Replacement of Glutamic Acid 29 with Glutamine Leads to a Loss of Cooperativity for AMP with Porcine Fructose-1,6-bisphosphatase*

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Mutations in the AMP binding site of porcine fructose-1,6-bisphosphatase were carried out by site-specific mutagenesis based on the crystal structure of the enzyme (Ke, H., Zhang, Y., and Lipscomb, W. L. (1989) Proc. Natl. Acad. Sci. U. S. A. 87, 5243–5247). The mutant and wild-type enzymes were characterized by SDS-polyacrylamide gel electrophoresis, circular dichroism spectroscopy, and initial rate kinetics. One of the mutant forms of fructose-1,6-bisphosphatase, Glu-29 → Gln, is ligated to the phosphoryl moiety of AMP, a potent inhibitor of the reaction, whereas the other mutant, Thr-31 → Val, is associated with the purine base of AMP. No discernible alteration in structure as measured by circular dichroism spectroscopy was noted for the mutants relative to the wild-type enzyme. As expected, major changes in kinetic parameters between the mutants and the wild-type enzyme were associated with inhibition by AMP. AMP, a competitive inhibitor with respect to Mg** in the fructose-1,6-bisphosphatase reaction, exhibits cooperativity in the case of the wild-type and the mutant Thr-31 → Val enzymes with a Hill coefficient of 2.0. On the other hand, cooperativity is completely lost in the case of Glu-29 → Gln fructose-1,6-bisphosphatase.

The activity of fructose-1,6-bisphosphatase, a key regulatory enzyme in gluconeogenesis, is controlled by fructose 2,6-bisphosphatase (Fru-2,6-P$_2$) and AMP (1–6). These two small molecules are also involved in the regulation of glycolysis through their action on phosphofructokinase (6). The biosynthesis and biodegradation of Fru-2,6-P$_2$ fluctuate in response to alterations in the concentration of glucagon and are responsible for the dynamic coordinated regulation of liver glycolysis and gluconeogenesis. On the other hand, levels of AMP remain relatively constant within cells under normal physiological conditions as a result of the adenylate kinase reaction. Nevertheless, AMP is an important component in fructose-1,6-bisphosphatase regulation because of the synergistic inhibition thought to exist between the nucleotide and Fru-2,6-P$_2$ (6).

Fructose-1,6-bisphosphatase from mammalian liver and kidney, two highly gluconeogenic tissues, has long been known to require divalent metal ions for activity (7). Although it had been reported that divalent metal ions are capable of raising the $K_a$ for AMP with fructose-1,6-bisphosphatase (8, 9), we have shown from binding (10) and kinetic studies (11) that these two ligands are in fact mutually exclusive in their binding to the enzyme. We have suggested that AMP inhibits fructose-1,6-bisphosphatase by denuding the enzyme of metal (10, 11). In addition, we reported from H NMR experiments that Fru-2,6-P$_2$ decreases the $K_a$ of AMP with fructose-1,6-bisphosphatase. This enhanced "stickiness" of AMP serves to increase the effectiveness of the nucleotide by removing divalent metal from the enzyme.

The structure of porcine kidney fructose-1,6-bisphosphatase is now available and shows that one AMP site exists per subunit (12). The enzyme is a homotetramer with a subunit molecular weight of 37,000 (13); however, studies indicate that AMP binding is cooperative with a Hill coefficient of approximately 2 (8, 9, 11). It is only at very high concentrations of AMP that crystallographic evidence exists for the binding of four molecules of AMP/tetramer of enzyme (12). These findings suggest that two molecules of nucleotide bind with positive cooperativity, whereas the other two molecules of AMP exhibit strong negative cooperativity.

We have carried out kinetic experiments with both bovine (11) and porcine fructose-1,6-bisphosphatase and have observed that the inhibition is a second-order function of AMP; this is in keeping with binding studies (8, 9, 10) and other kinetic studies (8, 9, 11) but not with our x-ray diffraction data (12). Unfortunately, it is not clear at the present time how cooperativity relative to AMP occurs.

