Mutations in the Hinge of a Dynamic Loop Broadly Influence Functional Properties of Fructose-1,6-bisphosphatase

Scott W. Nelson, Jun-Yong Choe, Richard B. Honzatko, and Herbert J. Fromm‡

From the Department of Biochemistry, Biophysics, and Molecular Biology, Iowa State University, Ames, Iowa 50011

Loop 52–72 of porcine fructose-1,6-bisphosphatase may play a central role in the mechanism of catalysis and allosteric inhibition by AMP. The loop pivots between different conformational states about a hinge located at residues 50 and 51. The insertion of proline separately at positions 50 and 51 reduces \( k_{\text{cat}} \) by up to 3-fold, with no effect on the \( K_m \) for fructose-1,6-bisphosphate. The \( K_m \) for Mg\(^{2+}\) in the Lys\(^{50} \) → Pro mutant increases ~15-fold, whereas that for the Ala\(^{51} \) → Pro mutant is unchanged. Although these mutants retain wild-type binding affinity for AMP and the fluorescent AMP analog 2′(3′)-O-(trinitrophenyl)adenosine 5′-monophosphate, the \( K_m \) for AMP increases 8000- and 280-fold in the position 50 and 51 mutants, respectively. In fact, the mutation Lys\(^{50} \) → Pro changes the mechanism of AMP inhibition with respect to Mg\(^{2+}\) from competitive to non-competitive and abolishes \( K^* \) activation. The \( K_m \) for fructose-2,6-bisphosphate increases ~20- and 30-fold in the Lys\(^{50} \) → Pro and Ala\(^{51} \) → Pro mutants, respectively. Fluorescence from a tryptophan introduced by the mutation of Tyr\(^{57} \) suggests an altered conformational state for Loop 52–72 due to the proline at position 50. Evidently, the Pro\(^{50} \) mutant binds AMP with high affinity at the allosteric site, but the mechanism of allosteric regulation of catalysis has been disabled.

Fructose-1,6-bisphosphatase (d-fructose-1,6-bisphosphatase 1-phosphohydrolase, EC 3.1.3.11; FBPase) catalyzes the hydrolysis of fructose 1,6-bisphosphate (Fru-1,6-P\(_2\)) to Fru-6-P and P\(_i\) (for a review, see Refs. 1–3). FBPase is a tetramer of identical subunits (\( M_r = 37,000 \)). Each subunit has binding sites for Fru-1,6-P\(_2\), Fru-2,6-P\(_2\), and metal ions (which, taken together, define the active site) and a distinct AMP-binding site (4–6). The two binding loci within the same subunit are 28 Å apart, lying for the most part in separate folding domains (called the AMP and Fru-1,6-P\(_2\) domains). The four subunits of the tetramer occupy the corners of a rectangle, labeled clockwise C1 through C4, starting with the upper left-hand corner (7). FBPase exists in at least two conformational states, called R and T, which differ by a 17° rotation of the C1-C2 subunit pair with respect to the C3-C4 subunit pair about one of three intersecting 2-fold axes of the tetramer (7, 8).

For Loop 52–72 to move between its various states, significant conformational change must occur in hinge elements at residues 50 and 51 and residues 71 and 72. The combined mutation of lysines 71 and 72 to methionine dramatically increases the \( K_{\text{in}} \) for AMP, presumably due to the stabilization of the R-state over the T-state conformer of FBPase (11). If the loop-mediated mechanism of AMP inhibition is valid, mutations at the second hinge element must qualitatively cause a similar increase in the \( K_{\text{in}} \) for AMP, yet mutations of Lys\(^{50} \) to methionine (17) and glutamine and alanine (18) cause virtually no change in the \( K_{\text{in}} \) for AMP. What is clear from crystal structures, however, is a large difference in main chain angles (\( \phi, \psi \)) at position 50 in the R- and T-states of FBPase (7, 9, 10). Here we explore the consequence of mutations Lys\(^{50} \) → Pro and...
Ala¹⁵¹ → Pro, which directly influence the backbone conformation of residues 50 and 51. The mutations have little effect on the binding affinity of a fluorescent analog of AMP, but increase the kinetic $K_m$ value for that analog by several orders of magnitude. The kinetic $K_m$ value for Fru-1,6-P$_2$ is unchanged by the mutations, but the $K_m$ for Mg$^{2+}$ rises 15-fold in the case of the Pro⁵⁰ mutant and is 40-fold less active under standard conditions of assay. The data suggest that Loop 52–72 is an essential element in the allosteric mechanism of FBPase.

