Site-directed Mutagenesis of Residues at Subunit Interfaces of Porcine Fructose-1,6-bisphosphatase*

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Mutation of Arg-15, Glu-19, Arg-22, and Thr-27 of porcine liver fructose-1,6-bisphosphatase was carried out by site-directed mutagenesis. These residues are conserved in all known primary sequences of mammalian fructose-1,6-bisphosphatase. On the basis of the crystal structure of the enzyme, Arg-15, Glu-19, and Arg-22 are located at the interface of the two dimers (C1-C2 and C3-C4), and Thr-27 is in the AMP binding site. The wild-type and mutant forms of the enzyme were purified to homogeneity and characterized by initial rate kinetics and circular dichroism (CD) spectrometry. No discernible differences were observed between the secondary structures of the wild-type and mutant forms of fructose-1,6-bisphosphatase on the basis of CD data. Kinetic analyses revealed similar kcat values for mutants R15A, E19Q, R22K, and T27A of fructose-1,6-bisphosphatase; however, a 2-fold increase of kcat was observed with R22M compared with that of the wild-type enzyme. Small changes in Km values for fructose-1,6-bisphosphate were found in the five mutants. 4- to 6-fold decreases in Km values for fructose-1,6-bisphosphate and 5-9-fold decreases in the binding affinity of Mg2+ relative to the wild-type enzyme were exhibited by R15A and E19Q. No alteration of Mg2+ cooperativity was found in the five mutants. Significant changes in Km values for AMP were obtained in the case of R22K (30-fold) and T27A (1300-fold) with a Hill coefficient of 2.0. Replacement of Arg-22 with methionine, however, caused the total loss of AMP cooperativity without changing AMP affinity. Modeling of the mutant structures was undertaken in an attempt to define the functional role of Arg-22. These studies link specific interactions between subunits in fructose-1,6-bisphosphatase to observed properties of cooperativity.

Fructose 1,6-bisphosphatase (d-fructose-1,6-bisphosphate 1-phosphohydrolase (EC 3.1.3.11), FBPase) is an allosteric enzyme located at a crucial control point in carbohydrate metabolism. In the presence of divalent metal ions, the enzyme catalyzes the hydrolysis of fructose-1,6-bisphosphate (FrU-1,6-P2) to fructose 6-phosphate (FrU-6-P) and inorganic phosphate (P). The reaction is noncompetitively inhibited by AMP and competitively inhibited by FrU-2,6-P1b and the action of these two inhibitors is synergistic (1-3). These two compounds are involved in the activation of phosphofructokinase (4) and are responsible for the coordinated regulation of glycolysis and glycogenolysis (5, 6).

FBPase is composed of four identical subunits, each of which contains a substrate and AMP binding domain separated by 28 Å, and metal binding sites in close proximity to the substrate binding site (7–9). AMP binding is cooperative with a Hill coefficient of approximately 2 (10–13). In addition, AMP inhibition exhibits “half of the sites” reactivity (14). Cooperativity with respect to metal ion binding to FBPase has also been recognized (10, 15, 16). The activity of FBPase, as a function of Mg2+ concentration, is sigmoidal at neutral pH but hyperbolic at pH 9.6. Furthermore, AMP and Mg2+ are mutually exclusive in their binding to the enzyme (10, 17). X-ray crystallographic studies have shown that an R- to T-state transition of FBPase is triggered by the binding of AMP, causing a shift of binding sites for metals to unfavorable positions (18, 19). The mutation of residues at the metal binding sites of FBPase affects the Mg2+ and AMP affinity (20); however, significant changes in Mg2+ cooperativity and affinity and/or AMP affinity were also a consequence of the mutation of active site residues (21–23). In addition, AMP cooperativity is completely lost when Glu-29, a residue in the AMP domain, is replaced by Gin (24).

X-ray diffraction studies of FBPase from porcine kidney have pinpointed a number of amino acid residues located at the interface of dimers that might contribute to cooperativity during the R- to T-state transition. In an attempt to associate functional properties of FBPase to specific interactions of the dimer interface, Arg-15, Glu-19, Arg-22, and Thr-27, which is located in the AMP binding site but interacts with Arg-22 in the T-state of FBPase, were the subjects of experiments in directed mutation. The kinetic properties of R15A, E19Q, R22K, R22M, and T27A were investigated. The most important finding of this investigation is that the replacement of Arg-22 with methionine causes a total loss of AMP cooperativity and increases the turnover number of the enzyme as measured by initial rate kinetics.