X-ray diffraction results with fructose-1,6-bisphosphatase have pinpointed a number of amino acid residues that are associated not only with the AMP moiety but also with specific portions of the nucleotide molecule. In an attempt to gain some insight into the role of these residues, we have prepared two mutants of fructose-1,6-bisphosphatase by site-specific mutagenesis and have studied their properties. One of the mutated residues (Thr-31) binds the purine base, whereas the other (Glu-29) is ligated to the phosphoryl portion of AMP. The findings of this investigation suggest that although the affinity of fructose-1,6-bisphosphatase for AMP is decreased with both mutants, in the case of the Glu-29 → Gln mutant there is a total loss of cooperativity as measured by initial rate kinetics. This particular mutant, when studied by x-ray diffraction crystallography, should provide insights into the mechanism of AMP cooperativity at the molecular level.

**EXPERIMENTAL PROCEDURES**

**Materials**—NADP, fructose-1,6-bisphosphatase (Fru-1,6-P$_2$), Fru-2,6-P$_2$, AMP, Hepes, and Tris were obtained from Sigma. Glucose-6-phosphate dehydrogenase and phosphoglucoisomerase were from Boehringer Mannheim. Distilled deionized water was used in all experiments.

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experiments, and reagents were of the highest purity available commercially. Recombinant and mutant forms of porcine liver fructose-1,6-bisphosphatase were prepared and purified as described elsewhere (14). Mutant forms of the enzyme were obtained in yields comparable with wild-type fructose-1,6-bisphosphatase. Porcine liver and kidney fructose-1,6-bisphosphatase are identical in their primary sequences (14).

Mutants of Fructose-1,6-bisphosphatase—Two mutants of recombinant porcine liver fructose-1,6-bisphosphatase (14), Glu-29 → Gln and Thr-31 → Val, were obtained by site-directed mutagenesis. Two mutagenic oligonucleotide primers, 5'-GGC-ACC-GGC-CAG-ATG-ACC-CAG-3' and 5'-GGC-GAG-ATG-GTC-CAG-CTG-3', were synthesized by using the 6-cyanoethylphosphoramidite method at the Nucleic Acid Facility at Iowa State University. The codons CAG and GTC were used to mutate Glu-29 to Gln and Thr-31 to Val, respectively. BamH1/Sph1 fragments encoding fructose-1,6-bisphosphatase from pEt-lla were ligated into a previously digested pUC118 plasmid as the template and synthesized oligonucleotides as primer. The oligonucleotidically-directed in vitro mutagenesis procedure was performed as described by Nakamaye and Eckstein (15). Mutagenesis was verified by dideoxy chain termination sequencing (16). The BamH1/Xbal fragments encoding the mutations were ligated back into the previously digested pEt-lla expression vector. pEt-lla was used to transform Escherichia coli.

Kinetic Studies—Fructose-1,6-bisphosphatase activity during purification was monitored using the phosphoglucosomerase-glucose-6-phosphate dehydrogenase-coupled spectrophotometric assay (11). Kinetic assays on the purified enzyme were determined specific activity and for evaluation of proteolysis employed the spectrophotometric assay. All other kinetic investigations made use of a fluorometric assay (11) at pH 7.5 (Hepes buffer) and 24 °C. Evaluation of the initial rate data for kinetic mechanisms was done on a personal computer using the MINITAB language with an a value of 2.0 (11). All kinetic analysis in this laboratory during the past 20 years was done using OMNITAB II and the main frame computer (11). MINITAB is similar to OMNITAB, and the previously published OMNITAB kinetics program was rewritten in MINITAB. This revision required only minor modification of the OMNITAB program. We currently investigated the kinetics of wild-type fructose-1,6-bisphosphatase and found the fundamental initial rate equation based upon stoichiometric evaluation to be as follows,

$$\frac{1}{v} = \frac{1}{V_m} \left( 1 + \frac{K_m}{A^2} + \frac{K_m}{B} + \frac{K_m}{A^2B} \right)$$

where $v$, $V_m$, $A$, and $B$ represent initial velocity, maximal velocity, Mg$^{2+}$, and Fru-1,6-P$_2$, respectively. $K_m$, $K_a$, and $K_b$ are taken to be kinetic constants associated with the substrate terms in the basic rate equation. Equation 1 formed the basis of the initial rate experiments presented in this report. The great advantage of using the MINITAB program is that it allows evaluation of large numbers of kinetic models in a relatively short period of time. Cooperativity was evaluated using the ENZFITTER program (17).

