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Generation of up-regulated allosteric variants of potato ADP-glucose pyrophosphorylase by reversion genetics

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ABSTRACT **Mutagenesis of the large subunit (LS) of the potato ADP-glucose pyrophosphorylase generated an enzyme, P52L, that was insensitive to 3-phosphoglycerate (3-PGA). To identify additional residues involved in 3-PGA interaction, we subjected P52L LS DNA to a second round of mutagenesis and identified second-site revertants by their ability to restore glycogen accumulation as assessed by iodine (I_2) staining. Enzymes from class I revertants with normal I_2-staining had an 11- to 49-fold greater affinity for the activator 3-PGA compared with the P52L mutant and a decreased sensitivity to the inhibitor orthophosphate. Sequence analysis of these class I revertants identified a P66L mutation in R4, an E38K mutation in R20, and a G101N mutation in R10 and R32. These mutations appear to restore 3-PGA binding by counteracting the effect of the P52L mutation because introducing E38K or G101N into the wild-type LS led to enzyme variants with higher affinity for the activator 3-PGA and increased resistance to the inhibitor orthophosphate. The generation of these revertant enzymes provides additional structure-function information on the allosteric regulation of higher plant ADP-glucose pyrophosphorylases and validates a strategy for developing novel variants of the enzyme that may be useful in manipulating starch biosynthesis in higher plants.**

Starch is the major storage form of photosynthetically fixed carbon in many agronomically important crops (1–3). A key enzymatic step in the biosynthesis of this carbohydrate polymer is catalyzed by ADP-glucose pyrophosphorylase (AGPase), which prepares the monomeric hexose for polymerization. Results from several *in vivo* and *in vitro* starch-labeling studies (4–7) suggest that AGPase is regulated by the relative levels of 3-phosphoglycerate (3-PGA; activator) and orthophosphate (Pi; inhibitor) in many plant tissues. These allosteric properties of AGPase support the view that this enzyme catalyzes a key regulatory step in the pathway of starch biosynthesis and is a potential site for altering the levels of starch in plants (2, 8). Hence, manipulation of this pathway could have a direct impact on crop yield and quality.

Much of the early information concerning the structure-function analysis of AGPase came from work on bacterial AGPases (reviewed in ref. 9). Biochemical-genetic analysis of mutant enzymes identified many amino acids with functional roles in the binding of allosteric effectors and substrates (reviewed in refs. 9 and 10). Gardiol and Preiss (11) showed that Lys-39 was essential for activator binding to the *E. coli* AGPase, thus proving the amino terminus has a direct role in normal allosteric regulation. This lysine is highly conserved in higher plant AGPases, but a similar role has not yet been demonstrated.

Studies with pyridoxal phosphate, an analog of the activator 3-PGA, identified three Lys residues on the LS of spinach leaf AGPase that were covalently modified under reductive conditions. These residues correspond to Lys residues at position 452, 414, and 124 of the potato AGPase LS (3, 12, 13). Previous studies by Morell *et al.* (14) identified a reactive Lys residue at position 440 on the small subunit (SS) of spinach leaf AGPase. All four Lys residues were protected from labeling by 3-PGA, but only two of the four residues were protected by Pi. That three of the four labeled Lys residues are positioned in the carboxyl terminus indicates that this region of AGPase has a prominent role in binding 3-PGA. This view also is supported by the site-directed mutagenesis of Lys-441 on the SS of potato to yield an enzyme with a reduced affinity for 3-PGA (3, 13). Taken as a whole, the current evidence indicates that the activator binds to the carboxyl terminus in higher plant AGPases.

A structure-function analysis using a bacterial expression system in combination with a random mutagenesis approach identified several residues that are important in allosteric regulation and catalysis of the higher plant AGPase (15, 16–18). Mutagenesis of Pro-52 in a highly conserved ProAla-Val (PAV) motif of the potato LS generated P52L, a mutant defective in 3-PGA activation (15), and gave the first evidence that the amino terminus of a higher plant AGPase was involved in allosteric regulation. The utility of this biochemical-genetic approach was further strengthened by the isolation of a second allosteric mutant, D413A, that was shown to have decreased affinity for 3-PGA (16). Primary sequence comparisons between potato and spinach leaf AGPases showed that D413A is located adjacent to Lys-414, a Lys residue reactive to pyridoxal phosphate in the spinach leaf AGPase. Isolation and analysis of D413A provided initial mutational and biochemical evidence for the role of this region in activator binding. It is important to note that the initial mutants of the potato LS generate alterations in the allosteric properties of the potato AGPase. This result correlates well with previous data suggesting that the LS may have a greater role in allosteric regulation than the SS (3, 19).

