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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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 (Fr-U-6-P). In the mammalian FBPases, Lys112 and Tyr113 play important roles in the AMP binding. To understand 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 $K_a$ and $K_1$ for Fr-U-6-P, indicating that the mutation causes minor effects on the enzyme catalysis and Fr-U-6-P binding, whereas resulted in higher than 50-fold increase of $[AMP]_0$, 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; Schützmann and Wolosiu, 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; Datie, 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, Fr-U-6-P [Herzog et al., 1984; Stitt et al., 1985; Stitt, 1990; Datie, 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 Kontowitz, 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
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 divergent 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 cFBBases in the photosynthetic sucrose synthesis, little is known about their structures and regulatory mechanisms. In the pea cFBBase, 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 cFBBase activity by an allosteric inhibitor AMP, the mutant cFBBases 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 cFBBases were then examined.

Materials and Methods

Site-directed mutagenesis and the expression vector construction. To generate the K115Q and Y116F cFBBases, a site-directed mutagenesis using an overlap extension PCR method [Ho et al., 1989] was carried out. The complementary pair of the primers containing mismatches bases (underlined) for the site-directed mutagenesis were 5'-TCTCTGCGTGGAGAGTATGTG-3' (K115Q-For) and 5'-TACAAACAATACTCTCCACG-3' (K115Q-Rev) for K115Q, and 5'-CTCGCTGGAAAGTGTTGTGTGTT-3' (Y116F-For) and 5'-AAATACACACACAAACTTTCCC-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'-GCCGCTTCCAGAGGCGCTTTCTCTT-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 Ncol and Xhol, followed by individual cloning into an expression vector pET28a (Novagen, Madison, WI).

Expression and purification of the recombinant cFBBases. 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 OD₆₀₀ 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 cFBBases were purified according to the manufacturer’s instructions. The recombinant cFBBases were eluted with the buffer containing 150 mM imidazole and stored at -70°C until use.

Assay of the recombinant cFBBase activity. The activities of the recombinant cFBBases were measured by monitoring the changes in the absorbance at 340 nm using a CARY 300 Bio UV/Vis spectrophotometer (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, on the enzyme activity, various concentrations of AMP (0-50 mM) and Fru-2,6-P (0-0.5 mM) in under the above reaction condition were applied.

Results and Discussion

Plant cFBBases and mammalian FBPAeses 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, [Pilkis et al., 1981; van Schaftingen and Hers, 1981; Herzog et al., 1984; Stitt et al., 1985; Stitt, 1990; Daele, 1993; Nielsen et al., 2004]. Upon comparison of the amino acid sequences, the pea cFBBase 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, 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
AMP Insensitive Mutants of Pea Cytosolic FBPase

Pea -MTAVCDEVSQDRKTKKTSQVSRKRTKESPSDSVKEDS 39
Pig MTRAVCDEHSQRKTKKTSQVSRKRTKESPSDSVKEDS 37
Pea TVLGFSTCVNAGAKHDKVSTKRCRCKKLKRDVL 79
Pig LCTAYAHSAKDATITAHFVKKMRKFNDVL 77
Pea RNLVIVCDEHSQRKTKKTSQVSRKRTKESPSDSVKEDS 117
Pig RNLVIVCDEHSQRKTKKTSQVSRKRTKESPSDSVKEDS 117
Pea ITPVFNKDEHSQRKTKKTSQVSRKRTKESPSDSVKEDS 119
Pig ITPVFNKDEHSQRKTKKTSQVSRKRTKESPSDSVKEDS 117
Pea HPVDAVETCSSKMLTSQVSRKRTKESPSDSVKEDS 199
Pig HPVDAVETCSSKMLTSQVSRKRTKESPSDSVKEDS 197
Pea GTMKZSDEHVRDQSQVSRKRTKESPSDSVKEDS 239
Pig GTMKZSDEHVRDQSQVSRKRTKESPSDSVKEDS 237
Pea NLKRVVHMCSKMLTSQVSRKRTKESPSDSVKEDS 277
Pig NLKRVVHMCSKMLTSQVSRKRTKESPSDSVKEDS 277
Pea VTVVYHKMCEHVRDQSQVSRKRTKESPSDSVKEDS 317
Pig VTVVYHKMCEHVRDQSQVSRKRTKESPSDSVKEDS 317
Pig PGYDVMIDKSHAEQSKKAER 341
Pig PGYDVMIDKSHAEQSKKAER 338

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-P2 (△) by hydrogen bonding [Ke et al., 1992; Xue et al., 1994]. Pea cFBPase (AAM14744), Pig cFBPase (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.

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total protein (mg)</th>
<th>Total activity (unit)</th>
<th>Specific activity (unit/mg protein)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell lysate</td>
<td>51.8</td>
<td>53.9</td>
<td>59.6</td>
<td>100</td>
</tr>
<tr>
<td>Ni-NTA</td>
<td>2.25</td>
<td>1.25</td>
<td>2.65</td>
<td>100</td>
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</table>
Table 2. Kinetic parameters of the wild-type and mutant cFBPases

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$K_v$ (Fru-1,6-P$_2$)</th>
<th>[AMP]$_{0.5}$</th>
<th>$K_i$ (Fru-2,6-P$_2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>10.8  µM</td>
<td>5810 µM</td>
<td>0.15 µM</td>
</tr>
<tr>
<td>K115Q</td>
<td>10.7  µM</td>
<td>5810 µM</td>
<td>0.10 µM</td>
</tr>
<tr>
<td>Y116F</td>
<td>2.8    µM</td>
<td>15960 µM</td>
<td>0.14 µM</td>
</tr>
</tbody>
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*Concentrations of AMP required for the half-inhibition of the maximal activity of the cFBPases.

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.

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References


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