AMP transforms fructose-1,6-bisphosphatase from its active R-state to its inactive T-state; however, the mechanism of that transformation is poorly understood. The mutation of Ala54 to leucine destabilizes the T-state of fructose-1,6-bisphosphatase. The mutant enzyme retains wild-type levels of activity, but the concentration of AMP that causes 50% inhibition increases 50-fold. In the absence of AMP, the Leu54 enzyme adopts an R-state conformation nearly identical to that of the wild-type enzyme. The mutant enzyme, however, grows in two crystal forms in the presence of saturating AMP. In one form, the AMP-bound tetramer is in a T-like conformation, whereas in the other form, the AMP-bound tetramer is in a R-like conformation. The latter reveals conformational changes in two helices due to the binding of AMP. Helix H1 moves toward the center of the tetramer and displaces Ile10 from a hydrophobic pocket. The displacement of Ile10 exposes a hydrophobic surface critical to interactions that stabilize the T-state. Helix H2 moves away from the center of the tetramer, breaking hydrogen bonds with a buried loop (residues 187–195) in an adjacent subunit. The same hydrogen bonds reform but only after the quaternary transition to the T-state. Proposed here is a model that accounts for the quaternary transition and cooperativity in the inhibition of catalysis by AMP.

Fructose-1,6-bisphosphatase (d-fructose-1,6-bisphosphate 1-phosphohydrolase, EC 3.1.3.11; FBPase) catalyzes a tightly regulated step of gluconeogenesis, the hydrolysis of fructose 1,6-bisphosphate (F16P2) to fructose 6-phosphate (Fru-6-P), and inorganic phosphate (Pi) (1, 2). AMP and fructose 2,6-bisphosphate (F26P2) binding to FBPase by crystallization. The precise sequence of events that attend the R- to T-state transition in FBPase has been elusive. Crystal structures of the R- and T-states are the endpoints of the allosteric transition and leave much to speculation regarding intermediate conformational states of FBPase. The immediate consequences of AMP binding to the R-state are unknown. Does the dynamic loop become disengaged in response to the binding of AMP or in response to the allosteric transition to the T-state? How does the binding of AMP destabilize the R-state and stabilize the T-state? A recent study (23) revealed the first intermediate state of porcine FBPase, a T-like conformation due to the binding of AMP or inorganic phosphate (Pi), all of which are in proximity to (and probably coordinate with) the 1-phosphoryl group of F16P2 (7–10).

FBPase is a homotetramer (subunit M, of 37,000 (11)) and exists in at least two distinct quaternary conformations called R and T (12–13). AMP induces the transition from the active R-state to the inactive (or less active) T-state. Substrates or products in combination with metal cations stabilize the R-state conformation. A proposed mechanism for allosteric regulation of catalysis involves three conformational states of loop 52–72 called engaged, disengaged, and disordered (14). AMP alone or with F26P2 stabilizes a disengaged loop (15, 16), whereas metals with products stabilize an engaged loop (10, 16–18). In active forms of the enzyme, loop 52–72 probably cycles between its engaged and disordered conformations (14, 17). Fluorescence from a tryptophan reporter group at position 57 is consistent with the conformational states for loop 52–72 observed in crystal structures (19, 20). Thus far, the engaged conformation of loop 52–72 has appeared only in R-state crystal structures and the disengaged conformer has appeared only in T-state structures; however, disordered conformations of the dynamic loop have appeared in both the R- and T-states (16, 17, 21, 22).

The precise sequence of events that attend the R- to T-state transition in FBPase has been elusive. Crystal structures of the R- and T-states are the endpoints of the allosteric transition and leave much to speculation regarding intermediate conformational states of FBPase. The immediate consequences of AMP binding to the R-state are unknown. Does the dynamic loop become disengaged in response to the binding of AMP or in response to the allosteric transition to the T-state? How does the binding of AMP destabilize the R-state and stabilize the T-state? A recent study (23) revealed the first intermediate state of porcine FBPase, a T-like conformation due to the binding of an allosteric effector to the center of the tetramer. The results of that study indicated the potential for trapping intermediate conformational states of FBPase by crystalization.

The mutation of Ala54 to leucine disrupts key packing interactions of the disengaged loop conformation. The resulting Leu54 enzyme has wild-type catalytic properties and retains cooperativity in AMP inhibition but exhibits a 50-fold increase in the IC50 for AMP. In the absence of AMP, Leu54 FBPase is in its canonical R-state. However, two crystal forms grow in the presence of saturating AMP. In one crystal form, the enzyme is in its T-state but with a disordered dynamic loop. In the other crystal form, the tetramer is in an R-like quaternary state with magnitude (6). Hence, although intracellular concentrations of AMP remain relatively constant, AMP should become a more potent inhibitor of FBPase as concentrations of F26P2 increase. AMP binds 28 Å away from the nearest active site and perhaps not surprisingly inhibits catalysis noncompetitively with respect to F16P2. Yet, AMP is a competitive inhibitor of catalysis with respect to essential divalent cations (Mg2+, Mn2+, or Zn2+), all of which are in proximity to (and probably coordinate with) the 1-phosphoryl group of F16P2 (7–10).
an engaged dynamic loop. The latter crystal form reveals the intermediate consequences of AMP association in the absence of an allosteric transition. The observed conformational changes suggest the mechanism by which AMP leverages the allosteric transition in FBPase.

