Sh2-UR1: AN ALLOSTERIC MUTANT OF ADPGLUCOSE PYROPHOSPHORYLASE

By

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Our understanding of starch synthesis in plants has benefitted from a broadening base of species studied. Discoveries made in one species may often translate to others. Previous work with potato tuber ADPglucose pyrophosphorylase (AGP), the enzyme responsible for synthesizing the substrate for starch synthesis, showed that a mutation from glutamate to lysine at position 38 in the large subunit of the enzyme increased sensitivity to 3-phosphoglycerate activation and reduced inhibition by phosphate.

Since the enzyme is highly conserved among species, this research tested the effect of this mutation in the maize endosperm AGP. Site-directed mutagenesis was used to change threonine to lysine at position 93 (comparable to the previously mentioned mutation in potato), and the resulting mutant enzyme was partially purified and molecularly and biochemically characterized. The mutation in maize did not affect the enzyme's affinity for substrates or phosphate, the inhibitor. Activation by 3-phosphoglycerate did increase by 27%. This amino acid position appears to be less important to allostery in maize endosperm AGP than it is to allostery in potato tuber AGP.

CHAPTER 1
INTRODUCTION

Starch Synthesis

Starch is the primary energy storage product in higher plants, as well as an important source of nutrition for humans. As such, it is a clear target for genetic engineering efforts to improve the world's food supply. Starch makes up 70% of the dry weight in the maize seed, and increased starch synthesis appears to provide a greater carbon sink in the seed (Giroux *et al*. 1996).

Starch Biosynthesis Pathway

ADPglucose is the glucosyl donor in starch biosynthesis (Recondo and Leloir 1961). In both prokaryotes and photosynthetic eukaryotes, ADPglucose is formed through the reaction:

ATP + alpha-glucose-1-P **<==>** ADPglucose + PPi (Espada 1962).

In higher plants, starch synthase then catalyzes the

addition of glucose from ADPglucose to growing amylose chains thus:

ADPglucose + $(glucosyl)_n$ **-->** ADP + $(glucosyl)_{n+1}$ (Espada 1962).

Amylopectin precursor is likely formed in a similar reaction by an unidentified enzyme. Starch branching enzyme then catalyzes the formation of alpha-1-6 linkages in phytoglycogen. Starch debranching enzyme removes about half the branch-chains in phytoglycogen to form amylopectin (reviewed in Hannah 1997).

Other Effects

Mutations that reduce starch synthesis have also been found to affect zein synthesis (Barbosa and Glover 1978), and in severe starch mutants, zein transcript levels actually increase (Giroux *et al*. 1994) though zein protein decreases (Tsai *et al*. 1978). Impairing synthesis of starch also increases transcription of the starch synthetic genes, depending on the mutation and each individual gene, as well as the tissue's function as source or sink (Giroux *et al*. 1994, Muller-Rober *et al*. 1990). In addition, the changes in sugar levels that can accompany starch reduction may affect signal transduction (Giroux *et al*. 1994), thus

precipitating other changes in the cell and tissue as a whole.

ADPglucose Pyrophosphorylase

ADPglucose pyrophosphorylase (ADP: alpha-D-glucose-1-phosphate adenyltransferase) represents the first unique step in starch synthesis and is important for the regulation of starch biosynthesis in plants and glycogen synthesis in bacteria (reviewed in Greene and Hannah 1998a). Loss of ADPglucose pyrophosphorylase (AGP) activity gives a shrunken, collapsed kernel phenotype in maize endosperm (Mains 1949), indicating lost capacity for starch production. AGP is an allosteric enzyme. Stark *et al.* (1992) have suggested that this allosteric property makes AGP rate-limiting in starch biosynthesis of higher plants, since they found that starch production varied with the allostery of *GlgC*-AGP transgenic potato tubers.

Early work with maize endosperm AGP mutants seemed to indicate the presence of a single form of AGP in maize endosperm, since AGP kinetics varied according to the mutant (Hannah and Nelson 1975, 1976). More recent work conducted by Giroux and Hannah (1994) using null *Bt2* and *Sh2* mutants

(having no detectable protein product) showed that 5 - 20% of wild type activity remains in the absence of a functional *Bt2* and/or *Sh2* gene. Giroux and Hannah (1994) concluded that a second form of AGP, independent of *Bt2* and *Sh2*, must account for this residual activity. These researchers then identified transcripts in the endosperm resembling those of *Agp*1 and *Agp2*, the large and small subunits of maize embryo AGP (Giroux and Hannah 1994).

Other plant tissues have been found to express multiple forms of AGP as well. For example, tomato fruit contains three isoforms of the AGP large subunit and two of the small subunit (Chen and Janes 1997). In *Arabidopsis*, the large subunit is encoded by *Adg2* (Lin *et al*. 1988, Wang *et al*. 1997), and the small subunit is encoded by *Adg1* (Wang, *et al*. 1998). *Adg2* mutants do not completely abolish activity, but retain 5% wild-type AGP activity levels and 40% wild-type starch levels (Lin *et al*. 1988), possibly due to the presence of multiple forms of the enzyme within the cell as in maize endosperm.

AGP Allosteric Properties

AGP is an allosteric enzyme. In higher plants, AGP is activated by 3-phosphoglycerate (3PGA) and inhibited by phosphate (Pi, or PO_4) (Preiss *et al.* 1966a), whereas bacterial AGP is activated by fructose-1,6-bisphosphate (FBP) and inhibited by cAMP (Preiss *et al*. 1966b). Known effector binding sites in bacteria are conserved in all species of bacteria and higher plants (Smith-White and Preiss 1992). Cereal endosperm AGP's are relatively insensitive to activation; at the extreme, wheat endosperm AGP is completely insensitive to 3PGA (reviewed in Preiss *et al*. 1991b). Leaf AGP's are highly sensitive to affector molecules. For example, spinach leaf AGP is activated 58 fold by 3PGA and inhibited 50% by 45 µM Pi (Copeland and Preiss 1981). For many plant species and isozymes, the ratio of 3PGA to Pi is postulated to be the critical regulator of starch accumulation (Singh *et al*. 1984, Kleczkowski 1999).

Allosteric Compensation for Reduced Activity

A study of dosage effects in *sh2* mutants of triploid maize endosperm suggested that AGP's allosteric properties can compensate for a one-third reduction in activity

conditioned by a mutant male crossed onto a wild-type female, since this combination caused no decrease in seed weight. Yet a two-thirds reduction conditioned by a wild-type male crossed onto a mutant female did decrease seed weight (Hannah and Greene 1998).

Identification of Effector Binding Sites

As an allosteric enzyme, AGP has multiple effector binding sites. Studies of amino acids important to enzyme activity are facilitated by the use of pyridoxal-5-phosphate, which covalently binds to lysines in the activator binding site, blocking 3PGA from binding (Preiss *et al*. 1987). The potato tuber large subunit has three lysines with which pyridoxal-5-P reacts, and the small subunit has one such lysine (Morell *et al.* 1988, Smith-White and Preiss 1992). Spinach leaf AGP also has three lysines that bind pyridoxal-5-P (Preiss *et al*. 1995), whereas maize endosperm AGP has only one (Shaw and Hannah 1992). While the number of identified lysines is correlated with 3PGA activation, addition of lysines to maize endosperm AGP at positions analogous to those in spinach AGP does not increase sensitivity to 3PGA (Shaw, personal communication).

Random mutagenesis of the AGP subunits has also helped identify AGP’s effector binding sites. Greene *et al*. (1996b) changed Asp413 to Ala in the potato large subunit

and the resultant mutant required six to ten fold more 3PGA than did the wild-type for maximum activation. Changing Lys417 and Lys455 of the large subunit also decreased 3PGA affinity nine and three fold, respectively (Ballicora *et al*. 1998) (Table 1).

Table 1. AGP large subunit mutations. Amino acid and position listed is maize endosperm AGP site relative to actual mutation where appropriate.

