Major Changes in the Kinetic Mechanism of AMP Inhibition and AMP Cooperativity Attend the Mutation of Arg49 in Fructose-1,6-bisphosphatase*

(Received for publication, June 6, 1997, and in revised form, August 11, 1997)

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The significance of subunit interface residues Arg49 and Lys50 in the function of porcine liver fructose-1,6-bisphosphatase was explored by site-directed mutagenesis, initial rate kinetics, and circular dichroism spectroscopy. The Lys50 → Met mutant had kinetic properties similar to the wild-type enzyme but was more thermostable. Mutants Arg49 → Leu, Arg49 → Asp, and Arg49 → Cys were less thermostable than the wild-type enzyme yet exhibited wild-type values for $k_{cat}$ and $K_m$. The $K_i$ for the competitive inhibitor fructose 2,6-bisphosphate increased 3- to 5-fold in Arg49 → Leu and Arg49 → Asp, respectively. The $K_i$ for Mg$^{2+}$ increased 4–8-fold for the Arg49 mutants, with no alteration in the cooperativity of Mg$^{2+}$ binding. Position 49 mutants had 4–10-fold lower AMP affinity. Most significantly, the mechanism of AMP inhibition with respect to fructose 1,6-bisphosphatase changed from noncompetitive (wild-type enzyme) to competitive (Arg49 → Leu and Arg49 → Asp mutants) and to uncompetitive (Arg49 → Cys mutant). In addition, AMP cooperativity was absent in the Arg49 mutants. The R and T-state circular dichroism spectra of the position 49 mutants were identical and superimposable on only the R-state spectrum of the wild-type enzyme. Changes from noncompetitive to competitive inhibition by AMP can be accommodated within the framework of a steady-state Random Bi Bi mechanism. The appearance of uncompetitive inhibition, however, suggests that a more complex mechanism may be necessary to account for the kinetic properties of the enzyme.

Fructose-1,6-bisphosphatase (o-fructose-1,6-bisphosphate 1-phosphohydrolase, EC 3.1.3.11, FBPase) governs a crucial step in carbohydrate metabolism, the hydrolysis of fructose 1,6-bisphosphate (Fru-1,6-P$_2$) to fructose 6-phosphate and inorganic phosphate (P$_i$) in the presence of required divalent cations such as Mg$^{2+}$ (1, 2). In mammals, FBPase is a homotetramer, inhibited competitively by fructose 2,6-bisphosphate (Fru-2,6-P$_2$) (3–6) and allosterically by AMP (7–9). Crystallographic complexes reveal 1 AMP binding site/subunit (10) and a single common binding site for Fru-2,6-P$_2$ and fructose 6-phosphate approximately 20 Å away from the AMP site. Metal ions bind at or near the active site. Activation of FBPase by Mg$^{2+}$ exhibits sigmoidal kinetics with a Hill coefficient of 2 at neutral pH but exhibits hyperbolic kinetics at pH 9.6 (11, 12).

In the context of FBPase kinetics, the binding of AMP results in a diverse and complex set of phenomena. The inhibition of FBPase by AMP and Fru-2,6-P$_2$ is synergistic (13, 14), and the Fru-2,6-P$_2$-induced enhancement of AMP binding is attributed to a decrease in the $k_{off}$ for the nucleotide (4). AMP and Mg$^{2+}$ are mutually exclusive in their binding to FBPase (11, 15). Crystallographic studies have shown that AMP indirectly perturbs metal binding sites (16). AMP inhibition is cooperative, with a Hill coefficient of 2 (7–9). The first two molecules of AMP putatively bind with positive cooperativity, whereas the last two molecules bind with negative cooperativity (17). The mechanism for AMP inhibition is nonlinear and noncompetitive, with respect to Fru-1,6-P$_2$ and nonlinear and competitive with respect to Mg$^{2+}$ (11).

