J. Appl. Biol. Chem. **51**(2), 45-49 (2008)

Article

Roles of the Residues Lys115 and Tyr116 in the Binding of an Allosteric Inhibitor AMP to Pea Cytosolic Fructose-1,6-bisphosphatase

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Received January 10, 2008; Accepted April 10, 2008

Cytosolic fructose-1,6-bisphosphatase (cFBPase) in plants is a key regulatory enzyme in the photosynthetic sucrose biosynthesis. Plant cFBPases, like the mammalian FBPases, are inhibited by adenosine 5'-monophosphate (AMP) and fructose-2,6-bisphosphate (Fru-2,6-P_2). In the mammalian FBPases, Lys112 and Tyr113 play important roles in the AMP binding. To understand roles of the corresponding residues, Lys115 and Tyr116, in pea cFBPase, the mutant cFBPases were generated by site-directed mutagenesis. The alterations of Lys115 to Gln and Tyr116 to Phe displayed small changes in K_m and K_i for Fru-2,6-P_2, indicating that the mutation causes minor effects on the enzyme catalysis and Fru-2,6-P_2 binding, whereas resulted in higher than 500-fold increase of $[AMP]_{0.5}$ compared with that of the wild-type enzyme. Results indicate the residues Lys115 and Tyr116 play important roles in the binding of AMP to the allosteric site of the pea cFBPase.

Key words: *adenosine 5'-monophosphate inhibition, cytosolic fructose-1,6-bisphosphatase, site-directed mutagenesis, sucrose biosynthesis*

FBPase (EC3.1.3.11) catalyzes the hydrolysis of Fru-1,6-P_2 into fructose-6-phosphate and inorganic phosphate. In plants, the enzyme exists as two isozymes, chloroplastic and cytosolic forms [Kelly *et al.*, 1982; Cho and Hahn, 1991; Nel and Terblanche, 1992; Lee *et al.*, 1994]. Chloroplastic FBPase plays a regulatory role in the photosynthetic CO_2 assimilation and is activated by the increase of pH and Mg^{2+} concentration in the chloroplast stroma and the reduction of the enzyme via ferredoxin-thioredoxin system in the light [Zimmermann *et al.*, 1976; Schürmann and Wolosiuk, 1978; Buchanan, 1980; Cho and Hahn, 1991]. On the other hand, cFBPase is a key regulatory enzyme in the photosynthetic sucrose biosynthetic pathway, in which the enzyme controls the first irreversible reaction in the pathway [Zimmermann *et al.*, 1978; Daie, 1993]. The reduced activity of cFBPase inhibits the sucrose synthesis in potato [Zrenner *et al.*, 1996] and Arabidopsis [Strand *et al.*, 2000] transformants, as well as the *Flaveria* mutants [Sharkey *et al.*, 1992]. The potato and Arabidopsis transformants also display the reduced photosynthetic activity under the saturated condition of CO_2 or light [Zrenner *et al.*, 1996; Strand *et al.*, 2000].

Just as the gluconeogenic FBPases in mammals, cFBPase is down-regulated by an allosteric inhibitor, AMP, and a signal metabolite, Fru-2,6-P_2 [Herzog *et al.*, 1984; Stitt *et al.*, 1985; Stitt, 1990; Daie, 1993; Nielsen *et al.*, 2004]. Both mammalian FBPases and plant cFBPases are known to be homotetramers [Ke *et al.*, 1989; Zimmermann *et al.*, 1978]. Extensive studies on the regulatory mechanism and structure of FBPase in the mammalian FBPases via X-ray crystallography and site-directed mutagenesis [Ke *et al.*, 1989; Ke *et al.*, 1990; Liang *et al.*, 1993; Xue *et al.*, 1994; Kelly-Loughnane and Kontrowitz, 2001; Chen *et al.*, 1994; Gidh-Jain *et al.*, 1994; Shyur *et al.*, 1996] revealed the enzyme could be present in two distinct quaternary conformations, the active R-state and the inactive (or low active) T-state [Ke *et al.*, 1990; Ke *et al.*, 1991]. Furthermore, the structural studies on the pig kidney FBPase demonstrated that the

