Analysis of Allosteric Effector Binding Sites of Potato ADP-glucose Pyrophosphorylase through Reverse Genetics*

ADP-glucose pyrophosphorylase (AGPase) is a key regulatory enzyme of bacterial glycogen and plant starch synthesis as it controls carbon flux via its allosteric regulatory behavior. Unlike the bacterial enzyme that is composed of a single subunit type, the plant AGPase is a heterotetrameric enzyme ($\alpha_4\beta_4$) with distinct roles for each subunit type. The large subunit (LS) is involved mainly in allosteric regulation through its interaction with the catalytic small subunit (SS). The LS modulates the catalytic activity of the SS by increasing the allosteric regulatory response of the hetero-oligomeric enzyme. To identify regions of the LS involved in binding of effector molecules, a reverse genetics approach was employed. A potato (Solanum tuberosum L.) AGPase LS down-regulatory mutant (E38A) was subjected to random mutagenesis using error-prone polymerase chain reaction and screened for the capacity to restore glycogen production when the LS containing only the second site mutations was co-expressed with the wild-type SS. Sequence analysis showed that most of the mutations were decidedly nonrandom and were clustered at conserved N- and C-terminal regions. Kinetic analysis of the dominant mutant enzymes indicated that the $K_m$ values for cofactor and substrates were comparable with the wild-type AGPase, whereas the affinities for activator and inhibitor were altered appreciably. These AGPase variants displayed increased resistance to $P_i$ inhibition and/or greater sensitivity toward 3-phosphoglyceric acid activation. Further studies of Lys-197, Pro-261, and Lys-429 in the potato SS, see Fig. 2), whereas three Lys residues (which align with Lys-124, Lys-414, and Lys-452 in the potato LS, Fig. 2) were labeled in the spinach leaf LS (8, 9). The multiple labeling patterns of the LS suggest that this subunit type plays a more dominant role in allosteric regulation than the small subunit, a view supported by genetic studies (10, 11).

A missense mutation in the LS gene of Arabidopsis caused only a partial reduction of AGPase activity and starch levels in leaves (12). The isolated enzyme, which was found to be composed of only the SS, required much higher levels of 3-PGA for activation as compared with the wild-type (wild type) Arabidopsis leaf AGPase (13). Similarly, bacterial expression of the SS and LS in glgC– Escherichia coli AC70R1-504 (an AGPase deficient strain) indicated that the SS alone is able to form an active homotetrameric enzyme, although requiring much higher levels of 3-PGA for activation and lower levels of $P_i$ for inhibition, as compared with the heterotetrameric wild-type enzyme (14, 15). These results suggest that the SS is involved in both catalytic and regulatory functions, whereas the LS is involved primarily in regulation.

The ability to produce an active plant enzyme in bacteria, capable of complementing a glgC (structural gene for AGPase) mutation and restoring glycogen production, enabled a biochemical-genetic approach to understand the role of the AGPase subunits in enzyme function. For example, the LS or SS cDNA of the potato AGPase was subjected to chemical mu-

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The abbreviations used are: AGPase, ADP-glucose pyrophosphorylase; 3-PGA, 3-phosphoglyceric acid; LS, large subunit; SS, small subunit; PCR, polymerase chain reaction.

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tagenesis with hydroxylamine and co-expressed with the wild-type counterpart subunit in a glgC–E. coli. With this technique, both substrate and effector binding mutants have been identified. Laughlin et al. (16, 17) isolated several potato SS mutants, which had defective substrate (ATP or Glc-1-P) binding properties. Additionally, random mutagenesis using the LS as a template yielded several up-regulated and down-regulated potato AGPase mutants (18–20). Overall, results from these biochemical and genetic studies indicate that the two subunit types play different roles in enzyme function. The SS contains both regulatory and catalytic sites, whereas the LS contributes a regulatory function by modulating the catalytic activity of the SS by increasing the allosteric regulatory response.