**EXPERIMENTAL PROCEDURES**

**Materials**—F16P2, F26P2, NADP⁺, and AMP were purchased from Sigma. DNA-modifying and restriction enzymes and 54 nucleotide kinase and ligase were from Promega. Glucose-6-phosphate dehydrogenase and phosphoglucone isomerase came from Roche Applied Science. Other chemicals were of reagent grade or equivalent. *Escherichia coli* strains BMH 71–18 mutS and XLI-Blue came from Clontech and Stratagene, respectively. The FBPase-deficient *E. coli* strain DP 657 came from the Genetic Stock Center at Yale University.

**Mutagenesis of Wild-type FBPase**—The mutation of Ala54 to leucine was accomplished by specific base changes in a double-stranded plasmid containing the gene coding for FBPase using the Transformer™ site-directed mutagenesis kit (Clontech). The mutagenic primer for Ala54→Leu was 5′-GGCGGCATCTGCAAGCTCT-3′ (the codon with the point mutation is underlined in boldface). The selection primer for mutagenesis, 5′-CAGCTCCGCTCAGAAGCCCA-3′ (digestion site underlined, changed and inserted into the BamH1 site) and a Deoxyribonuclease I site were added to a XhoI site. The mutation and the integrity of the construct were confirmed by sequencing the promoter region and the entire open reading frame. The Iowa State University sequencing facility provided data on sequences using the fluorescent dye-dideoxy terminator method.

**Expression and Purification of Wild-type and Leu54 FBPases**—Cell-free extracts of wild-type and Leu54 FBPases were subjected to heat treatment (63°C for 7 min) followed by centrifugation. The supernatant solution was loaded onto a Cibacron Blue-Sepharose column previously equilibrated with 20 mM Tris-HCl, pH 7.5. The column was washed first with 20 mM Tris-HCl, pH 7.5. Enzyme was eluted with a solution of 500 mM NaCl and 20 mM Tris-HCl of the same pH. After pressure concentration (Amicon PM-30 membrane) and dialysis against 10 mM Tris-HCl, pH 8.0, the protein sample was loaded onto a DEAE-Sepharose column equilibrated with 10 mM Tris-HCl, pH 8.0. Purified enzyme was eluted with a NaCl gradient (0–0.5 M) in 10 mM Tris-HCl, pH 8.0, and then dialyzed extensively against 50 mM Hepes, pH 7.4, for kinetic investigations and for crystallization experiments. Purity and protein concentrations of FBPase preparations were confirmed by SDS-polyacrylamide gel electrophoresis (24) and the Bradford assay (25), respectively.

**Kinetic Experiments**—Assays for the determination of *kₗₚ* and specific activity ratios at pH 7.5/9.5 employed the coupling enzymes, phosphoglucone isomerase, and glucose-6-phosphate dehydrogenase (1). The reduction of NADP⁺ to NADPH was monitored by absorbance at 340 nm. All of the other assays used the same coupling enzymes but monitored the formation of NADPH by its fluorescence emission at 470 nm using an excitation wavelength of 340 nm. Assays were performed at 22°C in 50 mM Hepes, pH 7.5, or in 50 mM CAPS, pH 9.5. Assay solutions contained EDTA and KCl at concentrations of 100 mM and 150 mM, respectively. Initial rates were analyzed with programs written either in MINTAB using an a value of 2.0 (26) or by ENZFITTER (27). The kinetic data for AMP inhibition with respect to Mg²⁺ and F26P₂ inhibition with respect to F16P2 were fit to several models, but only the parameters associated with the best-fitting mechanism of inhibition are reported under "Results."

**Crystallographic Data**—X-ray intensity data of crystals grown by the method of hanging drops were collected on a Rigaku R-AXIS IV+ rotating anode/image plate system using CuKα radiation from an Osmonic confocal optics system and a temperature-controlled CuKα source reduced with the program package CrystalClear provided with the instrument.

**Structure Determination, Model Building, and Refinement**—Crystals of Leu54 FBPase are isomorphous to either the AMP/Zn²⁺-product complex (16) or the Zn⁴⁺-product complex (10). Phase angles used in the generation of initial electron density maps were based on model LEY J or 1CNQ of the Protein Data Bank from which water molecules, metal cations, small molecule ligands, and residues 52–72 had been omitted. Residues 52–72 were built into the electron density of omit maps using the program XTALVIEW (28). Ligands were added to account for omit electron density at the active site and/or the AMP site. The resulting models underwent refinement using CNS (29) with force constants and parameters of stereochemistry from Engh and Huber (30). A cycle of refinement consisted of slow cooling of 1000 to 300 K in steps of 25 K followed by 120 cycles of conjugate gradient minimization and concluded by the refinement of individual thermal parameters. Thermal parameter refinement employed restraints of 1.5 Å² on nearest neighbor and next-to-nearest neighbor main chain atoms, 2.0 Å² on nearest neighbor side chain atoms, and 2.5 Å² on next-to-nearest neighbor side chain atoms. In subsequent cycles of refinement, water molecules were fit to difference electron density of 2.5σ or better and were added until no significant decrease was evident in the Rfree value. Water molecules in the final models make suitable donor-acceptor distances to each other and the protein and have thermal parameters under 60 Å². Stereochemistry of the models was examined by the use of PROCHECK (31).