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Abbreviations: AMP, adenosine 5'-monophosphate; cFBPase, cytosolic fructose-1,6-bisphosphatase; FBPase, fructose-1,6-phosphatase; Fru-1,6-P_2, fructose-1,6-bisphosphate; Fru-2,6-P_2, fructose-2,6-phosphate; PCR, polymerase chain reaction

binding of AMP induces a quaternary conformational transition from R- to T-state with a 19° rotation between the upper and lower dimers [Ke *et al.*, 1991]. The AMP binding also leads to the conformational changes in the divalent metal-binding site, a portion of the active site [Ke *et al.*, 1991]. The site-directed mutagenesis studies demonstrated that, among the residues in the AMP-binding site, Arg14, Ala24, Thr27, Glu29, Thr31, Lys112, and Tyr113 played important roles in the binding of AMP [Chen *et al.*, 1994; Gidh-Jain *et al.*, 1994; Shyur *et al.*, 1996; Kelly-Loughnane and Kantrowitz, 2001].

Despite the importance of cFBPases in the photosynthetic sucrose synthesis, little is known about their structures and regulatory mechanisms. In the pea cFBPase, the amino acid residues Lys115 and Tyr116, corresponding to Lys112 and Tyr113 in the pig kidney FBPase, are conserved. To elucidate the roles of these residues in the regulation of cFBPase activity by an allosteric inhibitor AMP, the mutant cFBPases were generated through the site-directed mutagenesis, in which the residues Lys115 and Tyr116 were changed into Gln (K115Q) and Phe (Y116F), respectively. The effects of these mutations on the AMP binding and the kinetic properties of the mutant cFBPases were then examined.

Materials and Methods

Site-directed mutagenesis and the expression vector construction. To generate the K115Q and Y116F cFBPases, a site-directed mutagenesis using an overlap extension PCR method [Ho *et al.*, 1989] was carried out. The complementary pairs of the primers containing mismatched bases (underlined) for the site-directed mutagenesis were 5'-TCTCTGCGTGGAGAGTATTGT-3' (K115Q-For) and 5'-TACAACACAATACTCTCCACG-3' (K115Q-Rev) for K115Q, and 5'-CTGCGTGGAAAGT TTTGTGTT-3' (Y116F-For) and 5'-AAATACAACACA AAACTTTCC-3 (Y116F-Rev) for Y116F. The 3' cDNA fragments for K115Q or Y116F were amplified with the primer sets of 5'-GCGCGCCAATGGATCATGCTGGG GAT-3' (cFBP-For, start codon is underlined) and either K115Q-Rev or Y116F-Rev from the pea (*Pisum sativum*) cDNA library. The primer sets of 5'-GCGCGCTCGAG GGCCGTCTTCTCTT-3' (cFBP-Rev, codon for the last amino acid is underlined) and either K116Q-For or Y116F-For were used for the amplification of the 5' cDNA fragments for K115Q or Y116F. The two resulting PCR products having the overlapping ends for each K115Q and Y116F were annealed and extended. The resulting full-length products were further amplified individually by PCR with the primers of cFBP-For and cFBP-Rev. The final PCR products were subcloned into pGEM-T vector (Promega, Madison, WI). After the sequence confirmation, each cDNA for K115Q and Y116F was digested with *Nco*I and *Xho*I, followed by individual cloning into an expression vector pET28a (Novagen, Madison, WI).

Expression and purification of the recombinant cFBPases. The resulting plasmids were individually transformed into *E. coli* BL21(DE3). The *E. coli* transformants were grown at 37°C in LB medium, and the expression was induced with 1 mM IPTG at an OD_{600} of 0.7. The cells were further grown for 6 h at 37°C and harvested by centrifugation. The cell pellets were resuspended in a small volume of the sonication buffer (50 mM Na-phosphate, pH 7.8, 300 mM NaCl) and disrupted by sonication. After removal of the cell debris by centrifugation, the resulting cell lysate was subjected to Ni-NTA affinity chromatography (Qiagen, Valencia, CA), and the recombinant His-tagged cFBPases were purified according to the manufacturer's instructions. The recombinant cFBPases were eluted with the buffer containing 150 mM imidazole and stored at –70°C until use.