In this study, we present the first isolation and characterization of up-regulated mutant forms of a higher plant AGPase. These mutants were isolated by using chemical mutagenesis and phenotypic screening in *Escherichia coli* to find second-site revertants of the P52L mutant. When these secondary mutations were introduced into a wild-type (wt) background, the new enzymes were more sensitive to 3-PGA activation and more resistant to Pi inhibition than wt. The isolation of these regulatory mutants further substantiates the power and versatility of this biochemical-genetic approach and has allowed us to isolate variant forms of AGPase that may be useful in enhancing starch synthesis in plants.

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Abbreviations: AGPase, ADPglucose pyrophosphorylase; G-1-P, glucose-1-phosphate; LS, large subunit; Pi, orthophosphate; 3-PGA, 3-phosphoglycerate; SS, small subunit; wt, wildtype.

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MATERIALS AND METHODS

Genetic Revertants. Cesium chloride density gradient-purified P52L LS plasmid DNA was used as the starting material for additional mutagenesis. DNA was treated with hydroxylamine-HCl as described earlier (15). Mutagenized DNA was transformed into the *glgC*$^-$ strain AC70R1–504, carrying the SS expression plasmid pML10. Revertants were found by their ability to complement the glgC$^-$ mutation and synthesize glycogen on media enriched with 2% glucose. Glycogen was detected by a dark staining phenotype when colonies were exposed to I_2 vapor (20).

AGPase Pyrophosphorylysis and Synthesis Assays. Pyrophosphorylysis assays that measure the incorporation of ^{32}PPi into ATP were performed as described (15). Kinetic-binding constants were determined by the synthesis assay, which measured the amount of ^{14}C-glucose-1-phosphate (G-1-P) label incorporated into ADP-glucose. The reaction mixture contained 100 mM glycylglycine (pH 7.5), 3 mM DTT, 5 mM $MgCl_2$, 0.4 mg/ml BSA, 0.2 unit inorganic pyrophosphatase, 1.5 mM ATP, 5 mM 3-PGA, 0.5 mM G-1-P (cold), and 100,000 cpm of ^{14}C-G-1-P in a final volume of 0.2 ml. Assays were processed and quantified as described (15).

Heat Stability. The crude extract (0.5 ml) was placed in a 60°C water bath for 6 min with constant mixing, quickly cooled on ice, and then clarified by centrifugation at 4°C. Protein concentrations of nonheated and heated samples were determined by Bradford assay (21). The pyrophosphorylysis assay was used to determine AGPase activity in each sample.

Enzyme Purification. AC70R1–504 cells expressing R4, R20, and R32 were grown, induced, and lysed as described (15). Crude extracts were subjected to a differential ammonium sulfate precipitation. The clarified 30–55% pellet containing the AGPase activity was resuspended in a small volume of buffer A (5 mM K_2HPO_4/KH_2PO_4, pH 7.5/50 mM glycylglycine, pH 7.5/20% sucrose/5 mM $MgCl_2$/1 mM EDTA/1 mM DTT) and heat treated at 60°C for 5 min as described earlier. Sample was clarified by centrifugation. Supernatant was adjusted to 1 M ammonium sulfate by addition of saturated ammonium sulfate. The sample was then loaded onto a Pharmacia HR 10/10 column packed with Tentacle Hydrophobic Interaction Chromatography media Fractogel EMD Propyl (EM Separations, Gibbstown, NJ) previously equilibrated with Buffer C [50 mM K_2HPO_4/KH_2PO_4, pH 7.0/20% sucrose/5 mM $MgCl_2$/0.1 mM EDTA/1 mM DTT/1 M $(NH_4)_2SO_4$]. Protein was bound and washed with five-column volumes of Buffer C. The column was then subjected to step gradient washes of 0.75, 0.5, 0.25, and 0 mM ammonium sulfate by adjusting the percentage of Buffer D (50 mM K_2HPO_4/KH_2PO_4, pH 7.0/20% sucrose/5 mM $MgCl_2$/0.1 mM EDTA/1 mM DTT). For each step in the gradient, the column was washed with approximately five-column volumes. Enzyme typically eluted in the 0.5 M fraction as determined by pyrophosphorylysis assay. Protein was precipitated by addition of ammonium sulfate to 70% and desalted over a G-25 Sephadex (Sigma) column equilibrated with buffer A. Desalted protein was then loaded onto a Millipore MEMSEP 1010 DEAE-Cellulose as described (15), except that 20% sucrose was added to buffers A and B. Fractions containing AGPase activity were concentrated to >1 mg/ml by using Centricell concentrators (Amicon), aliquoted, and stored at −80°C.