**EXPERIMENTAL PROCEDURES**

**Materials**—Fru-1,6-P$_2$, Fru-2,6-P$_2$, NADP$^+$, AMP, ampicillin, and isopropyl-$\beta$-thiogalactopyranoside were purchased from Sigma. DNA-modifying and restriction enzymes, T4 polynucleotide kinase, and ligase were from Promega. Glucose-6-phosphate dehydrogenase and phosphoglucone isomerase came from Roche Molecular Biochemicals. Trypomine, yeast extract, and agar were from Difco. Other chemicals were of reagent grade or equivalent. *Escherichia coli* strains BHM71-18 mutS and XL1-Blue came from CLONTECH and Stratagene, respectively. FBPase-deficient strain DF657 came from the Genetic Stock Center at Yale University.

**Mutagenesis of Wild-type FBPase**—Mutations were accomplished by specific base changes in double-stranded plasmid using the Transform-er™ site-directed mutagenesis kit (CLONTECH). The mutagenic primers for the Lys⁵⁰ → Pro, Ala¹⁵¹ → Pro, and Tyr⁷⁷ → Trp mutants were 5′-GTCGCCGGGGGGCACTC-3′, 5′-CCGGAAGCGGGCATCGC-3′, and 5′-CGCACTTGGGGAGATG3′, respectively (codons for mutations in boldface and underlined). The selection primer 5′-CAGGCTCGAGACCCG-3′ (the digestion site is in boldface and underlined) changed an original NcoI site on the plasmid into a XhoI site. The mutation and integrity of the gene were confirmed by sequencing the entire gene. The Iowa State University sequencing facility provided DNA sequences using the fluorescent dye dyeoxy terminator method.

**Expression and Purification of Wild-type and Mutant FBPases**—Protein expression and purification were performed as described previously (11), but with minor alterations. After cell breakage, FBPase was enriched and concentrated by 70% ammonium sulfate fractionation. The precipitate was taken up in 20 mM Tris (pH 7.5) and desalted on a Sephadex G-100 column using 20 mM Tris (pH 7.5). The active fractions were loaded directly onto a Cibacron blue column and eluted with a 0.5–1 M NaCl gradient. FBPase eluted at ~800 mM NaCl. The eluent was dialyzed against 20 mM Tris-HCl (pH 8.3), loaded onto a DEAE-Sephadex column, and then developed with a 0–300 mM NaCl gradient. FBPase eluted at a salt concentration of 150 mM, and its purity was confirmed by SDS-polyacrylamide gel electrophoresis (19). Protein concentration was determined using the Bradford assay (20) with bovine serum albumin as a standard.

**Circular Dichroism Spectroscopy**—CD studies on wild-type and mutant FBPases were done at room temperature on a Jasco J710 CD spectrometer in a 1-cm cell using a protein concentration of 0.35 mg/ml. Spectra were collected from 200 to 260 nm in increments of 1.3 nm. Each spectrum was blank-corrected and smoothed using the software package provided with the instrument.

**Kinetic Experiments**—Assays for the determination of specific activity, $v_{max}$, and activity ratios at pH 7.5 and 9.5 employed the coupling enzymes phosphoglucone isomerase and glucose-6-phosphate dehydrogenase (1). The reduction of NADP$^+$ to NADPH was monitored directly at 340 nm. All other assays employed the same coupling enzymes, but monitored NADPH evolution by its fluorescence emission at 470 nm (excitation wavelength of 340 nm). Initial rate data were analyzed using the Transform-er™ method (12) or ENZFITTER (21). The kinetic data were fit to a variety of models. Only models and parameters for the best fits are reported below.

**Steady-state Fluorescence Measurements**—Fluorescence data were collected using an SLM 8100C fluorometer (Spectronic Instruments). The single tryptophan in the Trp⁷⁷ mutant was excited selectively using a radiation of 295-nm wavelength. Fluorescence emission spectra were collected using an SLM 8100C fluorometer (Spectronic Instruments).

$$F = (F - B)/(V/V_0)10^{(A_{em}/A_{em})/2}$$ (Eq. 1)

where $F$ is the corrected fluorescence, $F$ is the fluorescence intensity experimentally measured, $B$ is the background, $V$ is the volume of the sample for a specific titration point, $V_0$ is the initial volume of the sample, $A_{em}$ is the absorbance measured at the wavelength of excitation (295 nm for AMP and 410 nm for TNP-AMP), and $A_{em}$ is the absorption measured at the wavelength of emission (535 nm for TNP-AMP). As a control, ligands by themselves caused no change in fluorescence emission from a solution of tryptophan (100 μM).