Circular Dichroism Spectrometry—CD studies on the wild-type and mutant forms of fructose-1,6-bisphosphatase were carried out at room temperature on an AVIV CD spectrometer model 62DS kindly supplied by Dr. Earl Stellwagen at the University of Iowa. Samples were prepared in 1-mm cuvettes, and data points were collected from 200 to 260 nm in 0.5-nm increments. Each spectrum was calibrated to remove the contributions of the buffer and smoothed using a program provided in the computer of the spectrometer.

RESULTS

AMP has long been recognized to be a potent inhibitor of fructose-1,6-bisphosphatase (8). It had been suggested from a variety of studies that binding of AMP to fructose-1,6-bisphosphatase is both cooperative and allosteric with a Hill coefficient of 2 (9, 18). X-ray diffraction investigations of porcine kidney fructose-1,6-bisphosphatase are in accord with the findings alluded to from solution studies except that at high levels of AMP site saturation is observed (12).

The crystal structure experiments pinpointed a number of amino acid residues involved in AMP binding to fructose-1,6-bisphosphatase. Among these are Glu-29 and Thr-31 (12). The latter residue is associated with the purine ring of AMP, whereas the former residue is ligated to the phosphoryl group of the nucleotide. Because AMP inhibits fructose-1,6-bisphosphatase by denuding the enzyme of activator metal ions, we investigated mutants of Glu-29 and Thr-31 prepared by site-specific mutagenesis. We were particularly interested in the effects of mutations in the AMP-binding site on the secondary structure and the kinetics of the fructose-1,6-bisphosphatase system.

Enzyme Purity—Fig. 1 illustrates an SDS-polycrylamide gel electrophoresis of wild-type recombinant porcine fructose-1,6-bisphosphatase and the two mutants of fructose-1,6-bisphosphatase. It can be seen that the proteins exhibit molecular weights of approximately 37,000 and are relatively pure using electrophoresis as a criterion of purity. These data, along with the pH activity ratios (Table I), suggest that the proteins have not undergone discernible degradation (19).

Circular Dichroism Spectrometry—In the study of mutants prepared by site-specific mutagenesis, it is essential to determine whether the structural alterations, if any, induced in the mutant are localized or global relative to the wild type-like protein. CD spectral data were obtained for the three forms of fructose-1,6-bisphosphatase used in these investigations. In data not presented, all three CD spectra are essentially superimposable. These findings suggest that major conformational changes, using CD as a criterion of structure, did not occur when wild-type fructose-1,6-bisphosphatase is mutated to Glu-29 → Gln and Thr-31 → Val.

Initial Rate Studies—In order to evaluate the effects of mutations in the AMP-binding site of fructose-1,6-bisphosphatase, kinetic studies were undertaken on the mutant and wild-type forms of fructose-1,6-bisphosphatase. The results of these investigations are summarized in Table I.

Site-specific mutations in the AMP-binding site involving Glu-29 and Thr-31 do not appear to alter the specific activity of fructose-1,6-bisphosphatase nor do they affect the $K_m$ for Fru-1,6-P$_2$. This latter finding is in keeping with an earlier suggestion that AMP inhibits fructose-1,6-bisphosphatase by preventing metal activator binding to the enzyme (10, 11). The $K_m$ for Fru-1,6-P$_2$ was determined at 5 mM levels of Mg$^{2+}$, which is considered to be saturating (20).