Site-Directed Mutagenesis. Site-directed mutations were introduced into the potato AGPase LS expression plasmid pML7 by using a Transformer Site-directed mutagenesis kit (CLONTECH). The protocol uses the unique site elimination method originally outlined by Deng and Nickoloff (22). The selection primer, 5′-GGAACAGTCATATGATAAGGGTATCGATGATAAGCTG-3′, was engineered to eliminate a unique *Hin*dIII restriction site located just after the double stop codons of pML7. A mutagenic primer, 5′-CATACTGGGAGGAGGAAAAGGGACCAAGTTATTCCC-3′, was engineered to bind to the same strand as the selection primer, and introduce the site specific mutation of Glu to Lys at position 38. Two additional mutagenic primers also were used to mutagenize position 38: 5′-CATACTGGGAGGAGGAGCAGGGACCAAGTTATTCCC-3′ introduced an Ala whereas 5′-CATACTGGGAGGAGGAAGAGGGACCAAGTTATTCCC-3′ introduced an Arg. The mutagenic primer 5′-CGAACATATTTTGGCAATAATGTGAGCTTTGGAGATGG-3′ was used to introduce an Asn at position 101. The presence of these site-directed mutations was confirmed by sequence analysis, and plasmid DNAs containing the site-directed mutations were transformed into AC70R1–504 carrying the SS expression plasmid, pML10.

RESULTS

Isolation and Molecular Characterization of Genetic Revertants. Although M345 carrying the P52L AGPase has near normal levels of activity in high concentrations of 3-PGA, it is unable to synthesize substantial glycogen in *E. coli* grown on glucose (15). Mutagenesis of the P52L LS plasmid and coexpression with pML10 (SS expression plasmid) allowed us to identify colonies containing second-site mutations in the coding sequence that restored the ability of the plant AGPases to complement the bacterial AGPase mutation and produce glycogen. Hydroxylamine, a mutagen that induces GC->AT mutations, was used to isolate P52L and its revertants to guarantee that the recovery of glycogen synthesis was not caused by restoration of the wt DNA sequence. Eight second-site revertants were isolated that displayed varying levels of complementation as determined by I_2 staining. Four of the eight revertants had wt-staining phenotypes and higher than wt AGPase activities (class I). Two revertants showed intermediate staining (class II), whereas two revertants had light staining (class III). Like P52L, all eight revertants had AGPase activities similar to or greater than the wt potato AGPase in the presence of excess 3-PGA (data not shown).

Molecular characterization of the LS plasmids of class I revertants R4, R10, R20, and R32 identified a single second-site mutation near the P52L mutation in each revertants (Fig. 1). R4 contained a single base pair mutation that replaced Pro at position 66 with Leu (Fig. 1). The loss of an additional Pro residue just 14 amino acids in the carboxyl terminus direction may reform the binding site for 3-PGA. Pro-66 is highly conserved among the higher plant AGPases but is not present in the bacterial AGPase (Fig. 1; ref. 23–26). R10 and R32

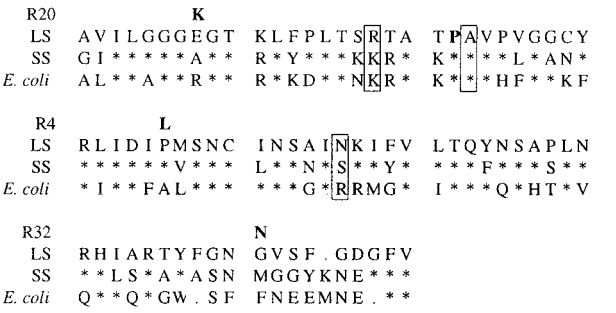

FIG. 1. Primary sequence alignments of the amino terminal sequences of potato LS, SS, and *E. coli* enzymes. Sequences shown begin with residue 31 for the LS, residue 21 for the SS, and residue 22 for the *E. coli* enzyme. Second-site mutations and their positions are indicated by the bold-faced amino acids above the residue of the LS. The P52L mutation of the potato LS that reduces the affinity for 3-PGA 45-fold and the complimentary residues on the SS and *E. coli* are also in bold face. Residues that alter the allosteric properties of the *E. coli* AGPase enzyme (26) and complimentary residues of the potato LS and SS are boxed.