The four identical subunits (C1, C2, C3, and C4) of FBPase each consist of single AMP (residues 1–200) and FBP (residues 200–335) binding domains (10). The tetramer is roughly a square with the upper left vertex occupied by subunit C1 followed by C2, C3, and C4 in a clockwise sense. Structures of AMP complexes of FBPase define the T-state, whereas structures of FBPase in complexes with substrates or substrate analogs without AMP define the R-state. To a first approximation, the transition from the T- to R-state is a 17° rotation of the lower subunit pair (C3-C4) relative to upper subunit pair (C1-C2) coupled with a 1.9° rotation of the AMP domain relative to the FBP domain within each subunit. The R- to T-state transition results in conformational changes at interfaces between C1 and C2, C1 and C4, and the AMP and FBP domains within a subunit (16). The C-terminal residues of helix H2, particularly residues 49 and 50, are strategically positioned at the C1-C2 interface, near the molecular center of the tetramer, approximately 20 Å from both the active site and the AMP binding site. The significance of Arg49 and Lys50 to the function of porcine liver FBPase is examined here by site-directed mutagenesis, circular dichroism (CD) spectroscopy, kinetics, and structural modeling. Our results clearly demonstrate the importance of position 49 in AMP cooperativity and inhibition. In addition, both residues are important determinants in the thermostability of the FBPase tetramer.

EXPERIMENTAL PROCEDURES

Materials—Fru-1,6-P$_2$, Fru-2,6-P$_2$, NADP, MgCl$_2$, AMP, and isopropyl-1-thio-β-D-galactopyranoside were purchased from Sigma. DNA-modifying and restriction enzymes were from Promega and CLONTECH Laboratories, Inc., respectively. Glucose-6-phosphate dehydrogenase and phosphoglucose isomerase came from Boehringer Mannheim. Other chemicals were of reagent grade or the equivalent.

Mutagenesis of FBPase—Mutations were accomplished by the intro-
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... was used to obtain homogeneous Arg 49 Cys, and Lys 50 - Met, respectively. A selective primer, 5'-CACCTGGCTCATGG-3', which exchanged the original Nru I site for a unique Xho I site on the pET-11a vector was used. The double-stranded FBPase expression plasmid (pET-FBP) (19) and mutagenic and selective primers were denatured, annealed, and polymerized as described by Deng and Nickoloff (18).

Mutations were confirmed by NruI/XhoI digestion and by fluorescent dyeoxo chain termination sequencing at the Nucleic Acid Facility at Iowa State University. The sequencing primer 5'-GTCATTGGAGAG-3' was used to confirm the mutations. The mutagenesis plasmid was finally transformed into Escherichia coli DF 657, a strain deficient in the FBPase gene.

Purification of Wild-type and Mutant FBPase—Wild-type, Arg 49 Cys, and Lys 50 - Met FBPases were purified by using 30–70% (NH 4 ) 2 SO 4 precipitation, a Sephadex G-100 column, and a CM-Sephadex C-50 column. Homogeneous Arg 49 Cys, and Lys 50 - Met was obtained by using an additional Cibacron blue column (from Sigma). The experimental details are described elsewhere (19, 20).

Protein concentration was assayed as described by Bradford (21) with bovine serum albumin (Sigma) as the standard. The protein purity was determined by using 12% SDS-polyacrylamide gel electrophoresis with bovine serum albumin (Sigma) as the standard. The protein purity was determined by using 12% SDS-polyacrylamide gel electrophoresis of purified FBPase in the presence or absence of ligands employed a Fasoe 4710 CD spectrometer and a 1-nm cell at room temperature. Spectra were collected from 200 to 260 nm in 1.5-nm increments, and each spectrum was blank-corrected and smoothed by using the software package provided with the instrument.