Because of the lack of structural information, the study of mutant enzymes generated by random mutagenesis is a plausible approach to understand enzyme function. However, this approach requires the ability to generate numerous mutations. Although hydroxylamine treatment has been used to mutagenize LS, it is limited by the narrow spectrum (GC to AT transitions) of mutations generated. To map amino acids involved in effector binding in the potato LS, a reverse genetics approach was employed where a “loss of function” LS (E38A) was subjected to error-prone PCR and co-expressed with the wild-type SS. Revertants that stained darkly by iodine vapor were selected and further studied for the presence of dominant second-site mutations that conferred elevated glycogen accumulation than the wild-type condition. Sixteen dominant LS mutants, which fell into 11 classes, were identified with most of the second-site mutations clustered in two regions of the primary sequence. Kinetic analysis of these 11 AGPase classes was performed and showed that the dominant LS mutations conferred an up-regulatory phenotype, i.e., increased sensitivity to the activator 3-PGA and/or increased resistance to the inhibitor Pi.

MATERIALS AND METHODS

PCR Mutagenesis

Random mutagenesis of the E38A LS cDNA was performed according to Cadwell and Joyce (21) with modifications. 30 fmol of E38A LS in pML7 plasmid was amplified using the primers LSUampS and PLS-2 (Table I). The PCR mixture contained 1× mutagenic buffer (7 mM MgCl2, 0.5 mM MnCl2, 50 mM KCl, 10 mM Tris-HCl, pH 8.3, and 0.1% (w/v) gelatin), 0.2 mM dGTP, 0.2 mM dATP, 1 mM dCTP, 1 mM dTTP, 30 pmol of each primer, and 5 units of Taq polymerase. PCR was performed using a Promega Rebo Cycler for 14 cycles at the following conditions: 40 s at 94 °C, 40 s at 61 °C, and 1.5 min 72 °C. PCR products were purified using a QiaGen PCR clean-up kit. The amplified products were digested with NcoI and HindIII, cloned into the corresponding sites of pML7 vector (22), and transformed into glgC–E. coli, carrying the SS expression plasmid pML10. Revertants were identified by their ability to complement the glgC– phenotype thereby restoring glycogen production, which is readily scored by iodine staining of the bacterial colonies.

Site-directed Mutagenesis

Site-directed mutagenesis was carried out using the Stratagene Quick-change Mutagenesis kit. The PCR reaction contained 30 fmol of DNA, 20 pmol of primers (Table I), 0.2 mM dNTPs, and 2.5 units of Pfu Turbo DNA polymerase. The PCR was carried out for 12 cycles under the following conditions: 40 s at 94 °C, 40 s at 61 °C, and 1.5 min 72 °C. PCR products were purified using DpnI to remove template plasmid DNA and transformed into E. coli DH5α. The presence of the site-directed mutations was confirmed by DNA sequencing through the Washington State University DNA sequencing facility.

Glycogen Quantitation—Glycogen was quantified using a glucose oxidase assay kit (Sigma). Cells were grown in 2% Kornberg media (23) containing 10 μg/ml of kanamycin. Cells were harvested by centrifugation, resuspended in water, and then lysed by incubating at 100 °C. The samples were solubilized in 2 ml of Me2SO and 0.5 ml of HCl at 60 °C for 30 min, and then the pH was adjusted to 4.5 using NaOH in sodium citrate buffer. The samples were then treated with 3 units of amyloglucosidase at 55 °C for 40 min to hydrolyze glycogen into D-glucose. The amount of D-glucose was measured spectrophotometrically by measuring the formation of the quinoneminine dye using a coupled enzyme assay containing glucose oxidase and peroxidase.

AGPase Assays

The activity of AGPase-glucose pyrophosphorylase was determined in both the reverse (pyrophosphorylase: Assay A) and the forward (synthesis: Assay B) directions.

Assay A—Pyrophosphorylase assays were used to monitor purification of the enzyme. 32P|ATP formation was measured from [32P]Pi, and ADP-glucose. The reaction mixture contained 50 mM HEPES, pH 7.5, 0.4 mM mg/ml bovine serum albumin, 5 mM MgCl2, 5 mM 3-PGA, 5 mM dithiothreitol, 0.5 mM Glc-1-P, 0.2 units of inorganic pyrophosphatase, 1.5 mM ATP, 0.5 μCi of [32P]Glc-1-P in a final volume of 0.1 ml. Reactions were incubated at 37 °C for 10 min. Calf intestinal alkaline phosphatase (2 units/reaction in 1× CIAP buffer) was added to each reaction and incubated at 37 °C for 1 h. A reaction sample volume of 0.055 ml was blotted onto DEAE-81 paper and washed 3 times with distilled water. The filter was dried and product formation quantified by liquid scintillation spectrometry.