**RESULTS**

**Rationale for the Leu54→Ala Mutation**—The Cβ atom of Ala54 is at the center of a cluster of hydrophobic side chains, which forms only when the dynamic loop is in its T-state-disengaged conformation. A mutation at position 54 to a large side chain would disrupt packing interactions and thereby destabilize the disengaged conformation of the dynamic loop. In contrast, ample room is available for large donor-acceptor distances to each other and the protein and have thermal parameters under 60 Å². Stereochemistry of the models was examined by the use of PROCHECK (31).

**Expression and Purification of Wild-type and Leu54 Enzymes**—Expression and isolation procedures described above provide wild-type and Leu54 FBPases in at least 95% purity, as judged by SDS-polyacrylamide gel electrophoresis (data not shown). Gels indicated no proteolysis of the purified enzymes.

**Kinetics Experiments**—Kinetics parameters for Leu54 and wild-type FBPases are in Table I. The determination of *kₗₚ* and *Kₘ* for F16P2 (listed as *K_F16P2* in Table I) at pH 7.5 employed a saturating concentration of Mg²⁺ (2 mM for Leu54 FBPase and 5 mM for wild-type FBPase) and concentrations of substrate ranging from 0.5 to 20 μM. A fit of the Michaelis-Menten equation to the data provided values for *kₗₚ* and *K^16P2_M* (Michaelis constant for F26P2). Ratios of specific activities at

---

**Table I**

| **Wild type** | **Leu54→Ala** |
|--------------|---------------|
| Activity ratio, pH 7.5/9.5 | 3.5 ± 0.5 | 3.9 ± 0.4 |
| *kₗₚ* (s⁻¹) | 20 ± 1 | 11.3 ± 0.6 |
| *K^F16P2_M* (μM) | 1.2 ± 0.5 | 0.94 ± 0.04 |
| *Aₗₜₚ* (mM) | 0.84 ± 0.04 | 0.14 ± 0.03 |
| Hill coefficient Mg²⁺ | 1.7 ± 0.1 | 2.1 ± 0.2 |
| *Iₗₜₚ* (μM) | 1.23 ± 0.04 | 62 ± 1 |
| Hill coefficient AMP | 2.2 ± 0.1 | 2.5 ± 0.1 |
| *Kₘ* (mM) | 0.78 ± 0.2 | 0.087 ± 0.005 |
| *K^F26P2_M* (μM) | 0.6 ± 0.1 | 3000 ± 200 |
| *K^F26P2_P_1* (μM) | 2.4 ± 0.4 | 3.2 ± 0.1 |
| *K^F26P2_P_2* (μM) | 0.25 ± 0.03 | 0.38 ± 0.04 |
pH 7.5–9.5 for wild-type and Leu54 FBPases (each above 3) are indicative of tetrameric enzymes with intact (nonproteolyzed) polypeptide chains.

The Hill coefficient for Mg\(^{2+}\) was determined at a saturating concentration of F16P2 (20\(\mu\)M) and concentrations of free Mg\(^{2+}\) ranging from 0.1 to 5.0 mM. Data were fit to Equation 1,

\[
\frac{v}{V_{m}} = \frac{1}{1 + \left(\frac{A_{0.5}}{A}\right)^{n}} \tag{Eq. 1}
\]

where \(v\) is the velocity, \(V_{m}\) is the maximum velocity at saturating concentrations of F16P2 and Mg\(^{2+}\), \(A\) is the concentration of Mg\(^{2+}\), \(n\) is the Hill coefficient for Mg\(^{2+}\), and \(A_{0.5}\) is the concentration of Mg\(^{2+}\) that gives \(v/V_{m}\) of 50%.

The Hill coefficient for AMP was determined at saturating F16P2 (20\(\mu\)M), Mg\(^{2+}\) concentrations of 0.8 and 0.15 mM for wild-type and Leu54 FBPases, and AMP concentrations ranging from 0 to 500\(\mu\)M. Data were fit to Equation 2,

\[
\frac{v}{V_{0}} = \frac{1}{1 + \left(\frac{I_{0.5}}{I}\right)^{n}} \tag{Eq. 2}
\]

where \(v\) is the velocity, \(V_{0}\) is the velocity at an AMP concentration of zero, \(I\) is the concentration of AMP, \(n\) is the Hill coefficient for AMP, and \(I_{0.5}\) is the concentration of AMP that gives \(v/V_{0}\) of 50%.

### Table II

Statistics of data collection and refinement for Leu54 FBPase

| Crystalline complex | R-state | I0-state | T-state |
|---------------------|---------|----------|---------|
| Resolution limit (Å) | 2.0     | 1.85     | 2.15    |
| Number of measurements | 155,545 | 124,926 | 193,057 |
| Number of unique reflections | 24,972  | 32,122  | 41,418  |
| Completeness of data (%) | 99.6    | 99.1    | 99.2    |
| Last shell/resolution-range (Å) | 96.5/2.07–2.0 | 91.1/1.96–1.85 | 98.6/2.25–2.15 |
| \(R_{\text{sym}}\) | 0.066   | 0.035   | 0.049   |
| Overall \(R_{\text{sym}}\) | 0.228/2.07–2.0 | 0.211/1.96–1.85 | 0.197/2.25–2.15 |
| Number of reflections in refinement | 22,691  | 29,277  | 37,089  |
| Number of atoms | 2,796   | 2,785   | 5,627   |
| Number of solvent sites | 176    | 296    | 510    |
| Mean B (Å²) for protein | 30     | 20     | 36     |
| Mean B (Å²) for AMP | 38     | 29/34  | 29/34  |
| Bond lengths (Å) | 0.006   | 0.005   | 0.005   |
| Bond angles | 1.4     | 1.3     | 1.3     |
| Dihedral angles | 22.8    | 22.5    | 22.9    |
| Improper angles | 0.75    | 0.70    | 0.72    |

\(a\) Space group and unit cell parameters provided under “Results.”

