Mutation of Arginine 276 to Methionine Changes Mg^{2+} Cooperativity and the Kinetic Mechanism of Fructose-1,6-bisphosphatase†

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ABSTRACT: Arginine 276 of porcine liver fructose-1,6-bisphosphatase (FBPase) was mutated to methionine by site-directed mutagenesis on the basis of the crystal structure of the enzyme [Zhang, Y., Liang, J.-Y., Huang, S., Ke, H., & Lipscomb, W. N. (1993) *Biochemistry 32*, 1844−1857]. The mutant and wild-type forms of the enzyme were purified to homogeneity and characterized by circular dichroism spectrometry (CD) and initial-rate kinetics. There were no discernible differences between the secondary structures of the wild-type and the mutant enzymes on the basis of the CD data. Replacement of Arg 276 with methionine caused a significant decrease in the enzyme's activity. The k_{cat} for the mutant enzyme was only about 0.67% of that of the wild-type enzyme. Most importantly, the mutation caused the total loss of cooperativity for Mg^{2+} and changed the kinetic mechanism to one in which the substrate adds to FBPase before Mg^{2+} and in which all steps equilibrate rapidly relative to the conversion of the ternary complex of enzyme, substrate, and Mg^{2+} to products. The K_a for Mg^{2+} increased by only about 5-fold relative to that of the wild-type enzyme. The mutation did not change the K_i for AMP or the Hill coefficient of this allosteric inhibitor. The K_i for fructose 2,6-bisphosphate was increased by 16-fold compared with that of the wild-type enzyme. The K_m for fructose 1,6-bisphosphate was similar to that of the wild-type enzyme. It is concluded that Arg 276 is critical for activity and Mg^{2+} cooperativity with FBPase and it determines the enzyme's kinetic mechanism.

Fructose-1,6-bisphosphatase (FBPase, EC 3.1.3.11) is located at a key regulatory position in the gluconeogenic pathway (Krebs, 1963; Marcus, 1981; Pilkis *et al.*, 1988; Hers & Hue, 1983). In the presence of a divalent metal ion, it catalyzes the hydrolysis of fructose 1,6-bisphosphate (Fru-1,6-P_2) to form fructose 6-phosphate (Fru-6-P) and inorganic phosphate (P_i) and exhibits a rapid-equilibrium, random Bi-Bi kinetic mechanism (Liu & Fromm, 1990). The reaction is competitively inhibited by fructose 2,6-bisphosphate (Fru-2,6-P_2) and noncompetitively inhibited by adenosine 5′-monophosphate (AMP) (Liu & Fromm, 1990). Both compounds act synergistically to inhibit FBPase (Hers & Van Schaftingen, 1982). It has been shown from both binding (Scheffler & Fromm, 1986) and kinetic studies (Liu & Fromm, 1990) that Mg^{2+} and AMP are mutually exclusive binding ligands. It is thought that one of the functions of Fru-2,6-P_2 is to make AMP stickier on the enzyme, thus enhancing the action of AMP (Liu & Fromm, 1988).

It is well-known that porcine kidney FBPase is a homotetramer with a subunit molecular weight of 37 000 (Marcus *et al.*, 1982; Burton *et al.*, 1993), and like all FBPases, it requires a divalent metal ion for activity (Gomori, 1943; Benkovic & deMaine, 1982). It has been shown from binding investigations that there are two metal-binding sites per enzyme subunit, a high-affinity "structural" site and a low-affinity "catalytic" site (Benkovic *et al.*, 1978), and catalysis requires the sequential addition of metal, substrate, and catalytic metal to form a catalytically competent quaternary complex of enzyme−M1−M2−Fru-1,6-P_2. Nimmo and Tipton (1975a,b) showed that pH could change the Mg^{2+} kinetics of bovine liver FBPase. At neutral pH, Mg^{2+} binding and activation of bovine liver FBPase show cooperativity, but the cooperativity is lost at pH 9.6. Chen *et al.* (1993) and El-Maghrabi *et al.* (1993) performed site-directed mutagenesis in the metal-binding sites of mammalian FBPase. Their results support the suggestion that there are two metal-binding sites associated with FBPase, but that the Mg^{2+} cooperativity is inter- rather than intrasubunit. Zhang *et al.* (1995) showed that porcine liver FPBase lost Mg^{2+} cooperativity at pH 7.5 when Gly 122 was mutated to alanine.