contained identical second-site mutations in which Gly at position 101 was replaced by Asn (Fig. 1). This Gly residue is conserved in maize, wheat, and barley LSs but is not found in any of the SSs or the *E. coli* enzyme (23, 24). Lack of conservation with the SS may indicate a unique binding site on the LS with respect to 3-PGA (24). In R20, the negatively charged Glu-38 residue was replaced with a positively charged Lys residue (Fig. 1). Glu-38 is not found in any of the other higher plant AGPases. The LS of AGPases from the endosperm tissues of wheat, barley, and maize contain a conserved Thr residue at the corresponding position (25). In contrast, the SSs of potato, rice, and wheat contain a conserved Ala at the corresponding position (23–25). Introduction of the positively charged Lys residue 14 amino acids in the amino-terminal direction from P52L could potentially enhance the enzyme's ability to interact with the negatively charged activator 3-PGA.

Heat Stability of Genetic Revertants. The native potato tuber AGPase retains the majority of its activity after incubation at 70°C for 5 min (27, 28), and this heat stability also was found with the wt recombinant AGPase (pML10/pMON 17336) (19). We tested the heat stability of the mutant AGPases to see whether the mutation(s) had a gross effect on enzyme structure. After heat treatment, AGPase enzyme activity of R20 was slightly higher (19%) than comparably treated recombinant wt enzyme. This gain in activity was reproducible in three different experiments and may indicate that the E38K reversion enhanced the stability of the enzyme. R4 and R32 AGPase enzymes were partially sensitive to heat treatment, losing 34% and 20% of their activities, respectively.

Purification and Enzymatic Characterization of Class I Revertants. To determine the kinetic parameters of the second-site revertants, each mutant enzyme was partially purified through a differential ammonium sulfate precipitation, heat treatment, amino-propyl (C_3) chromatography, and DEAE anion exchange chromatography. The final specific activities of the second-site revertants enzymes ranged from 16.0 μmol/min/mg to 24 μmol/min/mg (data not shown), which is 2.5- to 3.5-fold lower than the 56.9 μmol/min/mg determined for the near-homogeneous native potato AGPase (27). SDS/PAGE of the partially purified protein showed a dramatic increase in a broad band migrating in the 50–52 kDa range. Immunoblot analysis showed that only the large band migrating in 50–52 kDa range cross reacted with monospecific IgG against the recombinant potato AGPase LS or SS (data not shown).

Kinetic analysis of R4 revealed an AGPase with similar K_m values for G-1-P, ATP, and Mg^{2+} when compared with the previous values determined for the wt recombinant (pML10/pMON 17336) (Table 1). R4 AGPase required 91 μM 3-PGA to achieve one-half of the maximum activity ($A_{0.5}$), which was a 49-fold increase in affinity from the 4.5 mM $A_{0.5}$ determined for the P52L mutant (15). This increase in 3-PGA affinity is ≈two-fold greater than that exhibited by the wt recombinant enzyme (15). Inhibitor studies with R4 AGPase revealed that the Pi interaction also was altered in R4 (Table 2). At 0.25 mM 3-PGA, the concentration of Pi needed to achieve 50% inhibition ($I_{0.5}$) was 8 and 13.5-fold higher than for the native potato tuber and wt recombinant enzymes, respectively (Table 2). Thus, the addition of a P66L mutation to the P52L already on the LS not only increased the enzyme's affinity for the activator 3-PGA but also significantly decreased the enzyme's sensitivity to the inhibitor Pi.

Table 1. Kinetic parameters of partially purified AGPase from R4, R20, and R32 genetic revertants compared to the P52L and WT recombinant

	ATP, K_m	Glc 1-P, K_m	$MgCl_2$, K_m	3-PGA, $A_{0.5}$
WT	0.12	0.04	2.00	0.160
P52L	0.34	0.30	2.57	4.5
R4	0.13 (.03)	0.15 (.05)	1.88 (.4)	0.09 (.01)
R20	0.17 (.01)	0.08 (.04)	2.70 (.80)	0.12 (.05)
R32	0.12 (.01)	0.11 (.01)	1.36 (.38)	0.41 (.19)
Up-Reg-1	0.10 (.01)	0.11 (.04)	2.24 (.40)	0.002 (.0001)

Values in mM concentration are the average of at least two assays. Standard deviation for each measured value is in parenthesis.