Purification of Wild-type and Mutant Potato AGPases

Cells were grown in 1 liter of modified LB medium (23) to an A550 of 1.2. AGPase expression was induced by the addition of 10 μg/ml naldixic acid and 200 μM isopropyl-1-thio-β-D-galactopyranoside (final concentrations) and incubated with shaking at room temperature for 18 h. Cells were harvested by centrifugation at 6,000 × g for 5 min, and the pellet was resuspended in lysis buffer (50 mM HEPES, pH 8.0, 5 mM MgCl2, 5 mM dithiothreitol, 1 mM EDTA, 10% glycerol) containing 500 μg/ml lysozyme, 0.5 mg/ml pepstatin, 0.5 mg/ml leupeptin, 0.5 mM benzamidine, and 1 mM phenylmethylsulfonyl fluoride. The sample was sonicated 3 times for 45 s and centrifuged at 30,000 × g for 20 min at 4 °C. The supernatant was passed through a 0.2-μm filter, loaded onto an HQ-POROS anion exchange column equilibrated with buffer A (50 mM HEPES, pH 8.0, 5 mM MgCl2, 1 mM EDTA, 10% glycerol), and eluted from the column by a 200-mL linear gradient of buffer B (buffer A, pH 7.0, containing 1 M KCl). Active fractions were combined, and (NH4)2SO4 was added to a final concentration of 1 M. Samples were then loaded onto a C-4 hydrophobic interaction chromatography column, which was equilibrated with buffer C (50 mM HEPES, pH 7.0, 5 mM MgCl2, 1 mM EDTA, 1 M NaCl, 0.1% (w/w) CHAPS, and 10% glycerol). Fractionation was carried out using a linear 200-mL linear gradient with buffer D (50 mM HEPES, pH 7.0, 5 mM MgCl2, 1 mM EDTA, 10% glycerol). Active fractions were pooled and dialyzed against buffer A overnight. Aliquots of concentrated enzyme were stored at −80 °C. Protein levels were determined by Bradford analysis (24) using the Bio-Rad reagent. Purity and integrity of partially purified enzymes were determined by analysis on 12% SDS-polyacrylamide gels followed by Coomasie Brilliant Blue staining and immunoblot analysis with antibodies specific for the potato AGPase LS and SS.

Kinetic Studies

Kinetic parameters were determined by the synthesis assay, which measured [14C]Glc-1-P incorporation into ADP-glucose. The reaction mixture contained 50 mM HEPES, pH 7.3, 0.4 mg/ml bovine serum albumin, 5 mM MgCl2, 5 mM 3-PGA, 5 mM dithiothreitol, 0.5 mM Glc-1-P, 2 units of inorganic pyrophosphatase, 1.5 mM ATP, 0.5 μCi of [14C]Glc-1-P in a final volume of 0.1 ml. Reactions were incubated at 37 °C for 10 min. Calf intestinal alkaline phosphatase (2 units/reaction in 1× CIAP buffer) was added to each reaction and incubated at 37 °C for 1 h. A reaction sample volume of 0.055 ml was blotted onto DEAE-81 paper and washed 3 times with distilled water. The filter was dried and product formation quantified by liquid scintillation spectrometry.

RESULTS

Mutagenesis and Sequence Analysis of Revertants—Previous mutagenesis studies resulted in the identification of an LS mutant, which contained a single amino acid replacement, E38A. When co-expressed with the wild-type SS, the resulting AGPase required several hundred-fold higher levels of 3-PGA for activation than the wild-type enzyme (20). Bacterial cells expressing this mutant LS with wild-type SS were null for glycogen accumulation when analyzed by iodine staining. To map the peptide regions that participate in allosteric effector
Amino acid replacements in the 16 dominant mutants

| Clone | Wild-type residue | Position | Replacement residue |
|-------|------------------|----------|---------------------|
| 2     | Lys              | 420      | Arg                 |
| 11/30 | Lys              | 197      | Met                 |
| 12    | Asp              | 367      | Asn                 |
| 13/22 | Asn              | 175      | His                 |
| 14    | Pro              | 261      | Leu                 |
| 15/16 | Asn              | 145      | His                 |
| 17    | Phe              | 193      | Tyr                 |
| 18/20/23 | Ser      | 386 | Pro |
| 24    | Thr              | 11       | Ser                 |
| 25    | Ser              | 390      | Pro                 |
| 26    | Ser              | 188      | Cys                 |
|       | Met              | 377      | Thr                 |