\(b\) \(R_{\text{sym}} = \sum_{i} \sum_{j} |I_{ij} - \langle I_{ij}\rangle| / \sum_{i} \sum_{j} |I_{ij}|\), where \(i\) runs over multiple observations of the same intensity and \(j\) runs over all of the crystallographically unique intensities.

\(c\) \(R_{\text{factor}} = \sum |F_{\text{obs}}| - |F_{\text{calc}}| / \sum |F_{\text{obs}}|\), where \(F_{\text{obs}} > 0\).

\(d\) \(R_{\text{free}}\) based upon 10% of the data randomly pulled and not used in the refinement.

**Fig. 1. Overview of the R-like AMP-product complex of Leu54 FBPase.** The four subunits of the tetramer, labeled C1 through C4, are depicted with one molecule each of bound AMP (allosteric pocket) and Fru-6-P (active site). Active-site bound P, and Mg\(^{2+}\) and side chains of Leu54 from subunits C1 and C3 are omitted for clarity. Residues 52–72 (dynamic loop) and 187–195 (loop 190) are in black. The panel to the right shows electron density covering the AMP molecule from an omit map contoured at a level of 1\(\sigma\) with a cutoff radius of 1 Å. This drawing was prepared with MOLSCRIPT (38).
The kinetic mechanism of AMP inhibition with respect to Mg$^{2+}$ was determined from assays that employed saturating (20 μM) F16P2, five different Mg$^{2+}$ concentrations ranging from 0.8 to 3.0 mM for wild-type enzyme or 0.2 to 0.6 mM for Leu54 FBPase, and five different AMP concentrations ranging from 0 to 150 μM. A model for linear competitive inhibition (Equation 3) provided the best result (goodness-of-fit of <4%).

$$V_m/v = 1 + K_v/A^x + (K_v/K_{AMP}) (IA)^y$$

(Eq. 3)

where $v$ is the velocity, $V_m$ is the velocity at an inhibitor concentration of zero, saturating concentrations of F16P2 and Mg$^{2+}$, $A$ is the concentration of Mg$^{2+}$, $I$ is the concentration of AMP, $K_v$ is the Michaelis constant for Mg$^{2+}$, and $K_{AMP}$ is the dissociation constant for AMP from the enzyme-inhibitor complex. Equation 3 constrains the Hill coefficients for Mg$^{2+}$ and AMP to 2, consistent with independent determinations of these quantities.

The kinetic mechanism of F26P2 inhibition with respect to F16P2 was determined from assays that employed saturating Mg$^{2+}$ (5 mM for wild-type enzyme and 2 mM for Leu54 FBPase), five different concentrations (1–6 μM) of F16P2, and five different concentrations (0–1.0 μM) of F26P2. A model for linear competitive inhibition provided the best fit to the data (goodness-of-fit of <3%).

$$V_m/v = 1 + K_v/B + (K_v/K_{AMP}) (IB)^y$$

(Eq. 4)

where $V_m$ is the velocity at an inhibitor concentration of zero and saturating concentrations of F16P2 and Mg$^{2+}$, $B$ is the concentration of F16P2, $I$ is the concentration of F26P2, $K_v$ is the Michaelis constant for F16P2, and $K_{AMP}$ is the dissociation constant for F26P2 from the inhibitor-enzyme complex.

**Product Complex of Leu54 FBPase (Protein Data Bank code 1Y7Z)—Crystals belong to the space group I222 (α = 53.8, β = 82.6, and c = 166.6 Å). They contain one subunit in the asymmetric unit and are isomorphous to those of wild-type FBPase in its R-state (10, 16–18). Electron density for residues 1–9 is weak or absent. The model begins at residue 10 and continues to the last residue of the sequence. Thermal parameters vary from 10 to 75 Å$^2$. The model has stereochemistry comparable to that of structures of equivalent resolution (31).

The subunit of the AMP-product complex of Leu54 FBPase has one molecule each of Fru-6-P and P$_1$ with three atoms of Mg$^{2+}$ at the active site. In addition, strong electron density is present in the allosteric inhibitor pocket, which represents a bound molecule of AMP (Fig. 1). The dynamic loop (residues 52–72) adopts the engaged conformation. Superposition of the Leu54 tetramer onto canonical wild-type R- and T-states clearly indicates an R-state complex. We refer the reader to other descriptions of R-state product complexes (10, 16–18) for more detailed descriptions of comparable structures.

