covering sulfate$^{344}$ and residues in its vicinity. The arrow in Fig. 1 indicates the viewing direction for this figure. Parts of this drawing were prepared with XTALVIEW (35).

**FIGURE 3. AMP binding sites of porcine and E. coli FBPases.** AMP bound to the allosteric inhibitor site of porcine FBPase is shown (left) with the corresponding view of the presumed AMP binding site of E. coli FBPase (occupied by Phe$^{15}$) (right). Dotted lines are selected donor-acceptor interactions of 3.2 Å or less. The icon indicates viewing direction (arrow) and region of the tetramer being viewed (shaded black). This drawing was prepared with MOLSCRIPT (28).

Data Collection—Crystals were screened for diffraction 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. High resolution data were collected at 100 K on Beamline 4.2.2 of the Advanced Light Source, Lawrence Berkeley Laboratory. The program d’trek (31) was used to index, integrate, scale, and merge intensities, which were then converted to structure factors using the CCP4 (32) program TRUNCATE (33).

Structure Determination and Refinement—A molecular replacement solution was obtained using the program AMORE (34) and the model for R-state porcine FBPase (PDB accession identifier 1CNQ), from which residues 52–72 and all ligand and water molecules had been removed. Residues for the E. coli enzyme were manually fit to omit density, using the program XTALVIEW (35). The resulting model using CNS (36) underwent simulated annealing from 3000 to 300 K in steps of 25 K, followed by 100 cycles of energy minimization and thermal parameter refinement. Force constants and parameters of stereochemistry were from Engh and Huber (37). Restrictions 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 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.

Comparison of Porcine and E. coli FBPases—Construction of dimer and tetramer models from the monomer, as well as pair-wise least squares superpositions of E. coli FBPase and porcine liver FBPase were accomplished using the CCP4 programs DDBSET (38) and LSQKAB (39). The canonical R- and T-states of porcine FBPase used in superpositions have PDB identifiers 1CNQ and 1EYK, respectively, whereas 1Q9D and 1YYZ, respectively. The angle of rotation of subunit pairs in various quaternary states of FBPases is sensitive to the subset of residues used in the least-squares fit (40). By convention, Cα atoms of porcine FBPase residues 33–49, 75–265, 272–

centrifugation from 40–70% levels of saturated (NH$_4$)$_2$SO$_4$ and redissolved in 40 mM NaH$_2$PO$_4$, pH 7.2, and (NH$_4$)$_2$SO$_4$ (40% of saturation). Protein was loaded onto a phenyl-Sepharose column, washed with the same buffer, and eluted by a step gradient in (NH$_4$)$_2$SO$_4$ (20% followed by 14% of saturation). Combined fractions of highest specific activity were reduced in volume to 30 ml using an Amicon concentrator (YM 30,000 membrane). Protein was passed through a desalting column (Sephadex G-50 equilibrated with 0.1 M NaCl, 40 mM NaH$_2$PO$_4$, pH 7.2) and then eluted with a linear gradient in NaCl (0.1–0.5 M). Pooled fractions of highest specific activity were desalted (Sephadex G-50 column, equilibrated with 5 mM Tris malonate, pH 7.4). Enzyme (0.75 mg/ml) used for kinetic studies was frozen in this buffer, whereas enzyme used for crystallization experiments was concentrated to 15 mg/ml and filtered through a 0.22 μm filter. Protein was flash-frozen in 200-μl aliquots using a dry-ice ethanol bath and stored at −80 °C. Protein concentrations were determined by the method of Bradford (29), using bovine serum albumin as a standard. Protein purity was monitored by sodium dodecylsulfate-polyacrylamide gel electrophoresis (30). N-terminal sequencing and protein purity was monitored by sodium dodecylsulfate-polyacrylamide gel electrophoresis (30). N-terminal sequencing and residues in its vicinity. The arrow in Fig. 1 indicates the viewing direction for this figure. Parts of this drawing were prepared with XTALVIEW (35).
Kinetic Experiments—Assays for FBPase activity employed the coupling enzymes phosphoglucose isomerase and glucose-6-phosphate dehydrogenase and monitored formation of NADPH by either coupling enzymes phosphoglucose isomerase and glucose-6-phosphate dehydrogenase and monitored formation of NADPH by either...
Escherichia coli Fructose-1,6-bisphosphatase