Modeling Studies—Models for the Arg 49 Met mutants were built from the x-ray structures of the R- and T-states of the wild-type pig kidney enzyme (26, 27), whose amino acid sequence is identical to that of porcine liver FBPase, using the program XtalView (28). The Arg 49 Met T-state model was taken from the partially refined x-ray crystal structure determined at Iowa State University. 4 With the exception of the Arg 49 Met T-state model, the best conformations of the side chains of Leu 59 , Cys 49 , Asp 49 , and Met were obtained by a systematic search and then subjected to 20 steps of conjugate gradient energy minimization using XPLOR (29). The coordinates of all atoms except those within a 10 A radius around the residue of interest were fixed at their crystallographic positions. Solvent-accessible surface areas were calculated using GRASP (30) for residues 168–171 (turn T2) for the R- and T-state models after removal of all water molecules.

RESULTS

Purification of the Wild-type and Mutant Forms of FBPase—The elution profiles from the CM-Sephadex column were similar for mutant and wild-type enzymes, except that Arg 49 Leu Cys, and Lys 50 - Met mutants, were found in the mutant enzymes relative to the wild-type enzyme (Table I). Slight decreases in affinity (3–5-fold) for Fru-2,6-P 2 were found in Arg 49 Met and Lys 50 - Met relative to wild-type FBPase. Thus, Arg 49 Met and Lys 50 - Met are involved neither in Fru-2,6-P 2 inhibition nor in the discrimination between Fru-1,6-P 2 and Fru-2,6-P 2 . AMP inhibition is nonlinear and noncompetitive with respect to Fru-1,6-P 2 but nonlinear and competitive relative to Mg 2+ for the wild-type enzyme (11). The expected pattern for noncompetitive inhibition of AMP relative to Fru-1,6-P 2 was found in wild-type and Lys 50 - Met FBPases; however, the mechanism was changed to competitive inhibition in Arg 49 Met and Arg 49 Met and to uncompetitive inhibition in the Arg 49 Met enzyme. In addition, the cooperativity for AMP inhibition was lost in the three Arg 49 Met mutants. A double-reciprocal plot of 1/velocity versus 1/[Fru-1,6-P 2 ] at various concentrations of AMP for Lys 50 - Met gave an excellent fit to Equation 1 when n = 2 (data not shown). The “goodness of fit” was 4%. AMP inhibition, then, is noncompetitive and cooperative as for the wild-type enzyme (data not shown). Equation 1 is

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

where \( v \), \( V_m \), A, I, \( K_a \), \( K_m \), and \( K_i \) represent initial velocity, maximal velocity, the concentration of Fru-1,6-P 2 , the concentration of AMP, the Michaelis constants for Fru-1,6-P 2 , the dissociation constants for AMP from the enzyme AMP, and the enzyme-Fru-1,6-P 2 -AMP complexes, respectively. \( n \) represents the Hill coefficient for AMP with FBPase. When \( n = 2 \), the binding of AMP to FBPase exhibits cooperativity; on the other hand, there is no cooperativity when \( n = 1 \).

Double-reciprocal plots of 1/velocity versus 1/[Fru-1,6-P 2 ] at various fixed concentrations of AMP for the Arg 49 Leu mu-
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**Table I**

| Enzyme         | Specific activity | pH | Vcat | Kovan | KovaFru-1,6-P2 | AMP I50 | Hill coefficient |
|----------------|-------------------|----|------|-------|---------------|---------|-----------------|
| Wild type      | 30.9 ± 2.80       | 3.0| 18.0 ± 1.70 | 3.51 ± 0.24 | 0.24 ± 0.02 | 4.52 ± 1.26 | 0.49 ± 0.22 |
| Arg-49 → Leu   | 38.8 ± 9.52       | 4.1| 23.6 ± 5.99 | 3.44 ± 0.18 | 0.78 ± 0.08 | 32.9 ± 2.17 | 3.62 ± 0.13 |
| Arg-49 → Asp   | 33.1 ± 1.87       | 4.0| 20.1 ± 1.14 | 4.42 ± 0.41 | 1.13 ± 0.00 | 18.7 ± 1.48 | 4.02 ± 0.37 |
| Arg-49 → Cys   | 38.7 ± 2.93       | 2.2| 23.6 ± 1.78 | 4.07 ± 0.33 | 0.33 ± 0.04 | 45.2 ± 5.55 | 1.76 ± 0.09 |
| Lys-50 → Met   | 36.8 ± 2.09       | 2.0| 22.4 ± 1.27 | 4.02 ± 0.19 | 0.31 ± 0.02 | 6.34 ± 1.00 | 2.56 ± 0.09 |

