Directed Mutations in the Poorly Defined Region of Porcine Liver Fructose-1,6-bisphosphatase Significantly Affect Catalysis and the Mechanism of AMP Inhibition*

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Feruz T. Kurbanov, Jun-yong Choe, Richard B. Honzatko, and Herbert J. Fromm‡

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

Asn<sup>64</sup>, Asp<sup>68</sup>, Lys<sup>71</sup>, Lys<sup>72</sup>, and Asp<sup>74</sup> of porcine liver fructose-1,6-bisphosphatase (FBPase) are conserved residues and part of a loop for which no electron density has been observed in crystal structures. Yet mutations of the above dramatically affect catalytic rates and/or AMP inhibition. The Asp<sup>74</sup> → Ala and Asp<sup>74</sup> → Asn mutant enzymes exhibited 50,000- and 2,000-fold reductions, respectively, in $k_{cat}$ relative to wild-type FBPase. The pH optimum for the catalytic activity of the Asp<sup>74</sup> → Glu, Asp<sup>68</sup> → Glu, Asn<sup>64</sup> → Gln, and Asn<sup>64</sup> → Ala mutant enzymes shifted from pH 7.0 (wild-type enzyme) to pH 8.5, whereas the Lys<sup>71</sup> → Ala mutant and Lys<sup>71,72</sup> → Met double mutant had optimum activity at pH 7.5. Mg<sup>2+</sup> cooperativity, $K_c$ for fructose 1,6-bisphosphate, and $K_i$ for fructose 2,6-bisphosphate were comparable for the mutant and wild-type enzymes. Nevertheless, for the Asp<sup>74</sup> → Glu, Asp<sup>68</sup> → Glu, Asn<sup>64</sup> → Gln, and Asn<sup>64</sup> → Ala mutants, the binding affinity for Mg<sup>2+</sup> decreased by 40–125-fold relative to the wild-type enzyme. In addition, the Asp<sup>74</sup> → Glu and Asn<sup>64</sup> → Ala mutants exhibited no AMP cooperativity, and the kinetic mechanism of AMP inhibition with respect to Mg<sup>2+</sup> was changed from competitive to noncompetitive. The double mutation Lys<sup>71,72</sup> → Met increased $K_i$ for AMP by 175-fold and increased Mg<sup>2+</sup> affinity by 2-fold relative to wild-type FBPase. The results reported here strongly suggest that loop 51–72 is allosteric of catalytic activity and the mechanism of allosteric inhibition of FBPase by AMP.

Fructose-1,6-bisphosphatase (d-fructose-1,6-bisphosphate 1-phosphohydrolase, EC 3.1.3.11; FBPase<sup>1</sup>), a key regulatory enzyme in gluconeogenesis, catalyzes the hydrolysis of fructose 1,6-bisphosphate (Fru-1,6-P<sub>2</sub>) to fructose 6-phosphate and P<sub>i</sub>, in the presence of divalent metal ions such as Mg<sup>2+</sup>, Mn<sup>2+</sup>, and Zn<sup>2+</sup> (1, 2). The activation of the enzyme by Mg<sup>2+</sup> exhibits sigmoidal kinetics with a Hill coefficient of 2 at neutral pH, but is hyperbolic at pH 9.6 (3, 4). FBPase is inhibited synergistically by AMP (as an allosteric effector) and fructose 2,6-bisphosphate (Fru-2,6-P<sub>2</sub>; as a substrate analog) (5–7). In contrast, AMP and Fru-2,6-P<sub>2</sub> act as strong activators for phosphofructokinase, a major regulatory enzyme in the opposing glycolytic pathway.

In mammals, FBPase is a homotetramer with a subunit $M_s$ of 37,000 (8). Each subunit of the tetramer (designated C1, C2, C3, and C4) has an allosteric AMP domain (residues 1–200) and a catalytic Fru-1,6-P<sub>2</sub> domain (residues 201–335), with the AMP-binding site being ~28 Å away from the substrate-bind site (9–11). Structures of AMP complexes of the enzyme define the T-state, whereas structures in the presence substrate analogs and without AMP represent the R-state. The structural transition (R- to T-state) involves a 17° rotation of the C1-C2 dimer with respect to the C3-C4 dimer and a 1.9° rotation of the AMP domain relative to the Fru-1,6-P<sub>2</sub> domain within each subunit (10). The R- to T-state transition results in conformational changes at interfaces between subunits C1 and C2 and subunits C1 and C4 (as well as interfaces related to these by the symmetry of the tetramer). Metal-binding sites (up to three total) are at or near the active site at the interface between the two domains of the enzyme (12, 13). Mg<sup>2+</sup> and AMP are mutually exclusive in their binding to FBPase (14, 15). In fact, AMP inhibition is nonlinear and noncompetitive with respect to Fru-1,6-P<sub>2</sub> and nonlinear and competitive with respect to Mg<sup>2+</sup>. Yet crystallographic studies reveal similar metal coordination in the absence and presence AMP (either one Mg<sup>2+</sup> ion or two Zn<sup>2+</sup> ions or two Mn<sup>2+</sup> ions), although small perturbations in the active site are attributed to the 1.9° rotation of the AMP relative to the Fru-1,6-P<sub>2</sub> domain due to AMP binding (13).

