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

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Abstract
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 $K_a$ 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 $K_i$ 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 noncompetitive and abolishes K$^+$ activation. The $K_i$ 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.

Keywords
fructose bisphosphatase, enzyme activity, Adenosine Monophosphate, Alanine, Kinetics

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

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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 Km_cat by up to 3-fold, with no effect on the Km for fructose-1,6-bisphosphate. The Km for Mg2+ in the Lys50 → Pro mutant increases ~15-fold, whereas that for the Ala51 → Pro mutant is unchanged. Although these mutants retain wild-type binding affinity for AMP and the fluorescent AMP analog 2'-O-(trinitrophenyl)adenosine 5'-monophosphate, the Ki for AMP increases 8000- and 280-fold in the position 50 and 51 mutants, respectively. In fact, the mutation Lys50 → Pro changes the mechanism of AMP inhibition with respect to Mg2+ from competitive to non-competitive and abolishes Kcat activation. The Kt for fructose-2,6-bisphosphate increases ~20- and 30-fold in the Lys50 → Pro and Ala51 → Pro mutants, respectively. Fluorescence from a tryptophan introduced by the mutation of Tyr57 suggests an altered conformational state for Loop 52–72 due to the proline at position 50. Evidently, the Pro50 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-bisphosphate 1-phosphohydrolase, EC 3.1.3.11; FBPase) catalyzes the hydrolysis of fructose 1,6-bisphosphate (Fru-1,6-P2) to Fru-6-P and Pi, (for a review, see Refs. 1–3). FBPase is a tetramer of identical subunits (Mf = 37,000). Each subunit has binding sites for Fru-1,6-P2, Fru-2,6-P2, 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-P2 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).

Loop 52–72 could play an important role in the allosteric mechanism of AMP inhibition, existing in at least two conformational states (9, 10). In the absence of AMP, the loop interacts with the active site (engaged loop conformation), positioning the side chain of an essential catalytic residue, Asp74 (9, 11). The binding of AMP to its allosteric pocket putatively displaces the loop from the active site and stabilizes the disengaged loop conformation (9, 10). Subunits with disengaged loops evidently have reduced affinity for essential metal ions and, as a consequence, may be impaired with respect to catalysis. Mutations of conserved residues in Loop 52–72 have significant effects on catalysis and allosteric inhibition of catalysis by AMP (11).

The metabolic flux through FBPase is sensitive to prevailing levels of AMP and Fru-2,6-P2. AMP inhibits FBPase allosterically with a Hill coefficient of 2 (12, 13), whereas Fru-2,6-P2 inhibits FBPase by direct ligation of the active site with a Hill coefficient of unity (1–3). Inhibition of FBPase by AMP is nonlinear and competitive with respect to Fru-1,6-P2, but nonlinear and competitive with respect to Mg2+ (6, 14). In contrast, inhibition of FBPase by Fru-2,6-P2 is linear and competitive with respect to Fru-1,6-P2 and noncompetitive with respect to Mg2+. Due to the equilibrium reaction catalyzed by adenylate kinase, concentrations of AMP in vivo are nearly constant under normal physiological conditions, whereas the concentration of Fru-2,6-P2 varies in response to extracellular regulators such as glucagon and epinephrine. Hence, Fru-2,6-P2 is the probable dynamic regulator of FBPase in vivo, but AMP still plays a significant role. Fru-2,6-P2 lowers the apparent inhibition constant for AMP at least 10-fold (15) by decreasing the kcat of AMP (16). Levels of AMP association with the allosteric sites of FBPase will then necessarily change in response to Fru-2,6-P2 levels, even if the concentration of AMP in vivo is constant. AMP/Fru-2,6-P2 synergism may arise from the ability of each ligand to stabilize a common thermodynamic state of FBPase (disengaged Loop 52–72) through different mechanisms (9).

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 Kt 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 Kt for AMP, yet mutations of Lys50 to methionine (17) and glutamine and alanine (18) cause virtually no change in the Kt for AMP. What is clear from crystal structures, however, is a large difference in main chain angles (φ, ψ) at position 50 in the R- and T-states of FBPase (7, 9, 10). Here we explore the consequence of mutations Lys50 → Pro and

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1 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; TNP-AMP, 2'-O-(trinitrophenyl)adenosine 5'-monophosphate.

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Ala$^{51}$ → 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 $K_c$ 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 $K_c$ for Mg$^{2+}$ rises 15-fold in the case of the Pro$^{50}$ 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-$eta$-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 Boehringer Mannheim. Trypsine, yeast extract, and agar were from Difco. Other chemicals were of reagent grade or equivalent. Escherichia coli strains BMH71-18 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 Lys$^{57}$ → Ala$^{51}$ → Pro, and Tyr$^{57}$ → Trp mutants were 5'-GTCGGCCCGGCGGCATC-3', 5'-CCGCGAAGGCGGATC-3', and 5'-CGGACCTTCGGGAATGG-3', respectively (codons for mutations are in boldface and underlined). The selection primer 5'-CAGCCAGGGAGCTGCTGC-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 dideoxy 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 0–300 mM NaCl gradient. FBPase eluted at 800 mM NaCl. The eluent was dialysed against 20 mM Tris-HCl (pH 8.3), loaded onto a DEAE-Sephadex column, and then developed with a 0–300 mM NaCl gradient.