X-ray diffraction results (Zhang *et al.*, 1993) of porcine kidney FBPase showed that the side chain ϵ-NH_2 of Arg 276 in the C1 chain formes a salt bridge with the 1-phosphate oxygen O11 at the α-anomer of the substrate. But in the presence of metal ions, the side chain of Arg 276 no longer interacts with the 1-phosphate oxygen atom. This occurs because the binding of metal ions causes slight changes in orientation and position of the sugar ring and moves the 1-phosphoryl group of the substrate toward the metal-binding sites. Thus, the residue is postulated to play roles in enzyme catalysis and binding of metal ions. To verify this postulation, we altered Arg 276 of porcine liver FBPase to methionine by site-directed mutagenesis and studied its properties. In this study, we report that replacement of Arg 276 with methionine changes the Mg^{2+} cooperativity and the kinetic mechanism of porcine liver FBPase and causes a significant decrease in the enzyme's activity.

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EXPERIMENTAL PROCEDURES

Materials. NADP, fructose 1,6-bisphosphate (Fru-1,6-P_2), fructose 2,6-bisphosphate (Fru-2,6-P_2), AMP, Hepes, and Tris were purchased from Sigma (St. Louis, MO). Glucose-6-phosphate dehydrogenase and phosphoglucoisomerase were from Boehringer Mannheim (Indianapolis, IN). Distilled, deionized water was used in all experiments. All other reagents were of the highest purity available commercially. Recombinant and mutant forms of porcine liver FBPase were prepared and purified as described elsewhere (Burton *et al.*, 1993) with slight modifications. Mutant forms of the enzyme were obtained in yields comparable to the wild-type enzyme. Porcine liver and kidney FBPase are identical in their primary sequences (Burton *et al.*, 1993).

Mutant of Fructose-1,6-bisphosphatase. A mutant of recombinant porcine liver FBPase, R276M, was obtained by site-directed mutagenesis. A mutagenic oligonucleotide primer, 5′-GGA-AAG-TTA-**ATG**-CTG-CTA-TAC-3′, was synthesized by the β-cyanoethyl phosphoramidite method at the Nucleic Acid Facility at Iowa State University. The codon ATG was used to mutate Arg 276 → Met. *Bam*HI/*Sph*I fragments encoding FBPase from pEt-11a were ligated into a previously digested PUC118 plasmid. The mutagenesis was done by using single-stranded DNA from recombinant pUC118 plasmid as the template and synthesized oligonucleotide as primer. The oligonucleotide-directed *in vitro* mutagenesis procedure was performed as described by Nakamaye and Eckstein (1986). Mutagenesis was verified by dideoxy chain termination sequencing (Sanger *et al.*, 1977). The *Bam*HI/*Xba*I fragments encoding the mutations were ligated back into previously digested pEt-11a expression vector. pEt-11a was used to transform *Escherichia coli* strain BL21 (DE3).

Protein Assay. Total protein was measured by the Bio-Rad method with bovine serum albumin (from Sigma) as a standard.

Circular Dichroism Spectrometry. Circular dichroism spectroscopic (CD) studies on the wild-type and mutant form of FBPase were carried out in 5 mM Hepes buffer (pH 7.5) at room temperature in a JASCO CD spectrometer Model J-710. Samples were placed in a 1-mm cuvette, and data points were collected from 200 to 260 nm in 0.5-nm increments. Each spectrum was calibrated to remove the background of the buffer and smoothed by using a program in the computer of the spectrometer.

Kinetic Studies. FBPase activity during purification and the specific activity of pure enzyme were measured by using the phosphoglucoisomerase/glucose-6-phosphate dehydrogenase coupled spectrophotometric assay (Pontremoli & Traniello, 1975). All other kinetic experiments were done using a fluorometric assay (Liu & Fromm, 1990) at pH 7.5 (50 mM Hepes buffer) and 24 °C. The initial-rate data were analyzed for kinetic mechanisms by using a MINITAB language program with an α value of 2.0 (Liu & Fromm, 1990). Cooperativity was evaluated by using both the ENZFITTER program (Leatherbarrow, 1987) and the MINITAB program.

