Probing Allosteric Binding Sites of the Maize Endosperm ADP-glucose Pyrophosphorylase

Susan K. Boehlein,‡ Janine R. Shaw,‡ L. Curtis Hannah‡,* and Jon D. Stewart§,*

‡1117 Fifield Hall, Program in Plant Molecular and Cellular Biology and Horticultural Sciences, University of Florida, Gainesville, FL 32611 USA. Phone 352-392-6957, Fax 352-392-9905, E-mail lchannah@ufl.edu

§127 Chemistry Research Building, Department of Chemistry, University of Florida, Gainesville, FL 32611 USA. Phone 352-846-0743, Fax 352-846-2095, E-mail jds2@chem.ufl.edu

*Authors to whom correspondence should be addressed
Abstract

Maize (Zea mays) endosperm ADP-glucose pyrophosphorylase (AGPase) is a highly regulated enzyme that catalyzes the rate-limiting step in starch biosynthesis. Although the structure of the heterotetrameric maize endosperm AGPase remains unsolved, structures of a non-native, low activity form of the potato tuber (Solanum tuberosum) AGPase (small subunit homotetramer) revealed that several sulfate ions bind to each enzyme [Jin, X., Ballicora, M.A., Preiss, J., Geiger, J.H. EMBO J. 2005, 24, 694-704]. These sites are also believed to interact with allosteric regulators such as inorganic phosphate (P_i) and 3-phosphoglycerate (3-PGA). Several arginine side chains contact the bound sulfate ions in the potato structure and likely play important roles in allosteric effector binding. Alanine scanning mutagenesis was applied to the corresponding Arg residues in both the small and large subunits of maize endosperm AGPase to determine their roles in allosteric regulation and thermal stability. Steady state kinetic and regulatory parameters were measured for each mutant. All of the arginine mutants examined – in both the small and large subunits – bound 3-PGA more weakly than the wild type (A_{50} increased by 3.5 - 20 fold). By contrast, the binding of two other maize AGPase allosteric activators (fructose-6-phosphate and glucose-6-phosphate) did not always mimic the changes observed for 3-PGA. In fact, compared to 3-PGA, fructose-6-phosphate is a more efficient activator in two of the arginine mutants. Phosphate binding was also affected by arginine substitutions. The combined data support a model for the binding interactions associated with 3-PGA in which allosteric activators and P_i compete directly.
**Introduction**

ADP-glucose pyrophosphorylase (AGPase), a key enzyme in starch biosynthesis, catalyzes the formation of ADP-glucose from ATP and glucose-1-phosphate (G-1-P). Maize AGPase, like nearly all higher plant homologs, is a highly regulated heterotetramer containing two small and two large subunits. By contrast, virtually all bacterial forms of the enzyme are homotetramers. Evidence from eight independent plant transgenic or genetic experiments (Stark et al. 1992, Sakulsingharoj et al. 2004, Giroux et al. 1996, Hannah and Greene, unpublished, Smidansky et al. 2002, Smidansky et al. 2003, Obana et al. 2006, Wang et al. 2007) has shown that altering the allosteric properties and/or heat stability of AGPase can significantly increase starch content and starch turnover and, in turn, seed yield. Increased seed number giving rise to enhanced starch content occurs in some cases. Such observations have inspired efforts to understand AGPase regulation at a molecular level.

Virtually all known AGPases are subject to allosteric activation and inhibition by various metabolites associated with the specific carbon utilization pathway of the organism. For example, the bacterial AGPase from *Agrobacterium tumefaciens* is activated by fructose-6-phosphate (F-6-P) and inhibited by inorganic phosphate (P_i) whereas the *Escherichia coli* AGPase is activated by fructose-1,6-bisphosphate (FBP) but inhibited by AMP. *Rhodospirillum rubrum* AGPase is activated by both FBP and F-6-P, and inhibited by P_i, while anabaena AGPase mimics plant AGPases in its activation by 3-phosphoglycerate (3-PGA) and inhibition by P_i. Using both chemical modification and site directed mutagenesis, several arginine and lysine residues participating in allosteric regulation have been mapped to the C-terminal segments of the *Anabaena* and potato tuber enzymes (Charng et al. 1994, Sheng et al. 1996, Ballicora et al. 1998, Ballicora et al. 2002).

Unfortunately, only limited atomic-level structural data are available for AGPases. The three-dimensional structure of a bacterial homotetrameric enzyme from *A. tumefaciens* has recently been solved (Cupp-Vickery et al. 2008). Only one crystal structure is available for a higher plant AGPase: a non-native, low activity form of the enzyme from potato tuber (small subunit homotetramer) (Jin et al. 2005). Although both structures reflect inactive conformations due to high concentrations of ammonium sulfate in the crystallization buffer, important information about potential substrate binding sites was predicted by molecular modeling based on the known structures of thymidyltransferases. While this class of enzymes likely binds
sugar phosphates in the same manner as AGPases, thymidilyltransferases are not regulated allosterically. Both AGPase crystal structures suggest that the enzyme functions as a dimer of dimers, similar to the mechanism proposed for the \textit{E. coli} enzyme on the basis of ligand binding studies (Haugen and Preiss 1979). All available evidence leads to the conclusion that tetramers are required for AGPase catalytic activity.

