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Aspartic Acid 413 Is Important for the Normal Allosteric Functioning of ADP-Glucose Pyrophosphorylase[1]

Thomas W. Greene, Ronald L. Woodbury, and Thomas W. Okita*

Institute of Biological Chemistry (T.W.G., R.L.W., T.W.O.), Plant Physiology Program (T.W.G.), and Genetics and Cell Biology Program (R.L.W.), Washington State University, Pullman, Washington 99164–6340

As part of a structure-function analysis of the higher-plant ADP-glucose pyrophosphorylase (AGP), we used a random mutagenesis approach in combination with a novel bacterial complementation system to isolate over 100 mutants that were defective in glycogen production (T.W. Greene, S.E. Chantler, M.L. Khan, G.F. Barry, J. Preiss, T.W. Okita [1996] Proc Natl Acad Sci USA 93: 1509–1513). One mutant of the large subunit M27 was identified by its capacity to only partially complement a mutation in the structural gene for the bacterial AGP (*glg* C), as determined by its light-staining phenotype when cells were exposed to I_2 vapors. Enzyme-linked immunosorbent assay and enzymatic pyrophosphorylysis assays of M27 cell extracts showed that the level of expression and AGP activity was comparable to those of cells that expressed the wild-type recombinant enzyme. Kinetic analysis indicated that the M27 AGP displays normal Michaelis constant values for the substrates glucose-1-phosphate and ATP but requires 6- to 10-fold greater levels of 3-phosphoglycerate (3-PGA) than the wild-type recombinant enzyme for maximum activation. DNA sequence analysis showed that M27 contains a single point mutation that resulted in the replacement of aspartic acid 413 to alanine. Substitution of a lysine residue at this site almost completely abolished activation by 3-PGA. Aspartic acid 413 is adjacent to a lysine residue that was previously identified by chemical modification studies to be important in the binding of 3-PGA (K. Ball, J. Preiss [1994] J Biol Chem 269: 24706–24711). The kinetic properties of M27 corroborate the importance of this region in the allosteric regulation of a higher-plant AGP.

AGP catalyzes the initial step in starch biosynthesis in both photosynthetic and nonphotosynthetic tissues (Preiss, 1991; Okita, 1992; Preiss and Sivak, 1996). AGP synthesizes the formation of the sugar nucleotide ADP-Glc from ATP and G-1-P, and provides the activated glucosyl substrate that is utilized by starch synthase to form the linear α-1,4 linkage of the starch granule. The activity of AGP is tightly regulated in many higher-plant tissues, with 3-PGA being the most potent activator and Pi being the inhibitor (Sanwal et al., 1968; Preiss et al., 1991; Preiss, 1993).

Considerable genetic and biochemical evidence supports the importance of AGP in starch biosynthesis in higher plants (Preiss, 1991; Martin and Smith, 1995). This is best exemplified by mutations in *Shrunken* 2 and *Brittle* 2, the structural genes for the maize endosperm AGP, which result in a dramatic decrease in starch synthesis (Tsai and Nelson, 1966; Dickinson and Preiss, 1969; Hannah and Nelson, 1976). Likewise, mutations at the *Rb* locus of pea result in a 40-fold decrease in the AGP activity and a concomitant decrease in starch production (Hylton and Smith, 1992). A similar relationship between AGP activity and starch production was identified in the *Arabidopsis thaliana* mutants *adg-1* and *adg-2* (Lin et al., 1988a, 1988b). Antisense studies with potato (*Solanum tuberosum*) provided further biochemical evidence for a major role of AGP in starch production by establishing a direct relationship between AGP activity and the level of starch (Muller-Rober et al., 1992). These studies from both photosynthetic and nonphotosynthetic tissues confirm the important role of AGP in starch production.