EXPERIMENTAL PROCEDURES

Materials—FrU-1,6-P2, FrU-2,6-P1b, NADP, MgCl2, AMP, and isopropanol were purchased from Sigma. DNA-modifying and restriction enzymes were from Promega and Clontech Laboratories, Inc.. Glucose-6-phosphate dehydrogenase and phosphoglucone isomerase were from Boehringer Mannheim. Other chemicals were of reagent grade or the equivalent.

Mutagenesis of FBPase—Mutations were accomplished by the introduction of specific base changes into a double-stranded plasmid (25). Five mutagenic primers, as shown in Table I, were used to mutate Arg-15→Ala, Glu-19→Gin, Arg-22→Lys, Arg-22→Met, and Thr-27→Ala. A selective primer is also shown in Table I, which exchanged the original NruI site to a unique XhoI site on the pET-11a vector. The double-stranded FBPase expression plasmid (pET-FBP) and mutagenic and selective primers were denatured, annealed, and polymerized as described by Deng and Nickoloff (25).

Mutations were confirmed by NruI/XhoI digestion and by fluorescent dideoxy chain-termination sequencing at the Nucleic Acid Facility at
Iowa State University. The mutagenesis plasmid was finally transformed into Escherichia coli DE 657, a strain deficient in the FBPase gene.

FBPase Purification—The expression and purification of wild-type, R15A, E19Q, R22K, and T27A enzymes were carried out as described previously (21, 26). Because R22M did not bind to the CM-Sepharose C-50 column, a Cibacon blue 3GA (from Sigma) column, equilibrated with 25 mM malonate (pH 6.0), was used in the final step. Homogeneous R22M was eluted from the Cibacon blue column by 1 M NaCl, 25 mM malonate (pH 6.0).

Protein concentration was assayed as described by Bradford (27) with bovine serum albumin (from Sigma) as the standard. The protein purity was determined by using 12% SDS-polyacrylamide gel electrophoresis.

Kinetic Studies—Specific activity during purification was determined by the phosphoglucoisomerase and glucose-6-phosphate dehydrogenase-coupled spectrophotometric assay at either pH 7.5 or 9.6 (10.28). All other kinetic experiments were done at pH 7.5 (Hepes buffer) and 24 °C by using a coupled spectrofluorometric assay (10). Initial rate data were analyzed by using a computer program written in MINITAB language with an n value of 2.0 (10.29). Cooperativity was evaluated by using either the ENUMITTER program (30) or the MINITAB program.

Circular Dichroism Spectrometry—CD studies on the wild-type and mutant forms of FBPase were carried out in a jasco 710 CD spectrometer in a 1-mm cell at room temperature. Spectra were collected from 200 to 260 nm in 1.3-nm increments, and each spectrum was blank corrected and smoothed by using the software package provided with the instrument.

Model Building—Models for the mutants of FBPase were built using coordinates of the wild-type enzyme complexed with AMP (Protein Data Bank file 4FBP). The side chains of Arg-22 of subunit C1 and C4 were replaced with either methionine or lysine so that their torsion angle values were identical to those of arginine. Afterward, the models, including that of the wild type, were energy minimized using the program CHARMM of QUANTA (Molecular Simulations, Inc.).

RESULTS

Enzyme Purity—Enzyme purity of wild-type and the five mutant forms of porcine liver FBPase was evaluated by SDS-polyacrylamide gel electrophoresis (data not shown). Identical relative mobilities and homogeneities (≥96%) were found for these enzymes by using electrophoresis as a criterion. The pH 7.5/9.6 activity ratios shown in Table II, along with the SDS-polyacrylamide gel electrophoresis results, suggest that the enzymes had not undergone proteolysis (28).