*a* The half-inhibiting concentration of AMP (I50) was obtained from plots of 1/velocity versus [1/Fru-1,6-P2] at 5 mM Mg2+.  
*b* The Hill coefficient for AMP inhibition is 2.0.  
*c* The Hill coefficient for AMP inhibition is 1.0.

**FIG. 2.** Temperature sensitivity of wild-type and mutant forms of FBPases. The purified wild-type ( ), Lys50 → Met (•), Arg49 → Cys ( □), Arg49 → Leu (+), and Arg49 → Asp ( △) enzyme concentrations of 67 μg/ml were incubated 10 min at 30, 37, 47, 57, and 67 °C, respectively, in 50 mM malonate buffer at pH 6.0. Enzyme activity was assayed spectrophotometrically at 25 °C (as described under “Experimental Procedures”) immediately after the incubation and is expressed as a percentage of the relative activity at 30 °C. The protein concentration was 0.2 mg/ml in each assay. Each reaction was either in duplicate or triplicate.

**FIG. 3.** Plot of reciprocal of initial velocity in arbitrary fluorescent units versus [1/Fru-1,6-P2] for Arg49 → Cys FBPase. The concentrations of AMP are 0 ( ), 100 μM (+), and 300 μM ( △). The coupled spectrofluorometric assay was performed at 25 °C in 50 mM Hepes buffer (pH 7.5) containing 0.1 mM KCl, 5 mM Mg2+. The lines are theoretical based upon Equation 3 when n = 1, and the points were experimentally determined.

The Lys50 → Met mutation did not influence AMP affinity.  

**CD Spectroscopy of FBPase**—In the absence of ligands, the CD spectra from 200 to 260 nm of wild-type and the four mutant FBPases were superimposable (data not shown), indicating no major differences in secondary structure. In the presence or absence of saturating concentrations of Fru-1,6-P2 and Mg2+ (R-state), the CD spectra for the wild-type enzyme from 200 to 260 nm were superimposable (Fig. 4). The CD spectra of the wild-type enzyme in the presence of AMP and substrate or in the presence of AMP alone (data not shown) were identical to each other but differed in the region of 210 nm from the spectrum of the wild-type enzyme in the R-state (Fig. 4). CD spectroscopy is thus sensitive to differences in the R- and T-states of the wild-type enzyme.

The CD spectra of Lys50 → Met in the R- and T-state are identical to the CD spectra of wild-type FBPase in the R- and T-states, respectively (data not shown). On the other hand, the CD spectra of the R- and T-states of Arg49 → Leu, Arg49 → Asp, and Arg49 → Cys are identical (data not shown). Furthermore, the R- and T-state spectra of Arg49 → Leu are similar to the R-state spectrum of wild-type enzyme but dissimilar at 210 nm to the T-state spectrum of the wild-type enzyme (Fig. 5). These data indicate the existence of a CD-sensitive, R-like feature in the CD spectra from 200 to 260 nm of wild-type and the four mutant forms of fructose-1,6-bisphosphatase.  

**DISCUSSION**

Lipscomb and co-workers (16, 26) have proposed that FBPase exists in two different conformational states, an active R- and an inactive or less active T-state. Differences between the
two states are most apparent at the C1-C4 and C1-C2 inter-
faces (and interfaces related to these by molecular symmetry).
Residues in the AMP binding domain contribute to both inter-
faces (16). In particular, C1 Arg49 hydrogen bonds with C2
Gly168 and C2 Ser169 of turn T2, which itself is in contact with
loop L6 (residues 128–130 of C2) located at the C-terminal end
of helix H4 (residues 123–127 of C2). Helix H4 is near the
active site and may have an influence on the binding of metal
ions and/or the 1-phosphate group of the substrate (16). AMP
initiates the transition from the active R- to the inactive or less
active T-state or stabilizes the T-state relative to the R-state.
The R- to T-state transition involves significant intra- and
intersubunit rearrangements that are coupled in the wild-type
enzyme (31).