Structurally, the higher-plant AGPs exist as a heterotetramer that contains a pair of two distinct subunit types, large and small, that oligomerize to form the complex structure of the native enzyme. Alignment of the potato large and small subunit sequences revealed a sequence identity of 52%, indicating that the two subunits shared a common origin (Nakata et al., 1991). Despite their relative sequence conservation, current evidence indicates that the two subunit types do not possess identical roles in enzyme function. The capacity of the small subunit but not the large subunit to assemble into an active enzyme, albeit exhibiting reduced sensitivity to 3-PGA activation, suggests that the small subunit plays a more dominant role in catalysis (Ballicora et al., 1995). Alternatively, the large subunit may function to increase the sensitivity of the small subunit toward the allosteric effector molecules 3-PGA and Pi. The separate roles for these subunits are also supported by the degree of sequence divergence of AGPs from different plants (Nakata et al., 1991; Smith-White and Preiss, 1992). The primary sequences of the small subunits from different plants display a high degree of conservation (>85%), whereas the large subunit displays considerable divergence at levels that are similar to the extent of sequence

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* Corresponding author; e-mail tokita@mail.wsu.edu; fax 1–509–335–7643.

Abbreviations: AGP, ADPglucose pyrophosphorylase; Glc-1-phosphate, G-1-P; orthophosphate, Pi; 3-PGA, 3-phosphoglycerate; WT, wild-type.

divergence between the large and small subunits (Nakata et al., 1991; Smith-White and Preiss, 1992). For example, the large subunit from potato exhibits a sequence identity to the large subunits from maize and wheat endosperm of 58 and 61%, respectively (Nakata et al., 1991).

Chemical modification studies have been a useful approach in identifying potential binding sites for substrates and allosteric effectors in the higher-plant AGP. Pyridoxal-5-phosphate, which mimics the activator 3-PGA, was shown to specifically interact with a Lys residue that is located at position 440 near the carboxyl terminus of the small subunit of spinach-leaf AGP (Morell et al., 1988; Ball and Preiss, 1994). This Lys residue is highly conserved among the higher-plant AGPs (Smith-White and Preiss, 1992), and site-directed mutagenesis of the corresponding Lys^{441} on the potato AGP small subunit resulted in an enzyme with a decreased affinity toward the activator 3-PGA (Preiss and Sivak, 1996). Additional studies with pyridoxal-5-phosphate identified three Lys residues located in conserved regions of the large subunit of spinach-leaf AGP (Ball and Preiss, 1994). These sites correspond to Lys residues 452, 414, and 124 of the potato AGP large subunit. Lys^{452} of the potato large subunit and Lys^{441} of the potato small subunit are located approximately 10 amino acids from the carboxyl terminus and are referred to as activator site 1 (Preiss and Sivak, 1996). Lys^{414} of the potato large subunit is located 47 amino acids from the carboxyl terminus and is referred to as activator site 2. This region also shares a high degree of homology with the small subunit (Preiss and Sivak, 1996). Lys^{124} is located near the amino terminus of the potato large subunit and aligns with the Lys residue of activator site 3 (Ball and Preiss, 1994). Whether the Lys residues of activator sites 1, 2, or 3 on the large subunit are as important in the binding of 3-PGA as identified for the small subunit remains to be determined.

Chemical modification studies in combination with site-directed mutagenesis are beginning to identify residues that are important for the catalytic and allosteric properties of AGP, but additional information is needed to fully understand how the higher-plant AGP functions. We have established a novel random mutagenesis scheme that will help to elucidate these potential binding sites on AGP. Using this approach we have isolated both substrate (M.L. Laughlin and T.W. Okita, unpublished data) and allosteric (Greene et al., 1996) mutants. In this report we identify a second allosteric mutant, which displays reduced sensitivity to the activator 3-PGA. Molecular analysis indicates that the change in allosteric control is due to a single point mutation, which results in an amino acid replacement of a residue that is directly adjacent to Lys^{414} of the activator site 2 on the potato large subunit. The results provide direct experimental support for the role of the large subunit in the binding of 3-PGA and allosteric regulation of the heterotetrameric enzyme.

MATERIALS AND METHODS

Mutagenesis

Random chemical mutagenesis of the plasmid coding for the large subunit of potato (*Solanum tuberosum*) AGP was carried out as previously described (Greene et al., 1996). Mutations were identified by complete sequencing of the coding region of the large subunit using Sequenase (Amersham) and the manufacturer's recommendation for elongation-termination reactions.