**Results**

**Modeling of FBPase Mutants**—A structural overview of the region of interest is provided in Fig. 1. The main chain $\phi$ angle of Lys⁵⁰ differs significantly in the T- and R-states, neither value being compatible with that of proline, where $\phi$ must be close to ~60°. Insertion of proline then results in an initial model with poor stereochemistry at position 50. Energy minimization alone and energy minimization with simulated annealing at a starting temperature of 500 K (Protocol 1 and 2 in “Experimental Procedures”) leave the $\phi$ angle of Pro⁵⁰ some 30° away from its preferred value in both the T- and R-states. Further improvement in the stereochemistry of Pro⁵⁰ requires a starting temperature of 1000 K in simulated annealing (Protocol 3), the consequence of which is a significant drift of the Loop 52–72 away from either its T- or R-state conformation. Evidently, proline at position 50 is incompatible with the T-state disengaged and R-state engaged loop conformations.

Proline at position 51, on the other hand, is less disruptive. The $\phi$ angle of residue 51 is near ~60° in both the R- and T-states of FBPase. Backbone amide and hydrogen bonds with backbone carbonyls 47 and 48 in the R-state and with backbone carbonyl 46 in the T-state. Proline substitution at position 51 clearly disrupts the hydrogen bonds in both the R- and T-states. Moreover, the C⁶ atom of proline makes close contacts of 1.9 and 1.8 Å with backbone carbonyls 48 (R-state) and 46 (T-state), respectively. Either energy minimization alone or energy minimization with simulated annealing (initial temperature, 500 K) relaxes the contact in the R-state (the loop re-
mains engaged with the active site) by a 90° rotation of the plane of the polypeptide linkage between residues 47 and 48. The close contact of the T-state remains, however, at 2.4 and 2.8 Å after modeling Protocols 1 and 2, respectively. Contacts from within the subunit and from the neighboring subunit constrain the T-state conformation of residues 52–57 (Fig. 1). Furthermore, as backbone carbonyl 46 is an integral part of helix H2, it cannot relieve its close contact with Pro51 by a rotation of its polypeptide linkage. Hence, proline at position 51 probably increases the internal energy of the T-state, destabilizing it relative to the R-state of FBPase.

Expression and Purification of Wild-type and Mutant FBPases—Mutant and wild-type FBPases behaved identically throughout purification, including gel exclusion chromatography. The wild-type and mutant enzymes were at least 95% pure with no evidence for proteolysis on the basis of SDS-polyacrylamide gel electrophoresis.

Secondary Structure Analysis—The CD spectra of wild-type FBPase in the T- and R-states diverge minimally, but reproducibly in the vicinity of 210 nm (17). The CD spectra of the Ala51 → Pro, Lys50 → Pro/Tyr57 → Trp, and wild-type FBPases superimpose in the presence of Pi (5 mM), Fru-6-P (5 mM), and saturating Mg2+ (5 mM for the wild-type and Ala51 → Pro enzymes and 35 mM for Lys50 → Pro/Tyr57 → Trp). The addition of AMP (200 μM) to the wild-type enzyme produced small changes in the CD spectrum near 210 nm, as noted above, but caused no change in the CD spectra of the Lys50 → Pro/Tyr57 → Trp and Ala51 → Pro enzymes. (Concentrations of AMP in excess of 200 μM degraded CD spectra due to the elevated absorbance of radiation.) The CD spectra of the Lys50 → Pro mutant also differ from the corresponding wild-type spectra (Fig. 2). The CD spectrum of the Lys50 → Pro mutant did not change in the presence of Pi (5 mM), Fru-6-P (5 mM), and saturating Mg2+ (50 mM) in the presence or absence of AMP (200 μM).