TABLE 2
Conservation of the anion activation and AMP sites
Type-I FBPase sequences from more than 100 species of eukaryotes and bacteria were aligned using the program ClustalW (41). Listed below are results for a representative set of sequences for five residues, referenced by number to the porcine and FBPase was calculated by ALIGN (42).

| Species                        | Percent identity to E. coli FBPase | Residue number in porcine/E. coli FBPase |
|-------------------------------|-----------------------------------|------------------------------------------|
|                               | 14/5 | –/15 | 38/30 | 88/80 | 192/186 |
| Homo sapiens (liver)          | 42   | T    | —     | C     | S     | E     |
| Homo sapiens (muscle)         | 40   | T    | –     | L     | S     | E     |
| Sus scrofa (liver)            | 41   | T    | –     | C     | S     | E     |
| Rattus norvegicus (liver)     | 40   | T    | –     | C     | S     | E     |
| Xenopus tropicalis            | 44   | T    | –     | C     | S     | E     |
| Brachydanio rerio             | 43   | T    | –     | C     | S     | E     |
| Drosophila melanogaster       | 46   | T    | —     | F     | Q     | S     | E     |
| Schistosoma japonicum        | 44   | T    | F     | Q     | S     | E     |
| Caenorhabditis elegans        | 45   | Q    | H     | L     | S     | E     |
| Solanum tuberosum (cytoplasm) | 45   | T    | H     | V     | S     | E     |
| Arabidopsis thaliana (cytoplasm) | 44   | T    | Y     | V     | S     | E     |
| Oryza sativa (chloplast)      | 37   | T    | G     | S     | S     | E     |
| Pisum sativum (chloplast)     | 38   | T    | G     | V     | S     | E     |
| Saccharomyces cerevisiae      | 40   | P    | F     | Q     | S     | E     |
| Yarrowia lipolytica          | 41   | T    | V     | Q     | S     | E     |
| Leishmania major             | 40   | T    | H     | Q     | S     | E     |
| Trypanosoma brucei           | 40   | M    | M     | Q     | S     | E     |
| Toxoplasma gondii            | 52   | G    | L     | K     | R     | T     |
| Mannheimia succiniciproducens| 70   | D    | Y     | R     | R     | T     |
| Pasteurella multocida        | 70   | G    | Y     | R     | R     | V     |
| Haemophilus influenzae       | 70   | S    | Y     | R     | R     | T     |
| Haemophilus ducreyi          | 66   | G    | Y     | R     | R     | T     |
| Salmonella typhi              | 97   | G    | F     | K     | R     | V     |
| Escherichia coli             | 100  | G    | F     | K     | R     | V     |
| Photobacterius luminescens   | 84   | G    | F     | K     | R     | V     |
| Erwinia carotovar            | 87   | G    | F     | K     | R     | V     |
| Versinia pseudotuberculosis  | 83   | G    | F     | K     | R     | V     |
| Vibrio cholera               | 76   | T    | G     | F     | R     | R     |
| Photobacterium profundum     | 73   | G    | F     | K     | R     | V     |
| Chlorobium tepidum           | 52   | E    | Y     | A     | H     | E     |
| Bordetella parapertussis     | 47   | T    | A     | G     | S     |
| Leptospira interrogans       | 45   | G    | F     | K     | R     | V     |
| Nostoc punctiforme           | 40   | S    | F     | A     | S     | E     |
| Alcaligenes eutrophus        | 40   | T    | Y     | A     | G     | E     |
| Shewanella oneidensis        | 43   | A    | —     | A     | D     | E     |
| Geobacter sulfurreducens     | 37   | R    | —     | A     | S     | E     |
| Xanthobacter flavus          | 34   | H    | V     | A     | —     | L     |
| Campylobacter coli           | 31   | —    | —     | Q     | C     | N     |

a Dashes indicate no clear alignment partner.