Glycogen Staining

To stain for glycogen, colonies were streaked on a modified version of the enriched medium used by Govons et al. (1969), containing 0.85% KH_2PO_4, 1.1% K_2HPO_4, 0.6% yeast extract, 2% Glc, and 1.5% bacto agar. Cells were grown overnight at 37°C, stored at 4°C for 1 h, and exposed to iodine crystals for 1 min.

AGP Assay

AGP activity in the M27 crude extract was determined by assaying in the pyrophosphorylysis direction, which measured the incorporation of ^{32}P into ATP from the pyrophosphorylysis of ADP-Glc with labeled PPi (Okita et al., 1990). Contained in the assay mixture were 80 mM glycylglycine, pH 7.5, 5 mM DTT, 5 mM $MgCl_2$, 10 mM NaF, 1 mM ADP-Glc, 20 mM 3-PGA, 0.4 mg/mL BSA, 3×10^6 cpm/mL ^{32}PPi, 1.5 mM NaPPi, and 50 to 100 μg of protein in a final volume of 0.25 mL.

ADP-Glc Synthesis Assay

Kinetic parameters were defined by the synthesis (forward) assay, which measured the incorporation of [^{14}C]G-1-P into the sugar nucleotide ADP-Glc. Contained in the assay mixture were 80 mM glycylglycine, pH 7.5, 0.4 μg/μL BSA, 5 mM $MgCl_2$, 0.2 unit of inorganic pyrophosphatase, 1×10^5 cpm [^{14}C]G-1-P, 1.5 mM ATP, 0.5 mM G-1-P (cold), 5 mM DTT, and 5 mM 3-PGA in a final volume of 50 μL (Greene et al., 1996).

ELISA

M27 cells were lysed in 100 μL of Suc lysis buffer by repeated freeze-thawing and clarified by centrifugation as previously described (Greene et al., 1996). Large subunit antigen levels in the extracts were quantitated by an ELISA using rabbit monospecific anti-large subunit and goat anti-rabbit antibodies that were linked to horseradish peroxidase (Harlow and Lane, 1988).

Partial Purification

M27 cells were cultured and induced with isopropylthio-β-galactoside and nalidixic acid as described (Greene et al., 1996). Crude extracts were prepared and fractionated by ammonium sulfate. After desalting, the enzyme fraction was purified over a chromatography column (MEMSEP 1010 DEAE-Cellulose, Millipore) as described previously (Greene et al., 1996). Fractions containing enzyme activity were pooled and concentrated by the addition of ammonium sulfate to 70%. Enzyme concentrate was resuspended in Suc buffer, desalted by dialysis, and aliquoted for storage at −80°C.

Site-Directed Mutagenesis

A site-specific mutation was introduced into the large subunit expression plasmid using the Clontech Transformer site-directed mutagenesis kit (Clontech Laboratories, Palo Alto, CA). A selection primer, 5′-GGAACACAGTCATATGATAAGGGTATCGATGATAAGCTG-3′, eliminated a unique *Hin*dIII site 3′ of the protein coding sequence. The mutagenic primer 5′-GGAAATGTATCATTAAGAAGAACGCAAAGATAGG-3′ introduced the site-specific change of Asp to Lys at position 413.

RESULTS

M27 AGP

In our previous study (Greene et al., 1996) mutagenesis of the large subunit cDNA sequences of potato AGP generated a large population of mutants (>100) that were impaired in glycogen production, and these mutants were classified into six different groups depending on the extent of I_2 staining and enzyme activity levels. Mutants in the group IV category, which includes M27, stain weakly for glycogen but exhibit an in vitro AGP activity that is comparable with the WT recombinant when assayed in the presence of excess substrates and activator (Greene et al., 1996). The fact that low levels of glycogen are present in M27 indicates that some AGP activity remains in vivo, which enables the mutant enzyme to partially complement the mutation in the bacterial AGP of the *Escherichia coli* host AC70R1–504. Reduced glycogen levels could be the result of several different effects of the mutagenesis. Since the complete plasmid was subjected to the mutagenesis, mutations that altered the transcription or translation of the AGP large subunit in the cell could account for a decrease in the level of glycogen. An ELISA that was specific for the large subunit showed that the large subunit antigen levels were comparable to the WT recombinant, and that a decrease in the level of AGP expression was not the reason for the lowered glycogen levels in M27.