Assay of the recombinant cFBPase activity. The activities of the recombinant cFBPases were measured by monitoring the changes in the absorbance at 340 nm using a CARY 300 Bio UV/Vis spectrophotomer (Varian, Palo Alto, CA) as described previously [Jang *et al.*, 2003]. One unit of the enzyme activity was defined as 1 μmol NADPH formation per min. To investigate the effects of AMP and Fru-2,6-P_2 on the enzyme activity, various concentrations of AMP (0-50 mM) and Fru-2,6-P_2 (0-0.3 mM) in under the above reaction condition were applied.

Results and Discussion

Plant cFBPases and mammalian FBPases are involved in the anabolic pathways and exist as homotetramers in their native state [Zimmermann *et al.*, 1978; Ke *et al.*, 1989] as well as share common regulatory properties, including the inhibitions by AMP and Fru-2,6-P_2 [Pilkis *et al.*, 1981; van Schaftingen and Hers, 1981; Herzog *et al.*, 1984; Stitt *et al.*, 1985; Stitt, 1990; Daie, 1993; Nielsen *et al.*, 2004]. Upon comparison of the amino acid sequences, the pea cFBPase showed high homology to the pig kidney FBPase with 71.1% similarity (52.3% identity), and the residues interacting with AMP and Fru-2,6-P_2 by hydrogen bonding [Ke *et al.*, 1991; Xue *et al.*, 1994] were well conserved (Fig. 1). Of these residues, Lys112 and Tyr113 in the pig kidney FBPase are located at the beginning of the 113-118 β-strand that directly links the allosteric AMP-binding site and the metal-binding