Several of the individually isolated mutants shared identical amino acid changes (e.g. 11 and 30).
doxal phosphate, an analogue of 3-PGA. Kinetic analysis of 24A indicated that this single amino acid change conferred sensitivity to allosteric effectors, by requiring less 3-PGA for activation and more Pi for inhibition when compared with mutant 24 and wild-type AGPase (Table IV). To explore further the structure-function relationship of allosteric regulation, the effects of charge, size, shape, and hydrophobicity at the positions occupied by Lys-420, Lys-197, and Pro-261 were further investigated. These residues are strongly conserved in known plant AGPases (Fig. 2). Mutant (K420R) showed elevated resistance to Pi inhibition, which is likely responsible for its up-regulated phenotype. To observe the effects of neutral and negatively charged residues at that position, Lys-420 was replaced with Ala and Glu, respectively. Kinetic analysis indicated that replacement of Lys with Ala lowered the $K_{0.5}$ of 3-PGA about 8-fold while increasing $I_{0.5}$ over 2-fold. Enzymes containing Glu at position 420 showed a moderate increase in $I_{0.5}$ with a small effect on $A_{0.5}$. Hence, changes at this position altered either 3-PGA activation response or Pi inhibition response.

The K197M enzyme exhibited an up-regulatory phenotype containing both an increased affinity toward 3-PGA and increased resistance toward Pi inhibition. Replacement of this Lys residue by an Ile had only a modest change in 3-PGA activation, whereas Glu increased the affinity of the enzyme to 3-PGA (Table IV). Both amino acid replacements increased the resistance of the enzyme to Pi. Interestingly, these mutations change the 3-PGA and Pi reactivities in parallel as evident by the near constant $[3\text{-PGA}]I_{0.5}$ ratios measured for the different mutant enzymes.

Another up-regulated phenotype is due to a Pro-261 to Leu change in the LS. To investigate the contribution of this amino acid to allosteric control, Pro-261 was replaced with Gly, Lys, or Glu. Activator studies of these three mutants revealed that replacing Pro-261 with Gly, Lys, and Glu had a major impact on 3-PGA affinity. A Pro-261 to Gly change increased the $A_{0.5}$ for 3-PGA from 0.1 (wild-type AGPase) to 1.5 mM (Table IV). Although replacement of Pro-261 with Lys and Glu did not have as dramatic an effect on activator binding, the values were significant, requiring 3- and 6-fold more 3-PGA, respectively, to reach the 50% activation level.

### Table III

Kinetic parameters of the wild-type and mutant AGPases

| Wild-type | K420R | K197M | D367N/I412V | P261L | N175H/S390P | E403V | F193Y | S386P | T11S/S390P | S390P | S188C/M377T |
|-----------|-------|-------|-------------|-------|-------------|-------|-------|-------|-------------|-------|-------------|
| ATP ($K_m$) | 0.3 | 0.35 | 0.3 | 0.25 | 0.29 | 0.19 | 0.31 | 0.19 | 0.28 | 0.18 | 0.18 |
| Glc-1-P ($K_m$) | 0.26 | 0.25 | 0.23 | 0.18 | 0.35 | 0.24 | 0.25 | 0.17 | 0.26 | 0.23 | 0.23 |
| Mg$^{2+}$ ($K_m$) | 2.5 | 3.1 | 3.3 | 2.1 | 2.2 | 2.5 | 2.5 | 2.1 | 2.5 | 1.9 | 2.4 |
| 3-PGA ($A_{0.5}$) | 0.1 | 0.1 | 0.04 | 0.02 | 0.02 | 0.05 | 0.07 | 0.01 | 0.03 | 0.04 | 0.09 |
| P$_i$ (0.1 mM 3-PGA) | 0.09 | 0.7 | 0.19 | 0.5 | 0.6 | 0.2 | 0.6 | 0.2 | 0.3 | 0.3 | 0.24 |
| P$_i$ (0.25 mM 3-PGA) | 0.25 | 1.8 | ND* | ND | ND | 0.85 | ND | ND | 0.8 | ND | ND |
| P$_i$ (1.0 mM 3-PGA) | ND | ND | ND | 3.2 | 2.9 | ND | 3.5 | 5.9 | 2.9 | 2.9 | 3.3 |