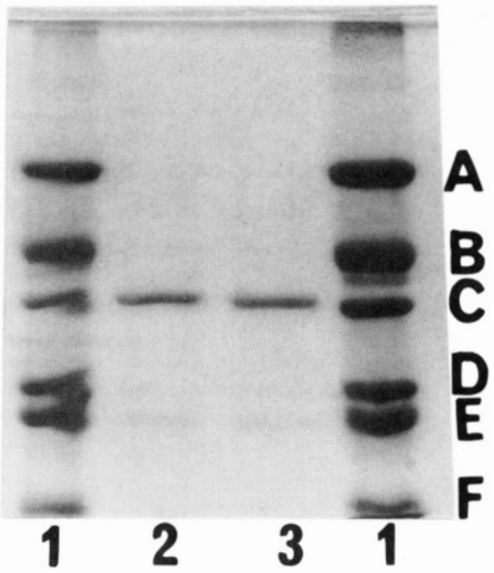


FIGURE 1: SDS−PAGE analysis of purified wild-type and mutant forms of porcine liver FBPase. All samples were run on a 12% polyacrylamide gel and stained with Coomassie Brilliant Blue R-250: lanes 1 and 4, protein standard; lane 2, wild-type; lane 3, R276M mutant. Molecular masses of protein standards: A, 66 kDa; B, 45 kDa; C, 36 kDa; D, 29 kDa; E, 20 kDa; F, 14 kDa.

RESULTS

Enzyme Quality. Figure 1 shows the SDS−PAGE of wild-type recombinant porcine liver FBPase and the R276M mutant FBPase. It can be seen that the proteins exhibit molecular masses of approximately 37 kDa and are greater than 95% pure using electrophoresis as a criterion. These data suggest that the proteins have not undergone discernible degradation.

Secondary Structure Analysis. The secondary structures of both recombinant wild-type and R276M mutant forms of FBPase were analyzed by CD spectrometry. The CD spectral data showed that the spectrum of the mutant enzyme was completely superimposable on that of the wild-type enzyme (data not shown). These results indicate that no major conformational changes occurred with FBPase when Arg 276 was mutated to methionine.

Initial-Rate Kinetics. To evaluate the effects of the mutation on the properties of FBPase, kinetic studies were carried out for the mutant and wild-type forms of FBPase at either saturating Mg^{2+} or Fru-1,6-P_2 concentrations. The kinetic parameters are summarized in Table 1. From Table 1, it can be seen that replacement of Arg 276 with methionine caused a significant decrease in the enzyme's activity. The

Table 1: Kinetic Parameters for Wild-Type and Mutant Forms of Fructose-1,6-bisphosphatase

				Mg^{2+}			
enzyme	specific activity (units/mg)	k_{cat} (s^{-1})	K_m Fru-1,6-P_2 (μM)	K_a[a]	Hill coeff	K_i[b] AMP (μM^2)	K_i[b] Fru-2,6-P_2 (μM)
wild-type	31 ± 1.5	20 ± 0.91	2.5 ± 0.31	0.50 ± 0.04	2.04 ± 0.09	17.8 ± 5.4	0.36 ± 0.05
R276M	0.21 ± 0.0	0.13 ± 0.0	3.1 ± 0.34	2.8 ± 0.32	1.18 ± 0.13	37.8 ± 6.7	5.8 ± 0.54

[a] The unit for K_a for wild-type FBPase is mM^2, and for R276M it is mM. [b] K_i values were determined at 5 and 8 mM Mg^{2+} for wild-type and R276M mutant FBPases, respectively.

k_{cat} for this mutant enzyme was only about 0.67% of that of the wild-type enzyme, whereas the K_m for Fru-1,6-P_2 was similar to that of the wild-type enzyme.

Mg^{2+} Ion Activation. As expected, the Hill coefficient of wild-type FBPase for Mg^{2+} was 2.0, and Mg^{2+} activation of the enzyme was sigmoidal (data not shown). These results indicate that Mg^{2+} activation of FBPase exhibits cooperativity. This is consistent with previous reports (Nimmo & Tipton, 1975a,b). However, it was found that the Hill coefficient of the mutant FBPase for Mg^{2+} was about 1, *i.e.*, Mg^{2+} activation of FBPase was hyperbolic instead of sigmoidal (data not shown). In these studies, the level of Mg^{2+} was varied 120-fold (0.1−12 mM) at a saturating concentration of Fru-1,6-P_2. No evidence of cooperativity, either positive or negative, was observed. These results indicate that the mutation of Arg 276 to methionine caused a total loss of Mg^{2+} cooperativity, although the K_a for Mg^{2+} increased only about 5-fold relative to that of wild-type FBPase.