Partial Purification and Characterization of M27

In vitro enzyme assays under saturating conditions showed comparable levels of AGP activity in the WT recombinant and M27, which did not correlate with the reduced glycogen levels in vivo. To investigate the catalytic and allosteric properties, M27 was partially purified by DEAE anion-exchange chromatography to a specific activity of 7.4 μmol min^{-1} mg^{-1} (data not shown). This is approximately 7.5-fold lower than the 56.9 μmol min^{-1} mg^{-1} value for the native potato tuber AGP that was determined to be 80% pure (Okita et al., 1990). Kinetic analysis of the partially purified enzyme was conducted to determine whether its affinity for substrates or allosteric effectors was altered. K_m values for the substrates ATP and G-1-P were very similar to those that were displayed by the WT recombinant, indicating that the mutation was not affecting the enzyme's affinity for its substrates (Table I). Likewise, no significant differences in the binding of Mg^{2+} were evident between the WT and M27 enzymes (Table I).

Further kinetic analysis revealed that the M27 AGP enzyme displayed altered allosteric regulatory behavior. A 3-PGA concentration of 0.6 mM was required to activate the enzyme to 50% (Fig. 1). This value is approximately 6- to 10-fold higher than previously published 50% enzyme activity values of 0.06 mM (Iglesias et al.) and 0.10 mM (Greene et al., 1996) for the WT recombinant. Thus, the light-staining phenotype identified in the cells expressing M27 AGP is likely to be due to the reduced allosteric activation by 3-PGA and, in turn, reduced catalytic activity in vivo. Other allosteric regulators were investigated. The *E. coli* activator Fru-1,6-bisP and PEP have previously been shown to slightly activate the potato enzyme (Iglesias et al., 1993). The M27 enzyme was totally insensitive to Fru-1,6-bisP (results not shown). This behavior is in contrast to that observed for the WT recombinant, which displayed a 3.3-fold stimulation with this effector (Iglesias et al., 1993). The M27 enzyme shows a 4.8-fold stimulation with PEP, a response similar to the 4.0-fold stimulation previously identified with the WT recombinant (Iglesias et al., 1993). These results show that 3-PGA is still the main activator. Furthermore, the mutation has not changed the enzyme's affinity for PEP, but it did affect its affinity for Fru-1,6-bisP.

Further kinetic analysis showed that the M27 enzyme is inhibited by increasing concentrations of Pi, but at lower levels than needed to inhibit the WT recombinant (Fig. 2). To obtain a measurable activity, Pi inhibition curves were determined in the presence of 0.6 and 0.3 mM 3-PGA, roughly the concentration required to activate the enzyme to 50% and one-half of this value (Fig. 2). At 0.6 mM 3-PGA, M27 was inhibited to fifty percent at a Pi concentration of 0.53 mM, and at 0.3 mM 3-PGA, a 50% inhibition value of 0.15 mM Pi was obtained. A Pi inhibition curve for the partially purified WT recombinant in the presence of 0.1 mM 3-PGA is included for comparison (Fig. 2). A 50% inhibition value of 0.66 mM was identified for the WT recombinant (Greene et al., 1996). The significant inhibition of M27 AGP at the higher concentrations of activator suggests that the enzyme is more sensitive to Pi inhibition. Previous studies with the activator mutant P52L showed that the mutant had a decreased affinity for the activator 3-PGA and displayed a concomitant increase in its sensitivity to inhibition by Pi (Greene et al., 1996), a condition similar to what we see here. Thus, our data correlate well with the 3-PGA mutant P52L and with data in the literature that suggest that the binding sites for 3-PGA and Pi are in close proximity (Ball and Preiss, 1994; Preiss and Sivak, 1996).

Table I. *Kinetic parameters for partially purified M27 and WT recombinant enzyme*

Reactions were run in the direction of ADP-Glc synthesis at 5 mM 3-PGA. K_m values for the WT recombinant were taken from Iglesias et al. (1993).