Initial Rate Studies—To evaluate the effects of mutations on the interface of FBPase, kinetic studies were undertaken on the wild-type and mutant forms of the enzyme. The kinetic parameters are summarized in Table II. Most of the data in Table II were obtained by measuring the initial rate at saturating Fru-1,6-P2 or 5 mM Mg2+, a concentration that does not cause substrate inhibition. It can be seen from Table II that mutation of Arg-22 to methionine increased the enzyme activity. The mutant has a 2.1-fold higher Km, values that of that of the wild-type enzyme, whereas the Km for Fru-1,6-P2 was similar to that of the wild-type enzyme. Specific activity and Km values for Fru-1,6-P2 were similar for R15A, R22K, and wild-type FBPase. Small decreases in Fru-1,6-P2, Km were found in E19Q (2.6-fold) and T27A (3.5-fold) relative to wild-type FBPase.

Loss of AMP Cooperativity with R22M Fructose-1,6-bisphosphatase

| Mutants | Primer sequences |
|---------|------------------|
| R15A    | 5′–ACCTTACGCCCGCTTCTGATG–3′ |
| E19Q    | 5′–TGCGTCAAGGAAGGCAG–3′ |
| R22K    | 5′–AGGCCGGAGACAGGCCAC–3′ |
| R22M    | 5′–TGCGTCATGGCCGGAGACAGGCCAC–3′ |
| T27A    | 5′–AGCGGTGGCTTCTGAGACAGGCCAC–3′ |

The candidate bases for mutation are shown in boldface and underlined.
Fru-1,6-P₂, AMP, the Michaelis constants for Mg²⁺ and Fru-1,6-P₂, dissociation constants for Mg³⁺, and the dissociation constants for AMP from the enzyme-AMP, the enzyme-AMP-AMP complexes, respectively. The coupled spectrofluorescence assay was performed at 25 °C in 50 mM Hepes buffer (pH 7.5) containing 0.1 M KCl, 10 mM Mg²⁺, and 4.5 mM AMP. The lines are theoretical based upon Equation 1 when n = 2, and the points were experimentally determined. The inset shows a plot of the slope of the family of curves in Fig. 1 versus [AMP]₂.

![Plot of reciprocal of initial velocity in arbitrary fluorescent units versus reciprocal of [Mg²⁺] for R22K FBPase. The concentrations of AMP are 0 (■), 2.0 μM (▲), 3.0 μM (▲), and 4.5 μM (□). The coupled spectrofluorescence assay was performed at 25 °C in 50 mM Hepes buffer (pH 7.5) containing 0.1 M KCl, 10 mM Fru-1,6-P₂. The lines are theoretical based upon Equation 1 when n = 2, and the points were experimentally determined. The inset shows a plot of the slope of the family of curves in Fig. 1 versus [AMP]².](image1)

It is clear from Equation 2 that I, a competitive inhibitor for substrate A will be a noncompetitive inhibitor of the other substrate (B) in the reaction. As expected, when a double-reciprocal plot of 1/velocity against 1/Fru-1,6-P₂ was made at different concentrations of AMP for wild type, R15A, E19Q, R22K, or T27A, a family of intersecting lines was obtained to the left of the 1/ν axis (data not shown). These data were best fit to Equation 2 when n = 2. In the case of R22M, the best fits were obtained when n = 1 in Equation 2. These results suggest that the positive charge on the side chain of Arg-22 is essential for maintaining AMP cooperativity. Mutation of Thr-27 to Ala, which interacts with O2A of the phosphate group of AMP, caused a significant decrease (1300-fold) in AMP affinity for FBPase without changing the AMP cooperativity. A dramatic change of AMP binding and cooperativity, however, has been reported for the Glu-29 → Gln mutant of FBPase (24). Glu-29 is also directly ligated to the phosphoryl portion of AMP.