Catalytic Rates, Michaelis Constants for Mg2+ and Fru-1,6-P2, and K+ Activation—Initial rate kinetics employ maximal substrate concentrations, sufficient to saturate the active site, but low enough to avoid substrate inhibition. The ratios of catalytic rate constants at pH 7.5 and 9.6 are comparable for wild-type and Ala51 → Pro FBPases (Table I), both consistent with reported activity ratios for an FBPase free of proteolysis. The activity ratios for the Lys50 → Pro and Lys50 → Pro/Tyr57 → Trp mutants are low, however; but as discussed below, the low value does not stem from limited proteolysis of that mutant.
All enzymes have comparable $K_m$ values for Fru-1,6-P$_2$ (Table I); but beyond this, the quantitative similarities end. Under standard conditions of assay for the wild-type enzyme (5 mM Mg$^{2+}$ and 20 μM Fru-1,6-P$_2$, pH 7.5), the mutation of Lys$^{60}$ to proline resulted in a 40-fold loss of specific activity. However, the decline in specific activity was due primarily to a 15-fold increase in the $K_m$ for Mg$^{2+}$ for the Pro$^{50}$ mutant. The $k_{cat}$ value for the Pro$^{50}$ mutant (saturated with Mg$^{2+}$) is one-third of that for the wild-type enzyme. The Hill coefficients for Mg$^{2+}$ for the Ala$^{51}$ → Pro, Lys$^{50}$ → Pro/Tyr$^{57}$ → Trp, and wild-type enzymes are similar, but are reduced significantly in the Lys$^{50}$ → Pro/Tyr$^{57}$ → Trp mutants at levels comparable to that of the wild-type enzyme, but is abolished altogether in the Pro$^{50}$ mutant.

Kinetics of AMP Inhibition—Concentrations of AMP needed for 50% inhibition increased 280-, 8000-, and 400-fold relative to that of the wild-type enzyme for the Ala$^{51}$ → Pro, Lys$^{50}$ → Pro, and Lys$^{50}$ → Pro/Tyr$^{57}$ → Trp mutants, respectively (Fig. 3 and Table I). Additionally, the Lys$^{50}$ → Pro/Tyr$^{57}$ → Trp double mutant displays biphasic behavior toward AMP. The data of Fig. 3 for the wild-type, Ala$^{51}$ → Pro, and Lys$^{50}$ → Pro enzymes were fit to Equation 3,

$$v = \frac{V_0}{1 + \left(\frac{1}{IC_{50-high}}\right)^n}$$

(Eq. 3)

where $v$ is the observed velocity at a specific concentration of AMP, $V_0$ is the fitted velocity in the absence of AMP, $I$ is the concentration of AMP, and $IC_{50}$ is the concentration of AMP that causes 50% inhibition. The exponent of 2 represents the Hill coefficient for AMP cooperativity, determined independently as described below. The data of Fig. 3 for Lys$^{50}$ → Pro/Tyr$^{57}$ → Trp FBPase were fit to Equation 4,

$$v = \frac{V_0}{1 + \left(\frac{1}{IC_{50-high}}\right)^2 + \left(\frac{1}{IC_{50-low}}\right)^2}$$

(Eq. 4)

where $v$, $V_0$, and $I$ are as defined above for Equation 3 and $IC_{50-high}$ and $IC_{50-low}$ represent concentrations of AMP that cause 50% relative inhibition due to the ligation of high and low affinity sites, respectively. Ligation of the high affinity sites, as shown below, is cooperative with a Hill coefficient of 2, whereas the cooperativity with respect to the ligation of low affinity sites is an adjustable parameter ($n$) in Equation 4. The fitted value for $n$ is 1.4.

AMP inhibition of wild-type FBPase is nonlinear and non-competitive with respect to Fru-1,6-P$_2$ and nonlinear and competitive with respect to Mg$^{2+}$. The Ala$^{51}$ → Pro mutant retains the nonlinear, competitive relationship between AMP and Mg$^{2+}$ (goodness-of-fit, 5.5%) (Fig. 4), consistent with Equation 5,

$$\frac{1}{v} = \frac{1}{V_0} + \frac{1}{V_0} \left(\frac{K_i}{K_a} + I\right)$$

(Eq. 5)

where $v$, $V_0$, $A$, $I$, $K_a$, and $K_i$ represent initial velocity, maximal velocity, Mg$^{2+}$ concentration, AMP concentration, the Michaelis constant for Mg$^{2+}$, the dissociation constant for AMP from the enzyme:AMP complex, and the Hill coefficient for AMP, respectively. The binding of AMP is cooperative at $n = 2$, whereas cooperativity is absent when $n = 1$. Data from the wild-type and Ala$^{51}$ → Pro enzymes are consistent with $n = 2$ (AMP cooperativity). When assayed at low AMP concentrations, Lys$^{50}$ → Pro/Tyr$^{57}$ → Trp retains the competitive mech-

fig. 2. CD spectra for wild-type and Lys$^{50}$ → Pro FBPases. Thin solid line, R-state wild-type enzyme with reaction products and Mg$^{2+}$; dotted line, T-state wild-type enzyme with reaction products, Mg$^{2+}$, and AMP; thick solid line, Lys$^{50}$ → Pro mutant under either R- or T-state conditions. mdeg, millidegrees.

fig. 3. AMP inhibition of wild-type and mutant FBPases. AMP titrations were of wild-type ( ), Ala$^{51}$ → Pro ( ● ), Lys$^{50}$ → Pro/Tyr$^{57}$ → Trp ( ■ ), and Lys$^{50}$ → Pro ( ▲ ) FBPases in saturating Fru-1,6-P$_2$ (20 μM) and a Mg$^{2+}$ concentration equal to the $K_m$ for Mg$^{2+}$ of each enzyme. See "Results" for details regarding the fitted curves.