Table 2. Pi Inhibition constants ($I_{0.5}$) of R4, R20, and R32 genetic revertants and the WT recombinant, and P52L enzymes

3-PGA, mM	wt	P52L	R4	R20	R32	Up-Reg-1
				$I_{0.5}$, mM		
0.025	n.d.	n.d.	n.d.	n.d.	n.d.	0.6 (0.2)
0.10	n.d.	0.68	n.d.	n.d.	n.d.	n.d.
0.125	n.d.	n.d.	n.d.	0.21 (.04)	n.d.	3.1 (0.1)
0.25	0.07	n.d.	1.20 (.35)	0.57 (.12)	0.32 (.11)	4.7 (1.0)
0.50	0.15	n.d.	1.38 (.25)	0.70 (.28)	0.58 (.11)	n.d.
2.5	n.d.	0.84	n.d.	n.d.	n.d.	n.d.
3.00	0.63	n.d.	13.0 (4.0)	n.d.	1.04 (.40)	n.d.
4.5	n.d.	2.5	n.d.	n.d.	n.d.	n.d.

Values (in mM) are the average of at least two assays. n.d., not determined.

Kinetic analysis of R20 AGPase showed that the K_m values for substrates and cofactor were identical to the wt recombinant enzyme (Table 1). The affinity for 3-PGA was increased 38-fold compared with the P52L mutant, which gave an $A_{0.5}$ of 117 μM (Table 1). The R20 AGPase was less sensitive to Pi, but the effect was not as great as with R4 (Table 2). The $I_{0.5}$ value determined for R20 AGPase at 0.25 mM 3-PGA was 4.5- and 8-fold higher than the value determined for the native potato and wt recombinant enzymes, respectively. The addition of a positive charge in this region of the enzyme appears to give R20 AGPase an enhance capacity to bind 3-PGA while decreasing the enzyme's affinity for the inhibitor Pi.

Substrate and cofactor-binding constants also were relatively unaffected for R32 AGPase (Table 1). R32 was more sensitive to 3-PGA activation, showing an $A_{0.5}$ of 414 μM, an 11-fold increase over the P52L mutant (15). Pi sensitivity also was lower in R32 AGPase (Table 2), but the $I_{0.5}$ values for this second-site revertants were more similar to the values determined for the wt recombinant enzyme than for the other second-site revertants. Thus, substitution of the polar amino acid Asn for the nonpolar Gly restored 3-PGA sensitivity in R32 whereas only slightly changing the enzyme's affinity for Pi.

Site-Directed Mutagenesis. The second-site mutations in the revertants might be acting by reversing the effect of the P52L mutation in an allele-specific way or they might have a phenotype that compensates for the binding defect in P52L. To determine the independent effect of amino acid replacements at positions 38 or 101 on AGPase activity, the wt potato AGPase LS was altered at these positions by site-directed mutagenesis. When coexpressed with the wt SS, the E38K site-directed mutant (specific activity of 19.7 μmol/min/mg) was unchanged in its affinity for the substrates and cofactors compared with R20 (Table 1), but its activation by 3-PGA was dramatically different. The E38K enzyme had an $A_{0.5}$ of 2 μM, a 58.5- and 80-fold increase in affinity for 3-PGA compared with R20 and wt recombinant, respectively (Table 1, Fig. 2). The difference in 3-PGA sensitivity of the P52L and E38K mutants was much larger with a factor of 2,250. It therefore appears that the E38K mutation, which increases the affinity for PGA, actually compensates for the negative effect caused by the P52L mutation. Inhibition studies showed that the E38K mutant was also less sensitive to Pi exhibiting $I_{0.5}$ values of 0.6, 3.1, and 4.7 mM in the presence of 25, 125, and 250 μM 3-PGA, respectively (Fig. 3). The E38K mutant has a 39- and 67-fold lower affinity for the inhibitor than the native potato and wt recombinant enzymes. Because the E38K mutation alone creates an up-regulated form of the enzyme by significantly