Both available AGPase crystal structures show two domains in each subunit: an \textit{N}-terminal catalytic domain, which resembles previously reported pyrophosphorylase structures (Jin et al. 2005, Cupp-Vickery et al. 2008) and a \textit{C}-terminal domain that makes strong hydrophobic interactions with the catalytic domain. In the potato small subunit homotetramer, two of the three bound sulfate ions (per monomer) are located in a crevice between the \textit{N}- and \textit{C}-terminal domains, separated by 7.24 Å. We have arbitrarily labeled these sites as sulfate 1 and sulfate 2, respectively. The third sulfate ion (in site 3) binds between two protein adjacent monomers. When ATP is included in the crystallization buffer, two substrate molecules are bound in two of the four presumptive active sites, consistent with the notion that the protein functions as a dimer of dimers. On the other hand, one of the sulfate ions originally found in site 3 is lost when ATP is bound, despite the large distance between their respective binding sites. The \textit{A. tumefaciens} AGPase homotetramer binds a single sulfate ion (per monomer) with 100% occupancy (Cupp-Vickery et al. 2008).

All known allosteric regulators of higher plant AGPases contain one or more phosphate moieties. Because of their structural similarity, it is likely that the sulfate ions found in AGPase crystal structures bind in sites normally occupied by \(P_i\) or anionic, phosphorylated ligands such as F-6-P, G-6-P and 3-PGA. Several studies suggest that all AGPase activators and inhibitors compete for binding to the same or closely adjacent sites within a subunit (Morell et al. 1988, Boehlein et al. 2008). Like \(P_i\), sulfate reverses 3-PGA-mediated activation for the potato, \textit{A. tumefaciens} and maize enzymes (\(I_{0.5} = 2.8\) mM in the presence of 6 mM 3-PGA, potato tuber AGPase; \(I_{0.5} = 20\) mM in the presence of 2.5 mM 3-PGA, maize endosperm AGPase) (Jin et al. 2005, Boehlein, SK, unpublished data). In addition, both sulfate and \(P_i\) significantly affect maize AGPase thermal stability. For these reasons, we analyzed sulfate ion binding to the potato small subunit homotetramer in order to guide alanine scanning mutagenesis studies on the analogous anion binding sites within the heterotetrameric maize endosperm AGPase. Replacements were made in both the small and the large subunits of the maize endosperm AGPase. More
conservative changes (Gln or Lys) were employed when Ala mutants displayed no catalytic activity. We chose not to create homology models of the maize subunits to help understand the behavior of Arg mutants. While computational models often predict core structures accurately, small details such as ligand binding sites and subunit-subunit contacts are less reliable. This is particularly important for sulfate ion binding site 3, which is located at the interface between two subunits. The problems are compounded by the lack of experimental data for an AGPase large subunit.

Our studies revealed that altering any Arg residue that participates in a sulfate ion binding – either in the small or the large subunits of maize AGPase – drastically altered the enzyme’s overall allosteric properties. This indicates that effector binding sites in both subunits function in concert in the native heterotetramer, reminiscent of their synergistic participation in catalysis. It also directly supports the notion that sulfate ion binding sites are also involved in binding allosteric effectors. On the other hand, while mutations at all sulfate ion binding sites affected allosterity, substantial variation was observed for the different Arg side chains. Finally we note that while the various AGPases of plant and bacterial origin exhibit vastly different allosteric properties, presumably due to differing selection pressures over evolutionary time, single amino acid changes of the maize endosperm enzyme can create allosteric properties that mimic those exhibited by bacterial and other AGPases.

Materials and Methods

*Site directed mutagenesis.* Except for the Sh2 R381A variant, site-directed mutagenesis involved PCR amplification of the entire plasmid as described previously (Boehlein et al. 2009). Two reverse complementary primers containing the desired mutations were used to initiate amplification by *vent* DNA polymerase. The resulting products were incubated with *Dpn*I to digest the template DNA prior to transforming competent cells. Mutant clones were selected from the resulting colonies and confirmed by sequence analysis. The Sh2R381A mutant was prepared by the overlap extension method as described by Cross *et al.* (2005).

*Iodine staining of AGPase overproducing colonies.* Colonies were grown on 2% glucose LB plates with spectinomycin and kanamycin at the levels described below. Following growth, plates containing bacterial colonies were inverted over iodine crystals for one minute to detect glycogen accumulation.
**Isolation of wild-type and Arg mutant maize AGPases.** *E. coli* AC70R1-504 cells (Iglesias et al. 1993) were transformed with the plasmids of interest, allowed to recover in SOC medium for 1 hr, then diluted into LB medium supplemented with 75 μg / mL spectinomycin and 50 μg / mL kanamycin. The culture was grown overnight at 37°C until it reached O.D.₆₀₀ = 0.7 – 1.0 (16 – 20 hr). After cooling to room temperature, expression of both AGPase subunits was induced by adding IPTG and nalidixic acid to final concentrations of 0.2 mM and 0.02 mg / mL, respectively. Protein overexpression continued for 3 hr at room temperature with constant shaking. Cells were harvested by centrifuging at 8,000 × g and stored at -80°C.

Wild type and mutant AGPases were purified as described by Boehlein *et al.* (2005). Purification of the wild type and mutant enzymes was monitored using assay A (below). Concentrated solutions of purified AGPases were stored at -80°C for many months with no appreciable loss of catalytic activity. Prior to kinetic analysis, proteins were desalted with Protein Desalting Spin Columns (Pierce), exchanged into 50 mM HEPES, 5 mM MgCl₂, 0.5 mM EDTA, pH 7.4 according to manufacturer’s instructions, then protein concentrations were determined and BSA was added to a final concentration of 1 mg / mL to maintain stability.