Table I Kinetic parameters for wild-type and mutant forms of fructose-1,6-bisphosphatase

| 7.5/9.5 ratio | Specific activity$^a$ | $k_{cat}$ | $K_m$ for Fru-1,6-P$_2$ | $K_m$ for Mg$^{2+}$ | Hill for Mg$^{2+}$ | $K_m$ for Fru-2,6-P$_2$ | IC$_{50}$ for AMP | $K^*$ activation$^d$ |
|---------------|-------------------|----------|-----------------|----------------|-----------------|-----------------|-----------------|----------------|
| Wild-type     | 3.3               | 35 ± 2   | 22 ± 1          | 1.7 ± 0.09     | 0.67 ± 0.04     | 1.9 ± 0.1       | 0.123 ± 0.005   | 1.61 ± 0.05         | 16.8 ± 0.3 |
| Lys$^{50}$ → Pro | 0.93             | 0.85 ± 0.06 | 6.7 ± 0.3      | 2.1 ± 0.2      | 10.7 ± 0.8      | 1.45 ± 0.08     | 2.3 ± 0.2       | 13400 ± 20         | -4.32 ± 0.04 |
| Ala$^{51}$ → Pro | 3.2              | 20 ± 1   | 12 ± 1.4       | 0.8 ± 0.2      | 0.8 ± 0.1       | 2.1 ± 0.1       | 3.4 ± 0.2       | 460 ± 7          | 15.9 ± 0.1 |
| Tyr$^{57}$ → Trp | 3.3              | 38.6 ± 0.1 | 24.0 ± 0.1     | 3.39 ± 0.09    | 0.53 ± 0.1      | 1.9 ± 0.1       | 0.84 ± 0.05     | 8.5 ± 0.4         | 16.6 ± 0.4 |
| Lys$^{50}$ → Pro/Tyr$^{57}$ → Trp | 1.9          | 8.7 ± 0.8   | 9.2 ± 0.5      | 1.3 ± 0.1      | 3.1 ± 0.2       | 1.80 ± 0.07     | 0.37 ± 0.03     | 650 ± 40          | 14.6 ± 0.1 |

$^a$ Specific activity was determined at 5 mM Mg$^{2+}$ and 20 μM Fru-1,6-P$_2$.

$^b$ $k_{cat}$ is defined as $V_{max}/E_0$, where $E_0$ is the total enzyme concentration.

$^c$ The Hill coefficient for AMP ligation of high affinity sites is 2. See "Results" for details.

$^d$ $K^*$ activation is the percent increase in $V_{max}$ using 150 mM KCl versus 0 mM KCl at saturating Mg$^{2+}$ and Fru-1,6-P$_2$.

$^e$ Data are from Ref. 25.

$^f$ The reported value is for the high affinity site (IC$_{50-high}$). IC$_{50-low}$ is 28 ± 6 mM with a Hill coefficient of 1.4. See "Results" for details.
Mutations in the Hinge of a Dynamic Loop

am with $n = 2$. In contrast, AMP inhibition of the Lys$^{50} \rightarrow$ Pro mutant fits best to a mechanism of noncompetitive inhibition with respect to Mg$^{2+}$ (goodness-of-fit, 3.1%) (Fig. 4), represented by Equation 6,

$$\frac{1}{v} = \frac{1}{V_m} \left( \frac{1}{1 + \frac{I}{K_i} + \frac{K_y}{A \left( 1 + \frac{I}{K_i} \right)} \right) \quad \text{(Eq. 6)}$$

where the terms of Equation 6 are defined as for Equation 5 and $K_y$ represents the dissociation constant for AMP from the enzyme-Mg$^{2+}$-AMP complex. The data are consistent with $n = 2$, reflecting AMP cooperativity.