Mutation	Organism/tissue	Effect
T93K	maize endosperm	3PGA activation increased 27% (this thesis)
P99L	potato tuber	3PGA activation decreased 45 fold (Greene *et al.* 1996)
R104T	maize endosperm	temperature stability increased 12% (Greene *et al.* 1998)
A177P	maize endosperm	temperature stability increased (Greene *et al.* 1998)
R217P	maize endosperm	temperature stability decreased in *Sh2-HS33* background (Greene *et al.* 1998)
K265A	potato tuber	no effect on activity; however, mutation of comparable Lys in small subunit (K222, Table I) decreased affinity for G1P (Fu *et al.* 1998)
H333Y	maize endosperm	temperature stability increased 51% (Greene *et al.* 1998)
D400H	maize endosperm	temperature stability increased (Greene *et al.* 1998)
V454I	maize endosperm	temperature stability increased (Greene *et al.* 1998)

Table 1 --Continued

Mutation	Organism/tissue	Effect
T460I	maize endosperm	temperature stability decreased in *Sh2-HS33* background (Greene *et al.* 1998)
D468A	potato tuber	sensitivity to both 3PGA and Pi decreased (Greene *et al.* 1996)
K469A	potato tuber	3PGA activation reduced 10-20 fold (Ballicora *et al.* 1998)
K469E	potato tuber	3PGA activation reduced 10-20 fold (Ballicora *et al.* 1998)
K506A	potato tuber	affinity for 3PGA did not change; affinity for Pi increased 4 fold (Ballicora *et al.* 1998)
K506E	potato tuber	affinity for 3PGA decreased 3 fold; affinity for Pi increased 4 fold (Ballicora *et al.* 1998)

However, mutating Lys404 and Lys441 of the small subunit decreased 3PGA affinity 3000 and 50 fold, respectively, and both mutations decreased Pi affinity 400 fold (Ballicora *et al*. 1998). (Table II) In this case, mutating the small subunit had greater significance for effector binding than did mutating the large subunit (Ballicora *et al*. 1998). These data agree with the suggestion by Greene *et al*. (1996a) that the allosteric domain is formed by interaction of both subunits.

Table 2. AGP small subunit mutations. Amino acid and position listed is maize endosperm AGP site relative to actual mutation where appropriate. FBP, fructose-1,6-bisphosphate.

Mutation	Organism/tissue	Effect
C36A	potato tuber	temperature stability decreased 65% (Ballicora *et al.* 1999)
C36S	potato tuber	temperature stability decreased 65% (Ballicora *et al.* 1999)
K64E	*E. coli*	affinity for FBP decreased (Gardiol and Preiss 1990)
A69T	*E. coli*	affinity for FBP decreased 25 fold; affinity for AMP decreased 7.7 fold (Meyer *et al.* 1993)
R92C	*E. coli*	affinity for FBP increased, affinity for cAMP decreased (Meyer *et al.* 1993)
K222A	potato tuber	affinity for G1P decreased 400 fold (Fu *et al.* 1998)
K222E	potato tuber	affinity for G1P decreased 550 fold (Fu *et al.* 1998)
K222R	potato tuber	affinity for G1P decreased 135 fold (Fu *et al.* 1998)
K296E	*E. coli*	no effect on activity (Kumar *et al.* 1989)
P324S	*E. coli*	affinity for FBP increased, affinity for cAMP decreased (Meyer *et al.* 1998)
G359D	*E. coli*	affinity for FBP increased (Meyer *et al.* 1998)
K428A	potato tuber	affinity for 3PGA decreased 3090 fold; affinity for Pi decreased 650 fold (Ballicora *et al.* 1998)

Table 2 --Continued

Mutation	Organism/tissue	Effect
K465A	potato tuber	affinity for 3PGA decreased 154 fold; affinity for Pi decreased 432 fold (Ballicora *et al.* 1998)
K465E	potato tuber	affinity for 3PGA decreased 191 fold; affinity for Pi decreased 216 fold (Ballicora *et al.* 1998)
K465Q	*Anabaena*	activator specificity changed: AGP activated by FBP rather than 3PGA (Charng *et al.* 1995)
K506A	*Anabaena*	affinity for 3PGA decreased 50 fold (Charng *et al.* 1994)
K506E	*Anabaena*	affinity for 3PGA decreased 140 fold (Charng *et al.* 1994)
K506Q	*Anabaena*	affinity for 3PGA decreased 150 fold (Charng *et al.* 1994)
K506R	*Anabaena*	affinity for 3PGA decreased 25 fold; affinity for Pi decreased 3 fold (Charng *et al.* 1994)

In addition, Joanna Cross (personal communication) expressed an AGP hybrid of the maize endosperm large subunit and potato tuber small subunit in *glgC- E. coli* lacking endogenous glycogen production. The hybrid exhibited reduced activation by 3PGA and intermediate inhibition by Pi compared to parental AGP's (Table 3), indicating the importance of both subunits for allosteric functions.

Table 3. Comparison of maize endosperm, potato tuber, and maize large subunit/potato small subunit hybrid AGP's. $A_{0.5}$ is concentration of 3PGA giving half-maximal activation in the absence of Pi; $I_{0.5}$ is concentration of Pi giving half-maximal inhibition in the absence of 3PGA.

Source	Fold Activation	$A_{0.5}$ (mM)	$I_{0.5}$ (mM)
Maize endosperm	2-6	2.0	3.0
Hybrid	1.6	1.3	0.8
Potato tuber	43	0.045	4.0

Activation by 3PGA

The extent of activation by 3PGA varies among isoforms of AGP. In barley endosperm, 3PGA activates the enzyme only 25 percent in the ADPglucose synthesis reaction, and 10 mM 3PGA actually inhibits activity 45 percent in the pyrophosphorolysis reaction (Kleczkowski *et al*. 1993a). In tomato fruit, AGP is half-activated by 0.2 mM 3PGA, and is almost completely inactive in the absence of 3PGA (Chen and Janes 1997). Potato tuber AGP is also highly activatable by 3PGA (Table 3) (Iglesias *et al.* 1993), as are the leaf AGP's of *H. carnosa* and *X. danguyi*, which are activated 26 fold by 3PGA, and half-maximally activated by 0.11 - 0.25 mM 3PGA (Singh *et al*. 1984). Reports of 3PGA activation of AGP in the maize endosperm typically vary from 2 to 6 fold (Dickinson and Preiss 1969). Maize endosperm AGP's K_m for

glucose-1-phosphate (G1P) in the presence of 3PGA is reportedly half that in the absence of 3PGA (Dickinson and Preiss 1969, Hannah and Nelson 1975).

Interestingly, there may be no physiological relevance of AGP activation by 3PGA outside the chloroplast (reviewed in Hannah and Greene 1998). 3PGA is found in the chloroplast starch synthesis pathway but is not used in starch synthesis in amyloplasts. Therefore, Greene and Hannah (1998a) have suggested that activation by 3PGA may be 'evolutionary baggage.'

Inhibition by Inorganic Phosphate

Phosphate is a strong inhibitor of AGP in higher plants (Ghosh and Preiss 1966). In maize endosperm AGP, 10 mM Pi causes 50% inhibition in the presence of 10 mM 3PGA (Dickinson and Preiss 1969). In barley endosperm AGP, which is relatively insensitive to effector molecules, 24 mM Pi causes 60% inhibition (Kleczkowski *et al*. 1993a). In contrast, potato tuber AGP is inhibited 50% by 0.33 mM Pi in the presence of 3.0 mM 3PGA (Sowokinos and Preiss 1982). However, Iglesias *et al.* (1993) have shown that low levels of 3PGA actually increase inhibition of potato tuber AGP by Pi ($I_{0.5}$ = 83 uM in the presence of 10 uM 3PGA, $I_{0.5}$ = 4 mM in the absence of 3PGA). Similarly, Singh *et al*. (1984) showed

that the presence of 3PGA increases AGP's sensitivity to Pi inhibition ($I_{0.5}$ = 500 uM Pi in the absence of 3PGA or 40 uM Pi in the presence of 0.15 mM 3PGA) in the leaves of *H. carnosa* and *X. danguyi*.