**Kinetic Experiments—**Assays for the determination of specific activity, $v_{o0}$, 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 software package provided with the instrument.

**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.

**Steady-state Fluorescence Measurements—**Fluorescence data were analyzed by nonlinear least-squares fitting using the software program provided with the instrument.

**Fluorimetric Data Analysis—**Initial rate data were analyzed using Equations 1 and 2,

$$F_i = \frac{F - F_0}{(V/V_0)^{1 - 1/2}}$$

and

$$\frac{\Delta F}{F_0} = \frac{(\Delta \text{F}_{\text{max}}/F_0) \cdot \lambda^2}{K_c + \lambda^2}$$

where $\Delta F$ is the change in fluorescence caused by the addition of ligand (L), $F_0$ is the fluorescence in the absence of ligand, $\Delta \text{F}_{\text{max}}$ is the theoretical maximum change in fluorescence at saturating ligand concentration, $K_c$ is the dissociation constant, and $\lambda$ is the Hill coefficient.

**RESULTS**

**Modeling of FBPase Mutants—**A structural overview of the region of interest is provided in Fig. 1. The main chain $\phi$ angle of Lys$^{57}$ differs significantly in the T- and R-states, neither value being compatible with that of proline, where $\phi$ must be close to $-60^\circ$. Insertion of proline then results in a low initial volume of the Loop 52–72 away from its preferred value in both the T- and R-states. Further improvement in the stereochemistry of Pro$^{50}$ requires a starting temperature of 1000 K. During simulated annealing and energy minimization, the C$^\alpha$ atoms (except for residues 50–72) were restrained to their initial crystallographic positions by a harmonic pseudo-potential with a force constant of 50 kcal/mol. The justification for restraining all C$^\alpha$ atoms outside of the loop is the 10-year history of FBPase crystal structures, which collectively reveal only small changes in the protein conformation outside of Loop 52–72.
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, Lys30 → Pro/Tyr57 → Trp, and wild-type FBPases superimpose in the presence of P1 (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 P1 (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.

**FIG. 1.** Wild-type FBPase in the T- and R-states. The upper panels show only the C3-C4 subunit pair from the R-state (left panel) and T-state (right panel) dimers. Loop 52–72 from subunit C4 is represented by a thick black line. The regions within the dashed boxes are enlarged in the lower panels, showing the relative positions of Lys50, Ala51, and Tyr57. This figure was prepared with MOLSCRIPT (32) and RASTER3D (33).
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 $\mu$M Fru-1,6-P$_2$, pH 7.5), the mutation of Lys$^{50}$ 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 $K_a$ for Mg$^{2+}$ for the Pro$^{50}$ mutant. The $V_{cat}$ 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 mutant under either R- or T-state conditions.

**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,

$$v = \frac{V_0}{1 + \left(\frac{1}{K_c}\right)^n}$$

(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, and $I$ 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 FBPass 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 FBPass 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,

$$\frac{1}{v} = \frac{1}{V_0} \left(1 + \frac{K_a}{K_c} \left(1 + \frac{I}{I_c}\right)^n\right)$$

(Eq. 5)

$K_a$ is the competitive 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 FBPasses. 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 FBPasses. AMP titrations were of wild-type (○), Ala$^{51}$ → Pro (●), Lys$^{50}$ → Pro/Tyr$^{57}$ → Trp (■), and Lys$^{50}$ → Pro (▲) FBPasses in saturating Fru-1,6-P$_2$ (20 $\mu$M) and a Mg$^{2+}$ concentration equal to the $K_a$ for Mg$^{2+}$ of each enzyme. See “Results” for details regarding the fitted curves.

<table>
<thead>
<tr>
<th>Table I</th>
<th>Kinetic parameters for wild-type and mutant forms of fructose-1,6-bisphosphatase</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.5/9.5</td>
<td>Specific activity$^a$</td>
</tr>
<tr>
<td>ratio</td>
<td>units/mg/s</td>
</tr>
<tr>
<td>Wild-type</td>
<td>3.3</td>
</tr>
<tr>
<td>Lys$^{50}$ → Pro</td>
<td>0.93</td>
</tr>
<tr>
<td>Ala$^{51}$ → Pro</td>
<td>3.2</td>
</tr>
<tr>
<td>Tyr$^{57}$ → Trp</td>
<td>3.3</td>
</tr>
<tr>
<td>Lys$^{50}$ → Pro/Tyr$^{57}$ → Trp</td>
<td>1.9</td>
</tr>
</tbody>
</table>

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

$^b$ $k_{cat}$ is defined as $V_{cat}/E_{tot}$, where $E_{tot}$ 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.
Kinetic mechanism of inhibition of Ala⁵¹ → Pro and Lys⁵⁰ → Pro FBPases. A, AMP competes with Mg²⁺ in the Ala³¹ → Pro mutant. Concentrations of AMP were 0 (○), 100 μM (■), and 225 μM (▲). B, AMP inhibition is noncompetitive with respect to Mg²⁺ in the Lys⁵⁰ → Pro mutant. Concentrations of AMP were 0 (○), 10 mM (■), 15 mM (▲), and 20 mM (▲). All assays were performed at saturating concentrations of Fru-1,6-P₂ (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.