In wild-type, Ala$^{51} \rightarrow$ Pro, and Lys$^{50} \rightarrow$ Pro/Tyr$^{57} \rightarrow$ Trp FBPases, the mechanism of AMP inhibition with respect to Fru-1,6-P$_2$ is nonlinear and noncompetitive (Equation 7),

$$\frac{1}{v} = \frac{1}{V_m} \left( 1 + \frac{I}{K_i} + \frac{K_y}{B \left( 1 + \frac{I}{K_i} \right)} \right) \quad \text{(Eq. 7)}$$

where $v$, $V_m$, $I$, $B$, $K_i$, and $K_y$ are as defined above, $K_y$ represents the dissociation constants for AMP from the enzyme-AMP and the enzyme-Fru-1,6-P$_2$-AMP complexes, respectively. $B$ is the concentration of Fru-1,6-P$_2$, and $K_y$ is the Michaelis constant for Fru-1,6-P$_2$. The high value for IC$_{50}$ associated with AMP inhibition of the Lys$^{50} \rightarrow$ Pro mutant (Table I) precludes the determination of a kinetic mechanism.

**Kinetics of Fru-2,6-P$_2$ Inhibition**—Fru-2,6-P$_2$ is a competitive inhibitor with respect to Fru-1,6-P$_2$ for the position 50 and 51 mutant FBPases. The data best fit to Equation 8 (goodness-of-fit, 4.0%),

$$\frac{1}{v} = \frac{1}{V_m} \left( 1 + \frac{I}{K_i} + \frac{K_y}{B \left( 1 + \frac{I}{K_i} \right)} \right) \quad \text{(Eq. 8)}$$

where $v$, $V_m$, $I$, $B$, $K_i$, and $K_y$ are as defined above. The kinetic mechanisms of Fru-2,6-P$_2$ inhibition are the same for mutant and wild-type FBPases, but the $K_i$ for Fru-2,6-P$_2$ is 180, 200-300, and 3-fold higher in the Lys$^{50} \rightarrow$ Pro, Ala$^{51} \rightarrow$ Pro, and Lys$^{50} \rightarrow$ Pro/Tyr$^{57}$ mutants, respectively, relative to the wild-type enzyme (Table I).

**Fru-2,6-P$_2$ and AMP Synergism**—AMP and Fru-2,6-P$_2$ synergistically inhibited wild-type and mutant FBPases (Fig. 5). For all enzymes, the Hill coefficient for AMP is 2. At high concentrations of Fru-2,6-P$_2$, the saturation curve for Fru-1,6-P$_2$ changes from hyperbolic to sigmoidal for mutant and wild-type enzymes.

**Steady-state Fluorescence Measurements**—TNP-AMP inhibited the wild-type enzyme competitively with respect to Mg$^{2+}$ and inhibited FBPase synergistically with Fru-2,6-P$_2$. Fluorescence emission from TNP-AMP increased significantly in the presence of FBPase. In addition, AMP reduced the fluorescence from TNP-AMP, presumably through competition for the allosteric effector site (data not shown). TNP-AMP bound to wild-type, Lys$^{50} \rightarrow$ Pro, and Ala$^{51} \rightarrow$ Pro FBPases with high

**Table II**

| IC$_{50}$ for TNP-AMP | $K_i$ for TNP-AMP |
|-----------------------|-------------------|
| Wild-type             | 7.4 ± 0.7         | 13.1 ± 0.6     |
| Lys$^{50} \rightarrow$ Pro | ND*          | 14 ± 4        |
| Ala$^{51} \rightarrow$ Pro | ND*      | 37 ± 2        |

*Not determined due to weak inhibition by TNP-AMP.

**Fig. 5.** AMP/Fru-2,6-P$_2$ synergism for wild-type (A) and Ala$^{51} \rightarrow$ Pro (B) FBPases. The concentrations of Fru-2,6-P$_2$ were 0 ($\cdot$) and approximately one-half of the $K_i$ for Fru-2,6-P$_2$ (0.05 μM for wild-type FBPase and 0.86 μM for the Ala$^{51} \rightarrow$ Pro mutant [A]). Assays had Mg$^{2+}$ concentrations equal to the $K_i$ for Mg$^{2+}$ of each enzyme and saturating levels of Fru-1,6-P$_2$ (20 μM).

### References

- **Mutations in the Hinge of a Dynamic Loop**
- **Kinetics of Fru-2,6-P$_2$ Inhibition**—Fru-2,6-P$_2$ is a competitive inhibitor with respect to Fru-1,6-P$_2$ for the position 50 and 51 mutant FBPases. The data best fit to Equation 8 (goodness-of-fit, 4.0%).