Substrate	WT Recombinant	M27
	mM	
ATP	0.24	0.11
G-1-P	0.087	0.083
Mg^{2+}	1.51	0.96

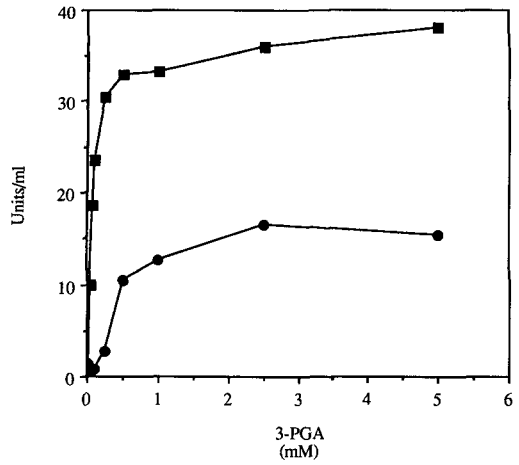

Figure 1. Activation of WT recombinant (■) and M27 (●) AGP in the presence of varying concentrations of 3-PGA. The WT recombinant and M27 AGPs were partially purified to specific activities of 14.0 and 7.4 units/mg protein, respectively.

Molecular Characterization

Complete sequence analysis of M27 large subunit cDNA identified a single point mutation that resulted in the replacement of Asp at position 413 with an Ala (Fig. 3). The Asp is located 48 amino acids from the carboxyl terminus of the large subunit and is highly conserved in every known AGP (Nakata et al., 1991; Smith-White and Preiss, 1992). Furthermore, primary sequence comparisons showed that Asp^{413} is located directly adjacent to the pyridoxylated Lys residue at activator site 2 on the spinach leaf large subunit (Preiss and Sivak, 1996) and the corresponding Lys^{414} of the potato large subunit (Fig. 3).

To further probe the possible interaction of this residue with the allosteric activator 3-PGA, Asp^{413} was replaced by Lys using a site-directed mutagenesis technique. Enzyme activity measurements of crude extracts indicated that the site-directed mutant D413K enzyme was only slightly activated by 3-PGA. Even at high levels (10 mM) of 3-PGA, the D413K enzyme showed only a 2-fold increase in activity (results not shown), compared with the >30-fold activation by the WT or M27 enzyme. These results further support the importance of this region in activator binding and suggest that charge interactions by Asp^{413} have a major effect on the interaction of 3-PGA.

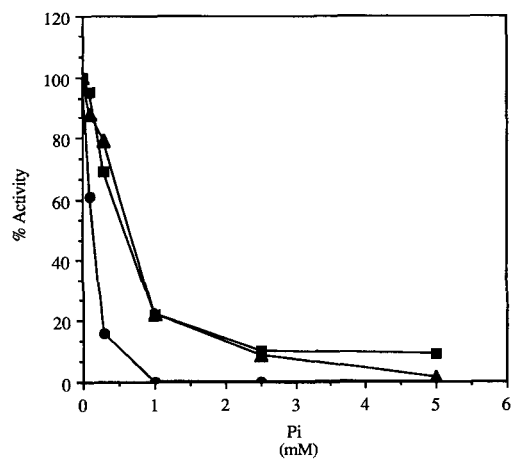

Figure 2. Inhibition curves for M27 at 0.6 (■) and 0.3 mM (●) 3-PGA and WT recombinant at 0.1 mM 3-PGA (▲).

M27	A	
L. S. Potato	G I G E N T K I R K C I I **D** **K** N A K I G K N V S I	424
S. S. Potato	G I G K N C H I K R A I I **D** **K** N A R I G D N V K I	413
L. S. Spinach	I K D A I I **D** **K** N A R	385

Figure 3. Primary sequence alignments of the potato large subunit, the potato small subunit, and the activator site 2 of the spinach leaf large subunit identified by Ball and Preiss (1994). The labeled Lys residue of activator site 2 and corresponding residues in the potato large and small subunits are in boldface. The corresponding residues to the Asp^{413}-to-Ala mutation that are identified in M27 are boxed. Primary sequences of the potato large and small subunits were taken from Nakata et al. (1991).