Inhibition by AMP. AMP is an allosteric inhibitor of FBPase (Nimmo & Tipton, 1975a; Taketa & Pogell, 1965; Stone & Fromm, 1980), and the function of AMP is to remove divalent metal ion from FBPase (Liu & Fromm, 1988). AMP binding to FBPase shows cooperativity (Chen *et al.*, 1994). When Arg 276 was mutated to methionine, the K_i for AMP increased only about 2-fold (Table 1) relative to the wild-type enzyme, and the AMP inhibition pattern still exhibited cooperativity. These data suggest that Arg 276 is not involved in AMP inhibition and cooperativity.

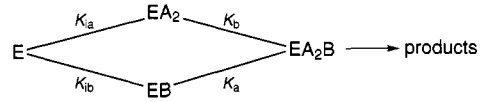
Inhibition by Fru-2,6-P_2. Fru-2,6-P_2 is a competitive inhibitor of Fru-1,6-P_2 and competes with the substrate for the active site of FBPase (Pilkis *et al.*, 1981; Pontremoli *et al.*, 1982; Ganson & Fromm, 1982; McGrane *et al.*, 1982; Liu & Fromm, 1989; Ke *et al.*, 1989, 1990; Scheffler & Fromm, 1986). From Table 1, it can be seen that the K_i of the R276M mutant FBPase for Fru-2,6-P_2 increased by about 16-fold relative to that of wild-type FBPase. These results indicate that Arg 276 is not as important as Lys 274 in permitting FBPase to discriminate between the substrate and inhibitor. Lys 274 is essential for Fru-2,6-P_2 inhibition of FBPase (El-Maghrabi *et al.*, 1992; R. Zhang and H. J. Fromm, unpublished observations).

Kinetic Studies in the Absence of Inhibitors. To gain some insight into the effects of the mutation on the kinetic mechanism, and to confirm the finding that altering Arg 276 to methionine results in the total loss of Mg^{2+} cooperativity, we studied the kinetic behavior of both wild-type and mutant forms of FBPase in the absence of inhibitors. Figure 2A shows a double-reciprocal plot of 1/initial velocity *versus* the concentration of 1/Fru-1,6-P_2. Figure 2B depicts a double-reciprocal plot of 1/initial velocity *versus* $1/[Mg^{2+}]^2$ with wild-type FBPase. From these two figures, it can be seen that, when the Fru-1,6-P_2 concentration was varied at different fixed levels of Mg^{2+}, a family of lines intersecting in the second quadrant was obtained (Figure 2A). The family of lines (Figure 2B) for various $1/[Mg^{2+}]^2$ concentrations at different fixed levels of Fru-1,6-P_2 was similar to that when Fru-1,6-P_2 was the varied substrate. The data shown in Figure 2A,B gave much better fits to eq 1 when $n = 2$ than when $n = 1$, e.g., the goodness of fit was 8% when $n = 1$ and 4% when $n = 2$. The form of eq 1 is

$$\frac{1}{V} = \frac{1}{V_m}\left[1 + \frac{K_a}{A^n} + \frac{K_b}{B} + \frac{K_{ia}K_b}{A^nB}\right] \quad (1)$$

where V, V_m, A, B, K_a, K_b, and K_{ia} represent the initial velocity, maximum velocity, concentration of free Mg^{2+}, concentration of free Fru-1,6-P_2, Michaelis constant for Mg^{2+}, Michaelis constant for Fru-1,6-P_2, and dissociation constant for Mg^{2+}, respectively; n represents the Hill coefficient for Mg^{2+} with FBPase. When $n = 1$, there is no cooperativity; when $n = 2$, the binding of Mg^{2+} to FBPase is cooperative. Equation 1 is the fundamental rate equation based upon stochastic evaluation for the sequential kinetic mechanism shown in Scheme 1.