Site-directed mutagenesis of the residues involved at subunit interfaces of porcine liver FBPase revealed significant changes in the kinetic mechanism of AMP inhibition and cooperativity (16–19). However, a detailed mechanism of catalysis and allosteric regulation based on changes in interacting residues has been elusive. Perhaps published structures of FBPase do not reveal all of the essential structural elements for catalysis and regulation. Mutation of Arg<sup>40</sup>, a residue that precedes a disordered loop (residues 54–71), causes dramatic changes in FBPase kinetics (17). Comparison of the amino acid sequences of mammalian FBPases reveals Gly<sup>59</sup>, Gly<sup>61</sup>, Asn<sup>64</sup>, Asp<sup>68</sup>, Asp<sup>74</sup>, Lys<sup>71</sup>, and Lys<sup>72</sup> as conserved residues within this disordered loop. Furthermore, loop 54–71 is proteolytically sensitive in all known FBPases (20). To determine whether loop 54–71 plays a major role in FBPase kinetics, mutant enzymes Asn<sup>64</sup> → Gln, Asn<sup>64</sup> → Ala, Asp<sup>68</sup> → Glu, Lys<sup>71</sup> → Ala, Lys<sup>71,72</sup> → Met (double mutation), Asp<sup>74</sup> → Ala, Asp<sup>74</sup> → Asn, and Asp<sup>74</sup> → Gln were expressed in Escherichia coli, purified to homogeneity, and evaluated by initial velocity kinetics. The above mutations have profound effects on both catalysis and regulation of FBPase.

**EXPERIMENTAL PROCEDURE**

*Materials—Fru-1,6-P<sub>2</sub>, Fru-2,6-P<sub>2</sub>, NADP, AMP, ampicillin, tetracycline, and isopropyl-β-D-thiogalactopyranoside were purchased from Sigma. DNA-modifying and restriction enzymes, T4 polynucleotide ki-
Enzyme & $k_{cat}$ & $K_m$, Fru-1,6-P$_2$ & $K_m$, AMP$^a$ & $K_m$, Fru-2,6-P$_2$ & $K_m$, Mg$^{2+}$ & Hill coefficient $Mg^{2+}$ $^{b}$
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Wild-type, pH 7.5 & 21 ± 2.2 & 2.8 ± 0.1 & 20.5 ± 5 & 0.24 ± 0.08 & 0.49 ± 0.01 & 1.94 ± 0.04
Lys$_{71,72}$ → Met, pH 7.5 & 34 ± 4.1 & 1.6 ± 0.2 & 3525 ± 296 & 1.78 ± 0.05 & 0.29 ± 0.01 & 1.78 ± 0.05
Lys$_{71}$ → Ala, pH 7.5 & 16 ± 1.9 & 2.8 ± 0.2 & 189 ± 25 & 0.29 ± 0.05 & 0.27 ± 0.01 & 2.3 ± 0.05
Asp$_{68}$ → Glu, pH 7.5 & 6.4 ± 0.8 & 5.31 ± 0.17 & 370 ± 4.5 & 0.24 ± 0.02 & 0.14 ± 0.03 & 2.2 ± 0.2
Wild-type, pH 8.5 & 9.1 ± 5.8 & 2.9 ± 0.08 & 537 ± 6.4 & 0.24 ± 0.04 & 0.27 ± 0.01 & 2.3 ± 0.1
Asp$_{74}$ → Ala, pH 8.5 & 0.0004 & ND & ND & ND & ND & ND
Asp$_{74}$ → Asn, pH 8.5 & 0.01 ± 0.002 & ND & ND & ND & ND & ND
Asp$_{74}$ → Glu, pH 8.5 & 1.2 ± 0.1 & 1.03 ± 0.1 & 5.25 ± 0.47$^b$ & 0.13 ± 0.04 & 1.7 ± 1.2 & 1.26 ± 0.06
Asn$_{64}$ → Ala, pH 8.5 & 7.1 ± 0.8 & 1.42 ± 0.08 & 127 ± 0.1$^c$ & 0.072 ± 0.01 & 5.45 ± 0.39 & 1.42 ± 0.07
Asn$_{64}$ → Glu, pH 8.5 & 5.4 ± 0.4 & 1.7 ± 0.1 & 141 ± 0.2 & 0.069 ± 0.01 & 6.6 ± 0.26 & 1.4 ± 0.04