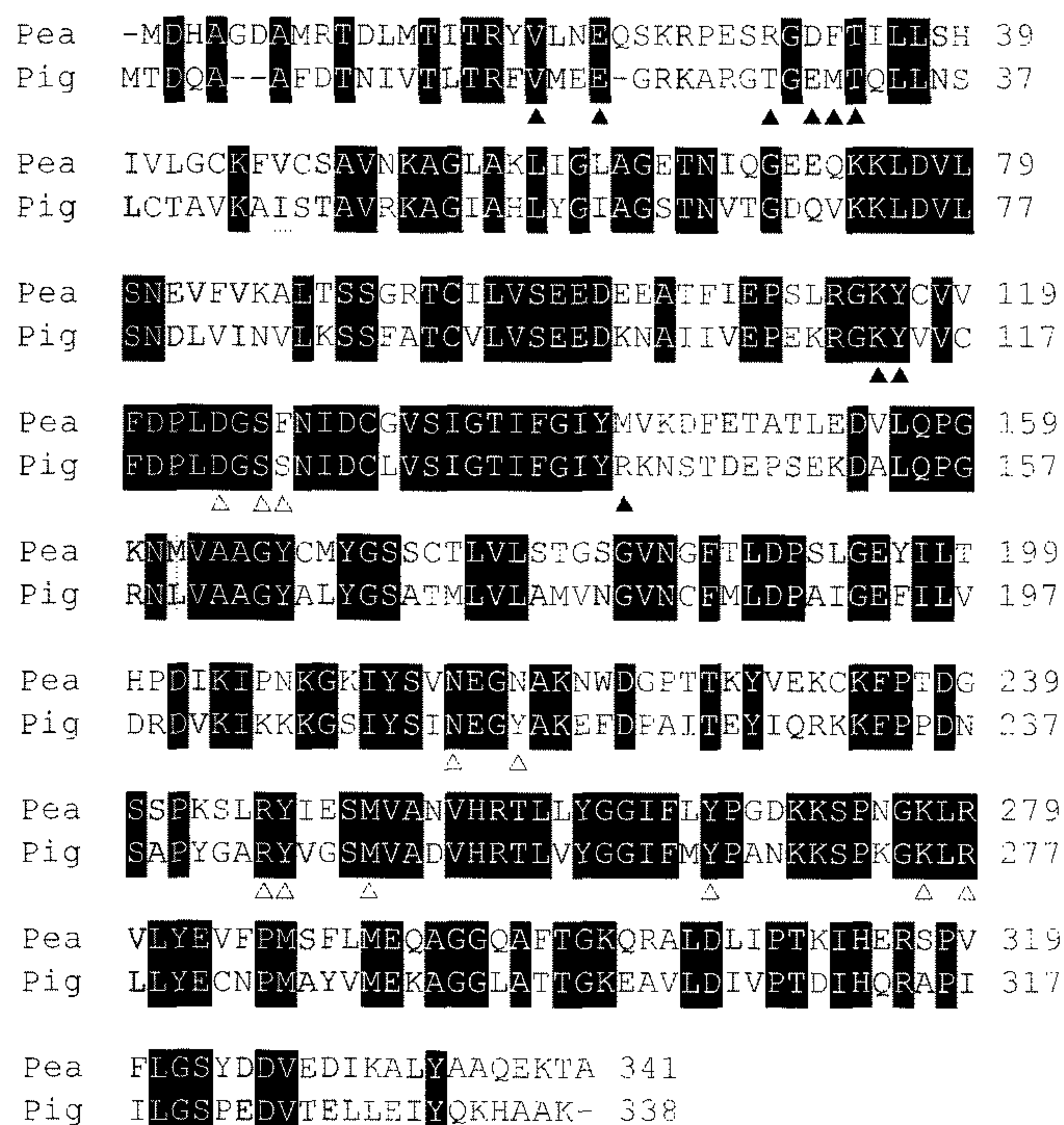

Fig. 1. Amino acid sequence alignment of pea cFBPase and pig kidney FBPase. Pea cFBPase shows high homology (71.1% similarity) with pig kidney FBPase. The amino acid residues that are identical and similar are shaded in black and grey, respectively. The symbols below the sequence indicate the potential amino acid residues interacting with AMP (▲) and Fru-2,6-P_2 (△) by hydrogen bonding [Ke *et al.*, 1991; Xue *et al.*, 1994]. Pea; pea cFBPase (AAM14744), Pig; pig kidney FBPase (AAA31035).

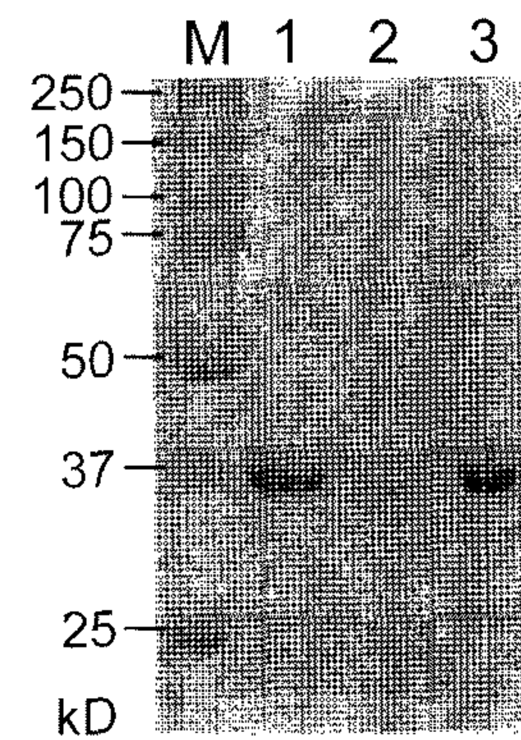

Fig. 2. SDS-PAGE analysis of the purified recombinant wild-type and mutant cFBPases. The recombinant cFBPases were expressed in *E. coli* BL21(DE3) and purified with Ni-NTA affinity chromatography. M, molecular weight marker; 1, purified wild-type cFBPase; 2, the purified K115Q cFBPase; 3, the purified Y116F cFBPase. The gel was visualized by Coomassie-brilliant blue staining.

pocket in the active site [Liang *et al.*, 1993]. Kelly-Loughnane and Kantrowitz [2001] demonstrated that the mutations of these two residues results in the AMP insensitivity of the pig kidney FBPase.