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**Fig. 4.** Kinetic mechanism of inhibition of Ala$^{51} \rightarrow$ Pro and Lys$^{50} \rightarrow$ Pro FBPases. A. AMP competes with Mg$^{2+}$ in the Ala$^{51} \rightarrow$ Pro mutant. Concentrations of AMP were 0 ($\cdot$), 100 μM [ ], and 225 μM [A]. B. AMP inhibition is noncompetitive with respect to Mg$^{2+}$ in the Lys$^{50} \rightarrow$ Pro mutant. Concentrations of AMP were 0 ($\cdot$), 10 μM [ ], 15 μM [A], and 20 μM [B]. All assays were performed at saturating concentrations of Fru-1,6-P$_2$ (20 μM) and at 150 mM KCl. The lines are based upon Equation 5 for A and Equation 6 for B using the parameters of Table I. A.U., arbitrary units.

**Fig. 5.** AMP/Fru-2,6-P$_2$ synergism for wild-type (A) and Ala$^{51} \rightarrow$ Pro (B) FBPases. The concentrations of Fru-2,6-P$_2$ were 0 ($\cdot$) and approximately one-half of the $K_i$ for Fru-2,6-P$_2$ (0.05 μM for wild-type FBPase and 0.86 μM for the Ala$^{51} \rightarrow$ Pro mutant [A]). Assays had Mg$^{2+}$ concentrations equal to the $K_i$ for Mg$^{2+}$ of each enzyme and saturating levels of Fru-1,6-P$_2$ (20 μM).
Polyacrylamide gel electrophoresis of the Lys50 mutant account for a diminished activity ratio in any FBPase. In SDS-lyzed tetramers. Proteolysis of one of eight subunits then could mutate, requires an equal mixture of intact and once-proteolyzed apoenzyme; open circles, product complex; asterisks, product complex + 400 μM AMP. B, Pro50/Trp57 mutant. Black line, apoenzyme; open circles, product complex; asterisks, product complex + 20 mM AMP. A.U., arbitrary units.

affinity, comparable to AMP. On the basis of fluorescence titration data (Table II), however, the Hill coefficient for TNP-AMP is near unity in all cases.

FBPase has no tryptophan, so the mutation of any single residue to tryptophan introduces a unique fluorophore. Fluorescence emission from tryptophan at position 57 is sensitive to the conformational state of Loop 52–72. The indole of Trp57 is exposed to solvent in the T-state disengaged loop conformation, whereas it resides in a hydrophobic pocket in the R-state engaged loop conformation. The Trp57 → Trp mutant has been thoroughly studied by x-ray crystallography and initial velocity kinetics (25). Fluorescence emission spectra from the Pro50/Trp57 double mutant differ significantly from those of the Trp57 single mutant (Fig. 6). Fluorescence emission from the single Trp57 mutant is maximum in the presence of products/metals, conditions that promote the R-state engaged loop conformation (9, 10). The addition of AMP reduced fluorescence emission to a level comparable to that observed from the Trp57 mutant in the absence of products and metals (apo form of FBPase). In contrast, the apo form of the Pro50/Trp57 double mutant has the highest fluorescence emission. The addition of either product/metals or product/metals/AMP caused only a decrease in fluorescence emission.

**DISCUSSION**

Low activity ratios (−0.3) are a property of wild-type FBPase after limited proteolysis by papain or subtilisin (26). The maximum effect on the activity ratio due to proteolysis occurs when one subunit per tetramer is cut. Thus, for the wild-type enzyme, an activity ratio of 1, as observed for the Lys50 → Pro mutant, requires an equal mixture of intact and once-proteolyzed tetramers. Proteolysis of one of eight subunits then could account for a diminished activity ratio in any FBPase. In SDS-polyacrylamide gel electrophoresis of the Lys50 → Pro mutant, however, no fragments typical of proteolysis were evident. Furthermore, loss of AMP inhibition due to proteolysis of wild-type FBPase varied proportionately with the extent of subunit cleavage. The 8000-fold increase in K_i for AMP far exceeds the effect on AMP inhibition due to proteolysis, even in a completely proteolyzed system. Hence, the kinetic properties of the Lys50 → Pro mutant are not the consequence of proteolysis, but rather the influence of a proline at position 50.