**Catalytic assay conditions.** Reverse direction (Assay A). A non-radioactive, endpoint assay was used to determine the amount of glucose-1-phosphate produced by coupling its formation to NADH production using phosphoglucomutase and glucose-6-phosphate dehydrogenase (Sowokinos 1976). Details of the reaction have been described previously (Boehlein *et al.* 2005).

Forward direction (Assay B). A non-radioactive, endpoint assay was used to determine the amount of pyrophosphate (PPᵢ) produced by coupling its formation to a decrease in NADH concentration. Standard reaction mixtures contained 50 mM HEPES pH 7.4, 15 mM MgCl₂, 1.0 mM ATP, and 2.0 mM G-1-P in a total volume of 200 μL. When activators were added to the reaction, their concentrations are specified in the appropriate Table or Figure Legend. Assay tubes were pre-warmed to 37°C and reactions were initiated by adding enzyme solution. Reactions were performed at 37°C and terminated by boiling for 1 min. To determine the quantity of PPᵢ formed, 300 μL of coupling reagent (described below) was added and reactions were incubated for 30 min at room temperature prior to determining A₃₄₀. Absorbance values from blanks lacking AGPase were also measured. The amount of PPᵢ produced was determined from a standard curve using freshly prepared PPᵢ in complete reaction mixtures lacking AGPase.
The difference in $A_{340}$ values between the blank and each assay reaction was used to calculate the amount of PP$_i$. Reactions were linear with respect to both time and enzyme concentration.

Coupling reagent contained 25 mM imidazole pH 7.4, 4 mM MgCl$_2$, 1 mM EDTA, 0.2 mM NADH, 0.725 U aldolase, 0.4 U triose phosphate isomerase, 0.6 U glycero-phosphate dehydrogenase, 1 mM fructose 6-phosphate and 0.8 $\mu$g purified PP$_i$-PFK per reaction (final concentrations; prepared as described previously (Boehlein et al. 2008).

**Kinetic constant determinations.** Steady state kinetic assays were performed using conditions described for Assay B (forward direction). Michaelis constants for ATP were determined by incubating purified AGPase with a constant, saturating level of G-1-P and varying the ATP concentration. Reactions were started by adding purified enzyme. After incubating for 10 min at 37°C, reactions were terminated by boiling for 2 min. When determining the allosteric modulator concentration providing 50% of the maximal activation level ($A_{50}$ values), reactions contained 1mM ATP and 2 mM G-1-P unless otherwise noted with varied concentrations of allosteric activators. All kinetic constants were obtained by nonlinear regression using equations derived from the full kinetic expression (Prism, Graph Pad, San Diego CA).

When determining the concentrations of Pi required to reverse allosteric activation ($K_{i,app}$ values), the activator concentration was maintained at approximately 10 $\times$ $A_{50}$ for that protein. If this would have required an activator concentration >100 mM, 100 mM was used. $K_{i,app}$ values were determined from Dixon plots ($1 / v$ versus $[P_i]$) using linear regression.

**Heat stability of purified maize AGPase and Arg mutants.** Resistance to thermal denaturation was determined using desalted enzymes (3.6 ng / $\mu$L of AGPase along with 0.5 mg / mL BSA) in a total volume of 10 $\mu$L. Samples were incubated at 42°C for 0 - 7.5 minutes, then immediately cooled with ice. The remaining catalytic activity of each sample was determined from the standard assay (forward direction) in the presence of 10 mM 3-PGA. Reactions were started by adding AGPase (36 ng) to the reaction mixture and incubating for 10 min at 37°C. Data were plotted as log % activity versus time and the inactivation constants for the fast and slow phases ($k_1$ and $k_2$) were calculated from slope = - $k$ / (2.3). Half life ($t_{1/2}$) values were calculated from $t_{1/2}$ = 0.693 / $k$. Dissociation constants for Pi ($K_D$) were determined by the method of Scrutton and Utter (1965), using the following scheme:
\[
E + A \rightleftharpoons E\cdot A \quad K_D = \frac{[E] \cdot [A]}{[E\cdot A]}
\]

\[
E \overset{k_1}{\rightarrow} D
\]

\[
E\cdot A \overset{k_2}{\rightarrow} D + A
\]

In this scheme, E represents free enzyme (catalytically active form), D corresponds to denatured enzyme (inactive), A is a stabilizing molecule, \(K_D\) is the dissociation constant for the E·A complex, \(k_1\) is the rate constant for denaturation of free E and \(k_2\) is the rate constant for denaturation of the E·A complex. If the equilibrium between E and A and E·A is rapid compared with denaturation, then the following relationship applies:

\[
\frac{v_a}{v_o} = \frac{k_2}{k_1} + K_D \cdot \left(1 - \frac{v_a}{v_o} \frac{[A]}{[A]}\right)
\]

Here, \(v_a\) and \(v_o\) describe the rates of inactivation of E and the presence and absence of stabilizing molecule A, respectively.