**AMP-Product R-like Complex of Leu54 FBPase (Protein Data Bank code 1Y7Z)—Crystals belong to the space group I222 (α = 53.8, β = 82.6, and c = 166.6 Å). They contain one subunit in the asymmetric unit and are isomorphous to those of wild-type FBPase in its R-state (10, 16–18). Electron density for residues 1–9 is weak or absent. The model begins at residue 10 and continues to the last residue of the sequence. Thermal parameters vary from 10 to 75 Å$^2$. The model has stereochemistry comparable to that of structures of equivalent resolution (31).**

The subunit of the AMP-product complex of Leu54 FBPase has one molecule each of Fru-6-P and P$_1$ with three atoms of Mg$^{2+}$ at the active site. In addition, strong electron density is present in the allosteric inhibitor pocket, which represents a bound molecule of AMP (Fig. 1). The dynamic loop (residues 52–72) adopts the engaged conformation. Superposition of the Leu54 tetramer onto canonical wild-type R- and T-states reveals a change in quaternary state (Table III). Subunit pair C1-C2 has rotated 3° relative to C3-C4 (Fig. 2). The dynamic loop lies between that of the canonical R-state (0° rotation) and T-state (15° rotation) and differs from the intermediate quaternary state (9° rotation) stabilized by the allosteric inhibitor OC252 (Fig. 2). Hereafter, we will use the label I$_T$ to represent the R-like state of the AMP-product complex of Leu54 FBPase and I$_R$ to represent the T-like state of the OC252 complex.

The I$_T$ structure reveals the effect of AMP binding in the absence of a complete quaternary transition. The superposition of the I$_T$ subunit onto the subunits of the R-state tetramer removes coordinate displacements due to the partial (3°) rotation of the subunit pairs in the I$_R$-state, revealing conformational changes at the tertiary level. In such a comparison, conformational changes are evident only in the N-terminal element and helices H1 and H2. The 6-amino group of AMP draws backbone carbonyl 17 (helix H1) toward itself while pushing away the side chain of Val17 in avoiding unacceptable interactions.
nonbonded contacts (Fig. 3A). The interactions between AMP and Val17 translate helix H1 by 0.5 Å toward the center of the tetramer and move the C-terminal end of helix H1 1.0 Å toward the bound AMP molecule. The movement compresses the N-terminal end of helix H1 into residues 193–195 of subunit C1 and displaces the side chain of Ile10 from a hydrophobic cluster of residues (Fig. 3, B and C). Helix H2 moves along its axis 0.5 Å away from the center of the tetramer. Helix movements sever hydrogen bonds between Thr14 and Asn35 and between Thr39 and Glu192, the latter being a linkage between subunits C1 and C4 (Table IV). Moreover, the hydrogen bond between Thr46 and backbone carbonyl 189 (another C1-C4 contact) may be weakened. Lys42 remains in its intersubunit salt link with Glu192 with little change to its other hydrogen-bonding interactions. The loss or weakening of hydrogen bonds involving Thr39, Thr46, and Glu192 observed in the IR structure is not evident in a direct comparison of the canonical R- and T-states (Table IV).

The superposition of the IR-state subunit onto the subunits of the T-state tetramer of wild-type FBPase reveals tertiary conformational change due to the 12° subunit-pair rotation. This includes the movement of the dynamic loop from its engaged to

![Image of tertiary conformational changes between R- and IR-states](Fig. 3. Tertiary conformational changes between R- and IR-states. Dotted lines represent selected donor-acceptor interactions of 3.2 Å or less. Solid green lines represent potential nonbonded contacts of 2.5 Å or less. Panels A–C, superposition of the IR-state subunit (red) onto each subunit of the R-state tetramer (black) reveals tertiary conformational changes induced by the binding of AMP and the resulting 3° subunit-pair rotation. Hydrogen bonds (dotted red lines) involving the 6-amino group of AMP and a nonbonded contact (green line) with the side chain of Val17 induce helix movement and shears hydrogen bonds between Thr14 and Asn35 (panel A) as well as Glu192 and Thr39 (panels A–C). In addition, the movement of helix H1 displaces Ile10 from its R-state hydrophobic contacts (panels B and C). This drawing was prepared with MOLSCIRPT (38). Inset, shown are regions of the tetramer (purple) and viewing directions (boldface arrows) corresponding to panels A–C. The viewing direction for panel C is 45° inclined to the plane of the tetramer.)

![Image of tertiary conformational changes between R- and IR-states](Tertiary conformational changes between R- and IR-states. Dotted lines represent selected donor-acceptor interactions of 3.2 Å or less. Solid green lines represent potential nonbonded contacts of 2.5 Å or less. Panels A–C, superposition of the IR-state subunit (red) onto each subunit of the R-state tetramer (black) reveals tertiary conformational changes induced by the binding of AMP and the resulting 3° subunit-pair rotation. Hydrogen bonds (dotted red lines) involving the 6-amino group of AMP and a nonbonded contact (green line) with the side chain of Val17 induce helix movement and shears hydrogen bonds between Thr14 and Asn35 (panel A) as well as Glu192 and Thr39 (panels A–C). In addition, the movement of helix H1 displaces Ile10 from its R-state hydrophobic contacts (panels B and C). This drawing was prepared with MOLSCIRPT (38). Inset, shown are regions of the tetramer (purple) and viewing directions (boldface arrows) corresponding to panels A–C. The viewing direction for panel C is 45° inclined to the plane of the tetramer.)
The enzyme in this crystal form is in the T-state (quaternary transition angle of 15°); however, unlike loop-disengaged AMP complexes of the wild-type enzyme, the dynamic loop in T-state Leu54 FBPase is disordered. Moreover, hydrogen bonds normally well established in the T-state of the wild-type enzyme seem to be marginally weaker in T-state Leu54 FBPase. The active site retains Fru-6-P and Mg2+ bound with low occupancy to site 1.