Phosphate appears to increase activator binding site interactions, making the 3PGA activation curve more sigmoidal (Ghosh and Preiss 1966). However, inhibition studies that identified Pi as the primary inhibitor have relied upon *in vitro* assays, and Greene and Hannah (1998a) have suggested that the true inhibitor *in vivo* may actually be a phosphate (or structurally similar) side-group of another molecule.

Expression and Structure of AGP

Expression

The embryo, leaf, and endosperm of maize have different forms of AGP (Preiss *et al.* 1971, Hannah and Nelson 1975, Hannah *et al.* 1976, Fuchs 1977). The maize embryo large subunit is encoded by *Agp1*, and the small subunit encoded by *Agp2* (Giroux and Hannah 1994). The maize leaf AGP small

subunit is encoded by *Agpszmlf* (previously known as *L2*) (Prioul *et al*. 1994).

Giroux and Hannah (1994) suggested that maize endosperm AGP is not localized to plastids since the *Bt2* cDNA does not encode a transit peptide. Denyer *et al*. (1996) later showed that at least 95% of maize endosperm AGP activity is localized to the cytoplasm.

General Structure

AGP in bacteria is a homotetramer, having four identical subunits encoded by the *GlgC* gene. Conversely, AGP in higher plants is a heterotetramer, having two pairs of unlike subunits (Hannah and Nelson 1976, Copeland and Preiss 1981, Morell *et al*. 1987). In maize endosperm, AGP has a molecular mass of 210kDa, with small and large subunits of 51kDa and 54kDa, respectively (Giroux and Hannah 1994). *Sh2* encodes the large subunit and *Bt2* encodes the small subunit (Hannah and Nelson 1976, Bae *et al.* 1990, Bhave *et al.* 1990). These two genes likely arose from a common progenitor, and have diverged until they are complementary rather than duplicate genes (Bhave *et al*. 1990).

AGP small subunits. The AGP small subunit is highly conserved in amino acid structure among maize endosperm,

spinach leaf, and potato tuber AGP's. Thorbjørnsen *et al*. (1996) have shown that the small subunits of barley leaf and endosperm AGP are encoded by a single gene and differ only in their first exon. Thorbjørnsen *et al*. (1996) hypothesized that alternative splicing would account for the different first exons, or the gene could have separate promoters for the two transcripts. In contrast, maize has two separate genes for these transcripts, termed *Bt2* for the endosperm and *Agpszmlf* for the leaf. They differ primarily in their first exon, and most likely are duplicate genes (Janine Shaw, personal communication).

AGP large subunits. In contrast to the small subunit, the sequence of the AGP large subunit varies greatly among source tissues (Smith-White and Preiss 1992). Giroux *et al*. (1996) showed that the large subunit C-terminus is important for wild-type activity levels and allostery in particular in a study of *Ds* insertion/excision in *Sh2*. The *Rev6* mutant, a Tyr-Ser insertion caused by imperfect *Ds* excision near the SH2 C-terminus, conditions an 11-18% increase in seed weight and insensitivity to Pi (30 mM Pi inhibits *Rev6* AGP 7% versus 70% in the wild-type) (Giroux *et al*. 1996).

Proteolysis. Plaxton and Preiss (1987) suggested that BT2 undergoes post-translational modification by proteolysis, and that this affected the allosteric

properties of AGP. However, Hannah *et al*. (1995) later showed that proteolysis of both subunits of maize endosperm AGP occurs over time with no effect on activity or 3PGA activation. Similar results were obtained with the large subunit of barley endosperm AGP, which underwent proteolysis from 60 to 51 kD without affecting specific activity (Kleczkowski *et al*. 1993a).

Subunit Interaction

The yeast two-hybrid system has been used to show that SH2 and BT2 do not form homodimers but interact with each other (Greene and Hannah 1998b). Stability of BT2 and SH2 is dependent upon subunit interaction (Greene and Hannah 1998b). Turnover of both subunits in the endosperm is more rapid in the absence of the other protein (Giroux *et al*. 1994). This was also demonstrated in *Arabidopsis* by Wang *et al.* (1998). Sequences involved in enzyme assembly have been found throughout both subunits (Greene and Hannah 1998b).

Homotetramers in potato tuber. In contrast to maize endosperm AGP, the potato tuber small subunit may form a homotetramer with very low activity (70 fold lower than that of the heterotetramer) (Iglesias *et al.* 1993). High concentrations (4 mM) of 3PGA increase homotetramer activity

to one-third the level of the heterotetramer (Ballicora *et al*. 1995).

Heat stability in maize endosperm AGP. Ninety-six percent of AGP in maize endosperm is heat-labile (Hannah *et al.* 1980), a trait which may have been selected for during cereal evolution (Greene and Hannah 1998c) to funnel resources to the embryo during periods of heat stress. Studies of maize yield have shown that increasing temperature from 22 C to 36 C during kernel development is accompanied by a decrease in seed weight (Singletary *et al.* 1994). Greene and Hannah (1998c) isolated a mutation in maize endosperm AGP, *Sh2-hs33*, which is more stable than the wild-type at elevated temperatures and has higher activity before heat-treatment. Increased heat-stability in this mutant was conditioned by a single His to Tyr change at amino acid position 333 in the large subunit (Table I), a change found repeatedly in mutagenesis experiments for heat-stability (Greene and Hannah 1998c).

Up-Reg1: An Allosteric Mutant of AGP

Up-Reg1 in *S. tuberosum*

Greene *et al*. (1996a) used hydroxylamine to mutagenize the large subunit of potato tuber AGP expressed in -*glgC E. coli* lacking endogenous glycogen production and selected mutants deficient in glycogen synthesis. Among these, they discovered a point mutation (P52L) that decreased AGP's affinity for 3PGA 45 fold (Greene *et al*. 1996a).

Greene *et al*. (1998) then used the hydroxylamine procedure to isolate second-site revertants that restored glycogen synthesis. Among several mutants, E38K conditioned the greatest increase in glycogen synthesis. Greene *et al*. (1998) placed this revertant (*Up-reg1*, or *UR1*) into the wild type potato large subunit, and expressed it with the potato small subunit in *glgC- E. coli* lacking endogenous glycogen production. *UR1* in a wild type background stained significantly darker than the wild-type when exposed to iodine vapors (Greene *et al*. 1998). The mutant enzyme was then partially purified by ammonium sulfate precipitation, heat treatment, C3 chromatography, and DEAE anion-exchange chromatography (Greene *et al*. 1998), and its kinetic properties were assayed. *UR1* had 80 fold higher affinity

for 3PGA than did the wild type enzyme and was 67 fold less sensitive to Pi inhibition. The K_m for substrates was unaffected (Greene *et al*. 1998). Also, an E38R mutant gave different activation properties from both wild-type and *UR1*, indicating that activation is dependent upon R-group charge and size (Greene *et al*. 1998).

Proposal

Based on the results obtained in potato with *UR1* and on the high degree of conservation between AGP's of various plant species including potato and maize, *UR1* could have a similar effect in maize endosperm AGP. Increasing AGP's sensitivity to 3PGA and decreasing its affinity for Pi would be significant for the ongoing efforts to improve the quality and yield of starchy maize.

Accordingly, site-directed mutagenesis was used to mutate the *Sh2* cDNA at position 93, analogous to E38 in the potato large subunit, from a Thr to a Lys (T93K). The mutated plasmid (*Sh2-UR1*) was coexpressed with *Bt2* in *glgC-E. coli* lacking endogenous glycogen production and exposed to iodine vapors to measure glycogen production qualitatively. The mutant was then partially purified and

its kinetics were characterized, with particular attention to changes in allostery.

Based on the results of these experiments, *Sh2-UR1* in maize endosperm AGP conditions a 27% increase in maximal activation by 3PGA and no apparent change in $A_{0.5}$ or Pi inhibition.