**Results and Discussion**

*Rationale for Mutagenesis.* In the substrate-free form of the potato small subunit homotetramer, the side chains of five residues (Arg 41, Arg 53, Asp 370, Lys 404 and Lys 441) form hydrogen bonds and/or electrostatic interactions with sulfate ion 1 in the potato tuber structure (Figure 1A). This ligand constellation with a net charge of +3 provides at least two interactions with each sulfate oxygen, forming a high-affinity binding site for tetrahedral anions. All of these residues are conserved in the maize AGPase small and large subunits, except for Lys 404, which is a Met in the maize large subunit (Figure 2). The *A. tumefaciens* AGPase also employs Arg side chains (at positions 33 and 45; Figure 2) to bind the single sulfate molecule found in this structure, but lacks the conserved lysine residue corresponding to Lys 441 (potato small subunit numbering) (Cupp-Vickery et al. 2008). The potato and *A. tumefaciens* AGPases respond to different allosteric effectors and these sequence differences may be required to accommodate the different ligands. Full alignments of these sequences have been published and analyzed previously (Georgelis et al. 2007, Georgelis et al. 2008, Georgelis et al. 2009).

In the potato small subunit, the second sulfate ion binding site involves the side chains of Arg 53, His 84, Gln 314 and Arg 316 (potato small subunit numbering; Figure 1A). All are
conserved in the maize endosperm AGPase except for Gln 314, which is replaced by Ala in the maize large subunit. While the overall charge of this site (+2) is less than that in site 1, the tetrahedral sulfate enjoys interactions with all four oxygens. The side chain of Arg 53 plays a key role in sulfate binding since it interacts with anions in both sites 1 and 2 simultaneously.

The third sulfate ion binding site is formed by four interactions: side chains of Arg 83, Lys 69, His 134 and the main chain N-H of Thr 135 (Figure 1B). The latter three residues are located in one subunit while Arg 83 is donated by a second subunit. There are fewer polar contacts with the bound sulfate, suggesting that the $K_D$ value for this site may be higher than that for binding sites 1 and 2. This is consistent with the observation that a sulfate ion from site 3 is lost upon ATP binding. Because guanidinium groups provide electrostatic and hydrogen bond interactions that might confer both affinity and specificity for anionic allosteric effectors such as P$_i$ and 3-PGA, we focused on the four Arg residues that participate in sulfate ion binding in the potato small subunit homotetramer (Figure 1).

*Expression and preliminary characterization of mutant enzymes.* Alanine scanning was carried out for the eight Arg residues (four each in the small and large subunits) likely to interact with sulfate and therefore the phosphate moieties of allosteric effectors in maize endosperm AGPase. Mutant subunit genes were prepared by standard methods and paired with counterparts encoding the complementary wild type subunit in *E. coli* overexpression strains. Bacterial colonies expressing the various mutants were exposed to iodine crystals and the extent of glycogen staining was compared to that of wild type AGPase to provide rough estimates of catalytic activity. Variations in staining levels were observed for several of the enzymes, including the R65A mutant that exhibited no detectable staining.

All mutant AGPases were partially purified and their catalytic activities were determined. All except the R65A (small subunit) variant possessed measurable catalytic ability at this level of purification, consistent with results of in situ iodine staining. Additional replacements for Arg 65 were evaluated. Even more conservative Lys or Gln substitutions for Arg 65 yielded no colony staining or detectable catalytic activity in partially purified samples, although western blots from partially-purified protein preparations showed that both subunits were expressed in soluble form (data not shown). The R77A (small subunit) mutant had very low catalytic activity and poor stability in the partially purified state. To overcome this problem, R77Q and R77K variants were also constructed. While the R77Q mutant could not be completely purified due to
its low activity and instability, the stability of the R77K variant was sufficient to allow purification. All other Ala mutants had considerable catalytic activity and were purified using standard protocols. We attempted to prepare double mutant proteins with Arg replacements in both subunits; unfortunately, such AGPase variants were too unstable to survive purification.

Steady state kinetic parameters were determined for each purified mutant AGPase in the presence of saturating levels of 3-PGA (chosen individually from measured $A_{50}$ values) (Table I). These data revealed how amino acid changes affected activator and inhibitor binding, as well as the interplay between substrate and allosteric effector binding characteristics. To assess the impact of Arg mutation on steady state kinetic parameters, values of $k_{cat} / K_{M,ATP}$ were calculated (Figure 3). ATP is known to be the first substrate bound by the two characterized AGPases in the literature (Paule and Preiss 1971 and Kleczkowski et al. 1993) as well as the maize endosperm enzyme (in preparation) and thus the calculated ratios represent the catalytic efficiencies of the variants.

We recently showed that the allosteric binding sites of the maize AGPase accommodate a variety of different activators in addition to the best-known effector, 3-PGA (Boehlelin et al. 2008). Both F-6-P and G-6-P activated the maize AGPase to a similar extent as 3-PGA, although their $A_{50}$ values were greater than that of 3-PGA. To monitor the molecular architecture of the allosteric binding site, the $A_{50}$ values for all three activators were determined for each mutant (Table II). Since $P_{i}$ competitively reverses allosteric activation by ligands such as 3-PGA, the apparent inhibition constant for $P_{i}$ was measured in the presence of each of the three activators (Table III). In the absence of allosteric activators, very little inhibition by $P_{i}$ is observed (Boehlein et al. 2009). Binding energies were calculated from the measured $A_{50}$ values ($\Delta G_{binding} = - R \cdot T \cdot \ln(A_{50})$) for the wild type and each mutant. Free energy changes ($\Delta \Delta G$ values) caused by each Arg mutation were determined by subtracting the wild type $\Delta G_{binding}$ value from each mutant (Figure 4). Values of $\Delta \Delta G$ for $P_{i}$-mediated reversal of allosteric activation were calculated in the same manner. It should be noted that $K_{i,app}$ values for $P_{i}$ were measured under condition where [activator] $\approx 10 \times A_{50}$. If $P_{i}$ competes with allosteric effectors for the same binding site, the $P_{i}$ equilibrium is coupled with that of the activator; keeping a constant fractional saturation makes the energetic contribution of activator binding identical for all proteins so that the observed binding energies for $P_{i}$ reflect only the interactions with this ligand. Given the
many assumptions and experimental uncertainties required for calculating binding energies, \( \Delta \Delta G \) values \( \leq 1 \text{ kcal / mole} \) were not considered in our analysis.