![Fig. 1. SDS-polycrylamide gel electrophoresis analysis of purified wild-type and mutant forms of porcine fructose-1,6-bisphosphatase. All samples were analyzed on a 12% polyacrylamide gel and stained with Coomassie Brilliant Blue R-250. Lane 1, protein standard; lane 2, wild type; lane 3, Glu-29 → Gln; lane 4; Thr-31 → Val. Molecular masses of protein standards are as follows: A, 66 kDa; B, 45 kDa; C, 36 kDa; D, 29 kDa.](image-url)
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**Table I**

| Enzyme          | Specific activity pH 7.5/9.6 | $k_{on}$ (Fru-1,6-P$_2$) s$^{-1}$ | $K_m$ (Fru-1,6-P$_2$) $\mu M$ | $K^*$ (AMP) $\mu M^2$ | $K_m$ (Mg$^{2+}$) mM$^2$ | Hill coefficient (Mg$^{2+}$) | $K_i$ (Fru-2,6-P$_2$) $\mu M$ |
|-----------------|-----------------------------|---------------------------------|-------------------------------|------------------------|--------------------------|-----------------------------|-----------------------------|
| Wild type       | 20                          | 3.0                             | 12.5                          | 1.96 ± 0.20            | 18.0 ± 3.2               | 0.300 ± 0.100               | 2.00 ± 0.05                 | 0.93 ± 0.15                |
| Glu-29 $\rightarrow$ Gln | 21                         | 3.1                             | 13.1                          | 2.03 ± 0.09            | 135 ± 25$^a$             | 0.278 ± 0.004               | 2.03 ± 0.14                 | 0.20 ± 0.02                |
| Thr-31 $\rightarrow$ Val   | 25                         | 3.1                             | 15.6                          | 1.96 ± 0.08            | 1170 ± 306               | 0.263 ± 0.003               | 2.00 ± 0.06                 | 0.76 ± 0.05                |

$^a$ The $K_m$ was obtained from plots of 1/velocity versus 1/Fru-1,6-P$_2$ at 5 mM Mg$^{2+}$.

$^b$ The $K_i$ for the Glu-29 $\rightarrow$ Gln mutant is micromolar.

**Mg$^{2+}$ Ion Activation**—Although it has been shown that Mg$^{2+}$ activation of fructose-1,6-bisphosphatase is sigmoidal at neutral pH and hyperbolic at alkaline pH (21), no mechanism has as yet been proposed to account for the cooperativity. In the studies contained in Table I, 12 $\mu M$ Fru-1,6-P$_2$ was used to determine the Hill coefficient and $K_m$ for Mg$^{2+}$. It is clear from the results shown in Table I that mutations in the AMP-binding site do not change the Hill coefficient for Mg$^{2+}$ nor do these mutations alter the Michaelis constant ($K_m$) for metal. On the other hand, there are very large and significant increases in the inhibition constant ($K_i$) for AMP when changes are effected in the AMP-binding site. These results support the x-ray diffraction studies, which pinpoint the residues in fructose-1,6-bisphosphatase that are ligated to AMP (12). In addition, these data are in keeping with the hypothesis that AMP and metal are mutually exclusive in their association with fructose-1,6-bisphosphatase (10, 11).

**Kinetics of Fru-2,6-P$_2$ Inhibition**—It is now well established that Fru-2,6-P$_2$, like Fru-1,6-P$_2$, binds within the active site of fructose-1,6-bisphosphatase (22, 23). On the other hand, one of the bisphosphates is a substrate, whereas the other is a potent inhibitor of the enzyme. Thus, these two ligands must be associated with different residues within the active site. This statement is validated from both the studies on the crystal structure of fructose-1,6-bisphosphatase (22, 23) and site-specific mutagenesis experiments with the rat liver enzyme (24). In these latter investigations, El-Maghrabi et al. (24) converted Lys-274 to Ala. The mutant showed a 20-fold increase in $K_m$ for Fru-1,6-P$_2$, but a 1000-fold increase in $K_i$ for Fru-2,6-P$_2$.

Table I shows that the $K_i$ for Fru-2,6-P$_2$ does decrease slightly in the case of Thr-31 but is almost 65-fold lower for Glu-29 than for the wild-type enzyme. This difference is somewhat surprising in light of the reported synergistic relationship between AMP and Fru-2,6-P$_2$ (6).