Secondary Structure Analysis—To determine whether the protein structure was changed by the mutations at the interface of the FBPase dimers, CD spectrometry was used to analyze the secondary structure of wild-type and mutant forms of FBPase. The CD spectra of the six enzymes are essentially the same, consistent with the random mechanism along with the following interactions:

\[
K_i \quad E + I = EI \quad (\text{Eq. 3})
\]

\[
K_{ii} \quad EI + I = EI_2 \quad (\text{Eq. 4})
\]

\[
K_{ii} \quad EB + I = EBI \quad (\text{Eq. 5})
\]

\[
K_{ii} \quad EBI + I = EBI_2 \quad (\text{Eq. 6})
\]
The major finding of this report is the complete loss of AMP cooperativity and increased enzymatic activity when FBPase Arg-22 is mutated to Met. It has long been recognized that the binding of AMP to wild-type FBPase exhibits cooperativity (10, 12, 13, 24). The kinetic data of AMP inhibition with wild-type, R15A, E19Q, R22K, and T27A FBPases gave excellent fits to a cooperativity model. In these cases, the Hill coefficient for the R15A, E19Q, R22K, and T27A FBPases gave excellent fits to a cooperative binding of AMP to wild-type FBPase exhibits cooperativity (10, 12, 13, 24). However, in the case of R22K mutant, the Hill coefficient of 1.0 for AMP indicates a competitive inhibitor of Mg
nucleotideis
. On the other hand, R22M shows a loss of AMP cooperativity with a Hill coefficient of 1.0 for AMP. AMP is a competitive inhibitor of Mg
 and noncompetitive inhibitor of Fru-1,6-P2 at neutral pH with the five mutant forms of FBPase as well as with the wild-type enzyme.

Data contained in this report suggest that mutation of porcine FBPase Arg-22 to Met causes the complete loss of cooperativity without changing AMP affinity; however, the affinity for AMP decreased markedly in the case of R22K without altering AMP cooperativity. We conclude that the Arg-22 residue of mammalian FBPase is essential for simultaneous cooperativity and binding of AMP. An understanding of this finding requires further investigations (e.g., solution of the crystal structure of R22M FBPase); however, modeling studies were undertaken to provide some insights into the effect of mutation of Arg-22 to Met and Lys. Fig. 3 is a stereoview of the subunit C1-C4 interface of FBPase in the T-state from the porcine kidney FBPase crystal structure reported by Ke et al. (38). AMP molecules and most of helix H1 and H2 are shown. In the T-state of FBPase, NE of Arg-22 of subunit C1 is hydrogen bonded (3.1 Å) to O of Thr-27, a residue located at the AMP binding site of subunit C4. Atoms N and OG1 of Thr-27 are hydrogen bonded to O2A of the phospholyl group of AMP. To determine the possible conformational changes in the area of the mutation, we used computer modeling to compare energy minimized R22M and R22K mutants with the wild-type enzyme. The 10-Å area around residue 22 of both subunits C1 and C4 was free to change, while the rest of the molecule was fixed. The conformation of residues in the energy minimized area changed slightly, but the changes were principally identical in all three models. This is why in our further analysis we kept all residues fixed to their positions in the x-ray structure except residues 22 and 27 and the nearest water molecules. In the R22K mutant, the side chain of Met-22 retained the conformation of the side chain of Arg (Fig. 4A). Thus, the only obvious change in subunit interactions is the loss of hydrogen bonding from NE of Arg-22 to O of Thr-27. The kinetic data suggest that the hydrogen bond is necessary for cooperativity but not AMP binding affinity. The importance of the interactions between Glu-19, Glu-29, and Arg-22 is not obvious from the x-ray structure. For example, Glu-19 is hydrogen bonded to Arg-22, but the symmetry related pair Glu-19 - Arg-22 is not. Moreover, the Glu-19 - Arg-22 interaction is within the same subunit and has no direct relation to intersubunit contacts. The interaction of Arg-22 with Glu-29 (and vice versa) may be more important, but these groups do not make a direct contact (Arg-22 NH2 is 4.74 Å from Glu-29 OE1). In the case of the R22K mutant, NZ of Lys is shifted 1.4 Å further from its C' atom than the NE of Arg. This difference puts NZ of Lys-22 close enough to OG1 of Thr-27 of the symmetry-related subunit so that they form a hydrogen bond (Fig. 4B). Our modeling suggests that the new hydrogen bond perturbs the interaction between OG1 of Thr-27 and the phospholyl group of AMP (the distance changes from 2.8 Å in the wild-type to 3.2 Å in the mutant enzyme). This finding correlates with kinetic data for the R22K and T27A mutants. In T27A, a complete loss of one hydrogen bond (from OG1 of Thr-27 to O2A of AMP) reduced its affinity for AMP by 1300-fold. The putative weakening of the same hydrogen bond in the R22K mutant also reduces AMP affinity but to a lesser extent. Despite the different positions for NZ of Lys-22 and NE of Arg-22, each side chain maintains an interaction with carbonyl 27. This interaction should be present as well in the T27A mutant. Thus, apparently, subtle changes in the position of OG1 of Thr-27 influences AMP affinity, whereas the loss of hydrogen bond to O of Thr-27 (as in R22M) results in the complete loss of cooperativity.