Scheme 1



$$E + 2A = EA_2, \quad K_{ia} = [E][A]^2/[EA_2] \quad (2)$$

$$E + B = EB, \quad K_{ib} = [E][B]/[EB] \quad (3)$$

$$EA_2 + B = EA_2B, \quad K_b = [EA_2][B]/[EA_2B] \quad (4)$$

$$EB + 2A = EA_2B, \quad K_a = [EB][A]^2/[EA_2B] \quad (5)$$

The data shown in Figure 2A,B are consistent with previous reports that showed that Mg^{2+} activation and binding to FBPase exhibit cooperativity (Benkovic & deMaine, 1982; Nimmo & Tipton, 1975a,b; Tejwani, 1983). However, the kinetic data (shown in Figure 3A,B) in the absence of inhibitors, with the mutant form of FBPase, gave excellent fits to eq 1 when $n = 1$ and did not fit as well to eq 1 when $n = 2$. In this case, the goodness of fit was 4% when $n = 1$ and 9% when $n = 2$. These results confirm the finding that altering Arg 276 to methionine results in the total loss of Mg^{2+} cooperativity with FBPase. Had Mg^{2+} ions bound cooperativity to FBPase, the data would have fit to eq 1 much better when $n = 2$ than when $n = 1$.

The mutation of Arg 276 to methionine not only changes the Mg^{2+} cooperativity but also changes the kinetic mechanism. From Figure 3B it can be seen that, when the Mg^{2+} concentration was varied at different fixed concentrations of Fru-1,6-P_2, the family of lines intersected *on* the 1/velocity axis rather than in the second quadrant. This finding is important because the data shown in Figure 3B represent a change in kinetic mechanism compared with the data shown in Figure 2B, *i.e.*, the data in Figure 2B represent either a rapid-equilibrium random Bi-Bi mechanism or a steady-state ordered Bi-Bi mechanism, whereas the data shown in Figure 3B represent a rapid-equilibrium ordered Bi-Bi mechanism (Fromm, 1975). For the case of the mutant FBPase, eq 1 can be written as

$$\frac{1}{V} = \frac{1}{V_m}\left[1 + \frac{K_b}{B} + \frac{K_{ia}K_b}{AB}\right] \quad (6)$$