**Reciprocal Relationship between AMP and Mg$^{2+}$**—In order to obtain the inhibition constant, $K_i$, for AMP it was necessary to evaluate the type of inhibition caused by the nucleotide. Taketa and Pogell (8) were the first to report that AMP is a noncompetitive inhibitor of Fru-1,6-P$_2$ with a Hill coefficient of 2.4. Stone and Fromm (18) reported in 1980 that AMP is a noncompetitive inhibitor of Fru-1,6-P$_2$ with bovine fructose-1,6-bisphosphatase; however, in their kinetic studies the rate equation requires that the concentration of AMP be squared, i.e. [AMP]$^2$. Liu and Fromm (11) also observed that inhibition at pH 9.5 was second order in AMP and that inhibition was competitive relative to Mg$^{2+}$.

We recently found that for wild-type fructose-1,6-bisphosphatase AMP inhibition with respect to Fru-1,6-P$_2$ is nonlinear and noncompetitive but nonlinear and competitive relative to Mg$^{2+}$. Fig. 2 illustrates a double-reciprocal plot of 1/velocity versus 1/(Mg$^{2+}$|$^{2+}$)$^2$ in the presence and absence of AMP for the Thr-31 $\rightarrow$ Val mutant of fructose-1,6-bisphosphatase. Excellent fits were obtained to the following rate expression, where $A$, $K_m$, $I$, and $K$ represent Mg$^{2+}$, $K_m$ for Mg$^{2+}$, AMP, and the apparent inhibition constant for AMP, respectively.

$$\frac{1}{v} = \frac{1}{V_1} \left(1 + \frac{K_i}{A} \left(1 + \frac{[I]}{K}ight)\right)$$

(Eq. 2)

Note that the only difference between Equations 2 and 3 is the order of inhibition relative to AMP. The experiments illustrated in Fig. 3 were repeated numerous times with identical results. The inset in Fig. 3 illustrates a plot of slopes of the family of curves in Fig. 3 versus the concentration of AMP. In addition, in data not presented, when 1/velocity versus [AMP] was plotted in the range of 0.33–120 $\mu M$ with Mg$^{2+}$ and Fru-1,6-P$_2$ at 0.4 mM and 12 $\mu M$, respectively, the curve was linear. These findings suggest that a mutation at the Glu-29 site causes a loss in the cooperative nature of AMP inhibition. The rationale behind this important finding must await further experimentation.

**DISCUSSION**

Although it has been recognized for 30 years that AMP is a potent allosteric inhibitor of fructose-1,6-bisphosphatase (8), it has only been within the last few years that its role in the coordinated regulation of glycolysis and gluconeogenesis has been established (1–6). The investigations of Taketa and Pogell (8) and Opie and Newsholme (25) established the fact that AMP binding to fructose-1,6-bisphosphatase decreases in the presence of Mg$^{2+}$, and the latter group attributed this effect to chelation of the metal by the nucleotide. Binding and kinetic experiments have established that Mg$^{2+}$ and AMP bind mutually exclusively to fructose-1,6-bisphosphatase (10, 11). Kinetic experiments demonstrated that AMP is a noncompetitive inhibitor of the substrate, Fru-1,6-P$_2$ (8). Binding studies involving AMP and fructose-1,6-bisphosphatase suggest that one molecule of nucleotide binds per subunit of
enzyme; however, kinetic studies have shown that there are only two molecules of AMP ligated per molecule of tetramer (8, 9, 18). A number of investigators have suggested that AMP binding to fructose-1,6-bisphosphatase is an example of "half-of-the-sites" reactivity (26).