CHAPTER 2
MATERIALS AND METHODS

Mutant Generation and Screening

Site-directed Mutagenesis

The Clontech Transformer site-directed mutagenesis kit (catalog number K1600-1) was used to change threonine 93 in the *Sh2* cDNA to lysine in order to generate the *Sh2-UR1* mutant. 2.0 ul Annealing buffer (0.2 M Tris-HCL [pH 7.5], 0.1 M $MgCl_2$, 0.5 M NaCl), 2.0 ul *Sh2* plasmid DNA (Monsanto vector pMON17336 bearing the *Sh2* cDNA [Giroux et al., 1996], 50 ng/ul), 2.0 ul selection primer (removes a unique SstI site to allow for selection of the mutant plasmid by restriction digestion to linearize parental DNA, sequence pGGGTCTGTCATATAGTGAGCACGGTACCCGGGG, obtained from GibcoBRL, 50 ng/ul), 2.0 ul mutagenic primer (introduces the mutation by a two-base pair change in the primer, sequence pGGGCGGAGGCAAGGGATCTCAGCTCTTTCC, obtained from GibcoBRL, 50 ng/ul), and 12.0 ul dH_2O were added to a 0.5 ml

microcentrifuge tube. The mixture was centrifuged briefly, boiled 3 minutes, chilled on ice immediately for five minutes, then centrifuged briefly again.

The mutant strand was then synthesized by adding 3.0 ul 10X synthesis buffer (A, T, C, and G nucleotide mixture in 10mM Tris-HCl, pH 8.0), 1.0 ul T4 DNA polymerase (2-4 U/µl), 1.0 µl T4 DNA ligase (4-6 U/µl), and 5.0 µl dH_2O to the above reaction. The reaction was mixed and centrifuged briefly, then incubated two hours at 37 C. The mixture was incubated five minutes at 70 C to stop the reaction, then cooled to room temperature.

The newly-mutated plasmid was then digested with *SstI* to linearize parental DNA by adding 3.0 ul *SstI* restriction endonuclease (10U/ul, GibcoBRL catalog number 15222-011) to the above reaction. The mixture was incubated at 37 C for two hours. The mutant plasmid was separated from parental DNA on a 0.8% agarose minigel. The bands representing non-linearized plasmid DNA were eluted from the gel. To ethanol-precipitate the plasmid DNA, 1/10 volume 3.0 M sodium acetate was added to a microcentrifuge tube containing the eluted plasmid DNA, and the mixture was vortexed. Then two volumes 100% ice-cold ethanol was added, and the mixture was vortexed again and frozen for 30 minutes at -80 C. The plasmid was thawed for five minutes at 37 C,

centrifuged five minutes at 12,000 rpm in an Eppendorf microcentrifuge (catalog number 05-400-10), and the supernatant was discarded. Ethanol (400 ul 70%) was added to the tube, mixed by inversion, and centrifuged five minutes at 12,000 rpm in an Eppendorf 5415-C microcentrifuge. The supernatant was discarded, and the DNA pellet was resuspended in 5 ul TE buffer (10mM Tris-HCl, 1mM EDTA, pH 8.0).

To amplify the *Sh2-UR1* plasmid (Monsanto vector pMON17336 bearing the *Sh2* cDNA with the T93K mutation), *mutS E. coli* (deficient in DNA repair to prevent reversion of the mutant plasmid, provided in Transformer site-directed mutagenesis kit) was transformed with the *Sh2-UR1* plasmid by adding 40 ul electrocompetent *mutS E. coli* cells (Clontech, catalog number C-2020-1) and 5 ul (about 100 ng) *Sh2-UR1* plasmid DNA to an electroporation cuvette. The mixture was shaken to the bottom of the cuvette, inserted into a Bio-Rad *E. coli* Pulser electroporator (catalog number 165-2101) and electroporated at 2.5 V. The transformed cells were then used to inoculate 800 ul liquid Luria broth medium (10 g tryptone, 5 g yeast extract, 5 g NaCl per liter) in a 14 ml Falcon tube (Fisher Scientific, catalog number 35-2059). The cells were incubated one hour in a 37C shaker at 220 rpm. This culture was then used to inoculate 4 ml Luria

broth containing 100 ug/ml spectinomycin and cultured overnight in a 37C shaker at 220 rpm.

The *Sh2-UR1* plasmid was isolated from the *mutS E. coli* using a 5Prime-3Prime PerfectPrep plasmid DNA miniprep kit (catalog number 1-323085). The isolated plasmid DNA was then digested with *SstI* again to linearize remaining parental DNA. 5.0 ul *Sh2-UR1* plasmid DNA (about 100 ng), 2.0 ul 10X restriction enzyme buffer #2 (GibcoBRL, catalog number 16302-010), 2.0 ul *SstI* restriction endonuclease (10 U/ul), and 11.0 ul dH_2O were added to a 0.5 ml microcentrifuge tube. The reaction was incubated three hours at 37 C, with 1.0 ul fresh SstI (10U/ul) added after the second hour. The digested *Sh2-UR1* plasmid DNA was then ethanol-precipitated (as above) to stop the reaction and to remove salts for electroporation.

AC70R1-504 *E. coli* cells (a mutant *glgC* strain lacking endogenous ADPglucose pyrophosphorylase activity) expressing the pMON17335 vector containing wild-type (WT) *Bt2* were transformed with the pMON17336 vector containing *Sh2-UR1*. Electrocompetent AC70R1-504 cells (40 ul, O.D.=0.6) and 5 ul *Sh2-UR1* plasmid (20 ng/ul) were added to an electroporation cuvette, and the cuvette was subjected to 2.5 V current in a Bio-Rad *E. coli* Pulser electroporator (Bio-Rad, catalog number 165-2101). The transformed cells were then used to

inoculate 800 ul liquid Luria broth medium in a 14 ml Falcon tube. The cells were incubated one hour in a 37 C shaker at 220 rpm. 100 ul of this culture was plated out on selective solid Luria broth containing spectinomycin (100 ug/ml) and kanamycin (75 ug/ml). The cells were cultured overnight at 37 C, and individual colonies were selected for analysis.

Glycogen Staining

Single isolated colonies of AC70R1-504 *E. coli* expressing pMON17335 and pMON17336 plasmids containing *Bt2* and *Sh2-UR1* cDNA's, respectively, were plated out on solid Luria broth containing glucose (0.1%), spectinomycin (100 ug/ml), and kanamycin (75 ug/ml), concurrently with cells expressing wild-type maize endosperm AGP. The cells were cultured overnight at 37 C, then stored at 4 C for one hour. Speed and intensity of glycogen staining after exposure to iodine vapors for 60 seconds were monitored and compared.

Sequencing to verify point mutations

The vector pMON17336 containing *Sh2* cDNA that had undergone site-specific mutagenesis to introduce the T93K mutation was isolated from AC70R1-504 *E. coli* using the 5Prime-3Prime Perfectprep plasmid miniprep kit. The isolated plasmid DNA was then sent to the University of Florida/Interdisciplinary Center for Biotechnology Research

sequencing core at the University of Florida. Both strands of the gene were sequenced to verify the presence of the mutation.

Purification of AGP

Culture and Induction of AGP

Cells from a glycerol stock (stored at -80 C) of AC70R1-504 *E.coli* expressing the pMON17335 plasmid containing *Bt2* and pMON17336 containing either WT *Sh2* or *Sh2-UR1* were plated on solid Luria broth containing spectinomycin (100 ug/ml) and kanamycin (75 ug/ml) and cultured overnight at 37 C. A single colony was selected to inoculate 20 ml liquid Luria broth containing spectinomycin (100 ug/ml) and kanamycin (75 ug/ml), then the culture was grown overnight in a 37 C shaker at 250 rpm. 10 ml of this culture was used to inoculate 1000 ml liquid Luria broth containing spectinomycin (100 ug/ml) and kanamycin (75 ug/ml). The one-liter culture was divided into two-500 ml cultures in 2 l flasks and grown in a 37 C shaker at 250 rpm to an optical density of 0.5 - 0.6. Synthesis of AGP was

induced for eight hours by the addition of 0.2 mM isopropyl beta-D-thiogalactoside (25.4 mg/500 ml) and 25 ug/ml nalidixic acid (12.5 mg/500 ml) to the cell cultures, and incubation at 24 C. The cells were then harvested by centrifugation (five minutes at 2300 x g) in 500 ml centrifugation tubes and stored at -80 C for protein extraction.