where all of the terms in eq 6 have the same meaning as in

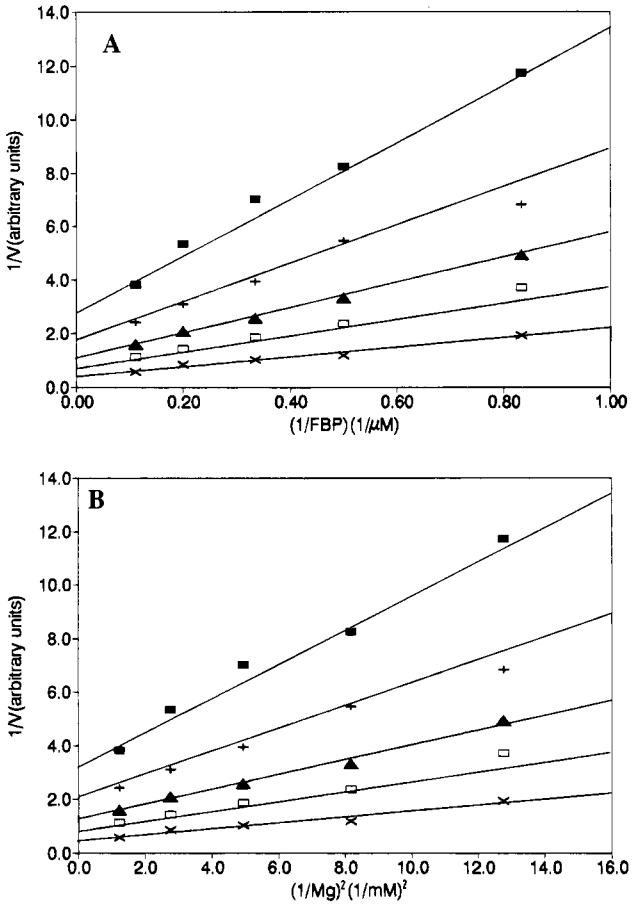


FIGURE 2: (A) Plot of reciprocal of initial velocity with wild-type FBPase in arbitrary fluorescent units *versus* reciprocal of [Fru-1,6-P_2] concentration. The concentrations of Mg^{2+} are 0.28 (■), 0.35 (+), 0.45 (▲), 0.60 (□), and 0.90 mM (×). The lines are theoretical based on eq 1 when $n = 2$, and the points are experimentally determined. (B) Plot of reciprocal of initial velocity with wild-type FBPase in arbitrary fluorescent units against reciprocal of $[Mg^{2+}]^2$ concentration. The concentrations of [Fru-1,6-P_2] are 1.2 (■), 2.0 (+), 3.0 (▲), 5.0 (□), and 9.0 μM (×). The lines are theoretical based on eq 1 when $n = 2$, and the points are experimentally determined.

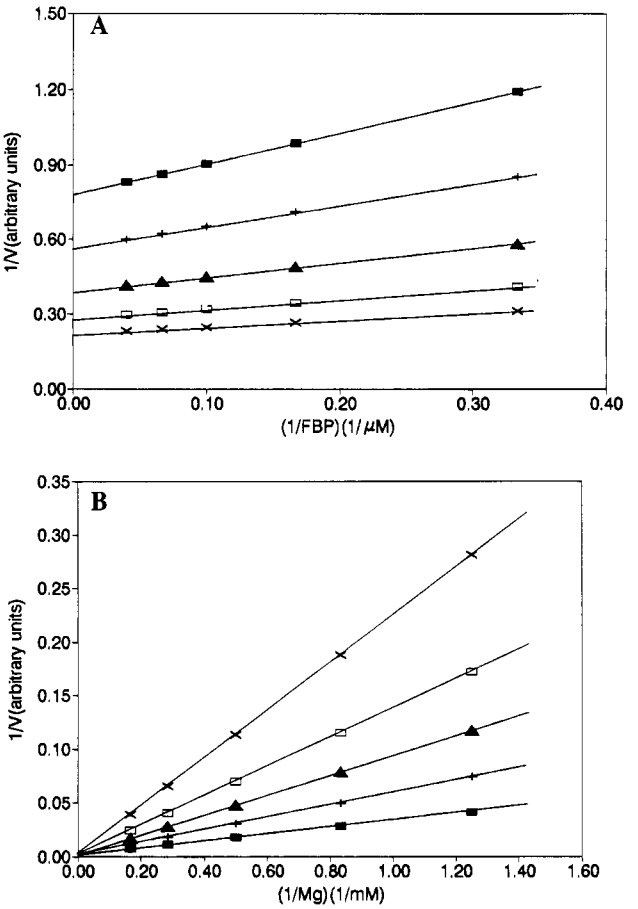
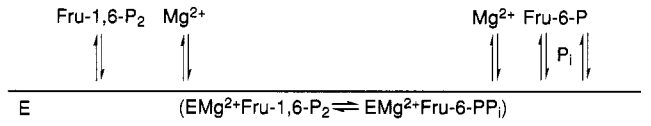


FIGURE 3: (A) Plot of reciprocal of initial velocity with R276M mutant FBPase in arbitrary fluorescent units versus reciprocal of [Fru-1,6-P_2] concentration. The concentrations of Mg^{2+} are 0.8 (■), 1.2 (+), 2.0 (▲), 3.5 (□), 6.0 mM (×). The lines are theoretical based on eq 1 when $n = 1$, and the points are experimentally determined. (B) Plot of reciprocal of initial velocity with R276M mutant FBPase in arbitrary fluorescent units *versus* reciprocal of Mg^{2+} concentration. The concentrations of [Fru-1,6-P_2] are 25 (■), 15 (+), 10 (▲), 6 (□), and 3 μM (×). The lines are theoretical based on eq 1 when $n = 1$, and the points are experimentally determined.

eq 1. Equation 6 is the initial rate equation for the rapid-equilibrium ordered kinetic mechanism shown in Scheme 2.