Finally, because phosphate also plays a role in stabilizing maize endosperm AGPase against thermal deactivation, enzyme half lives with respect to inactivation, both in the presence and absence of Pi, were also determined for each of the mutants (Table IV).

Arg 65 (small subunit) / Arg 104 (large subunit). While an Arg residue is highly conserved at this position in small subunits of all sugar phosphate-activated enzymes, and Arg is also present in the maize endosperm large subunit, other amino acids occupy this position in some large subunits of heterotetrameric AGPases (Figure 2). As noted above, we were unable to identify a functional replacement for Arg 65 of the maize small subunit, and this may be due to an additional role in maintaining protein structure. The side chain of Arg 65 forms a salt bridge with an Asp residue near the \( C \)-terminus in the crystal structure of the potato small subunit homotetramer (Asp 403). An Asp residue is present at this position in all known AGPase sequences (including both maize subunits), making it highly likely that this Arg - Asp salt bridge is also conserved. It has been proposed that this interacting pair of residues forms part of the allosteric binding cleft in AGPases, and altering the Asp residue in the large subunit of the potato tuber enzyme significantly impacted 3-PGA-mediated regulation (Greene et al. 1996).

Replacing Arg 104 in the large subunit with Ala substantially increased both the \( K_M \) value for ATP (by >10-fold; Table I) and the \( A_{50} \) value for 3-PGA (by 20-fold; Table II). This alteration also decreased the \( k_{cat} \) value by an order of magnitude (Table I, Figure 3). To further probe the link between \( K_{M,ATP} \) and \( A_{50} \) for 3-PGA, the latter values were determined in the presence of sub-saturating (1 mM) and saturating (6 mM) levels of ATP. As the ATP concentration approaches saturation, the \( A_{50} \) value for 3-PGA is reduced (Table II), again showing the interaction between the allosteric and catalytic sites that has been noted in many previous studies. This effect is particularly noteworthy since the mutant large subunit was paired with a wild type small subunit and hints at significant cross-talk between the catalytic machinery in the types of subunits. Such an effect is difficult to reconcile with the proposal that the maize large subunit serves a primarily regulatory function, as has been proposed for other AGPases (Ballicora et al. 2004).

The large subunit R104A mutant also revealed a surprising difference between binding affinities of different allosteric activators: while the \( A_{50} \) value for 3-PGA was increased almost 20-fold, the corresponding values for F-6-P and G-6-P were increased by less than two fold.
(Table II, Figure 4) when all were measured at saturating concentrations of ATP. The presence of an additional negative charge in 3-PGA is an obvious difference between these classes of allosteric activators, and it is tempting to speculate that the side chain of Arg 104 may interact with the carboxylate of 3-PGA. These differences are also manifest in the more efficient activation of the large subunit R104A mutant by F-6-P compared to 3-PGA, which is reversed from the wild type. Given the coupling between allosteric activator binding and $K_{M,ATP}$ values, we hypothesized that the large subunit R104A mutant would therefore have greater affinity for ATP in the presence of F-6-P as compared to 3-PGA. The observed data are consistent with this hypothesis (Table V). For example, $K_{M,ATP}$ in the presence of saturating 3-PGA is almost 10 fold higher compared to values measured in the presence of saturating F-6-P or G-6-P. This alteration in substrate binding affinity is the major cause of more efficient allosteric activation by F-6-P and G-6-P as compared to 3-PGA. Whether this reflects intra- or intersubunit ATP binding remains unknown.

$K_{i,app}$ values were measured for $P_i$ reversal of activation caused by 3-PGA, F-6-P and G-6-P to better define changes to the large subunit allosteric binding site caused by the R104A mutation (Table III). As expected, values for $K_{i,app}$ were uniformly higher, regardless of allosteric activator, although the effect is small ($\Delta \Delta G < 1$ kcal/mole) (Figure 5). The consistent changes in $K_{i,app}$ values for $P_i$ when all were measured at saturating levels of allosteric activators argues strongly that all of these ligands compete with one another for the same or closely-linked binding sites. Interestingly, loss of the Arg 104 side chain had a relatively larger impact on the binding of $P_i$ to the allosteric site than on the other allosteric effectors. The latter are larger molecules that likely enjoy additional protein contacts, so that loss of a single contact (the guanidinium moiety of Arg 104) is less detrimental than for $P_i$, whose small size limits its number of protein contacts.

**Arg 77 (small subunit) / Arg 116 (large subunit).** Arginine is found at this position in all bacterial and plant AGPase subunits. In the potato small subunit, the guanidinium moiety forms key hydrogen bond and electrostatic interactions with sulfate ions in both sites 1 and 2 (Figure 1A). In the *A. tumefaciens* structure, the corresponding arginine side chain (Arg 45) interacts with the single bound sulfate and also contributes to activator binding since its replacement by Ala desensitizes the enzyme to F-6-P (Kaddis et al. 2004). These data suggest that both Arg 77
and Arg 116 might play similarly important roles in the maize endosperm small and large subunits, respectively.