Preliminary crystal structures of Arg 49
3
Asp in the pres-
ence of AMP reveal a T-state tetramer, which has R-like sub-
units.2 The FBP binding domains of the C1-C2 dimer (the
asymmetric unit of the crystal) can be superimposed on the
corresponding domain of the canonical T- and R-states of FBP-
ase. The orientation of the AMP binding domain relative to the
FBP binding domain of the Arg49
3
Asp mutant approximates
those of the R-state subunit. Thus, the T-state tetramer of
Arg49
3
Asp has R-like subunits in the context of the canonical
T-state quaternary arrangement of those subunits.

The CD spectra of position 49 mutants in the presence and
absence of AMP are identical to each other and to the spectrum
of the R-state of the wild-type enzyme. In contrast, the CD
spectra of wild-type R- and T-states are different. Invariant CD
spectra (with or without AMP) of Arg49
3
mutants may reflect the
absence of conformational change in the mutant or be the
result of conformational changes that are CD-insensitive. The
Arg49
3
mutants are not locked into an R-state quaternary ar-
rangement of subunits, as the crystal structure of Arg49
3
Asp
clearly reveals a T-state subunit arrangement. CD spectro-
copy may be sensitive then to changes in relative orientation
of the AMP and FBP domains within a subunit. Thus, an R-like
subunit may produce an R-state CD spectrum regardless of the
quaternary subunit arrangement.

The most significant findings reported here are the loss of
AMP cooperatively and the change in mechanism of AMP in-
hibition in the Arg49 mutants. Loss of AMP cooperatively has
been observed by Shyur et al. (32) in an Arg22
3
Met mutant
(C1-C4 interface) and more recently by Lu et al. (33) in muta-
tions of Glu192 to alanine and glutamine (C1-C4 interface).
The Arg49 mutants are the first instances of mutations at the C1-C2
interface that abolish AMP cooperativity. Presumably the sta-
bilization by AMP of a T-like conformation of one subunit (say
C1) can be communicated through the C1-C2 interface to sub-
unit C2 or the C1-C4 interface to subunit C4. If subunit C1
cannot adopt a T-like conformation and remains R-like in the
presence of AMP, subunit C2 and/or C4 may not be influenced
by the binding of AMP to subunit C1, hence the loss of positive
cooperativity in AMP association. The modest decrease in AMP
affinity observed in position 49 mutants may represent the loss
in synergy between a pair of AMP molecules bound to C1/C2 or
C1/C4. As mutations at the C1-C2 interface (Arg49) and the
C1-C4 interface (Arg22 and Glu192) independently abolish AMP
cooperativity, we cannot however unambiguously assign the

FIG. 4. CD spectra for the R-state and T-state of wild-type
fructose-1,6-bisphosphatase. The CD spectra of the enzyme in the
presence (●) or absence (○) of Fru-1,6-P2 and Mg2
1
are shown (R-state).
The CD spectrum for fructose-1,6-bisphosphatase complexed with Fru-
1,6-P2, Mg2
1
, and AMP (■) represents the T-state of the enzyme.
Details are under “Experimental Procedures.”