*ND, not determined.
Hence, these mutant enzymes required lower levels of P_i for 50% inhibition have a [3-PGA]/I_{0.5} ratio in elevated levels of 3-PGA for 50% activation or decreased were 0.6, 0.4, and 0.16 mM P_i, respectively, when measured in tritated by the ratio of [3-PGA]/I_{0.5}. The wild-type enzyme has 50% inhibition than the wild-type enzyme. This is best illus-

| Position | Mutation | 11 | 145 | 175 | 188 | 193 | 197 | 261 |
|----------|----------|----|-----|-----|-----|-----|-----|-----|
| Potato, stem | T27P | Y28F | V34M | N35D | K36 | E37 | R39 | V40 |
| Tomato, stem | V34M | D35N | K36 | E37 | R39 | V40 | *  | |
| Arabidopsis, stem | T27P | Y28F | V34M | N35D | K36 | E37 | R39 | V40 |
| Tomato, leaf | V34 | S35 | K8 | E37 | R39 | V40 | *  | |
| Arabidopsis, leaf | T27P | Y28F | V34M | N35D | K36 | E37 | R39 | V40 |
| Pea, leaf | T27P | Y28F | V34M | N35D | K36 | E37 | R39 | V40 |
| Barley, leaf | T27P | Y28F | V34M | N35D | K36 | E37 | R39 | V40 |
| Pea, cotyledon | Y28F | V34M | N35D | K36 | E37 | R39 | V40 | *  |
| Pea, cotyledon | Y28F | V34M | N35D | K36 | E37 | R39 | V40 | *  |
| Wheat, endosperm | Y28F | V34M | N35D | K36 | E37 | R39 | V40 | *  |
| Barley, endosperm | Y28F | V34M | N35D | K36 | E37 | R39 | V40 | *  |
| Maize, endosperm | Y28F | V34M | N35D | K36 | E37 | R39 | V40 | *  |

Fig. 2. Primary sequence alignments of potato LS with Perilla seed (GenBankTM accession number AF249917), tomato LS (acces-

| LS | \(I_{0.5, \text{mut}}\) (mm) | [3-PGA]/\(I_{0.5}\) | \(I_{0.5, \text{mut}}\) (mm) | [3-PGA]/\(I_{0.5}\) |
|----|----------------------------|------------------|----------------------------|------------------|
| Wild-type | 0.09 at 0.1 mM 3-PGA | 1.11 | 0.26±0.25 | 0.096 |
| K240R | 0.68 at 0.1 mM 3-PGA | 0.15 | 1.80±0.25 | 0.14 |
| K240A | 0.22 at 0.1 mM 3-PGA | 0.45 | 0.49±0.25 | 0.50 |
| K240E | 0.26 at 0.1 mM 3-PGA | 0.38 | 0.75±0.50 | 0.67 |
| K197M | 0.19 at 0.04 mM 3-PGA | 0.21 | | |
| K197L | 0.50 at 0.1 mM 3-PGA | 0.22 | | |
| K197E | 0.60 at 0.1 mM 3-PGA | 0.16 | 2.40±0.50 | 0.20 |
| P261L | 0.19 at 0.04 mM 3-PGA | 0.21 | 0.85±0.25 | 0.29 |
| P261G | 0.60 at 0.15 mM 3-PGA | 2.5 | 0.90±0.25 | 3.34 |
| P261E | 0.40 at 0.6 mM 3-PGA | 1.5 | 1.20±0.25 | 2.1 |
| P261K | 0.16 at 0.30 mM 3-PGA | 1.9 | | |
| 24 (T11S/S73C) | 0.29 at 0.1 mM 3-PGA | 0.33 | 0.79±0.25 | 0.31 |
| 24 A (T11S) | 0.40 at 0.1 mM 3-PGA | 0.25 | 1.90±0.50 | 0.25 |

| LS | \(I_{0.5, \text{mut}}\) (mm) | [3-PGA]/\(I_{0.5}\) | \(I_{0.5, \text{mut}}\) (mm) | [3-PGA]/\(I_{0.5}\) |
|----|----------------------------|------------------|----------------------------|------------------|
| Wild-type | 0.09 at 0.1 mM 3-PGA | 1.11 | 0.26±0.25 | 0.096 |
| K240R | 0.68 at 0.1 mM 3-PGA | 0.15 | 1.80±0.25 | 0.14 |
| K240A | 0.22 at 0.1 mM 3-PGA | 0.45 | 0.49±0.25 | 0.50 |
| K240E | 0.26 at 0.1 mM 3-PGA | 0.38 | 0.75±0.50 | 0.67 |