$^a$ The $K_m$ was obtained from plots of ln(velocity) versus 1/[Fru-1,6-P$_2$] at 5 mM Mg$^{2+}$.
$^b$ The Hill coefficient for AMP inhibition is 1.0.
$^c$ ND, not determined.

### Results

**Purification of Wild-type and Mutant Forms of FBPase**

Purification of mutant and wild-type enzymes followed previous protocols (22). Mutant enzymes exhibited elution patterns similar to wild-type FBPase, except for Lys$_{71}$ → Ala and Lys$_{71,72}$ → Met, which eluted from the CM-Sepharose column at 100 mM NaCl. On the basis of SDS-polyacrylamide gel electrophoresis, all enzymes exhibit identical mobilities (M$_s$ ~ 37, 500) with no evidence of proteolysis (27) and purity greater than 95% (data not shown). Activity ratios (pH 7.5:9.6) for the wild-type, Lys$_{71}$ → Ala, and double mutant Lys$_{71,72}$ → Met enzymes confirmed the absence of proteolysis.

**Secondary Structure Analysis**

The CD spectra of wild-type and mutant FBPases are essentially superimposable from 200 to 260 nm (data not shown), indicating the absence of global structural alteration changes caused by the mutations.

**Catalytic Rates of FBPase Mutants**

Initial rate studies were done at saturating concentrations of Fru-1,6-P$_2$ or Mg$^{2+}$ that do not cause substrate inhibition. Kinetic parameters are in Table I. The $k_{cat}$ values for the Asp$_{74}$ → Ala and Asp$_{74}$ → Asn mutants decreased 50,000 and 2,000 times, respectively, whereas a shift in the pH of optimum activity from pH 7.5 to 8.5 was exhibited by the Asp$_{74}$ → Glu mutant with only a 20-fold reduction in $k_{cat}$. A similar alteration in the pH of optimum activity occurred with mutants Asn$_{64}$ → Glu, Asn$_{64}$ → Ala, and Asp$_{68}$ → Glu. On the other hand, replacements Lys$_{71}$ → Ala and Lys$_{71,72}$ → Met did not alter the pH of optimum activity.

In Table I, the kinetic parameters for the Asp$_{74}$ → Glu, Asp$_{68}$ → Glu, Asn$_{64}$ → Ala, and wild-type FBPase were measured at pH 8.5, and for mutants Asp$_{68}$ → Glu, Lys$_{71}$ → Ala, and Lys$_{71,72}$ → Met at pH 7.5. A slight change in Fru-1,6-P$_2$ affinity was observed in all mutants relative to wild-type FBPase.

**Mg$^{2+}$ Activation**

The activity of wild-type FBPase as a function of Mg$^{2+}$ concentration is sigmoidal at neutral pH with a Hill coefficient of ~2, but is hyperbolic at pH 9.6 (3, 4). The Hill coefficient for Mg$^{2+}$ and the $K_m$ for Mg$^{2+}$ of the wild-type and mutant forms of FBPase were determined here at a saturating Fru-1,6-P$_2$ concentration (30 μM) at pH 7.5 or 8.5. Hill coefficients at pH 8.5 dropped from 2.2 for the wild-type enzyme to ~1.4 for the Asp$_{74}$ → Glu, Asn$_{64}$ → Glu, and Asn$_{64}$ → Ala mutants, and at pH 7.5 from 2.0 to 1.4 for the Asp$_{68}$ → Glu mutant. Increases of 126, 51, 47, and 39-fold in $K_m$ values for Mg$^{2+}$ were found for the Asp$_{74}$ → Glu, Asp$_{68}$ → Glu, Asn$_{64}$ → Glu, and Asn$_{64}$ → Ala mutants, respectively, relative to the wild-type enzyme. The Lys$_{71}$ → Ala and Lys$_{71,72}$ → Met mutant enzymes as well as wild-type FBPase showed sigmoidal kinetics for Mg$^{2+}$ at pH 7.5. On the other hand, the affinity for Mg$^{2+}$ at pH 7.5 increased 2-fold for the Lys$_{71}$ → Ala and Lys$_{71,72}$ → Met mutant enzymes relative to wild-type FBPase.