Maize small subunit mutants in which Arg 77 was replaced with Ala, Lys or Gln exhibited reduced, but measurable, catalytic activity in partially purified preparations, but only the Lys variant was sufficiently stable to survive full purification. This conservative substitution retains a positive charge and hydrogen bonding ability, although the geometry differs from that of Arg. By contrast, Ala substitution for Arg 116 was successful and R116A mutant was purified by standard methods.

Replacing Arg 77 in the maize small subunit with Lys significantly impacted both steady state kinetic and allosteric binding properties. The \( K_{\text{M,ATP}} \) value was increased by nearly an order of magnitude and \( k_{\text{cat}} \) declined by nearly the same factor (Table I, Figure 3). The mutant also had significantly weaker affinities for 3-PGA, F-6-P and G-6-P, with so little affinity for the last that a reliable \( A_{50} \) value could not be determined (Table II). These changes were paralleled in values for \( P_i \) reversal of activation (Table III, Figure 4). The R77K mutant’s diminished binding affinities for all allosteric effectors is very different from the large subunit R104A variant that had weakened affinity only for 3-PGA. This suggests that Arg 77 interacts with all allosteric effectors, possibly by binding to the phosphate moieties common to each. Such interactions may also be important in maintaining protein stability. As noted above, we were unable to purify maize AGPase variants that lacked a positive charge at position 77 in the small subunit. Tetrahedral oxyanions such as \( P_i \) and sulfate are generally required for all steps in AGPase purification procedures to prevent loss of catalytic activity and our results suggest that a positive charge at position 77 plays a key role in anion-mediated stabilization, perhaps via direct hydrogen bonding and electrostatic interactions.

In contrast to the dramatic, pleiotropic impacts of altering Arg 77 in the small subunit, the corresponding position of the maize large subunit (Arg 116) tolerated Ala substitution with little significant changes to enzyme properties. Steady state kinetic parameters (Table I) and binding constants for allosteric activators (Table II) and \( P_i \) reversal of activation (Table III) were all similar to those of the wild type protein (Figures 3, 4). It is difficult to reconcile the proposed role of Arg 116 (interacting simultaneously with anions in binding sites 1 and 2) with these observations. Clearly, the allosteric binding sites in the small and large subunits are not equivalent, even when the same residues are present in the amino acid sequences.
Arg 107 (small subunit) / Arg 146 (large subunit). Arginine is highly conserved at this position, and its side chain interacts directly with a bound sulfate ion in the third anion binding site of the potato small subunit (Figure 1B). Replacing Arg 107 in the maize small subunit with alanine had negligible effects on steady state kinetic parameters (Table I, Figure 3), although this mutant did possess interesting allosteric properties: while the A_50 value for 3-PGA increased by 5.5 fold, those for F-6-P and G-6-P both decreased by approximately 2-fold (Table II, Figure 4). Interestingly, this was the only variant examined in this study with a greater affinity for G-6-P than the wild type. Like the large subunit R104A mutant, F-6-P more effectively activates the small subunit R107A variant compared to 3-PGA. Taken together, these data suggest that Arg 107 (small subunit) and Arg 104 (large subunit) are more critical for binding a C_3 allosteric activator than for C_6 phosphorylated sugars.

We had originally hypothesized that anion binding site 3 might be critical for sulfate- or P_i-mediated structural stabilization. This proved not to be the case, however, since the Ala mutant had essentially the same thermal inactivation kinetics as the wild type in the presence or absence of added P_i (Table IV). It is also worth noting that – like the wild type enzyme – thermal inactivation of the R107A mutant proceeded with biphasic kinetics featuring a fast and slow phase. While we cannot yet explain the biphasic inactivation of maize AGPase, these results eliminate the possibility that the presence of slightly different anion binding site 3’s in the small and large subunits are the source.

Based on the available structural data, Arg 146 likely occupies an analogous position in the maize large subunit. Changing this residue to Ala modestly increased K_M,ATP and diminished k_cat by nearly 10-fold (Table I). The net result is that, even in the presence of saturating 3-PGA, k_cat / K_M,ATP is diminished by ca. 50-fold by the mutation (Figure 3). This hints at close communication between Arg 146 and the active site, which is also reflected in changes in 3-PGA and P_i affinity caused Ala substitution (Tables II and III). It is not clear why P_i is relatively more effective at reversing 3-PGA-mediated allosteric activation compared to that of F-6-P or G-6-P, particularly since this residue is not predicted to interact directly with any allosteric effectors (Figure 5). Like its small subunit counterpart (Arg 107), mutation of Arg 146 alone does not eliminate P_i-mediated thermal stabilization (Table IV). We cannot eliminate the possibility that the small and large subunit anion binding site 3’s are redundant, and simultaneous mutation at both sites may be required in order to observe a phenotypic change.
Arg 340 (small subunit) / Arg 381 (large subunit). In the potato small subunit homotetramer, this Arg side chain forms a hydrogen bond with a sulfate in anion binding site 2 (Figure 1A). Arginine is found at this position in all higher plant AGPases, although it is replaced by Glu in the A. tumefaciens AGPase, where it forms part of the allosteric cleft (Figure 2). Replacing Arg at this position in the Anabaena AGPase with Glu altered inhibitor selectivity and lowered the affinity for P$_i$ by 100-fold (Frueauf et al. 2002). It appears that this residue plays a crucial role in governing the selectivity of the AGPase allosteric site.