Kinetic Parameters—Native and selenomethionine-substituted enzymes were kinetically indistinguishable: k_cat K_m for Fru-1,6-P_2 and K_m for Mg_2^+ are 24 s^-1.1, 1.7 ± 0.1 μM and 1.0 ± 0.1 mM, respectively. The Hill coefficients for both Fru-1,6-P_2 and Mg_2^+ are 1.1 ± 0.1. These values are similar to those reported previously for E. coli FBPase (4, 14, 16), and differ from the porcine enzyme principally in the Hill coefficient for Mg_2^+, which for porcine FBPase is 1.9 ± 0.1 (45).

Structure of E. coli FBPase (PDB identifier 2GQ1)—Type I FBPase from E. coli has the same overall fold and tetrameric subunit organization as other Type I FBPases for which structural information is available (Fig. 1) (11, 46, 47). Crystals belong to the space group I222 (α = 45.6, b = 81.3, c = 170.1 Å) with one subunit of the tetramer in the asymmetric unit. Data also were collected from a native protein crystal in-house to a resolution of 2.2 Å. The native and selenomethionine-substituted structures exhibited no differences beyond coordinate uncertainty. Hence, we report here only the results of the high resolution, selenomethionine-substituted structure (Table 1).

Each subunit has 7.5 bound sulfate ions. The “half-sulfate” (sulfate^341) is on the crystallographic 2-fold axis that relates subunits C1 and C2 (and subunits C3 and C4) (Fig. 1). Sulfate^343 is at a lattice contact between adjacent tetramers, and sulfate^346 is at a locus that maps onto the AMP binding side of porcine FBPase (48). Sulfate^346 and sulfate^345 are at the 1-phosphoryl- and 6-phosphoryl-binding sites of Fru-1,6-P_2, respectively, as defined by structures of porcine FBPase (11, 49). An additional sulfate ion (sulfate^342) binds at a locus occupied by the side-chain of Asp^38 of the porcine R-state structure (49). Sulfate^344 binds at the C1-C4 interface, roughly at the location of the carboxyl group of Glu^192 in porcine FBPase (The corresponding residue is Val^186 in the E. coli enzyme), and 8.5 Å away from a symmetry-related partner (Fig. 2). Sulfate^347 binds on the surface of the subunit at a position of no known significance. No other ligands are present, even though fructose-1,6-bisphosphates were present in the crystallization experiments.

The deposited model is in good electron density; however, residues 42–63, which correspond to the dynamic loop of the porcine enzyme, are without electron density and have been omitted. The dynamic loop in porcine FBPase takes on either an engaged or disordered conformation in the absence of AMP (48, 49). The disordered loop in the E. coli structure may be a consequence of the high concentration of sulfate.

E. coli FBPase is in an intermediate quaternary state relative to the canonical R- and T-states of porcine FBPase, being 6° from the R-state and 8° from the T-state. (Quaternary states of porcine FBPase are defined by rotations of the C1-C2 subunit pair with respect to the C3-C4 subunit pair about the vertical molecular 2-fold axis in Fig. 1.) The quaternary state of E. coli FBPase is most similar to the I_R-state of the porcine enzyme (40); however, no conclusion is possible regarding ligand-induced changes in the quaternary state of the E. coli enzyme.