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**Structure/Function of Fructose-1,6-bisphosphatase**

Expression and Purification of Wild-type and Mutant FBPases—

The expression and purification of wild-type and mutant forms of FBPase were carried out as described previously (22) with slight modification. After centrifugation to remove cell debris, the supernatant was subjected to heat treatment, 30–70% ammonium sulfate precipitation, Sephadex G-200 column chromatography, and CM-Sepharose column chromatography. Wild-type and mutant FBPases eluted as a single peak from the CM-Sepharose column by a NaCl gradient from 20 to 400 mM in 10 mM malonate buffer, pH 6.0. Protein purity was evaluated by 12% SDS-polyacrylamide gel electrophoresis according to Laemmli (23).

**Circular Dichroism Spectrometry**—CD studies on the wild-type and mutant FBPases were carried out on a Jasco J710 CD spectrometer in a 1-mm cell at room temperature using a 0.2 mg/ml concentration of the enzyme. Spectra were collected from 200 to 260 nm in increments of 1.3 nm, and each spectrum was blank-corrected and smoothed using the software package provided with the instrument.
Fru-2,6-P2 Inhibition—Fru-2,6-P2 is a competitive inhibitor of Fru-1,6-P2 and competes with the substrate for the active site of FBPase (7, 29). K values for Fru-2,6-P2 decreased by <4-fold for all mutant enzymes compared with the wild-type enzyme. On the other hand, the Lys71,72 → Met mutant exhibited a 7-fold increase in K for Fru-2,6-P2.

Kinetics of AMP Inhibition—AMP is an allosteric regulator of FBPase (30). The action of AMP inhibition is nonlinear and noncompetitive with respect to Fru-1,6-P2 (31), but nonlinear and competitive relative to Mg2+ for wild-type FBPase at either neutral or alkaline pH (15). AMP binding to FBPase is cooperative with a Hill coefficient of −2 (4). The wild-type and Lys71 → Ala, Lys71,72 → Met, Asp68 → Glu, and Asn64 → Gln FBPases exhibited competitive inhibition patterns for AMP relative to Mg2+, whereas the Asp74 → Glu and Asn64 → Ala mutants showed noncompetitive inhibition. Fig. 1 shows double-reciprocal plots of 1/velocity versus 1/[Mg2+] for various fixed concentrations of AMP for the Lys71,72 → Met mutant of FBPase. The data of Fig. 1 are consistent with a steady-state random mechanism represented by Equation 1,

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

(Eq. 1)

where v, V_m, A, B, I, K_a, K_i, K_{ii}, K_i, K_{ii}, K_{i}, and K_{ii} represent initial velocity, maximal velocity; the concentrations of Mg2+, Fru-1,6-P2, and AMP; the Michaelis constants for Mg2+ and Fru-1,6-P2; the dissociation constant for Mg2+; and the dissociation constants for AMP from the enzyme:AMP, enzyme:AMP:AMP, enzyme:Fru-1,6-P2:AMP, and enzyme:Fru-1,6-P2:AMP:AMP complexes, respectively. n represents the Hill coefficient for AMP. When n = 2, the binding of AMP to FBPase is cooperative; on the other hand, there is no cooperativity when n = 1. In the case of the wild-type, Lys71 → Ala, Asp68 → Glu, and Asn64 → Gln FBPases, the kinetic data (similar to those shown in Fig. 1) fit best to Equation 1 when n = 2. The “goodness of fit” was 4% when n = 2 as opposed to 11% when n = 1.