Extraction of Protein from Cells

Frozen pellets of AC70R1-504 *E.coli* expressing the pMON17335 plasmid containing *Bt2* along with pMON17336 containing either WT *Sh2* or *Sh2-UR1* were resuspended in 5 ml (total) of sucrose buffer (50 mM HEPES, 10 mM KPi, 5 mM $MgCl_2$, 5 mM EDTA, 20% sucrose, and 30% ammonium sulfate; dithiothreitol (DTT, 1 mM), 50 ug/ml lysozyme, 1 ug/ml pepstatin, 1 ug/ml leupeptin, 1 ug/ml phenylmethylsulfonyl fluoride, 10 ug/ml chymostatin, 1 ug/ml antipain, and 1 ug/ml benzamidine were added just before use). The cell suspension was combined in one 14 ml Falcon tube and then centrifuged five minutes at 3600 x g to re-pellet the cells for sonication. The re-pelleted cells were sonicated in three ten-second bursts by a Branson Sonifier 450 at setting 3.5. Lysed cells were cooled on a slurry of ice for at least one minute between bursts to prevent protein denaturation. The lysed cells were centrifuged 10 minutes

at 22,000 x g and 0 C to clarify the crude extract, and the supernatant was placed on ice. The cell pellet was resuspended in 2 ml sucrose buffer, and the sonication-centrifugation procedure was repeated as above. The supernatants were then combined for purification.

Separation by Hydrophobicity

Crude extract was immediately purified by a Pharmacia FPLC superose 10 mm x 10 cm hydrophobic column (catalog number 17-0530-01) using a step gradient of 1.0 M, 0.6 M, 0.3 M, and 0.0 M ammonium sulfate created by changing the ratio of buffer A (50 mM HEPES pH 7.5, 0.1 mM EDTA, 5 mM $MgCl_{2,}$ 20% sucrose, and 1.0 M ammonium sulfate) to buffer B (50 mM HEPES pH 7.5, 0.1 mM EDTA, 5 mM $MgCl_{2,}$ and 20% sucrose). All solutions were filtered at 0.45 µm before use with columns, and fresh DTT was added to 1 mM before use.

The pumps were primed with buffer A, and the column was equilibrated with the same buffer. The sample (8 ml) was filtered through a 0.45 um Acrodisc filter (Gelman Sciences, catalog number 4184), then loaded onto the hydrophobic column by use of a superloop. Fractions were collected in 50 ml Oakridge tubes (Fisher Scientific, catalog number 05-529C) at 1.0 M (18 ml), 0.6 M (11 ml), 0.3 M (7 ml) and 0.0 M (3 ml) ammonium sulfate. A pyrophosphorylysis assay (described in the next section) was immediately performed to

determine which fraction contained the greatest activity per ml of AGP, and that fraction was concentrated to 2 ml in an Amicon Centriplus concentrator (catalog number 4412). The partially purified enzyme was quickly separated into 50 ul aliquots and immediately frozen at -80 C. Bradford assays (described below) were used to determine protein concentrations at each step of extraction and purification.

Bradford Assay to Measure Protein Concentration

Enzyme samples were diluted 5, 10, or 20 fold with 0.15 M NaCl to a final volume of 20 ul, then added to a 0.5 ml microcentrifuge tube. 20 ul samples of 25, 50, 75, or 100 ug/ml Bovine Serum Albumin (BSA) in 0.15 M NaCl, and a blank with 20 ul 0.15 M NaCl were used as standards. 180 ul Coomassie blue (5 mg Coomassie brilliant blue G-250 [Kodak, catalog number 14360], 2.5 ml 95% ethanol, 5 ml 85% phosphoric acid, bring to 50 ml with dH_2O) was then added to each microcentrifuge tube. The mixture was vortexed and stored two minutes at 24 C. The A_{595} of each sample was measured using a Beckman DU-68 spectrophotometer.

Assays of Enzyme Activity

Pyrophosphorolysis Assay

The pyrophosphorolysis reaction mixture (0.08 M Glycyl-glycyl, 5 mM DTT, 5 mM $MgCl_2$, 10 mM NaF, 1 mM ADPglucose, 10 mM 3PGA, 0.4 mg/ml BSA, and 1.5 mM NaPPi) was added to a 1.5 ml Eppendorf microcentrifuge tube (Fisher Scientific, catalog number 05-406-15). Then an amount of $Na^{32}PPi$ (4.4 Ci/mmol, 4.9 mCi/ml) needed to give 5 x 10^6 cpm per reaction was added. The reaction mixture was then aliquotted (approximately 85 ul) to individual 1.5 ml Eppendorf microcentrifuge tubes, and dH_2O was added to a final volume of 245 ul (usually 150-160 ul per reaction). 5 ul enzyme diluted five-fold with dH_2O was added to each tube, and the reaction was mixed by tapping gently with a finger. The reaction mixtures were then incubated for 10 minutes at 37 C, after which 1 ml 5 % TCA, 10 mM NaPPi was added to stop the reaction. 150 ul activated 15 % charcoal solution was added to each reaction to bind the radioactive product. The mixture was centrifuged five seconds at 14,000 rpm in an Eppendorf microcentrifuge to pellet the charcoal, and the supernatant was aspirated. The charcoal was resuspend in 1 ml 5 % TCA, 10 mM NaPPi and mixed by vortexing, then finger-vortexing. This centrifugation / aspiration / resuspension

procedure was repeated twice. 1 ml of 1.0 M HCl was added to each tube, mixed, and boiled five minutes to release radioactive ATP from the charcoal. The mixture was then centrifuged 10 seconds to clarify the supernatant. 500 ul samples of the supernatant were collected in vials of 5 ml ScintiSafe Plus 50% scintillation fluid (Fisher Scientific, catalog number SX25-5), and the radioactivity measured in a Rackbeta 1214 liquid scintillation counter. Background cpm was determined by assaying AGP without ADPglucose (substrate); total counts were determined by assaying AGP with no ADPglucose and no wash step.

ADPglucose-Synthesis Assay

The reaction mixture (80 mM HEPES buffer, 2 mM glucose-1-phosphate [G1P], 4 mM $MgCl_2$, 0.5 mg/ml BSA, 8.6 uM alpha-[^{14}C]-G1P [233.9 mCi/mmol, 0.02 mCi/ml], 1.5 mM ATP, 10 mM 3PGA) was divided into 85 ul aliquots in 0.5 ml microcentrifuge tubes. 10 ul enzyme diluted five fold (80 fold for K_m determination) was added to each tube. Reactions were incubated at 37 C for 30 minutes, boiled for two minutes, and cooled to room temperature. Then 0.6 U (5 ul) bacterial alkaline phosphatase (Worthington, catalog number LS004081), diluted five fold with dH_2O, was added to each reaction tube, and the reactions were incubated at 37 C for 1+ hours or overnight to dephosphorylate remaining G1P.

20 ul of each reaction was then spotted onto labeled squares of DE81 filter paper (Fisher Scientific, catalog number 05-7171B) and washed by placing the squares in a sieve and dunking the sieve in dH_2O 16 times, then discarding the dH_2O and adding fresh dH_2O. The wash procedure was repeated three times. Water from the first wash was discarded in ^{14}C waste. The DE81 filters were removed from the sieve, dried under a heat lamp for 20 minutes, then placed in scintillation vials with 5 ml ScintiSafe Plus 50% scintillation fluid. Radioactivity was measured in a Rackbeta 1214 liquid scintillation counter.

Enzyme Kinetics

The ADPglucose-synthesis assay was used to measure the kinetic properties of partialy purified WT and *Sh2-UR1* AGP. Unless noted, Pi was not added to the reactions.