All LS mutants were co-expressed with the wild-type SS in glgC E. coli and partially purified for kinetic analysis. All values are determined from synthesis assay data of at least two iterations, and the difference was <10% in all cases. The average values are shown below. The [3-PGA]/\(I_{0.5}\) ratio is an indicator of the general regulatory properties of the enzyme. It was obtained by measuring the \(I_{0.5}\) value (i.e. the amount of P_i that inhibits enzyme activity 50%) in the presence of a known quantity of 3-PGA.

Table IV

DISCUSSION

AGPase is the one of the key regulatory enzymes of the starch biosynthetic pathway. Plant AGPases are structurally complex and are composed of two large and small subunits. With few exceptions (26–28), plant AGPases are allosterically regulated with 3-PGA being the primary activator and P_i as the main inhibitor (29). Biochemical and genetic studies have assigned the roles for the SS as catalytic and regulatory, whereas the LS appears to be involved in regulation by modulating the response of the SS toward the allosteric effectors (13, 17–20, 22, 30, 31) and substrate (32). Although there is evidence suggesting the involvement of the N and C termini of the LS in effector binding (16, 19, 33), there is limited information pertaining to specific regions and amino acids that may be involved. In this study we applied a second-site genetic reversion approach to identify peptide region(s) and amino acids that may compose part of the binding site(s) for effector molecules. In this approach, a down-regulatory LS mutant (E38R) was subjected to error-prone PCR to generate second-site mutations. The mutated LSs were then co-expressed with the wild-type SS and screened for the restoration of AGPase activity and, in turn, glycogen production.

Two general types of second site mutations were expected. They included suppressor mutations that act in conjunction with the primary mutation to counteract the down-regulatory phenotype imposed by the primary mutation and dominant mutations that act independently of the primary mutation. Of the initial positive clones obtained, those containing dominant mutations were identified by “curing” the primary defect by site-directed mutagenesis and re-screening for glycogen production. Sixteen independent clones, grouped into 11 different mutation classes, showed more intense iodine staining and elevated glycogen levels than wild-type cells. Although several of the lines, e.g. clones 18, 20 and 23, contain identical amino acid replacements at the same site (Table II), they were independently derived as they contain unique silent mutations as well. The generation of multiple independent mutants of the same class suggest that our mutagenesis and screening procedure was close to saturation.

Analysis of the location of these dominant mutations revealed that they were not randomly distributed. Other than two amino acid replacements, one near the N terminus and properties as demonstrated by its low [3-PGA]/\(I_{0.5}\) ratio (Table IV).
another toward the middle of the protein sequence, the mutations were clustered in two regions as follows: one located between residues 145 and 197 and the other close to the C terminus. Both regions have been implicated in allosteric control based on results from chemical labeling studies (8). Pyridoxal phosphate, which serves as a 3-PGA analogue for enzyme activation, interacts with three Lys residues of the spinach leaf enzyme large subunit, two located near the C terminus (positions 414 and 452 of the potato sequence, Fig. 2), whereas a third is located toward the N terminus (position 124, Fig. 2).

Results from this study confirm the role of the Lys-414 containing region in allosteric effector binding. Four mutations, which result in the formation of enzymes displaying up-regulatory properties, flank Lys-414 at positions 403, 412, 420, and 424. Residues at these positions are strongly conserved among the three types (stem, leaf, and endosperm) of AGPase families of enzymes (Fig. 2) suggesting that they play an important role in effector binding. The location of Lys-414 at or near the effector binding site(s) is also supported by mutations at the adjacent Asp-413, which resulted in a deficiency in 3-PGA activation (19).