Fig. 2 illustrates double-reciprocal plots of 1/velocity versus 1/[Mg2+] for various fixed concentrations of AMP for the Asp74 → Glu mutant of FBPase. The family of lines intersect to the left of the 1/velocity axis in Fig. 2. A similar result was found with the Asn64 → Ala mutant (data not shown). The data for both mutants are indicative of noncompetitive inhibition by AMP relative to Mg2+ and are consistent with Equation 2,

\[
\frac{1}{v} = \frac{1}{V_m A} \left( \frac{I + S}{K_i + B} + \frac{I}{K_i} \right) + \frac{B}{S} \left( \frac{I + S}{K_i + B} + \frac{I}{K_i} \right) 
\]

(Eq. 2)

where v, V_m, A, I, K_a, K_i, and K_{ii} are defined as above, and n represents the Hill coefficient for AMP. The data for Asp74 → Glu and Asn64 → Ala all fit best to Equation 2 when n = 1. The “goodness of fit” is 5% and 6% when n = 1 for Asp74 → Glu and Asn64 → Ala, respectively, and >18% when n = 2.

The double-reciprocal plots of 1/velocity versus 1/[Fru-1,6-P2] at various fixed concentrations of AMP for the wild-type, Lys71 → Ala, Lys71,72 → Met, and Asp68 → Glu enzymes gave a family of lines intersecting to the left of the 1/velocity axis (data not shown). Consequently, the mechanism of AMP inhibition for the Lys71 → Ala, Lys71,72 → Met, Asp68 → Glu, and wild-type FBPases is noncompetitive. The K values for the Lys71,72 → Met and Lys71 → Ala mutants increased 175- and 10-fold.
relative to the wild-type enzyme, respectively. AMP inhibition relative to Fru-1,6-P$_2$ for Asp$_{74}^{3}$ Glu, Asn$_{64}^{3}$ Gln, and Asn$_{64}^{3}$ Ala gave a family of parallel lines (Fig. 3), indicating uncompetitive inhibition. Equation 3 with $n = 1$ best accounts for the data,

$$\frac{1}{v} = \frac{1}{V_m} \left( 1 + \frac{I}{K_i} + \frac{K_a}{A} \right)$$  \hspace{1cm} (Eq. 3)

where $v$, $V_m$, $I$, $n$, $K_i$, and $K_a$ represent initial velocity, maximal velocity, the concentration of Fru-1,6-P$_2$, the concentration of AMP, the Hill coefficient, the Michaelis constants for Fru-1,6-P$_2$, and the dissociation constant for AMP from the enzyme-AMP complex, respectively. The goodness of fit was <6%. Thus, cooperativity for AMP inhibition is lost in addition to the change of the kinetic mechanism.

DISCUSSION

Amino acid sequences of all known mammalian FBPases are well conserved, particularly in segment 52–72, where 10 amino acids are highly conserved, suggesting an important functional or structural role. In all published crystal structures of FBPase (9–13), however, little or no electron density is present for residues 52–72, which is indicative of conformational disorder or proteolytic damage. The latter possibility is unlikely as microsequencing detected no proteolysis (10). Reduced $k_{cat}$ values for the Asp$_{74}^{3}$ Ala, Asp$_{74}^{3}$ Asn, and Asp$_{74}^{3}$ Glu FB-Pases by 50,000-, 2,000-, and 20-fold, respectively, along with the shift in the pH of optimum activity from neutral to alkaline for the latter mutant, demonstrate the importance of position 74 in catalysis. Given the sensitive nature of position 74, conformational changes in the adjacent segment 52–72 could le-
verage significant alterations in the catalytic properties of FBPase. Mutations Asn64 → Ala, Asn64 → Gln, and Asp68 → Glu, for instance, change the pH of optimum activity from neutral to alkaline and decrease kcat values from 3- to 4-fold relative to that of the wild-type enzyme, and the double mutation Lys71,72 → Met elevates the Kf for AMP nearly 175-fold. The activity profiles of the Asp74 → Glu, Asn64 → Ala, Asn64 → Gln, and Asp68 → Glu mutants are very similar to those obtained with the proteolyzed enzyme in which residues 1–64 and 1–25 are missing (32–34).

The analysis above is strengthened by the crystal structure2 (2.3-Å resolution, R-factor = 0.165, R-free = 0.24) of recombiant wild-type porcine liver FBPase with the products Fru-6-P, P0, and Zn2+, which reveals strong electron density for loop 52–72 and a significant network of hydrogen bonds involving Asp68, Asn64, and other residues of the active site. In the proposed catalytic mechanism of hydrolysis of Fru-1,6-P2 (12, 13), a water molecule, bound to a metal cation, is activated for a nucleophilic attack of the 1-phosphate. The side chains of Asp74 and Glu98, relative to the attacking water and to the metal ion at the "catalytic" site (called metal site 2 in crystal structures of FBPase) (Fig. 4), are in position to act as general base catalysts. The distance between OD2 of Asp74 and OE2 of Glu98 and the cation at site 2 is ~3.3 Å, but neither side chain is in the inner coordination sphere of that cation. Replacement of Glu98 by glutamine resulted in a 1,600-fold reduction in kcat relative to wild-type FBPase (35). The Asp74 → Asn mutation caused a decrease in catalytic rate of the same order of magnitude, whereas the Asp74 → Ala mutation resulted in almost the total loss of catalytic activity. Thus, Asp74 and Glu98 may act in concert to remove a proton from the attacking water molecule.