Research with the potent inhibitor of fructose-1,6-bisphosphatase, Fru-2,6-P$_2$, has demonstrated so-called "synergistic" interactions between these two ligands. The tightening of AMP binding to the enzyme by Fru-2,6-P$_2$ has been attributed to a decrease in the $k_{cat}$ for the nucleotide (27). Recent binding and kinetic investigations have demonstrated that Mg$^{2+}$ and AMP are mutually exclusive in their binding to fructose-1,6-bisphosphatase (10, 11). These observations have led to the proposal of a molecular mechanism of fructose-1,6-bisphosphatase regulation (10, 11). This hypothesis suggests that AMP inhibits fructose-1,6-bisphosphatase by precluding binding of the essential divalent activator ion. Fru-2,6-P$_2$, in addition to being a potent competitive inhibitor of the substrate, makes AMP stickier. This later effect enhances the ability of AMP to prevent divalent metal ion binding to the enzyme. Recent x-ray diffraction crystallography studies suggest that a complex of fructose-1,6-bisphosphatase, AMP, and Mg$^{2+}$ can exist; however, kinetic investigations and the results of the present study, which demonstrate that AMP is a competitive inhibitor of metal ion activation, are clearly at variance with this suggestion. An explanation for this discrepancy has been advanced elsewhere (11).

The cloning and expression of porcine fructose-1,6-bisphosphatase (14) has permitted the preparation of site-specific mutations in the AMP-binding site of fructose-1,6-bisphosphatase based upon its crystal structure. Glu-29 is believed to bind the phosphoryl moiety of AMP, whereas Thr-31 --- Val fructose-1,6-bisphosphatase. The concentrations of AMP are 0 (■), 200 µM (+), 400 µM (▲), and 600 µM (□). The lines are theoretical based upon Equation 2, and the points are experimentally determined. The inset shows a plot of the slope of the family of curves in Fig. 2 versus [AMP]$^2$.

FIG. 3. Plot of reciprocal of initial velocity in arbitrary fluorescent units versus reciprocal of [Mg$^{2+}$]$^3$ for Glu-29 → Gln fructose-1,6-bisphosphatase. The concentrations of AMP are 0 (■), 5 µM (+), 10 µM (▲), 15 µM (□), and 20 µM (×). The lines are theoretical based upon Equation 3, and the points are experimentally determined. The inset shows a plot of the slope of the family of curves in Fig. 3 versus [AMP].
31 is associated with purine ring binding (12). Mutation of these residues to Gln-29 and Val-31 causes a significant decrease in the affinity for AMP by the enzyme. This is to be expected based upon the crystal structure of fructose-1,6-bisphosphatase. On the other hand, these mutations do not cause significant effects on enzyme activity nor do they affect Mg$^{2+}$ activation significantly. Enhanced inhibition by Fru-2,6-P$_2$ with the mutant forms of fructose-1,6-bisphosphatase was observed; however, this finding is difficult to rationalize with the well recognized synergism reported for AMP and Fru-2,6-P$_2$ (6).

Clearly the most significant finding of this study concerns the complete loss of AMP cooperativity in the Glu-29 $\rightarrow$ Gln mutant. In the case of the Glu-29 $\rightarrow$ Gln mutant, the Hill coefficient for the nucleotide is 1.0. On the other hand, the Thr-31 $\rightarrow$ Val mutant exhibits an increase in its $K_i$ for AMP of some 65-fold relative to the wild-type enzyme with no loss of cooperativity. In addition, with both mutant forms of fructose-1,6-bisphosphatase as well as the wild-type enzyme (data not presented) AMP is a competitive inhibitor of Mg$^{2+}$ at neutral pH.

The origin of AMP cooperativity, like that for Mg$^{2+}$, is unknown. We have made three site-specific mutations at the Mg$^{2+}$ binding site of fructose-1,6-bisphosphatase (28). Although there is a dramatic decrease (approximately 1000-fold) in the activity of these mutants as well as a marked enhancement in AMP sensitivity, no significant alteration in metal ion cooperativity was noted (28). The results of the present investigation may lead to a fuller understanding of the basis of the cooperative phenomenon for AMP binding to fructose-1,6-bisphosphatase at the molecular level, provided the Glu-29 $\rightarrow$ Gln mutant can be crystallized and its structure established. These investigations will be initiated in the near future.

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