When Arg 340 in the maize small subunit was changed to Ala, the $K_{M,ATP}$ value was increased slightly and the $k_{cat}$ value declined by 6-fold in the presence of saturating 3-PGA (Table I, Figure 3), supporting the notion that this residue participates in allosteric effector binding. Larger changes were observed for binding of allosteric activators (Table II) and P$_i$, which was weakened substantially (Table III). In fact, the values of $K_{i,app}$ for P$_i$ were the highest observed for any protein examined in this study.

In contrast to the very large impact caused by replacing Arg 340 with Ala in the maize small subunit, the analogous mutation of its counterpart in the large subunit yielded a protein whose properties were close to that of wild type. Steady state kinetic parameters were essentially unchanged (Table I, Figure 3). Allosteric activator binding was somewhat weaker (Table II), as were $K_{i,app}$ values (Table III), although the differences from wild type were small.

Effect of P$_i$ on enzyme thermal stability. As noted above, the presence of tetrahedral oxyanions such as sulfate and P$_i$ significantly stabilizes maize endosperm AGPase against loss of catalytic activity. In its substrate-free form, the potato small subunit homotetramer binds twelve sulfate ions. We hypothesize that the maize heterotetramer complexes the same number. To determine which one(s) play a dominant role in stabilizing the three dimensional structure, we measured the thermal denaturation profiles for the wild type and each arginine mutant, using catalytic activity to assess the retention of native structure. As described previously (Boehlein et al. 2008), activity loss proceeds with a biphasic profile with each segment representing approximately 50% of the original catalytic activity. Logarithmic plots of the data provide values for the initial fast phase of activity loss ($k_1$) and for the slower phase ($k_2$) and data are collected in Table IV. Like the wild type, all of the Arg mutants display biphasic activity loss with a nearly constant ratio of rate constants ($k_1 / k_2 \approx 3$). Moreover, in the absence of added P$_i$, extracted t$_{1/2}$ values are essentially unchanged by any of the mutations studied. There may be a
slight stabilization of some Arg mutants (most notably R104A, R116A and R107A), although the effects are small. Finally, we note that the observed binding constant for Pi-mediated protein stabilization is essentially unchanged by any of the Arg mutations.

**Conclusions**

Our goal in these studies was to identify protein residues in maize endosperm AGPase that play key roles in allosteric effector binding. Because the maize small subunit shares high sequence identity with its potato counterpart, protein-ligand contacts are likely to be conserved. By contrast, the maize large subunit has weaker sequence similarity with the potato small subunit, making our predictions of protein-ligand interactions more tenuous. It is also important to note that the structures and functions of allosteric effector binding sites may not be identical between the maize large and small subunits. Because all of our mutant subunits were paired with wild type counterparts, only half of the effector binding sites were altered in the resulting heterotetramers.

Taken together, our results suggest that the small subunit allosteric site minimally includes the side chains of Arg 77 and Arg 340 and that both anion binding sites 1 and 2 are crucial for modulation. In addition to binding activators such as 3-PGA, F-6-P and G-6-P, these side chains competitively interact with Pi. The large subunit allosteric site involves Arg 104 and possibly Arg 381. Compared to the small subunit site, large subunit Arg side chains are relatively less important in activator and Pi binding. This parallels the observations of Ballicora et al. (1998), who investigated the roles of Lys residues in allosteric effector binding by the native potato AGPase. Unfortunately, since structural information is only available for the small subunit, our conjectures concerning the large subunit allosteric site are more tenuous. Once X-ray data for a plant heterotetrameric large subunit become available, a molecular interpretation of these data will become possible.

It is also noteworthy that two of our mutants reversed the relative affinities of maize AGPase for allosteric activators. Like other plant AGPases, the wild type maize enzyme has the greatest affinity for 3-PGA and this is considered the most efficient activator for this class of enzymes. Two single amino acid changes (large subunit R104A and small subunit R107A) yielded variants whose affinity for F-6-P exceeded that for 3-PGA (Table II). In both cases, the major change was a significant decrease in affinity for 3-PGA, rather than increased binding of F-6-P. Given
that a switch in activator efficiency can occur by a single mutation and the majority of plant AGPases are most efficiently activated by 3-PGA, we suggest that 3-PGA activation is under positive evolutionary selection. This is significant since mutationally altered AGPases with enhanced 3-PGA activation giving rise to enhanced starch synthesis or seed yield have not been described in plants.

Acknowledgements
We gratefully acknowledge support from the National Science Foundation (L.C.H. IBM 0444031 and IOS 0815104) and the USDA Competitive Grants Program (L.C.H. 2006-35100-17220 and 2008-35318-18649).
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Figure Legends

Figure 1. Close-up views of polar contacts with sulfate ions in the potato small subunit homotetramer. Carbon atoms are colored by subunit and dotted lines indicate calculated polar interactions. Figures were rendered with PyMOL (DeLano 2002). A. Sulfate ion binding sites 1 and 2. Black labels correspond to residues in the potato small subunit; those in yellow refer to maize small (ss) or large (ls) subunits. B. Sulfate ion binding site 3. Calculated polar interactions are predicted for the side chains of three residues and the main chain amide of Thr 135. Label colors are the same as in Figure 1A.