In the present study, Lys115 and Tyr116 in the pea cFBPase corresponding to Lys112 and Tyr113 in the pig kidney FBPase were changed into Gln and Phe by site-directed mutagenesis, respectively, to unravel the roles of these residues in the regulation of the pea cFBPase activity. The His-tagged wild-type and the mutant cFBPases were successfully expressed in *E. coli* BL21(DE3) and purified by the Ni-NTA affinity chromatography (Table 1 and Fig. 2). The K115Q and Y116F cFBPases displayed the electrophoretic motilities identical to that of the wild-type enzyme with a molecular mass of 38 kDa (Fig. 2).

At the saturating concentration of Mg^{2+}, the saturation curves of the K115Q and Y116F cFBPases to the substrate, Fru-1,6-P_2, showed a hyperbolic shape, indicating the enzymes follow the Michaelis-Menten kinetics as the wild-type enzyme does (data not shown). The calculated K_m values of the K115Q and Y116F enzymes were 10.7 and 2.8 μM, respectively (Table 2), indicating the affinity of the K115Q enzyme to the substrate was almost identical, whereas that of the Y116F enzymes increased slightly compared to the wild-type enzyme. These small changes in K_m suggest that the mutations of K115Q and Y116F have a minor effect on the catalysis, consistent with the report that the corresponding mutations of the pig kidney FBPase display small changes on the affinity to the substrate [Kelly-Louhgnane and Kantrowitz, 2001].

The influences of the K115Q and Y116F mutations on the binding of the inhibitor molecules, AMP and Fru-2,6-P_2, were examined. The K_i values for Fru-2,6-P_2 binding of the K115Q and Y116F cFBPases were 0.1 and 0.14 μM, which were comparable to that (0.15 μM) of the wild-type enzyme (Table 2), indicating that the alteration of these residues have little effect on the binding of Fru-

Table 1. Purification of the recombinant wild-type and mutant cFBPases

Purification step	Total protein (mg)			Total activity (unit)			Specific activity (unit/mg protein)			Yield (%)		
	WT	K115Q	Y116F	WT	K115Q	Y116F	WT	K115Q	Y116F	WT	K115Q	Y116F
Cell lysate	51.8	53.9	59.6	47.6	47.9	63.8	0.92	0.89	1.07	100	100	100
Ni-NTA	2.25	1.25	2.65	31.7	21.1	47.9	14.1	16.9	18.1	66.6	44.1	75.1

Table 2. Kinetic parameters of the wild-type and mutant cFBPases

Enzyme	K_m (Fru-1,6-P_2) μM	$[AMP]_{0.5}$[a] μM	K_i (Fru-2,6-P_2) μM
Wild-type	10.8	10.2	0.15
K115Q	10.7	5810	0.10
Y116F	2.8	15960	0.14

[a]Concentrations of AMP required for the half-inhibition of the maximal activity of the cFBPases.