The segment (residues 12–18) connecting the first and second α-helices ( helices H1 and H2, respectively) of E. coli FBPase differs signifi-
stantly from the corresponding element of the porcine enzyme. The connecting loop in porcine FBPase bears residues (such as Thr\(^{27}\)) critical to the recognition of AMP, and in fact, Thr\(^{27}\), Gly\(^{28}\), Glu\(^{29}\), Thr\(^{31}\), Lys\(^{112}\), Tyr\(^{113}\), and Arg\(^{140}\) of porcine FBPase have close analogs in \(E.\ coli\) FBPase. Notwithstanding sequence conservation, the AMP pocket in \(E.\ coli\) FBPase is not open to AMP ligation. Although sulfate\(^{346}\) maps near the 5'-phosphoryl group of AMP in porcine FBPase, Phe\(^{15}\) of the helix H1-H2 connecting loop occupies what would be the binding pocket for AMP (Fig. 3). Moreover, residue types flanking Phe\(^{15}\) differ significantly in the \(E.\ coli\) and porcine enzymes. Hence, the local fold of the polypeptide chain sterically blocks AMP. AMP ligation, as observed as a mechanism of allosteric regulation of catalysis.

Speculate in the discussion, that such a conformational change is part of the catalytic mechanism of the enzyme that would likely perturb helices H1 and H2. We speculate in the discussion, that such a conformational change is part of a mechanism of allosteric regulation of catalysis.

Many residues are conserved between porcine and \(E.\ coli\) FBPases, but a number of critical residues are missing from the C1-C4 interface, most notably residues corresponding to Asn\(^{35}\), Thr\(^{39}\), and Glu\(^{192}\) of the porcine enzyme (Fig. 4). Glu\(^{192}\) in porcine FBPase forms critical hydrogen bonds between subunits C1 and C4 in the R- and T-states, interacting with Thr\(^{39}\) and Lys\(^{42}\). Glu\(^{192}\) is valine in \(E.\ coli\) FBPase, and Thr\(^{39}\) a leucine. Sulfate\(^{346}\) binds in place of the carboxyl group of Glu\(^{192}\), filling a positively charged cavity between subunits C1 and C4. Eight residues define this anion-binding site, six of which differ in type from corresponding residues in porcine FBPase. Porcine residues Thr\(^{14}\), Asn\(^{35}\), Cys\(^{38}\), Thr\(^{39}\), Ser\(^{88}\), and Glu\(^{192}\) are Gly\(^{7}\), Ser\(^{27}\), Lys\(^{30}\), Leu\(^{31}\), Arg\(^{80}\), and Val\(^{186}\) in \(E.\ coli\) FBPase. Only Lys\(^{34}\) and Thr\(^{12}\) in the porcine enzyme are conserved as Lys\(^{34}\) and Thr\(^{3}\) in \(E.\ coli\) FBPase. Five residues, Thr\(^{3}\), Gly\(^{5}\), Lys\(^{30}\), Lys\(^{34}\), and Arg\(^{80}\), contribute interactions to sulfate\(^{344}\) (Fig. 4). In the R-state porcine enzyme, Asn\(^{35}\) hydrogen bonds across the C1-C4 interface through water molecules and interacts directly with Thr\(^{14}\) of the same subunit. These interactions are missing in \(E.\ coli\) FBPase; the corresponding residues (Ser\(^{27}\) and Gly\(^{5}\)) are correspondingly smaller and allow sulfate\(^{344}\) to interact with backbone amide 5 of the first turn of helix H1. The presence of several water molecules in the cavity suggests a binding site for a larger ligand.

**Activation of \(E.\ coli\) FBPase**—The specific activity of \(E.\ coli\) FBPase decreases with dilution in the standard assay mixture, whereas that of porcine FBPase is constant (data not shown). The decline in specific activity of the \(E.\ coli\) enzyme in response to dilution occurs in minutes and is not temperature-dependent, but is affected by certain salts. PEP and ammonium sulfate activate \(E.\ coli\) FBPase in assays initiated by the addition of Mg\(^{2+}\) (enzyme is at equilibrium prior to the initiation of assays). The activity of \(E.\ coli\) FBPase increases more than 300% in the presence of 20 mM (NH\(_4\))\(_2\)SO\(_4\) (Fig. 5). Activation is caused by the sulfate anion; NH\(_4\)Cl only causes inhibition of the \(E.\ coli\) enzyme (Fig. 5); Na\(_2\)SO\(_4\) and KH\(_2\)PO\(_4\) exhibit similar activation effects (data not shown). Under identical conditions, porcine FBPase is also activated to a lesser extent by (NH\(_4\))\(_2\)SO\(_4\), but the observed activation is caused by NH\(_4\)\(^+\) (50, 51), and can be reproduced by the addition of NH\(_4\)Cl. Ammonium sulfate concentrations above 20 mM progressively inhibit the \(E.\ coli\) enzyme, presumably because of the binding of sulfate anions to the active site. PEP is a far more potent activator of \(E.\ coli\) FBPase, causing ~300% activation at a concentration of 2 mM (Fig. 5). Fifty percent of maximum activation occurs at a concentration of ~40 \(\mu\)M PEP.