Dilution Series

To determine the linearity of activity, the enzyme was diluted with dH_2O zero, five, 10, 20, and 40 fold in series and assayed. The five fold dilution was selected for subsequent assays.

E x T Assay

A series of reactions of *Sh2-UR1* were run at 15, 30, and 60 minutes and zero, five, and 10 fold dilution of AGP with dH_2O to determine the linearity of activity at each incubation time/dilution combination. Five fold dilution and 30 minutes incubation was selected for subsequent assays.

Activation by 3PGA

AGP was assayed with varying amounts of 3PGA to determine $A_{0.5}$ and maximal activation by 3PGA. Concentrations of 3PGA used were zero, 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, 10.0, and 20.0mM.

Inhibition by Pi

Likewise, Pi concentrations of zero, 1.0, 2.0, 3.0, 4.0, 5.0, 10.0, and 20.0 mM were used to determine $I_{0.5}$. 10 mM 3PGA was added to each reaction.

Measurement of K_m

G1P concentrations of 8.6, 18.6, 28.6, 38.6, 48.6, 58.6, 108.6, 208.6, and 408.6 uM were used for K_m determinations. 10 mM 3PGA was added to each reaction.

CHAPTER 3
RESULTS

Initial Screening and Purification

Glycogen Staining

AC70R1-504 *glgC- E. coli* bacteria expressing the pMON17336 vector containing *Sh2-UR1* and the pMON17335 vector containing *Bt2* were cultured on solid Luria broth medium containing 0.1% glucose, then stained with iodine vapors and compared to AC70R1-504 *glgC- E. coli* expressing wild-type maize endosperm AGP. Differences in staining intensity were not detected. Subsequent sequencing verified the expected nucleotide change (CT to AG at nucleotides 364-5 of *Sh2* cDNA).

Purification

Specific activity for the wild-type was 2.8 U/mg, while that for *Sh2-UR1* was 1.1 U/mg, where U = 1 µmol ADPglucose formed/min. The wild-type had 4.2 U total activity after

purification and *Sh2-UR1* had 1.7 U. The wild-type enzyme was purified 16.5 fold, while the *Sh2-UR1* mutant was purified 9.4 fold. Final recovery of AGP activity as determined by pyrophosphorolysis assay was 200% for the wild-type and 70.8% for *Sh2-UR1*. Bradford assays performed on the partially purified enzyme indicated the protein concentration of wild-type was 1.0 mg/ml (Table 4), and that of *Sh2-UR1* was 0.62 mg/ml (Table 5).

Table 4. Purification of wild-type AGP. U = µmol ATP formed/min. Crude = crude extract; Filtered = filtered extract; FPLC = 0.0 M ammonium sulfate fraction from hydrophobic column; Concentrated = concentrated hydrophobic fraction (partially purified enzyme).

	Crude	Filtered	FPLC	Concentrated
activity (U)	2.1	1.7	0.32	4.2
yield (%)	100	81	15.2	200
protein (mg)	12.2	7.8	1.5	1.5
specific activity (U/mg)	0.17	0.22	0.21	2.8
purification fold	1	1.3	1.2	16.5

Table 5. Purification of *Sh2-UR1* AGP. See Table 4 for definitions.

	Crude	Filtered	FPLC	Concentrated
activity (U)	2.4	1.2	0.11	1.7
yield (%)	100	50	4.6	70.8
protein (mg)	20.4	13.6	1.5	1.5
specific activity (U/mg)	0.12	0.09	0.07	1.13
purification fold	1.0	0.75	0.58	9.4

E x T Determination

Sh2-UR1 was assayed at varying enzyme dilutions and incubation times to select a dilution/incubation combination in the linear range of increasing activity over time, at adequate levels of activity for measurement. (Table 6) The five fold dilution was selected at 30 minutes incubation time.

Table 6. AGP activity as E x T determination. U = 1 nmol ADPglucose formed. *Adjusted for fold dilution and incubation time.

Dilution (fold)	Time (min)	Activity (U)	E x T (U)*
5	15	2.9	11.6
5	30	4.0	8.0
5	60	5.8	5.8
10	15	1.6	12.5
10	30	2.9	11.4
10	60	5.5	11.0
20	15	0.7	10.8
20	30	1.5	11.9
20	60	1.7	6.8

Kinetic Properties

K_m Determination

Wild-type and *Sh2-UR1* AGP's were assayed with a series of substrate concentrations (Figure 1). Based on a Hanes-Woolf plot (Figure 2), the K_m for wild-type was 3.9×10^{-5} M and that for *Sh2-UR1* was 3.7×10^{-5} M in the presence of 10 mM 3PGA. The previously published K_m for maize is 5×10^{-5} M in the presence of 10 mM 3PGA (Dickinson and Preiss 1969). Hill plots of the two genotypes (Figure 3) showed little or

no cooperativity for either the wild-type (n=1.2) or *Sh2-UR1* (n=1.0).

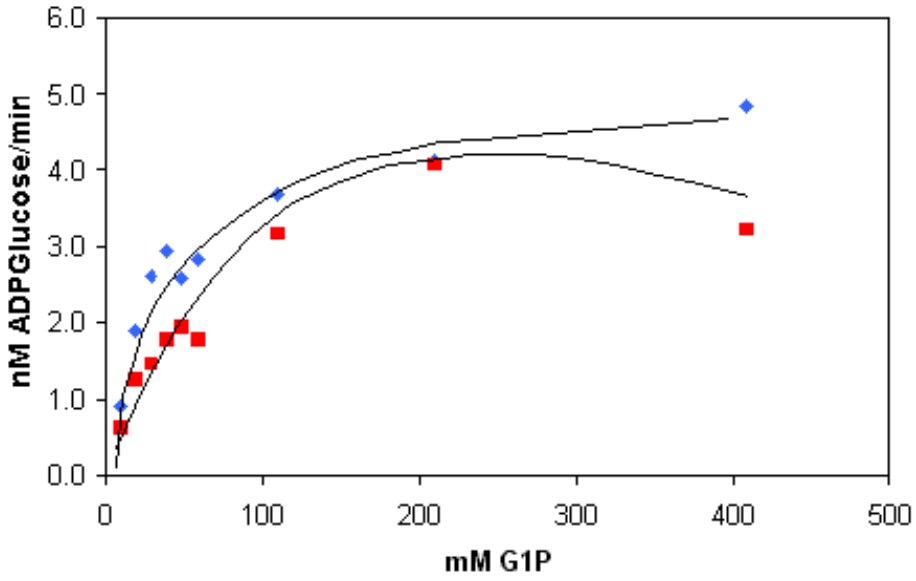

Figure 1. Glucose-1-P saturation of wild-type AGP (diamonds) and *Sh2-UR1* (squares) in the presence of 10 mM 3PGA.

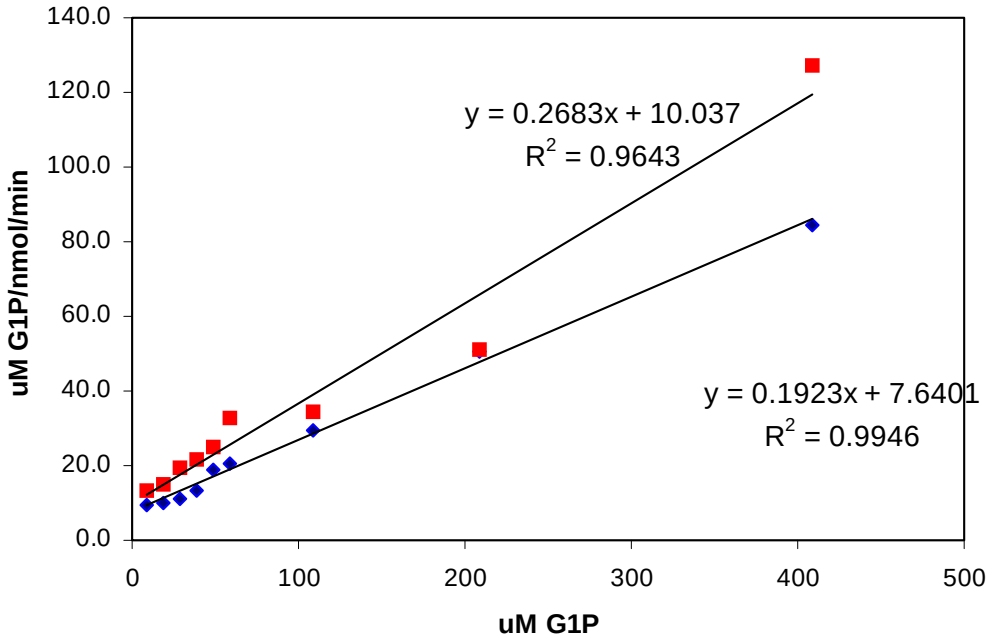

Figure 2. Hanes-Woolf plot of wild-type (diamonds) versus *Sh2-UR1* (squares) AGP. Wild-type K_m was 3.9×10^{-5} M and *Sh2-UR1* K_m was 3.7×10^{-5} M. Assays were performed in the presence of 10 mM 3PGA.