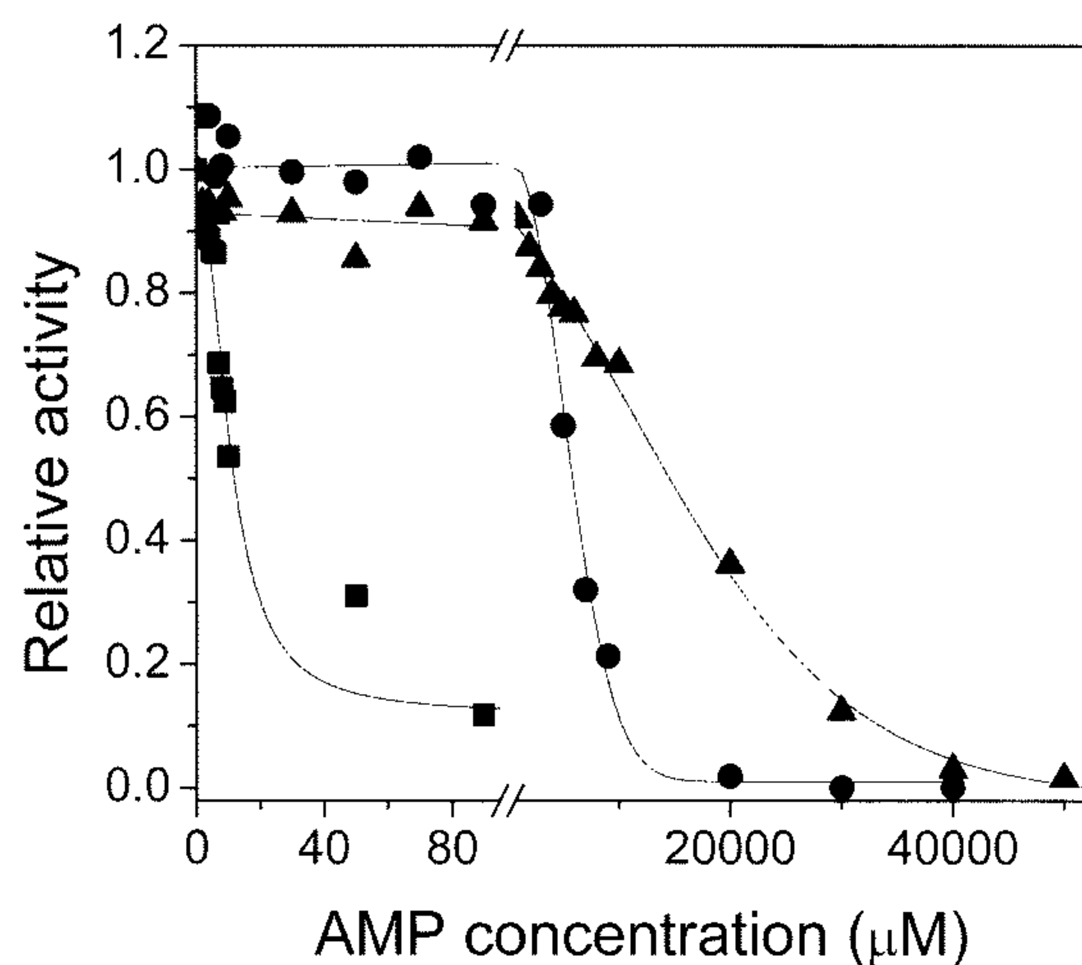

Fig. 3. AMP inhibition of the purified recombinant wild-type and mutant cFBPases. In the K115Q (●) and Y116F (▲) cFBPases, the concentrations of AMP for the inhibition of the enzyme activities increased dramatically compared with the wild-type enzyme (■).

2,6-P_2 to cFBPase. On the other hand, the binding properties of AMP to the K115Q and Y116F cFBPases changed dramatically compared with the wild-type enzyme (Fig. 3). The K115Q and Y116F cFBPases, respectively, required 5810 and 15960 μM of AMP for the half-inhibition of their maximal activities ($[AMP]_{0.5}$) (Table 2), approximately 570- and 1560-fold higher than that of the wild-type enzyme (10.2 μM), indicating that the mutant cFBPases are apparently AMP insensitive. This result was comparable to the mutation of the corresponding residues (Lys112 and Tyr113) in the pig kidney FBPase, resulting in higher than 1200-fold increase of the $[AMP]_{0.5}$ [Kelly-Loughnane and Kantrowitz, 2001]. The mammalian FBPase residues are known to interact directly with AMP; Lys112 forms a hydrogen bond with the phosphate group of AMP, and Tyr113 interacts not only with the phosphate group, but also with the hydroxyl group in the ribose ring [Ke *et al.*, 1991]. Results of the present study thus indicate that the residues Lys115 and Tyr116 in the pea cFBPase play important roles in the binding of AMP to the allosteric site of cFBPase.

In summary, the mutant pea cFBPases with an alteration in either Lys115 or Tyr116 were generated by site-directed mutagenesis. Examination of the kinetic properties revealed that the mutation causes only minor effects on the enzyme catalysis and the binding of an inhibitor Fru-2,6-P_2, whereas the mutant cFBPases were apparently AMP insensitive. The present study provides a clue to understanding the regulatory mechanism of plant cFBPases by AMP. To further understand the regulatory mechanism of this key enzyme in the photosynthetic sucrose synthesis, more structural and biochemical studies should be performed.

Acknowledgments. This research was supported by the SRC program of MOST/KOSEF (R11-2000-081) through the Plant Metabolism Research Center, Kyung Hee University.

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