DISCUSSION

Starch is the major carbohydrate storage form in many agronomically important crops (Okita et al., 1993; Preiss et al., 1994). AGP catalyzes the initial, allosterically regulated reaction in starch biosynthesis in both photosynthetic and nonphotosynthetic tissues and provides a pivotal point at which manipulation of this pathway can take place (Kleczkowski et al., 1991; Okita et al., 1993; Nakata and Okita, 1994). Before we can fully manipulate this enzyme and starch biosynthesis, we must begin to understand how AGP interacts with its substrates and effectors. The enzymatic defects exhibited by M27 and other mutants generated by the random mutagenesis approach should help define these interactions.

E. coli cells expressing the M27 enzyme are unable to accumulate significant levels of glycogen as determined by the weak-staining phenotype when cells are exposed to I_2 vapors. Analysis by ELISA and glycerol density gradient centrifugation showed that M27 AGP was expressed and assembled at levels comparable to that observed for the WT recombinant enzyme (results not shown). Kinetic studies determined that the M27 enzyme had K_m values for ATP, G-1-P, and Mg^{2+} similar to those exhibited by the WT enzyme. Studies with the activator 3-PGA, however, showed that M27 required 6- to 10-fold greater levels of the activator 3-PGA compared with the WT recombinant enzyme. M27 also appeared to be more sensitive to inhibition by Pi. These results indicate that the mutation specifically alters the enzyme's ability to interact with its allosteric regulators without any effect on its substrate interactions.

A single point mutation near the carboxyl terminus of the large subunit that changed Asp^{413} to Ala (D413A) was responsible for the altered allosteric properties. Asp^{413} is highly conserved in all AGPs, and the region surrounding this residue also shows a high degree of sequence similarity (Fig. 3) (Nakata et al., 1991; Smith-White and Preiss, 1992). This conservation is consistent with a role for this region in the normal allosteric functioning of AGP, as first shown by the activator analog studies on the spinach leaf enzyme (Ball and Preiss, 1994). Three Lys residues on the

large subunit and one Lys residue on the small subunit were modified by pyridoxyl-5-phosphate under reductive conditions (Ball and Preiss, 1994; Preiss and Sivak, 1996). All four Lys residues were protected by 3-PGA, but only the Lys residue of activator site 2 on the large subunit and the Lys residue of the small subunit were protected by Pi (Ball and Preiss, 1994). Asp^{413} is immediately adjacent to Lys^{414} of activator site 2 on the potato large subunit (Fig. 3). The altered allosteric properties of D413A provide direct biochemical evidence that this region is important in 3-PGA activation, and that the carboxyl terminus of the large subunit is involved in activation, as indicated by the labeling studies. The importance of this region is further supported by the D4113K site-directed mutant, which results in almost total abolishment of 3-PGA activation. Thus, two independent studies using different techniques have identified this region as important in the interaction of AGP with its allosteric regulators. Additional site-directed mutagenesis of the Asp and Lys residues of the small subunit may allow us to determine whether this region of the small subunit also has a functional role in activation.

Ballicora et al. (1995) recently showed that expression of the small subunit of potato AGP alone gave measurable catalytic activity, but the homotetrameric enzyme required significantly higher levels of 3-PGA to achieve activation. In contrast, the large subunit is unable to assemble into a catalytically active enzyme. These observations suggest that the two subunit types possess distinct roles in the functioning of this enzyme. The small subunit may play a more dominant role in catalysis and the large subunit may serve to increase the sensitivity of the small subunit toward activation by 3-PGA. The data presented here and in our previous study (Greene et al., 1996) support such a role for the large subunit in the activation of AGP. Further studies on the mutants generated by this biochemical-genetic approach should provide additional information on how the allosteric and catalytic properties are distributed between the two subunits of potato AGP.

D413A is the second activator mutant identified by the random chemical mutagenesis, and confirms this strategy as a viable approach for identifying amino acid residues that are important in the allosteric regulation of AGP. The two mutants D413A and P52L (Greene et al., 1996) show that both the amino and carboxyl termini of the large subunit are important in allosteric activation of potato AGP. Data from these two mutants also provide initial evidence that indicates an interaction between the amino and carboxyl termini in the normal allosteric functioning of AGP. A similar approach with the small subunit will be essential in determining the roles of the individual subunits as well as identifying residues involved in catalytic or allosteric functioning.

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