**DISCUSSION**

Conformational changes between the R- and IR-states of Leu54 FBPase are consistent with two models for quaternary change: 1) a concerted model in which AMP drives the quaternary transition by acting on a set of interconnected levers and 2) a sequential model in which AMP raises the energy level of the R-state while simultaneously lowering that of the nascent T-state (Fig. 5). The point of departure for each model is an AMP-induced translation of helices H1 and H2 in opposite directions, helix H1 toward and helix H2 away from the center of the tetramer. The two models differ in regard to the consequences of helix H2 movement. For the concerted model, helix H2 retains its interactions with loops 190, whereas in the sequential model, interactions between helix H2 and loops 190 are broken.

In the concerted model, two sets of coupling interactions distribute forces throughout the entire tetramer due to the binding of one AMP molecule. One set of interactions involves Thr39, Lys42, and Thr46 of helices H2 with Glu192 and backbone carbonyls 189 and 190 of loops 190 (Table IV). These interactions link subunits C1-C4 and C2-C3. A second set of interactions defines the C1-C2 and symmetry-related C3-C4 subunit interfaces (13). The binding of one molecule of AMP, for instance subunit C1, results in the aforementioned movements of helices H1 and H2. The movement in helix H2 of subunit C1 exerts an outward force on loops 190 of subunits C1 and C4. The C1-C2 and C3-C4 subunit interactions, however, constrain loops 190 to a fixed distance from the center of the tetramer. Loops 190 can only follow the outward movement of helix H2 by the rigid body rotations of subunit pairs C1-C2 and C3-C4 (Fig. 5). The two sets of coupling interactions ensure that all loops 190 and their associated subunits undergo rigid-body motions and that all helices H2 undergo an outward movement in response to the binding of one or more molecules of AMP.

The concerted model suffers from two significant shortcomings. First, the coupling interactions between helix H2 and loops 190 have weakened in the IR state. Only the interactions involving Lys42 appear unaffected by movements in helices H2, and we suggest below that even this critical salt link may rupture during the quaternary transition. The weakened linkages between helices H2 and loops 190, however, may be the consequence of having four AMP molecules bound to an R-state tetramer. Two bound molecules of AMP convert R-state hybrid tetramers of FBPase into their T-states (32). Hence, the IR-state of the Leu54 FBPase may represent an “over-torqued” tetramer, the existence of which is possible only because the mutation at position 54 eliminates the T-state as a low-energy alternative. The second shortcoming of the concerted model is not so easy to dismiss. A concerted mechanism for FBPase requires cooperativity in the binding of AMP molecules to any pair of sites. A hybrid tetramer of FBPase that constrains AMP binding to subunits C1 and C2, however, exhibits noncooperative inhibition, even though it undergoes a quaternary transition (32).

In considering the sequential model for the quaternary transition, we note first that the subunit rotation observed in the R-
to T-state transition cannot happen as a rigid-body motion. In
the R-state, loop 190 from subunit C1 is in contact with the
symmetry-related loop from subunit C4. Progress toward the
T-state results in unacceptable contacts between loops 190
from subunits C1 and C4. Loop 190 must undergo confor-
mati onal change, but multiple hydrogen bonds fix its conformation
in both the R- and T-states (Table IV). The movement of helix
H2 releases a conformational restraint on loop 190 in a neigh-
boring subunit by the disruption or weakening of hydrogen
bonds involving Thr39 and Thr46. In this environment of fewer
restraints, loop 190 is more likely to relax unfavorable contacts
that occur during the quaternary transition. Moreover, the
movement in helix H2 favorably positions Thr39 and Thr46 for
the formation of strong hydrogen bonds in the T-state. In fact,
the hydrogen bond involving Glu192 and Thr39 ruptured in the
R- to T-state transition reforms in the T-state. The mechanism
is fully reversible. The loss of AMP from the T-state presum-
ably causes helix H2 to move back toward the center of the
enzyme, breaking or weakening hydrogen bonds between sub-
units C1 and C4 and repositioning Thr39 and Thr46 in favor of
R-state interactions.

The sequential model can accommodate both cooperative and
noncooperative mechanisms of AMP inhibition. A mixture of
hybrid mutants of FBPase that force AMP binding to opposite
halves of the tetramer exhibits cooperative inhibition (32).
Hence, subunit coupling must exist between top and bottom
halves of the tetramer. Coupling interactions necessarily in-
volve subunits C1 and C4, because subunits C1 and C3 have no
direct interactions in the R-state. The binding of AMP to sub-
unit C1 may not only disrupt hydrogen bonds between helix H2 of
subunit C1 and loop 190 of subunit C4, but it may also
weaken symmetry-related hydrogen bonds between helix H2 of
subunit C4 and loop 190 of subunit C1. Hence, a second mole-
cule of AMP would divert less of its binding energy to the
movement of helix H2 in subunit C4 and, as a consequence,
bind with higher affinity. All of the reported mutations of
Lys42, Arg49, Glu192, Ile190, and Gly191 and some of the muta-
tions of Lys50 abolish AMP cooperativity (22, 33–35). These
residues are part of helix H2 or loop 190 and are near to or part
of the coupling interactions between subunits C1 and C4.