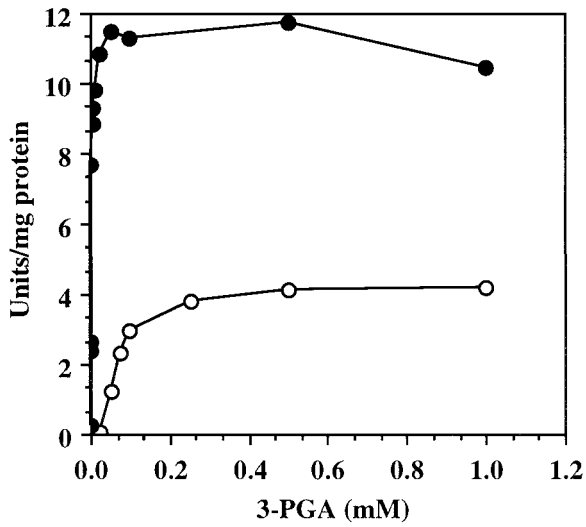

FIG. 2. 3-PGA activation curve of (○) wt recombinant (15) and (●) UpReg-1.

increasing the affinity for the activator while decreasing the affinity for the inhibitor, we henceforth referred to it as UpReg-1. Phenotypically, colonies of the UpReg-1 mutant coexpressed with wt SS stain significantly darker on glucose media compared with the wt recombinant and R20 (Fig. 4).

Additional site-directed mutagenesis of Glu-38 was conducted. An Ala residue is highly conserved at the analogous position on the SS. Substitution of Ala for Glu at position 38 generates an enzyme insensitive to activation by 3-PGA (Fig. 5). In *E. coli* there is an Arg residue at the analogous position. Introduction of an E38R mutation also generated an enzyme whose activity is not activated by low concentrations of 3-PGA (Fig. 5). An AGPase activity comparable with the wt recombinant enzyme, however, can be achieved with the E38A and E38R mutant enzymes at very high concentrations (50 mM) of 3-PGA (data not shown). The difference between the E38K and E38R enzyme activities at low concentrations of 3-PGA indicates that activation is not strictly charge dependent but also constrained by R-group size. The 3-PGA insensitive E38A and E38R mutants will provide new starting material for new

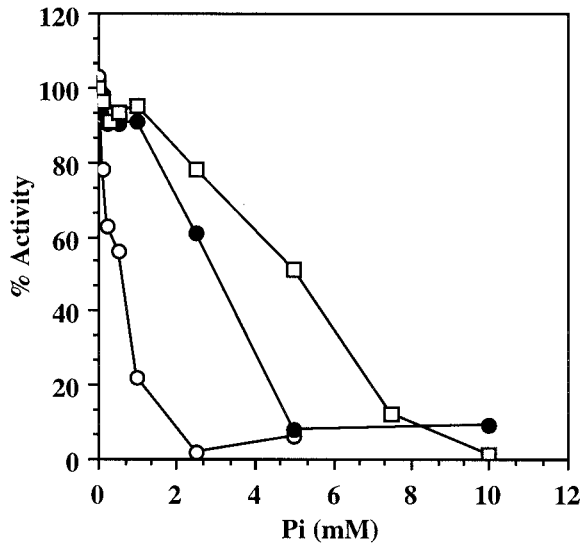

FIG. 3. Pi inhibition curves of UpReg-1 at (○) 25, (●) 125, and (□) 250 μM 3-PGA.

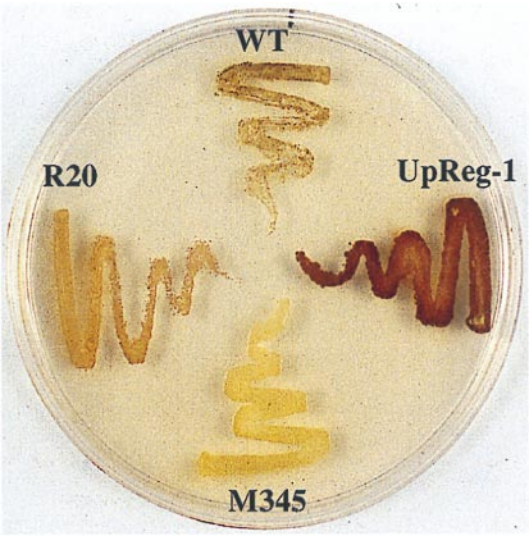

FIG. 4. I_2 staining of UpReg-1 cells compared with the wt recombinant and R20. The glycogen-deficient mutant line M345, which contains the P52L mutation on the LS is included for a negative control. Note the increase I_2 staining and, in turn, increase glycogen levels, exhibited by UpReg-1 cells compared with wt and R20 cells.

mutagenesis experiments to identify additional residues important for 3-PGA interaction.