Scheme 2



$$E + A + EA, \quad K_{ia} = [E][A]/[EA] \tag{7}$$

$$EA + B = EAB, \quad K_b = [EA][B]/[EAB] \tag{8}$$

$$EAB \rightarrow E + \text{products} \tag{9}$$

DISCUSSION

The findings of this report demonstrate the loss of Mg^{2+} cooperativity and the change in kinetic mechanism when Arg 276 of porcine liver FBPase is mutated to methionine by site-directed mutagenesis. In addition, the mutation caused a significant decrease in enzyme activity, *i.e.*, the k_{cat} of the mutant enzyme was only about 0.67% that of the wild-type enzyme.

On the basis of the crystal structure data (Zhang *et al.*, 1993), the ϵ-NH_2 group of Arg 276 forms a salt bridge with the 1-phosphoryl oxygen O11 in the absence of metal ions. In the presence of metal ions, however, this salt bridge no longer exists because metal binding causes slight changes in the orientation and position of the sugar ring and moves the 1-phosphoryl group toward the metal-binding sites. The repositioning of the 1-phosphate is believed to be essential for activation and catalysis. Because Arg 276 does not bind Fru-1,6-P_2 in the presence of metal ions, mutation of Arg 276 to methionine should not affect substrate binding. Table 1 shows that the K_m of the mutant FBPase is similar to that of the wild-type enzyme. These results support the suggestion that no binding occurs between the substrate and Arg 276 in the presence of metal ions. On the other hand, Arg 276 must play important roles in the processes of activation and catalysis. First, it may anchor the 1-phosphoryl group in the required position for metal binding, which is absolutely required for enzyme activity. Also, it may contribute to the orientation of various groups into proper positions for

activation and catalysis. In addition, this residue may aid in the protonation of the ester oxygen O1, thus weakening the P−O bond so that nucleophilic attack by OH^- on the 1-phosphorus atom would be facilitated. These arguments are supported by the data shown in Table 1. It can be seen from Table 1 that porcine liver FBPase loses more than 99% of its activity when Arg 276 is mutated to methionine.

It has long been recognized that metal ions activate and bind cooperatively to FBPase (Benkovic & deMaine, 1982; Nimmo & Tipton, 1975a,b; Tejwani, 1983). A significant finding associated with this report is the complete loss of Mg^{2+} cooperativity with R276M mutant FBPase. The Hill coefficient of this mutant enzyme for Mg^{2+} is about 1. This conclusion is alluded to from the kinetic data evaluated by using either the ENZFITTER or MINITAB program. These results indicate that replacing Arg 276 of FBPase by methionine essentially causes the complete loss of Mg^{2+} cooperativity, but the rationale behind the metal cooperativity and its loss is unclear. Nimmo and Tipton (1975a,b) showed that the plots of velocity against Mg^{2+} are sigmoidal at neutral pH, but that they are hyperbolic at pH 9.6. Site-directed mutagenesis at the metal-binding sites (Chen *et al.*, 1993; El-Maghrabi *et al.*, 1993) did not significantly alter the Mg^{2+} cooperativity. However, Mg^{2+} cooperativity was totally lost when Gly 122 was mutated to alanine (Zhang *et al.*, 1995). It is believed that this mutation can reduce the flexibility of the H4 (residue 123−127) region or lock the enzyme in a conformation that blocks signal transmission and suppresses the cooperativity for Mg^{2+}. All of these findings suggest, but do not provide conclusive proof, that divalent metal ion cooperativity is inter- rather than intra-subunit. The X-ray diffraction studies of FBPase by the Lipscomb group (Zhang *et al.*, 1994; Ke *et al.*, 1991) have provided a basis for AMP cooperativity. The enzyme forms a dimer of dimers in which the R and T states are in equilibrium. When AMP binds, the T state is induced, and the substrate binds to the R state. This model provides a rational explanation for a Hill coefficient of 2 for AMP. The results of the present study may lead to a better understanding of the cooperativity phenomenon for divalent ion binding to FBPase at the molecular level when the three-dimensional structure of the R276M mutant FBPase becomes available.