A second coupling pathway between AMP binding sites may
involve Arg22. The mutation of Arg22 to methionine eliminates
cooperativity in AMP inhibition (36). Arg22 is near the AMP
pocket, has high thermal parameters in the R-state, and does
not participate in intersubunit hydrogen bonds in either the R-
or T-state structures. Replacing subunit C1 of R-state Leu54
FBPase with an I5 subunit generates a model that approxi-
mates FBPase with one bound molecule of AMP (Fig. 6). In that
model, Arg22 of subunit C1, due to conformational changes
induced by AMP, makes a strong hydrogen bond with backbone
carbonyl 108 of subunit C4. The formation of the symmetry-
related hydrogen bond involving Arg22 of subunit C4 should

Fig. 5. Models of concerted and sequential conformational change. The subunits of the FBPase tetramer are simplifi ed to helix H2 (rectangle) and loop 190 (oval). The viewing direction is down a molecular axis of 2-fold symmetry with subunit C1 and C2 above the plane (boldface lines) and subunit C3 and C4 below the plane. In the concerted model (left), AMP molecules bind successively in any order to the subunits of tetramer until the combined torque (represented by open arrows) on each subunit pair exceeds the energy barrier that separates the R- and T-states. The binding of at least two AMP molecules is necessary for the concerted conformational change. In the sequential model (right), the first molecule of AMP can bind to any subunit with equal affinity, causing the outward movement of helix H2 of only that subunit (filled arrow). If binding occurs at subunit C1, interactions between subunit C1 and C4 are weakened. The second molecule of AMP binds to subunit C4, because less binding energy is spent in the movement of helix H2 in that subunit. The R- to T-state transition can occur in response to the binding of at least two molecules of AMP. For the sequential mechanism, transition to the T-state restores hydrogen bonds lost by the AMP-induced movements of helices H2 in the R-state.
induce conformational changes in subunit C4 that favor the binding of AMP.

The concerted and sequential models both assume an energy barrier between R- and T-states. In the sequential model, hydrogen bonds involving Thr39 and Thr46 contribute significantly to the barrier but not so in the concerted model because these interactions are retained. At least one other interaction may contribute significantly to the energy barrier between R- and T-states. Unacceptable contacts between the side chains of Glu192 (subunit C4) and Lys42 (subunit C1) are likely during the quaternary transition (Fig. 4B). A conformational change in the side chain of Glu192 eliminates bad contacts with Lys42 and re-establishes its hydrogen bond with Thr39. The conformational change in Glu192 may require a transitory loss or weakening of its salt link with Lys42. The presumed loss of this salt link could favor the dissociation of the tetramer into subunit pairs (C1-C2 from C3-C4) and thereby represent the first step in FBPase subunit exchange kinetics (32, 37). The models above have yet to consider conformational change in the dynamic loop (residues 52–72). In all of the reported structures of FBPase, the dynamic loop is either engaged or disordered in R-like states and either disordered or engaged in T-like states. The AMP-induced movement of helix H1, which probably occurs in concert with that of helix H2, displaces Ile10 from a hydrophobic surface. That surface interacts with side chains of the dynamic loop in its disengaged T-state conformation (Fig. 4C). In the R-state, Ile10 effectively blocks the disengaged conformer of the dynamic loop. The formation of the disengaged conformer appears as a significant thermodynamic driving force in the quaternary transition to the T-state. A modest change in the conditions of crystallization (substitution of glycerol for 2-methy-2,4-pentanediol) transforms the AMP-product complex of Leu54 FBPase from a loop-disordered T-state to a loop-engaged R-state. Direct interactions between cryoprotectant and enzyme are unlikely, because no bound cryoprotectant molecules appear in either crystal structure. The AMP-product complex of Leu54 FBPase probably has substantial populations of Iγ- and T-states in solution, allowing the growth of different crystal forms under nearly identical conditions.

The potential significance of C1-C4 interactions in FBPase has been suggested by others (13, 22), but crystal structures of the R- and T-states did not reveal the transitory loss of hydrogen bonds across the C1-C4 subunit interface. As a consequence, the basis for a sequential mechanism of quaternary change remained hidden. The sequential model presented here reconciles properties of AMP inhibition in wild-type, mutant, and hybrid FBPases with known conformational changes in the tetramer. The present study also suggests that AMP-ligation of the R-state does not displace the dynamic loop from its engaged conformation. Instead, the dynamic loop leaves the engaged conformation in the T-state for reasons now poorly understood.