According to the x-ray diffraction data (19), AMP induces localized conformational changes, which lead to the global rearrangement of the C1-C4 (C2-C3) subunit interface. This rearrangement involves significant movement of side chains along the C1-C4 (C2-C3) interface and the formation of new hydrogen bonds among residues at that interface. The rearrangement is putatively responsible for the cooperativity of AMP binding and the stabilization of the T-state conformation of FBPase. The kinetic properties of the three mutants can be explained simply by the disruption of interactions in the T-state. Studies of the R22M mutant demonstrates that despite the reorganization of significant numbers of hydrogen bonds during the transition to the T-state, the elimination of interactions produced by the mutation of a single residue abolishes AMP cooperativity. The loss of AMP cooperativity in R22M, without a concomitant change in AMP affinity and inhibition, demonstrates that these properties can be separated. Based on the mechanism of allosteric regulation of FBPase by AMP (19), we can suggest two mechanisms for the loss of cooperativity: 1) the loss of cooperativity is associated with the destabilization of the T-state. As a result, a subunit with bound AMP is transformed from the R-state to the T-state but without altering the...
conformation of other subunits; 2) the binding of AMP to the mutant still forces all the subunits into the T-state, but the lack of an Arg-22-Thr-27 interaction leaves the symmetry-related AMP binding sites unaltered.

In the absence of AMP, $k_{\text{cat}}$ of the R22M is higher than that of the wild-type enzyme. The increase in activity of R22M may be associated with changes in the conformation of the R-state due to the loss of two hydrogen bonds from the guanidinium of Arg-22 in the mutant FBPase. In the R-state, NH1 of Arg-22 is hydrogen bonded to the carbonyls of Arg-110 and Glu-108, each belonging to a neighboring subunit (C1-C4 or C2-C3 interfaces). Alternatively, FBPase may exist as an equilibrium of R- and T-states, with a significant amount of T-state even in the absence of AMP. The loss of hydrogen bonds in the T-state mutants would shift the equilibrium to the R-state and, thus, increase the activity. Both alternatives can provide an explanation of the observed Mg$^{2+}$ cooperativity. On one hand, Mg$^{2+}$ cooperativity may be due to the transformation of FBPase to some "activated" R-state after it binds one Mg$^{2+}$ ion, whereas, on the other hand, Mg$^{2+}$ cooperativity may be explained by the shift of the equilibrium between R- and T-states toward the R-state.

The hydrogen bonding between the side chains of Arg-15 and Ser-87 (C1-C4 and C4-C1) is abolished in the Ala-15 mutant, thus causing the increase in Fru-2,6-P$_2$ binding affinity (6-fold) and in the $K_M$ for Mg$^{2+}$ (9-fold) relative to that of wild-type FBPase. A similar effect was found with the E19Q mutant, which exhibits about 5-fold changes in Fru-2,6-P$_2$ and Mg$^{2+}$ binding affinities. X-ray diffraction studies (18, 19) have demonstrated that the relative movements between the Fru-1,6-P$_2$ and AMP domains during the R- to T-state transition also shifts the metal binding sites to unfavorable positions, the result of which is the inhibition of enzyme activity.

This study demonstrates that residues located at the interfaces of subunits C1 and C4 and of subunits C2 and C3 have different effects on AMP binding, substrate binding, and Mg$^{2+}$ binding, i.e. Arg-22 is essential for AMP cooperativity and affinity, and Arg-15 and Glu-19 effect Mg$^{2+}$ and Fru-2,6-P$_2$ binding. This report also shows that the helix H1 (resides 12–24) are somehow involved in the communication between the active site and the AMP site during the R- to T-state transition, a finding consistent with the results of x-ray diffraction studies (19).

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