In wild-type, Ala⁵¹ → Pro, and Lys⁵⁰ → Pro/Tyr⁵⁷ → Trp FBPases, the mechanism of AMP inhibition with respect to Fru-1,6-P₂ is nonlinear and noncompetitive (Equation 7),

\[
\frac{1}{v} = \frac{1}{V_m(n)} \left( \frac{1 + \frac{I}{K_a} + \frac{I}{K_i} + \frac{B}{K_B} + \frac{1 + I}{K_i} }{1 + \frac{1}{K_i}} \right)
\]  

(Eq. 7)

where \( v, V_m, I, B, K_i, \) and \( n \) are as defined above, \( K_i \) and \( K_B \) represent the dissociation constants for AMP from the enzyme-Mg²⁺:AMP complex. The data are consistent with \( n = 2 \), reflecting AMP cooperativity.

In wild-type, Ala⁵¹ → Pro, and Lys⁵⁰ → Pro/Tyr⁵⁷ → Trp FBPases, the mechanism of AMP inhibition with respect to Fru-2,6-P₂ is nonlinear and noncompetitive (Equation 8),

\[
\frac{1}{v} = \frac{1}{V_m(n)} \left( \frac{1 + \frac{I}{K_a} + \frac{I}{K_i} + \frac{B}{K_B} + \frac{1 + I}{K_i} }{1 + \frac{1}{K_i}} \right)
\]  

(Eq. 8)

where \( v, V_m, I, B, K_i, \) and \( K_B \) are as defined above. The kinetic mechanism of Fru-2,6-P₂ inhibition is the same for mutant and wild-type FBPases, but the \( K_i \) for Fru-2,6-P₂ is ~20-, 30-, and 3-fold higher in the Lys⁵⁰ → Pro, Ala⁵¹ → Pro, and Lys⁵⁰ → Pro/Tyr⁵⁷ → Trp mutants, respectively, relative to the wild-type enzyme (Table I).

Fru-2,6-P₂ and AMP Synergism—AMP and Fru-2,6-P₂ synergistically inhibited wild-type and mutant forms of fructose-1,6-bisphosphatase

For all enzymes, the Hill coefficient for AMP is 2. At high concentrations of Fru-2,6-P₂, the saturation curve for Fru-1,6-P₂ 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 Mg²⁺ and inhibited FBPase synergistically with Fru-2,6-P₂. Fluorescence emission from TNP-AMP increased significantly in the presence of FBPase. In addition, AMP reduced the fluorescence from bound TNP-AMP, presumably through competition for the allosteric effector site (data not shown). TNP-AMP bound to wild-type, Lys⁵⁰ → Pro, and Ala⁵¹ → Pro FBPases with high
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-proteolysed 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 mutant, requires an equal mixture of intact and once-proteolysed products/metals. Noncleavable fragments typical of proteolysis were evident. Furthermore, no fragments typical of proteolysis were evident. The 8000-fold increase in $K_a$ for AMP far exceeds the effect on AMP inhibition due to proteolysis, even in a completely proteolysed 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 Mg$^{2+}$). 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 $K_a$ for Mg$^{2+}$ is elevated 15-fold in the Pro50 mutant, and $K^+$ activation is lost. The kinetic mechanism of AMP inhibition with respect to Mg$^{2+}$ is competitive in wild-type FBPase. Hence, in wild-type FBPase, AMP elevates the apparent dissociation constant of metals. A greatly elevated $K_a$ for Mg$^{2+}$, 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 absence 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 $K_a$ 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 Met51/72 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$_{50}^{30}$ → Pro and Ala$_{51}^{51}$ → 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 hyperbolic to sigmoidal (3, 30) at low concentrations of Fru-2,6-P$_2$, the Hill coefficient for AMP inhibition changes from 2 to 1, and the saturation curve for Fru-1,6-P$_2$ changes from hyperbolic to sigmoidal (31). At low concentrations of Fru-2,6-P$_2$, the Hill coefficient for Mg$^{2+}$ changes from 2 to 1 (31). Finally, Fru-2,6-P$_2$ protects FBPase against loss of activity (31). Although a complete understanding of the above phenomena will come with further study, mutations of the loop underscore a fundamental difference in FBPase in its Fru-2,6-P$_2$- and Fru-1,6-P$_2$-ligated states.

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

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