REFERENCES

1. Benkovic, S. T., and de Maine, M. M. (1982) Adv. Enzymol. Relat. Areas Mol. Biol. 53, 45–82
2. Tejwani, G. A. (1983) Adv. Enzymol. Relat. Areas Mol. Biol. 54, 121–194
3. Van Schaftingen, E. (1987) Adv. Enzymol. Relat. Areas Mol. Biol. 59, 45–82
4. Piliks, S. J., El-Maghrabi, M. R., and Claus, T. H. (1988) Ann. Rev. Biochem. 57, 755–783
5. Okar, D. A., and Lange, A. J. (1999) Biofactors 10, 1–14
6. Pillus, S. J., El-Maghrabi, R. M., McGuire, M. M., Piliks, J., and Claus, T. H. (1981) J. Biol. Chem. 256, 3619–3622
7. Liu, F., and Fromm, H. J. (1991) J. Biol. Chem. 266, 11774–11778
8. Liu, F., and Fromm, H. J. (1988) J. Biol. Chem. 263, 9122–9128
9. Scheffler, J. E., and Fromm, H. J. (1986) Biochemistry 25, 6659–6665
10. Choe J.-Y., Poland B. W., Fromm H. J., and Honzatko R. B. (1998) Biochemistry 33, 11441–11450
11. Stone, S. R., and Fromm, H. J. (1990) Biochemistry 19, 620–625
12. Ke, H., Zhang, Y., Liang, J.-Y., and Lipscomb, W. N. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 2989–2993
13. Zhang, Y., Liang, J.-Y., Huang, S., and Lipscomb, W. M. (1994) J. Mol. Biol. 240, 609–624
14. Nelson, S. W., KurbanoF, H., Honzatko, R. B., and Fromm, H. J. (2001) J. Biol. Chem. 276, 6119–6124
15. Xue, Y., Huang, S., Liang, J.-Y., Zhang, Y., and Lipscomb, W. N. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 12482–12486
16. Choe, J.-Y., Fromm, H. J., and Honzatko, R. B. (2000) Biochemistry 39, 8965–8974
17. Choe, J.-Y., Nelson, S. W., Fromm, H. J., and Honzatko, R. B. (2003) J. Biol. Chem. 278, 16008–16014
18. Choe, J.-Y., Iancu, C. V., Fromm, H. J., and Honzatko, R. B. (2003) J. Biol. Chem. 278, 16015–16020
19. Nelson, S. W., Choe, J.-Y., Iancu, C. V., Honzatko, R. B., and Fromm, H. J. (2000) Biochemistry 39, 11100–11106
20. Wen, J., Nelson, S. W., Honzatko, R. B., Fromm, H. J., and Petrich, J. W. (2001) Photochem. Photobiol. 74, 679–685
21. Ke, H. M., Thorpe, C. M., Seaton, B., Lipscomb, W. N., and Marcus F. (1990) J. Mol. Biol. 233, 11100–11106
22. Lu, G., Stee, B., Giroux, E. L., and Kuntrowitz, E. R. (1996) Protein Sci. 5, 2333–2342
23. Choe, J.-Y., Nelson, S. W., Arienti, K. L., Asse, F. U., Collins, T. L., Jones, T. K., Kimmich, R. D. A., Newman, M. J., Norvell, K., Ripka, W. C., Romano, S. J., Short, K. M., Stee, D. H., Fromm, H. J., and Honzatko, R. B. (2003) J. Biol. Chem. 278, 51175–51183
24. Laemmli, U. K. (1970) Nature 227, 680–685

FIG. 6. Stereoview of the proposed role of Arg22 in cooperativity of AMP inhibition. The view is down a molecular 2-fold axis toward the center of the tetramer. The superposition of the Iγ-state subunit (boldface lines) onto subunit C1 of the R-state (fine lines) represents possible relaxation events due to the binding of AMP to subunit C1 (top). AMP-induced conformational change in helix H1 would allow the formation of a hydrogen bond between Arg22 (subunit C1) and backbone carbonyl 108 (subunit C4) and stack the side chains of Arg22 and Phe49 (subunit C4). A second superposition of an Iγ-state subunit (boldface lines) onto subunit C4 of the R-state (fine lines) represents the relaxation of subunit C4 to the altered conformation of AMP-bound subunit C1 (bottom). The proposed interaction involving Arg22 (subunit C1) carries over to the symmetry-related Arg22 (subunit C4). As a consequence, the AMP pocket of subunit C4 may adopt a conformation that approximates the AMP-bound conformation of subunit C1, even in the absence of AMP.
25. Bradford, M. M. (1976) Anal. Biochem. 72, 248–252
26. Liu, F., and Fromm, H. J. (1990) J. Biol. Chem. 265, 7401–7406
27. Leatherbarrow, R. J. (1987) ENZFITTER: A Non-Linear Regression Data Analysis Program for the IBM PC, pp. 13–75, Elsevier Science Publishers B.V., Amsterdam
28. McClure, D. E. (1990) J. Mol. Graph. 10, 44–46
29. Bruenger, A. T., Adams, P. D., Clore, G. M., DeLano, W. L., Gros, P., Grosse-Kunstleve, R. W., Jiang, J.-S., Kuszewski, J., Nilges, M., Pannu, M. S., Read, R. J., Rice, L. M., Simonson, T., and Warren, G. L. (1998) Acta Crystallogr. Sect. D 54, 905–921
30. Engblom, R., and Huber, R. (1991) Acta Crystallogr. Sect. A 47, 392–400
31. Laskowski, R. A., MacArthur, M. W., Moss, D. S., and Thornton, J. M. (1993) J. Appl. Crystallogr. 26, 283–291
32. Nelson, S. W., Honzatko, R. B., and Fromm, H. J. (2002) J. Biol. Chem. 277, 15539–15545
33. Lu, G., Giroux, E. L., and Kantrowitz, E. R. (1997) J. Biol. Chem. 272, 5076–5081
34. Carcamo, J. G., Yañez, A. J., Ludwig, H. C., Lein, O., Pinto, R. O., Reyes, A. M., and Slebe, J. C. (2000) Eur. J. Biochem. 267, 2242–2251
35. Shyur, L.-F., Aleshin, A. E., Honzatko, R. B., and Fromm, H. J. (1996) J. Biol. Chem. 271, 33301–33307
36. Shyur, L.-F., Aleshin, A. E., Honzatko, R. B., and Fromm, H. J. (1996) J. Biol. Chem. 271, 3005–3010
37. Nelson, S. W., Honzatko, R. B., and Fromm, H. J. (2001) FEBS Lett. 492, 254–258
38. Kraulis, J. (1991) J. Appl. Crystallogr. 24, 946–950

Fructose-1,6-bisphosphatase 19745