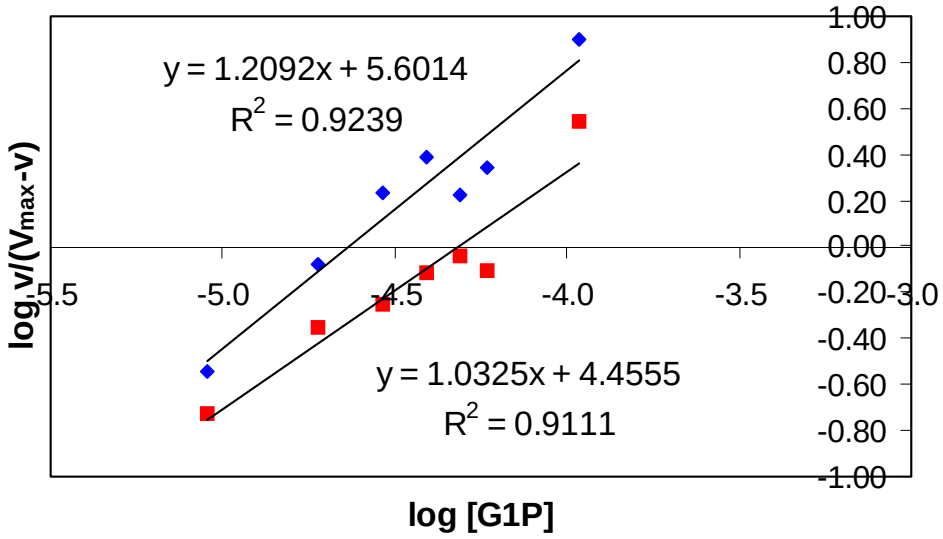

Figure 3. Hill plot of wild-type (diamonds) and *Sh2-UR1* (squares) AGP. Wild-type n = 1.2 and *Sh2-UR1* n = 1.0. V_{max} and v measured in nmol ADPglucose formed per minute.

Activation by 3PGA

Sh2-UR1 and wild-type AGP were assayed at different concentrations of 3PGA in the absence of Pi. Maximal activation of *Sh2-UR1* was 3.3 fold, while that of wild-type was 2.5 fold. $A_{0.5}$ was 0.5 mM 3PGA for both genotypes.

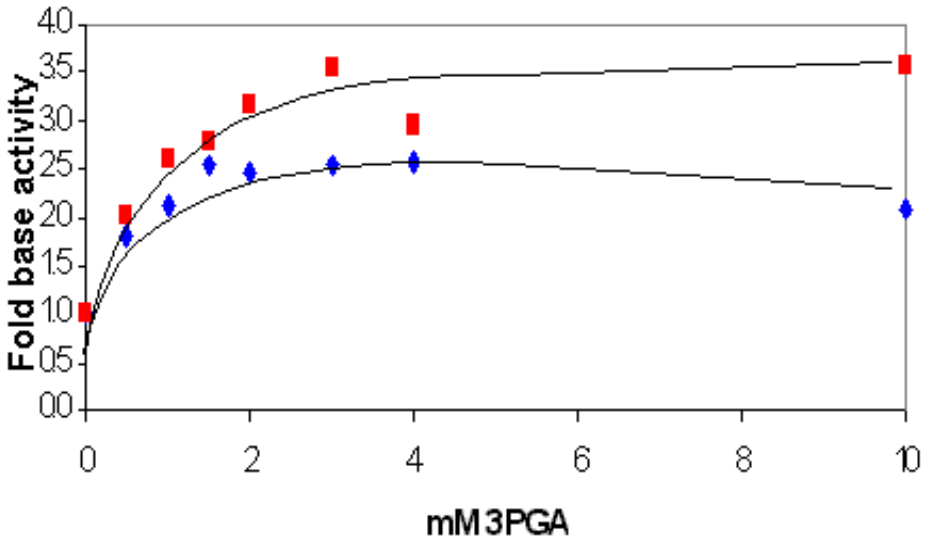

Figure 4. 3PGA activation curve. Wild-type (diamonds) was activated 2.5 fold, while *Sh2-UR1* (squares) was activated 3.3 fold. $A_{0.5}$=0.5 mM for both. Data are averages of at least two assays on separate days, each with two repetitions.

Inhibition by PO_4

Wild-type and *Sh2-UR1* were assayed at different Pi concentrations. $I_{0.5}$ was approximately 10 mM PO_4 for both wild-type and *Sh2-UR1* in the presence of 10 mM 3PGA.

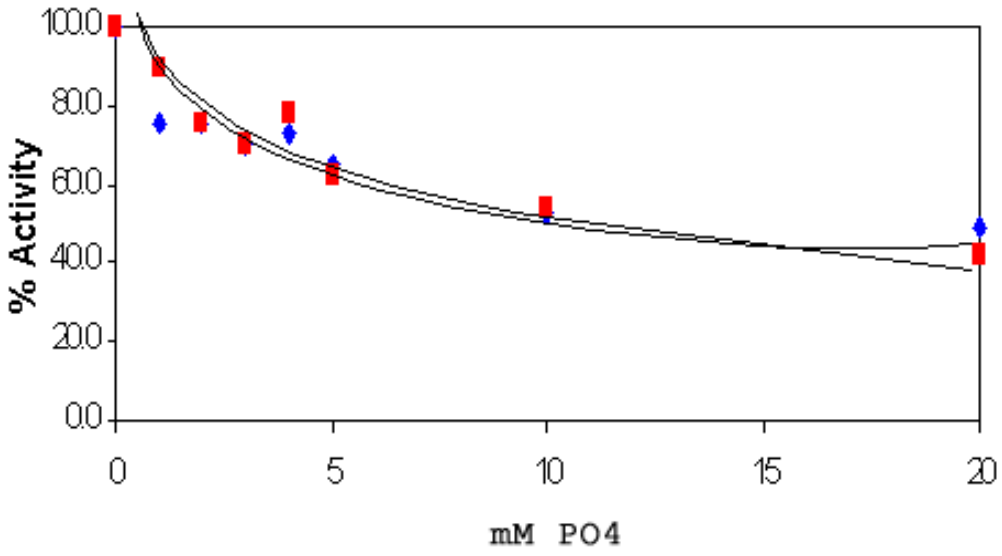

Figure 5. PO_4 inhibition curve. Wild-type (diamonds) and *Sh2-UR1* (squares) both were inhibited 50% by 10 mM PO_4 in the presence of 10 mM 3PGA. Data are averages of at least two repetitions in a single assay.

CHAPTER 4
DISCUSSION

Summary

Starch as a storage product for plants and as a food source for humans has become the focus of intensified research. AGP, a regulatory enzyme of starch biosynthesis in higher plants, is a prime target for engineering to improve starch production and thus crop yield. Mutation of the genes encoding this enzyme has provided insight into the function of various regions the AGP proteins.