Like the UpReg-1 mutant, replacement of Gly-101 by Asn generated a variant enzyme, UpReg-2, with up-regulated allosteric properties. 3-PGA activation studies of the partially purified enzyme (specific activity of 9.1 μmol/min/mg) yielded an $A_{0.5}$ of 0.035 mM, an 11.8- and 4.6-fold enhancement over the parental revertant R32 and wt (Table 1). Although the increase in affinity for 3-PGA was less pronounced for UpReg-2 than for UpReg-1, the response to Pi was more dramatic for UpReg-2 than for UpReg-1. Pi inhibition studies in the presence of 0.1 and 0.001 mM 3-PGA revealed $I_{0.5}$ constants of 6.2 and 1.3 mM, respectively. Hence, UpReg-2 shows a higher resistance to Pi inhibition than UpReg-1. In addition to altered allosteric properties, kinetic analysis revealed that UpReg-2 had a lower affinity for the substrate G-1-P (Table 1). This change in G-1-P binding

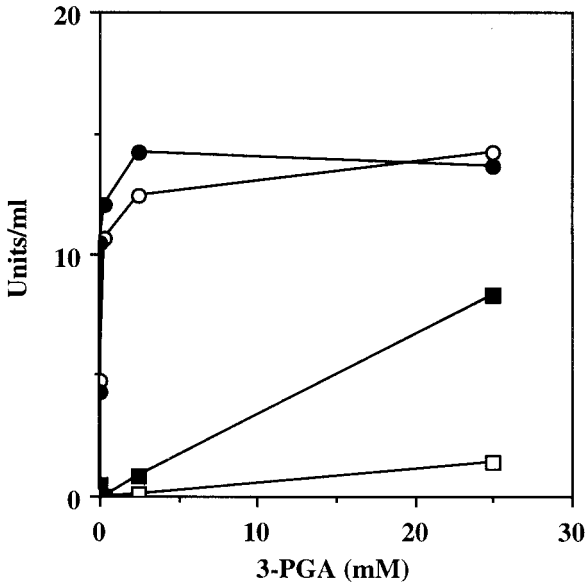

FIG. 5. 3-PGA activation of (○) wt recombinant and site-directed mutants (●) UpReg-1 (E38K), (□) E38A, and (■) E38R.

affinity is consistent with the view that the G101N mutation is close to a putative G-1-P-binding site (reviewed in ref. 24). These results also indicate that the LS has a role in substrate binding.

DISCUSSION

Starch is the major storage form of carbohydrate in many plants (8, 29). The initial reaction in the biosynthesis of this storage polymer is catalyzed by AGPase, and the observed allosteric properties of AGPase identifies it as a potential site for manipulating starch biosynthesis in plants (1, 3, 8). Precedence for this view is supported by the increased starch in transgenic potato tubers that express an allosterically altered AGPase from *E. coli* (30, 31) and more recently in maize endosperm in which a germinal revertant of *Sh2*, *Rev 6*, conditioned an 11–18% increase in seed weight (32). Given the general public distrust of genetically engineered food products, manipulation of starch levels in plants by the expression of a modified plant gene may prove to be more commercially viable. We present here the isolation and characterization of up-regulated forms of potato AGPase by using a reversion genetics approach.

The P52L mutant of the potato AGPase LS (15), which requires high levels of 3-PGA for activation, was the starting point for additional mutagenesis experiments to study 3-PGA activation of AGPase. Expression of mutagenized P52L plasmid DNA in *E. coli* together with the SS enabled us to use I_2 staining to identify AGPase LS second-site revertants that restored glycogen production. Eight second-site revertants were isolated that varied in their ability to restore glycogen production. The four class I genetic revertants exhibited an I_2-staining phenotype comparable to cells expressing the wt recombinant enzyme, whereas the four class II and III genetic revertants showed only partial complementation.

DNA sequencing of the four class I revertants R4, R10, R20, and R32 identified three different second-site mutations. All three of the second-site mutations are close to the original P52L mutation, which gives additional evidence that the amino terminal region interacts with 3-PGA. Further, the close proximity of the identified mutations to the Lys39 residue of *E. coli* further supports the possibility of a conserved activator-binding site between the bacterial and higher plant AGPases.