**Sequence Alignments of Type 1 FBPases**—Sequence alignments of more than 100 Type 1 bacterial and eukaryotic FBPases appear in an abbreviated format in Table 2. FBPases fall into three groups: (i)
sequences that have the signature residues of the anion binding site and blocked AMP site (residues corresponding in type and position to Gly⁵, Phe¹⁵, Lys⁴⁰, and Arg⁸⁰ of E. coli FBPase), (ii) sequences that have a glutamate corresponding to Glu¹⁹² of porcine FBPase, and (iii) sequences that have neither signature residues of an anion binding site nor a residue corresponding to Glu¹⁹² of the porcine enzyme. Notably, no sequence falls into a fourth category, matching both Glu¹⁹² of porcine FBPase and signature residues of the anion binding site. Hence, glutamate at position 192 and the anion binding site may be mutually exclusive. Approximately 65% of bacterial sequences lack glutamate at positions corresponding to 192 of porcine FBPase, and of these approximately one-half have the signature residues of an anion-binding site. Only one eukaryotic organism, the pathogenic protozoa Toxoplasma gondii, has residues corresponding in position and type to the anion-binding site. Curiously, all species known to produce Fru-2,6-P₂ always have FBPases with glutamate at positions corresponding to 192 of porcine FBPase. No evidence supports the existence of Fru-2,6-P₂ in T. gondii.

With the exception of T. gondii, organisms that have FBPases with signature residues of the anion-binding site are heterotrophic bacteria, and like E. coli, are members of the phylum Proteobacteria and class Gammaproteobacteria. Most are pathogens, a bias due perhaps to the disproportionate number of FBPase sequences available for pathogenic organisms. The anion-binding site may be present in all known Type I FBPases from the orders Enterobacteriales, Vibroniales, and Pasteurellales, all of which include important pathogens.

DISCUSSION

The structure here indicates a subunit arrangement for the E. coli enzyme, similar to that of mammalian FBPase tetramers. Enzyme concentrations in crystallization experiments, however, are ~20,000-fold higher than those of assays. The E. coli enzyme remains a tetramer at levels 20-fold less than those of crystallization experiments (14, 16), but its apparent mass decreases slightly with concentration (14), suggesting the possibility of a dissociated state of the enzyme.

Porcine FBPase undergoes spontaneous subunit exchange (45, 52, 53). In the presence of Fru-1,6-P₂ or Fru-2,6-P₂, exchange reactions involve only dimers of the tetramer (45, 53). As the active site is shared between subunits C1 and C2, dimer exchange probably entails separation at the C1-C4 interface. Protein-protein hydrogen bonds between subunits C1 and C4 are few in number in the E. coli enzyme, suggesting that in the absence of an anion at the C1-C4 interface the E. coli tetramer may be unstable relative to the mammalian tetramer. At low protein concentrations, and in the absence of specific anionic ligands, the bacterial enzyme may dissociate into less active (or inactive) dimers. Sulfate ions form extensive connections between subunits C1 and C4, and presumably activate the enzyme by stabilizing a tetramer.