In addition to the peptide region containing Lys-414, these studies identified a second putative peptide region important for allostery located at the N-terminal side of the protein sequence. Amino acid replacements at Ser-188 (with a second mutation at Met-377), Phe-193, and Lys-197 resulted in an up-regulatory phenotype affecting both 3-PGA and P\(_i\) binding characteristics. Lys-197 is conserved among all AGPase types, whereas Ser-188 and Phe-193 show varying degrees of conservation among stem, leaf, and endosperm isoforms.

Among the mutant sites generated in this study, Thr-11 in the potato LS was the least conserved. Alignment of the LS N-terminal sequences of higher plants indicated that this region is quite variable between monocot and dicot plants (Fig. 2). It is noteworthy that kinetic analysis of the barley endosperm and wheat endosperm AGPases shows that these enzymes are insensitive toward 3-PGA and P\(_i\) regulation (26, 27). Thr-11 is present and conserved in pea and tomato, whereas a Ser is present in that position in the LS of barley endosperm and pea embryo AGPases (Fig. 2).

Slight changes in the N and C termini may mediate different sensitivities toward effectors for their respective AGPase isoforms (Fig. 2). As shown here, several of the mutations were relatively conservative changes but nevertheless mediated significant alterations in allosteric regulatory properties of the enzyme. In particular, F193Y caused a dramatic change in both activator and inhibitor binding. Interestingly, a Tyr at an equivalent position of 193 is observed in the seed AGPases from Perilla and pea cotyledons and wheat and barley endosperms (Fig. 2). These seed AGPases have been suggested to have high catalytic activity in the absence of 3-PGA and are not sensitive to P\(_i\) inhibition. Hence, the apparent loss of allosteric control by the seed AGPases may be accounted for by subtle changes in residues between the allosteric responsive stem and leaf enzymes and the allosteric, independent seed isoforms.

The highly conserved residues Lys-197, Pro-261, and Lys-420 were studied in more detail by site-directed mutagenesis. K420R increased the resistance of the enzyme toward P\(_i\) inhibition while having no effect on 3-PGA activation. Similar to that observed for K420R, K420E only affected P\(_i\) binding although not to the same degree. Hence, the net charge at this location has little effect on P\(_i\) binding. In contrast, replacement of Lys-420 with Ala enhanced the affinity of the enzyme toward 3-PGA about 8-fold while having a much smaller (>2-fold) effect on P\(_i\) compared with the wild type. These results indicate that the nature of the side chain at this position can selectively affect either 3-PGA or P\(_i\) binding. These observations are consistent with the view that these effectors interact at distinct but nearby binding sites. The existence of distinct 3-PGA- and P\(_i\)-binding sites is also supported by labeling studies with the activator analogue pyridoxal phosphate. Whereas all three Lys residues in the LS (positions 124, 414, and 452 in potato sequence, Fig. 2) were protected from phosphopyridoxylation by the activator 3-PGA, only Lys-414 was protected by P\(_i\) (8).

K197M affected the interaction of the enzyme with both 3-PGA and P\(_i\). Comparison of this residue with known LS (Fig. 2) and SS amino acid sequences in GenBankTM (data not shown) indicate that this residue is conserved at this position among the higher plant enzymes. Replacement of Lys-197 with Ile or Glu enhanced the affinity of the enzyme toward 3-PGA while decreasing its affinity to P\(_i\). These results suggest that a conserved Lys is important to maintain the stringent allosteric regulatory properties of the potato AGPase.

The P261L containing AGPase shows up-regulatory properties as it is approximately 2-fold more sensitive to 3-PGA activation and 5-fold more resistant to P\(_i\) inhibition. Replacement by residues, which impose less steric constraints on conformation, resulted in the formation of enzymes displaying just the opposite, i.e. down-regulatory, properties. The greatest effect was evident with Gly indicating that the loss of allosteric regulative properties is correlated with increased flexibility in structure (Table IV).

Previous studies (34, 35) have demonstrated that the introduction of up-regulated forms of AGPase led to a 20–30% increase in starch and overall productivity. These results indicate the feasibility of increasing the yield of crops by manipulating AGPase. In this study, we have generated a wide range of allosteric mutants, which required different levels of effectors for activation and inhibition. The introduction of these mutants into crop plants may allow for the manipulation of sink and/or source tissue, and in turn increase crop production.

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