Greene *et al.* (1996a) implicated the AGP large subunit's highly conserved PAV sequence (Figure 6) in 3PGA binding by mutating the Pro52 to Leu in a study of glycogen-deficient mutants of *E. coli* expressing potato tuber AGP. This mutation decreased AGP's sensitivity to 3PGA 45 fold. Greene *et al*. (1998) then used hydroxylamine to generate second-site revertants that restored glycogen synthesis. The E38K mutation gave the greatest increase in glycogen synthesis.

Greene *et al.* (1998) used site-directed mutagenesis to make the E38K change in wild type potato AGP large subunit, increasing activation by 3PGA 80 fold and decreasing inhibition by Pi seven fold compared to the wild type. The change in AGP's sensitivity to effectors indicated that Glu38 is important for binding effector molecules.

The highly conservative nature of AGP suggests that this site could be important for the allostery of AGP in other species. Thus, site-directed mutagenesis was used to change Thr93 in the maize large subunit (analogous to position 38 in the large subunit of potato) to a Lys, and the T93K mutant was characterized.

The T93K mutation had no detectable effect on the K_m for G1P compared to wild-type AGP. No change was expected, since the original E38K mutation in potato only affected the binding of effector molecules (Greene *et al.* 1998). However, inhibition by PO_4 was also unaffected by the mutation in maize, in contrast to the decrease in sensitivity to PO_4 conditioned by the E38K mutation in potato. Sensitivity to 3PGA did increase in the maize mutant, as in potato, by 27% compared to potato AGP's 8000% increase.

Comparison with E38K in *S. tuberosum*

The contrasting results obtained from this mutation in potato tuber AGP and maize endosperm AGP indicate that the importance of this position to allostery likely varies among AGP isozymes. These differences could be due to slight variations in tertiary structure, e. g. the residue may be more exposed to binding 3PGA molecules in potato tuber AGP than in maize endosperm AGP.

This residue in the large subunit is in a region of otherwise highly conserved amino acids but is itself not conserved (Table 7), suggesting that this position is not crucial for activity but does have the potential to enhance activation. An Ala is conserved throughout higher plants at this analogous position in the small subunit, indicating some importance for activity in the small subunit.

Other Possible Amino Acid Substitutions

Greene *et al.* (1998) mutated Glu38 further to study this amino acid position. Among these mutants, E38R

decreased activation at low concentrations of 3PGA, and E38A almost eliminated the enzyme's sensitivity to 3PGA, but E38G

Table 7. Amino acid sequence of the region surrounding the residue analogous to Glu38 in the potato tuber large subunit. Bold = 95% conserved in plants. Position 38 and PAV sequence are labeled at top of figure.

Species/tissue	Sequence
	38 PAV
potato tuber	**V**AAV**ILGGG**E **G**TK**LFPLT**SR T**AT**P**AVP**V**GG**
tomato stem	**V**AAV**ILGGG**E **G**TK**LFPLT**SR T**AT**P**AVP**V**GG**
tomato leaf	**V**VAI**ILGGG**G **G**TR**LFPLT**KR R**AK**P**AVP**I**GG**
potato leaf	**V**ASV**ILGGG**V **G**TR**LFPLT**SR R**AK**P**AVP**I**GG**
tomato root	**V**ASV**ILGGG**V **G**TR**LFPLT**SR R**AK**P**AVP**I**GG**
barley endosperm	**V**AAV**ILGGG**T **G**TQ**LFPLT**ST R**AT**P**AVP**I**GG**
wheat endosperm	**V**AAV**ILGGG**T **G**TQ**LFPLT**ST R**AT**P**AVP**I**GG**
maize embryo	**V**AAV**ILGGG**T **G**TQ**LFPLT**ST R**AT**P**AVP**I**GG**
maize endosperm	**V**SAI**ILGGG**T **G**SQ**LFPLT**ST R**AT**P**AVP**V**GG**
sorghum seed	**V**SAI**ILGGG**T **G**SQ**LFPLT**ST R**AT**P**AVP**V**GG**
rice endosperm	**V**SAV**ILGGG**T **G**VQ**LFPLT**ST R**AT**P**AVP**V**GG**
barley leaf	**V**VAV**ILGGG**A **G**TR**LFPLT**KR R**AK**P**AVP**I**GG**
beet leaf	**V**AAIV**LGGG**A **G**TR**LFPLT**SR R**AK**P**AVP**I**GG**
oriental melon	**V**ASI**ILGGG**A **G**TH**LFPLT**KR S**AT**P**AVP**A**GG**
watermelon	**V**ASI**ILGGG**A **G**TH**LFPLT**RR S**AT**P**AVP**V**GG**
sweet potato	**V**AAI**ILPGG**A **G**TH**LFPLT**NR A**AT**P**AVP**L**GG**
pea cotyledon	**V**ISIV**LGGG**P **G**TH**LY**P**LT**KR A**AT**P**AVP**V**GG**
Arabidopsis leaf	**V**AAI**ILGGG**D **G**AK**LFPLT**KR A**AT**P**AVP**V**GG**
E. coli	SVAL**IL**A**GG**R **G**TR**L**KD**LT**NK R**AK**P**AV**HF**GG**

produced effects similar to those of E38K (the *UR1* mutation). Based on these observations, the researchers concluded that both charge and the size of the R-group at this position are important to 3PGA binding. The loss of activation caused by the change to Ala is further evidence of variations in enzyme conformation. Ala is found at this relative position in the large subunit of various AGP's,

some of which are highly activatable (Table 8). Thus, it may also follow that a different amino acid at this position in maize endosperm AGP would have a more positive effect on 3PGA binding than did lysine.

Table 8. Sensitivity to 3PGA of AGP from various species and tissues, and large subunit residue analogous to position 38 in potato tuber AGP. **, no detectable activity in the absence of 3PGA; n/a, no analogous amino acid.

Species/organ	**Amino acid**	**Activation by 3PGA**
Tomato fruit	E	**
Spinach leaf chloroplast	A	58x
Potato tuber	E	43x
Hoya sp.	A	27x
A. thaliana	A	24x
Barley leaf	A	13x
Maize endosperm	T	4x
Barley endosperm	T	1/4x
Wheat endosperm	T	none

Since size and charge of the residue in potato tuber AGP both play a part in sensitivity to 3PGA, amino acids with different characteristics should be placed in this site in the maize endosperm enzyme to verify the site's importance to 3PGA binding. Particularly, mutants should alter the charge, polarity, and/or size of the residue. The Thr normally located at this position in maize endosperm AGP

and in all cereal endosperm AGP's has a small, polar R-group. Alternative mutations should include Glu, the original residue in the potato large subunit, which has a large acidic R-group. Ala, which has a small nonpolar R-group, is commonly found in species outside the grass family (Table 7), and would yield a change in R-group polarity from Thr. Tyr is another possible substitution, bearing a large polar R-group, and Trp would represent a mutant with a large nonpolar R-group. One of these amino acids may prove to be a superior candidate for improving 3PGA binding.

The mutation of Thr93 in maize endosperm AGP represents translation of discoveries in one species to knowledge about another by the use of site-directed mutagenesis. Thus, the genetics of starch biosynthesis benefits from the broad base of species studied, and as important amino acids are identified in model species, those analogous positions can be mutated and studied in other species. In an agronomically important crop such as maize, this translation of information leads to further improvement of starch production for the world's food supply.

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BIOGRAPHICAL SKETCH

Joel Caren was born in Gainesville, Florida, on July 31, 1973, and has lived in the Gainesville area all his life. He graduated from Bradford High School as Valedictorian in 1991; then he attended Santa Fe Community College from 1991 to 1993, where he graduated with honors.

Joel attended Stetson University in DeLand, Florida, for one year, then returned to Gainesville to attend the University of Florida, from which he received the degree of Bachelor of Science with high honors in botany in 1996. He was accepted into the graduate school of the University of Florida in the plant molecular and cellular biology program that same year. In the spring of 2000, he was conferred the degree of Master of Science.