Allosteric inhibition of wild-type FBPase by TNP-AMP is essentially identical to that caused by AMP, the only difference being the absence of cooperativity in the kinetics and in the binding of the fluorescent analog. In fact, TNP-AMP is indistinguishable from formycin 5′-monophosphate in its inhibition and binding to wild-type FBPase. Formycin 5′-monophosphate also exhibits a Hill coefficient of unity (27), and crystal structures of complexes of formycin 5′-monophosphate and AMP with human FBPase are identical (28). The difference in Hill coefficient may stem from subtle differences in conformation, evident perhaps only in partially ligated states of FBPase. Nonetheless, the fluorescence data clearly demonstrate tight binding of TNP-AMP and the displacement of that analog from FBPase by the addition of AMP. Hence, wild-type and Pro50 mutant FBPases are indistinguishable in their binding of TNP-AMP and its displacement by AMP.

On the basis of kinetics, however, Pro50 mutant FBPase is altogether insensitive to AMP. Inhibition of the Pro50 mutant by AMP requires an 8000-fold increase in ligand concentration and exhibits a different kinetic mechanism (noncompetitive versus competitive with respect to Mg2+). The apparent change in kinetic mechanism may arise from the unmasking of a secondary mechanism, due to the complete loss of allosteric inhibition by AMP. That secondary mechanism may be the direct coordination of AMP to the metal-ligated active site of Pro50 mutant FBPase. Hence, if AMP binds tightly to the Pro50 mutant, as evidenced by the fluorescence data, but does not inhibit, then proline at position 50 must interrupt the transmission of the allosteric signal.

Kinetic data indicate the disruption of Loop 52–72 as the root cause for the loss of allosteric properties in Pro50 mutant FBPase. The K_i for Mg2+ is elevated 15-fold in the Pro50 mutant, and K+ activation is lost. The kinetic mechanism of AMP inhibition with respect to Mg2+ is competitive in wild-type FBPase. Hence, in wild-type FBPase, AMP elevates the apparent dissociation constant of metals. A greatly elevated K_i for Mg2+, accompanied by the complete loss of AMP inhibition, is consistent with an FBPase unable to achieve its high affinity state for metal cations. On the basis of recent crystal structures (10), the R-state engaged conformation of Loop 52–72 represents the high affinity state for metal cations and the catalytically productive state of FBPase. Pro50 mutant FBPase then cannot achieve an engaged loop conformation.

Molecular modeling, CD spectroscopy, and fluorescence emission from Trp57 strengthen the argument above. The mutation of Lys50 to proline destabilizes the R-state engaged loop and T-state disengaged loop conformations in slow cooling/energy minimization protocols. The CD spectrum of Pro50 mutant FBPase supports an altered state, conformationally unresponsive to the binding of ligands. The indole group of Trp57 in the Pro50/Trp57 double mutant does not enter its hydrophobic pocket, as evidenced by the low fluorescence emission in the presence of saturating products/metals. The several lines of evidence provided here suggest that Loop 52–72 in Pro50 mutant FBPase can achieve neither its T-state disengaged nor its R-state engaged loop conformation. The complete loss of allosteric inhibition in Pro50 mutant FBPase suggests furthermore, that Loop 52–72 is an essential element in the allosteric regulation of FBPase.

The effects of the Ala51 → Pro mutation are less dramatic, in harmony with the results of modeling and CD spectroscopy. The Pro51 mutant retains K+ activation and wild-type affinity for divalent cations, but exhibits a significant increase in the K_i for AMP. The mutation evidently destabilizes the T-state disengaged conformation of Loop 52–72 relative to the R-state engaged conformation, again consistent with the results of modeling. In this respect, the Pro51 mutant and the Met71/72 double mutant (11) have similar effects on FBPase function. In
both instances, lessened AMP inhibition probably arises from a perturbation in the equilibrium between engaged and disengaged loop conformations in favor of the engaged state. The $K_i$ for Fru-2,6-P$_2$ increases 20- and 30-fold in the Lys$_{50}^{\rightarrow}$ Pro and Ala$_{51}^{\rightarrow}$ Pro mutants, respectively. The change in binding affinity for Fru-2,6-P$_2$ in these mutants must be indirect and is most likely through a perturbation of conformational states accessible to Loop 52–72. The increase in $K_i$ for Fru-2,6-P$_2$ due to loop mutations does not come with an increase in the $K_m$ for Fru-1,6-P$_2$, as has been observed in other mutations that increase the $K_i$ for Fru-2,6-P$_2$ (29). The observation above is consistent with findings that suggest that Fru-2,6-P$_2$ and Fru-1,6-P$_2$, although binding at the same site, evoke different conformational responses from FBPase. For instance, at high concentrations of Fru-2,6-P$_2$, the Hill coefficient for Mg$_2^{2+}$ against loss of activity (31). Although a complete understanding of the above phenomena will come with further study, mutations in the Hinge of a Dynamic Loop

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