It is well documented that AMP and Fru-2,6-P_2 are potent synergistic inhibitors of FBPase (Chen *et al.*, 1994; Van Schaftingen *et al.*, 1980a,b). The binding of AMP to wild-type FBPase exhibits cooperativity (Taketa & Pogell, 1965; Nimmo & Tipton, 1975a; Liu & Fromm, 1990; Chen *et al.*, 1994). Site-directed mutagenesis at the AMP-binding site leads to a total loss of AMP cooperativity with FBPase (Chen *et al.*, 1994). The kinetic data of AMP inhibition with R276M mutant FBPase gave excellent fits to a cooperative model. Also, the K_i of R276M enzyme for AMP increased by only 2-fold relative to that of wild-type enzyme (shown in Table 1). These results suggest that Arg 276 may not be involved in AMP inhibition and cooperativity. The other regulator, Fru-2,6-P_2, is believed to have two functions in regulating FBPase: one is that it is a competitive inhibitor of Fru-1,6-P_2 and competes with the substrate for the enzyme's active site (Van Schaftingen & Hers, 1980; Pilkis *et al.*,1981; Ganson & Fromm, 1982; Ke *et al.*, 1989, 1990), and the other function is to enhance the effect of AMP by making AMP "stickier" to the enzyme (Liu & Fromm, 1988). Alteration of Arg 276 to methionine did not cause a significant increase in K_i for Fru-2,6-P_2 (Table 1). The results suggest that Arg 276 may not be directly involved in Fru-2,6-P_2 inhibition.

The kinetics of both the forward and reverse reactions of FBPase have been studied in detail with enzymes from a number of sources (Nimmo & Tipton, 1975a; Marcus *et al.*, 1973; Dudman *et al.*, 1978; Caperelli *et al.*, 1978; Casazza *et al.*, 1979; Stone & Fromm, 1980; Ganson & Fromm, 1982). Liu and Fromm (1990) studied the kinetic mechanism at alkaline pH (9.6) and found it to be rapid-equilibrium random Bi-Bi. Kinetic results of this study have shown that double-reciprocal plots involving magnesium ion intersect (Figures 2B and 3B), indicating a sequential mechanism for Mg^{2+} and Fru-1,6-P_2. However, the plots of 1/velocity *versus* $1/[Mg^{2+}]^2$ in the case of wild-type FBPase intersect in the second quadrant (Figure 2B), whereas the plots of 1/velocity *versus* $1/[Mg^{2+}]$ in the case of R276M mutant FBPase intersect *on* the 1/velocity axis (Figure 3B). The data presented in Figure 2B are consistent with either a rapid-equilibrium random or a steady-state ordered Bi-Bi mechanism. These results are in harmony with previous reports (Zhang *et al.*, 1995; Liu & Fromm, 1990; Marcus *et al.*, 1973). On the other hand, the data shown in Figure 3B support a rapid-equilibrium ordered Bi-Bi mechanism, as shown in Scheme 2. From Scheme 2 and eq 6, it can be seen that Fru-1,6-P_2 binds to the enzyme before Mg^{2+} adds. Another example of switching kinetic mechanisms from rapid-equilibrium random Bi-Bi to ordered Bi-Bi was reported by Schimerlik and Cleland (1973), who found that lowering the pH from 8 to 7 changed the kinetic mechanism for creatine kinase.

Benkovic *et al.* (1978) suggested that catalysis requires the sequential addition of metal and substrate in the order structural metal, substrate, and catalytic metal to form a catalytically competent quaternary complex of enzyme−M_1−M_2−Fru-1,6-P_2 with respect to Mn^{2+} or Zn^{2+} binding. On the other hand, no direct evidence from binding studies supports the existence of structural and catalytic sites for Mg^{2+}. Also, crystallographic data (Zhang *et al.*, 1993) suggest that two metal ions with respect to Mn^{2+} or Zn^{2+} are associated with each enzyme subunit; however, only one Mg^{2+} per subunit was found to bind FBPase. The structural and catalytic sites for Mg^{2+} can be inferred from kinetic studies (Benkovic & deMaine, 1982). On the basis of the experimental data of this report, we postulate that one of the roles of Arg 276 might be to direct and ensure the binding of the structural metal. The enzyme loses its ability to bind the structural metal when Arg 276 is replaced by methionine, and the catalytic metal can only bind to the enzyme after the binding of Fru-1,6-P_2. If this is true, it can explain not only the change in kinetic mechanism but also the loss of metal cooperativity and the significant decrease in enzyme activity, *i.e.*, without the structural metal, the catalytic metal cannot efficiently direct the 1-phosphoryl group to the orientation required for catalysis.

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