FIG. 5. Comparison of the CD spectra of the wild-type and
Arg49
3
Leu FBPases. All spectra were normalized based on protein
concentration. A, superposition of CD spectra of the R- (●) and T-state
(●) of Arg49
3
Leu and the R-state of the wild-type enzyme (○). B, superposition of the CD spectra of the T-state of Arg49
3
Leu (●), the
T-state of Lys50
3
Met (○), and the wild-type enzyme (○).
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phenomenon of cooperativity to a specific pair of subunits. Nonetheless, the crystal structure of the AMP complex of the Arg^{29} → Met mutant reveals a canonical T-state, implying that the loss in cooperativity is due to an effect (perhaps electrostatic in origin) localized to the C1-C4 interface (31). In contrast, the Arg^{49} → Asp mutant has an impact on both C1-C2 and C1-C4 interfaces. As only the C1-C4 interface is perturbed by both mutations, the subunit pair most likely associated with positive cooperativity is C1-C4. Lu et al. (33) also ascribed the origin of AMP cooperativity to the C1-C4 interface. The involvement of the C1-C2 interface in AMP cooperativity, however, was not considered by Lu et al., perhaps because their work predates the discovery of a C1-C2 interface mutant that abolishes AMP cooperativity.

It is possible to rationalize the data for noncompetitive inhibition and competitive inhibition within the context of the steady-state Random Bi Bi mechanism proposed for FBPase (34) and the suggested relationship of interactions of FBPase with AMP and Mg^{2+} (15). If it is assumed that AMP can bind to the free enzyme and the FBPase-AMP complex, it is possible to reduce the steady-state Random Bi Bi initial rate equation to the competitive and noncompetitive models by simply making assumptions regarding the relationships that must exist among rate constants. Similar manipulations were made by Rudolph and Fromm (35) for hexokinase by computer simulations. On the other hand, the steady-state Random Bi Bi mechanism cannot readily account for the results of Fig. 3 (uncompetitive inhibition). The kinetic data infer that AMP inhibits wild-type FBPase by several related (and coupled) mechanisms and that the kind of side chain at position 49 determines the dominant mechanism of inhibition.

Although other explanations are possible, the decrease in thermal stability of the Arg^{49} mutant can be explained by structural perturbations localized to the C1-C2 interface. In the R-state, C2 Arg^{49} hydrogen bonds with C1 Gly^{168} and is in van der Waals contact with C1 Arg^{49} (16). In the T-state, C2 Arg^{49} hydrogen bonds with C2 Gly^{168}, C2 Ser^{169}, and C2 Thr^{171} (16). The position-49 mutations eliminate the C1 Arg^{49}-C2 Arg^{49} van der Waals contacts and the hydrogen bonds in both the R- and T-states. Another consequence of the mutation may be an increased exposure of residues 168–171 (turn T2) of the C1-C2 interface to water (Table II). In fact, for the Arg^{49} → Asp mutation, nonbonded contacts are not only lost but replaced with repulsive electrostatic interactions between C1 Asp^{49} and C2 Asp^{49}, possibly accounting for the increased thermosensitivity of the Arg^{49} → Asp mutant (Fig. 2). Both the loss of intersubunit hydrogen bonds and the increase in solvent-accessible surface area of the C1-C2 interface may contribute significantly to the decreased thermostability of the other Arg^{49} mutations.

The enhanced thermostability of the Lys^{50} → Met mutant, however, is not readily apparent from an examination of the R- and T-states. The energy-minimized model for the Lys^{50} → Met mutant in the R-state retains van der Waals contacts but replaces the salt-link of C1 Lys^{50} to C2 Asp^{187} with a nonbonded contact. The T-state model for Lys^{50} → Met retains similar nonbonded contacts to those of the wild-type enzyme. The enhanced thermostability of the Lys^{50} → Met mutant must originate from either a kinetic phenomenon (an increase in the energy of activation of a fundamental step in the unfolding process) or a difference in the free energy between the unfolded states of the mutant and wild-type enzymes.

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| Table II | Solvent-accessible surface area (Å²) of turn T2 (168–171) |
|---------|---------------------------------|
| R-state | 21 |
| T-state | 14 |
| Wild Type | |
| Arg^{49} → Asp | 50 |
| Arg^{49} → Leu | 35 |
| Arg^{49} → Cys | 53 |

V. Villaret, L.-F. Shyur, H. J. Fromm, and W. N. Lipscomb, unpublished data.