If indeed E. coli FBPase requires an anion to assemble into a fully active tetramer, then enzyme activity should be sensitive to concentrations of anions and protein. Large losses in activity have been observed after dialysis steps that remove ammonium sulfate (4), or by dilution of the enzyme (4, 14). Most striking is the sensitivity of E. coli FBPase to the method of assay. The enzyme loses as much as 70% of its activity upon a 1000-fold dilution/incubation in the standard assay, a behavior not exhibited by porcine FBPase. This loss of activity is even more severe in (in excess of 80%) after dialysis of the (NH₄)₂SO₄ from coupling enzymes used in assays.

Pep relieves AMP inhibition (14, 15) and activates FBPases from E. coli (Fig. 5) and other prokaryotic organisms (18, 24–26). Pep levels in E. coli change more than 10-fold between glycolytic and gluconeogenic growth (54), hence the suggestion by Babul et al. (14) that Pep is a physiological regulator of E. coli FBPase. If Pep is a physiological regulator of FBPase, then it must bind to the enzyme, but where? Observations of similar phenomena in Type III FBPases have led others to suggest competition between Pep and AMP for the same site (25), but such a mechanism is unlikely for E. coli FBPase. AMP-binding residues of porcine FBPase are present in E. coli FBPase and yet Pep has no effect on AMP inhibition of mammalian systems (2). The binding of Pep to the active site can only cause inhibition. So the anion-binding site at the C1-C4 interface remains as the most probable site for Pep association. A Pep-bound structure of E. coli FBPase will require new crystallization conditions that avoid high salt concentrations, but using the electron density of the sulfate anion to anchor its phosphoryl group, one can easily model the remaining atoms of Pep into the void of the C1-C4 anion binding pocket (Fig. 6).

A binding site for Pep, distinct from AMP, however, creates a new challenge: What mechanism causes the apparent mutual exclusivity in the binding of Pep and AMP? Unlike the R-state of porcine FBPase, which can bind AMP (40, 53), the sulfate-ligated state of E. coli FBPase cannot bind AMP because Phe¹⁵ occupies the binding pocket for the nucleotide. Important recognition elements for AMP are at the C-terminal side of helix H₁, whereas important recognition elements for the sulfate anion (the proposed binding site of Pep) are at the N-terminal side of helix H₁ (Fig. 7). As Amp binds to E. coli FBPase, the conformational perturbation on helix H₁ probably disrupts hydrogen bonds to the ligand at the C1-C4 interface. In fact, one of the effects of AMP ligation of porcine FBPase is the conformational change in helix H₁ that displaces Ile¹⁰ (which is at the N-terminal side of helix H₁) from a hydrophobic pocket (40). Hence, certain features of the allosteric mechanism of AMP inhibition in mammalian FBPases may be evolutionary adaptations of an ancient mechanism of allosteric regulation prevalent in bacteria.

Sequence alignments of Type I FBPases from eukaryotic and bacterial sources reveal a putative activation site common to heterotrophic bacteria from the class Gammaproteobacteria, specifically the orders Enterobacteriales, Vibrionales, and Pasteurellales, all of which include important pathogens.

Glu¹⁹² of porcine FBPase is conserved among eukaryotic organisms, and is prevalent in Bacteria as well. Approximately 35% of the bacterial FBPases investigated here have glutamate at positions corresponding to 192 of porcine FBPase and lack the signature residues of an anion activation site. The side-chain of Glu¹⁹² probably accomplishes the task of an anion in stabilizing an active tetramer. Hence, the introduction of Glu¹⁹² probably resulted in a constitutively active enzyme, thus requiring a new strategy of regulation. In bacterial FBPases that have glutamate at position 192, that mechanism of regulation is unclear. In eukaryotic organisms, a permanently activated FBPase is subject to dynamic inhibition by Fru-2,6-P₂. At some unknown point in evolution dynamic inhibition of FBPase by Fru-2,6-P₂ became synergistic with AMP.
Escherichia coli Fructose-1,6-bisphosphatase

Acknowledgments—We thank Dr. Scott W. Nelson for helpful discussions regarding molecular cloning, protein purification, and initial rate enzyme kinetics. We also thank 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.

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