Sequence analysis of R4 identified a P66L mutation in addition to the original P52L mutation. The second mutation may compensate for the structural changes made to the P52L containing 3-PGA-binding site demonstrated earlier (15). R10 and R32 both contained a G101N mutation, suggesting that the targets for second-site reversions of glycogen production by hydroxylamine may be limited and that less specific mutagenesis techniques may be needed to isolate additional genetic revertants of P52L. The G101N mutation was located approximately 23 amino acids from a Lys identified in activator site 3 of the spinach leaf LS (ref. 3; Fig. 1) and provides additional evidence that this region may be important in binding 3-PGA. Moreover, the lack of conservation of this residue in the SS may indicate this is a unique binding site on the LS. In R20, Glu at position 38 was replaced with a Lys. As positively charged residues such as Lys are important in binding the negatively charged phosphate ester and carboxylate moieties of 3-PGA (31), increasing the positive charge of this region may further increase the ability of AGPase to bind the negatively charged 3-PGA.

Partial purification and kinetic analysis showed that the k*m*s for ATP, G-1-P, and Mg^{2+} were unaffected in each of the second-site revertants. However, the allosteric properties were changed by dramatic increases in the affinity for 3-PGA (Table 1). In addition, each of these revertants showed an increased resistance to Pi inhibition as compared with the recombinant wt enzyme (Table 2). The only other higher plant AGPase altered in its affinity for Pi is the germinal revertant of *Sh2 Rev 6* of maize endosperm (32). This antagonistic effect in allosteric effector response was related to how strongly the mutation reverted the 3-PGA insensitivity of the P52L mutant. For example, R4 is not only the most sensitive to 3-PGA activation but also the least sensitive to Pi inhibition. That binding of both effectors is changed by a single mutation implies that the binding sites for 3-PGA and Pi influence each other.

To determine whether the second-site reversions of R20 and R32 influenced enzyme activity in the absence of the P52L mutation, we performed kinetic analysis of mutant enzymes containing just the E38K or the G101N mutations. UpReg-1 containing the E38K mutation alone or UpReg-2 containing the G101N alone showed an increased sensitivity to 3-PGA activation and decreased sensitivity to Pi inhibition as compared with the double mutants R20 and R10/R32. The positive allosteric effects mediated by either E38K or G101N alone indicate that the up-regulated phenotypes caused by these mutations are partially dominant over the down-regulated effects of P52L. The increase in affinity of E38K for 3-PGA with simultaneous decrease in sensitivity to Pi clearly suggests that the N-terminal site is important in allosteric interaction. Further site-directed mutagenesis and kinetic studies with the E38R mutant revealed that a positively charged moiety by itself is insufficient for the observed up-regulatory properties mediated by E38K.

In potato, a 32-fold increase in AGPase activity by 3 mM 3-PGA is reduced by 50% with the addition of only 0.33 mM Pi (27). The antagonistic effect of Pi on AGPase activity is probably further enhanced at equal molar concentrations of 3-PGA and Pi, which is a closer approximation of *in vivo* concentrations (1). This would indicate that under normal physiological conditions AGPase is acting well below its maximum level of activity. Thus, an obvious choice for manipulating AGPase activity is to alter the enzyme's sensitivity to the allosteric effectors 3-PGA and Pi. The importance of allosterically altered forms of AGPase has already been established in higher plants (30–32). Potato plants expressing an allosteric variant of the bacterial AGPase showed a 25 to 60% increase in starch over control plant tubers. In contrast, expression of a wt bacterial AGPase failed to elevate the starch content in tubers (30, 31). Further, the germinal revertant of *Sh2*, *Rev 6*, that conditioned an 11–18% increase in seed weight was shown to be highly insensitive to Pi inhibition. This result indicates that using allosterically altered forms of AGPase has promise for manipulating starch biosynthesis *in vivo*.

The isolation of UpReg-1 and UpReg-2 represents a novel approach to generate allosteric variants of higher plant AGPases. From a single down-regulated mutant, we have used a bacterial expression system to isolate several unique up-regulated allosteric mutations by combining a rapid and sensitive color screen and random and site-directed mutagenesis. These mutants are some of the first higher plant AGPases altered in Pi sensitivity. Further manipulations similar to those described here may be useful in fine-tuning the sensitivity of AGPase to its allosteric regulators and lead to increased partitioning of photosynthate into starch. The ability of UpReg-1 to manipulate starch biosynthesis in plants currently is being tested.

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