Asn64 and Asp68 are also in the active site (Fig. 5), with the former hydrogen bonding with Glu98 and Glu92 and with the latter with Arg276. Evidently, Asn64 maintains Glu97 and Glu98 in proper orientations for metal binding and the activation of the attacking water molecule. The above is consistent with the reduction in the Hill coefficient for Mg2+ and the 50-fold decrease in affinity for Mg2+, exhibited by the Asn64 → Ala and Asn64 → Gln mutants relative to wild-type FBPase. Mutation of Arg276 to methionine reduces activity by 2,000-fold, abolishes Mg2+ cooperativity, and alters the kinetic mechanism of FBPase (36). Evidently, the Asp68→Arg276 hydrogen bond is a determinant for many of the kinetic properties of FBPase; its loss could destabilize loop 52–72 and impair the catalytic function of Asp74.

AMP induces a modest structural change in the helix (H2) immediately preceding loop 52–72, and in all AMP-bound complexes of FBPase, loop 52–72 is disordered (13, 28). The new crystal structure has an ordered loop in the absence of AMP. Hence, AMP could inhibit FBPase by the disruption of interactions between loop 52–72 and the active site. As Asn64 and Asp68 probably stabilize metal binding to the active site, the observed competition between AMP and Mg2+ in kinetics and NMR investigations may stem from the displacement of loop 52–72 from the active site due to the AMP-induced perturbation of helix H2.

The 175- and 10-fold increases in Kf of AMP inhibition with respect to Fru-1,6-P2 for the Lys71,72 → Met and Lys71 → Ala enzymes, respectively, could originate from the destabilization of loop 52–72 in its disordered, AMP-induced conformation. AMP may be less effective in the displacement of loop 52–72 of the double mutant (in which positions 71 and 72 are methionine instead of lysine) because of the unfavorable thermodynamics of exposing two methionyl side chains to the solvent. Indeed, the double mutant apparently exhibits preference toward the active R-state, relative to the less active or inactive T-state. Its kcat/Km increases 3-fold, and its Kf for Mg2+ decreases 2-fold, whereas its Kf for AMP and Fru-2,6-P2 increases 175- and 7-fold, respectively. In fact, the double mutant may represent the kinetic properties of FBPase locked into the R-state.

Mutations that bring about a change in the kinetic mechanism of FBPase from competitive to noncompetitive (AMP inhibition relative to Mg2+) are possible in the context of a steady-state random mechanism (16, 17) and now can be understood in terms of a conformational mechanism. To a first approximation, loop 52–72 is the instrument by which AMP exerts its allosteric effect on the active site. In the wild-type system with a fully functional loop, AMP and Mg2+ (presumably at site 2) are probably antagonists with respect to the conformation that they stabilize for loop 52–72. Hence, in wild-type FBPase, loop 52–72 strongly couples the AMP- and Mg2+-binding sites. In some mutants of loop 52–72, however, the strong coupling of the AMP and the cation site is diminished; AMP binds and perturbs the metal at site 2, thereby causing inhibition, but is no longer completely effective in displacing the cation from a catalytically productive association with the active site.

A rational basis for an uncompetitive mechanism is more challenging, however. Four separate mutations (Asp74 → Glu, Asn64 → Ala, Asn64 → Gln, and Arg276 → Cys) change AMP inhibition with respect to Fru-1,6-P2 from noncompetitive to uncompetitive. The uncompetitive mechanism implies that AMP binds as an inhibitor only when Fru-1,6-P2 is productively bound at the active site. Hence, these mutations may stabilize a conformation of loop 52–72 that does not favor the binding of AMP (as an inhibitor), but permits the association of Fru-1,6-P2 with the active site. Productively bound Fru-1,6-P2 could then induce a conformational change in loop 52–72 that re-establishes the coupling between the AMP and the substrate-binding site. A more detailed explanation of the above phenomenon, however, must await crystallographic and ligand binding studies of mutant FBPases.

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