Figure 2. Alignment of AGPase sequences. CLUSTAL was used to generate a multiple alignment from the mature AGPase sequences from the indicated organisms and selected regions are shown (accession numbers: potato small subunit, X61186; maize small subunit, AF334959; maize large subunit, M81603; potato large subunit, X61187; Anabaena, Z11539; A. tumefaciens, P39669; E. coli, V00281; R. rubrum, YP_427333). Residues predicted to make polar contacts with sulfate ion binding sites 1 – 3 are shown in black shading for the potato small subunit sequence (numbers reflect potato small subunit numbering). Identical residues in other AGPases are colored in the same manner; conservative substitutions are shaded grey. The arginine residues examined by mutagenesis are marked with ▼; the corresponding residue numbers for maize small and large subunits are listed as well. Other residues predicted to form polar interactions are marked by ● and numbered according to the potato small subunit sequence.

Figure 3. Changes in kinetic properties from Arg mutations. Values of $k_{cat} / K_{M,ATP}$ calculated from the data in Table I are plotted against a logarithmic axis. The wild type value is depicted in black and data for small and large subunit mutants are shown in white and grey, respectively.

Figure 4. Schematic diagram of presumed arginine-phosphate contacts and binding energies for allosteric modulator interactions with mutant AGPases. Predicted polar contacts between Arg side chains and $P_i$ are taken from Figure 1. Phosphate binding sites are numbered as in the text. Free energies for allosteric activator binding to the wild type and
mutant enzymes were calculated from $\Delta G = - R \cdot T \cdot \ln (A_{50})$. Free energies for $P_i$ binding in the presence of each allosteric activator were calculated from $\Delta G = - R \cdot T \cdot \ln (K_{i,app})$. Free energy differences ($\Delta \Delta G$) were calculated by subtracting the appropriate wild type value from each mutant. All graphs utilize the same y-scale and x-axis arrangements. Allosteric activator binding data are shown with black bars while $P_i$ data are shown in white. In the case of G-6-P binding to the R77K small subunit mutant, no evidence of saturation was observed, and $\Delta \Delta G >> 3 \text{kcal/mole}$ (indicated by the broken line).
Table I. Kinetic constants of wild type AGPase and arginine mutants. $K_M$ values for ATP were measured in the presence of 2 mM G-1-P and saturating levels of 3-PGA as indicated.

| Enzyme   | Mutant Subunit | [3-PGA] (mM) | $K_{M,ATP}$ (mM) | $k_{cat}$ (s$^{-1}$) |
|----------|----------------|--------------|-----------------|---------------------|
| wild type| ---            | 10           | $0.12 \pm 0.003$| $98 \pm 1$         |
| R104A    | large          | 40           | $1.4 \pm 0.2$   | $11 \pm 1$         |
| R77K     | small          | 40           | $1.1 \pm 0.2$   | $11 \pm 1$         |
| R116A    | large          | 10           | $0.19 \pm 0.02$ | $33 \pm 1$         |
| R107A    | small          | 20           | $0.13 \pm 0.01$ | $70 \pm 1$         |
| R146A    | large          | 20           | $0.42 \pm 0.05$ | $11 \pm 1$         |
| R340A    | small          | 40           | $0.36 \pm 0.02$ | $16 \pm 1$         |
| R381A    | large          | 20           | $0.26 \pm 0.02$ | $69 \pm 2$         |
Table II. Activation of wild type and mutant maize endosperm AGPase by allosteric effectors. Activities in the presence of F-6-P and G-6-P are ca. 60% - 80% of what is observed with 3-PGA for all enzymes.

| Enzyme      | Mutant Subunit | 3-PGA  | A<sub>s0</sub> (mM) | F-6-P  | G-6-P  |
|-------------|----------------|--------|---------------------|--------|--------|
| wild type   | ---            | 0.22 ± 0.03 | 0.6 ± 0.1          | 4.0 ± 0.8 |
| R104A       | large          | 7 ± 1<sup>a</sup> | 6 ± 2<sup>a</sup> | 19 ± 4<sup>a</sup> |
|             |                | 4 ± 1<sup>b</sup> | 0.9 ± 0.2<sup>b</sup> | 5 ± 1<sup>b</sup> |
| R77K        | small          | 4.3<sup>c</sup> | 65 ± 20            | N.D.<sup>d</sup> |
| R116A       | large          | 0.8 ± 0.2 | 1.4 ± 0.1          | 6.2 ± 0.9 |
| R107A       | small          | 1.2 ± 0.3 | 0.30 ± 0.07        | 2.1 ± 0.8 |
| R146A       | large          | 1.5 ± 0.4 | 2.7 ± 0.5          | 6 ± 3  |
| R340A       | small          | 3.5 ± 0.7 | 7 ± 2              | 18 ± 3 |
| R381A       | large          | 2.0 ± 0.5 | 3.2 ± 0.4          | 13 ± 3 |

<sup>a</sup> Activity was determined in the presence of 1 mM ATP.
<sup>b</sup> Activity was determined in the presence of 6 mM ATP.
<sup>c</sup> 3-PGA inhibits the R77K mutant at concentrations above 10-15 mM.
<sup>d</sup> Not determined since activity increased linearly with increasing [G-6-P] up to 50 mM.
Table III. Values of $K_{i,\text{app}}$ for $P_i$ in the presence of 3-PGA, F-6-P and G-6-P. The activator concentrations were chosen on the basis of the respective $A_{50}$ values (Table I).

| Enzyme | Mutant Subunit | 3-PGA   | F-6-P   | G-6-P   |
|--------|----------------|---------|---------|---------|
|        |                | [3-PGA] | [F-6-P] | [G-6-P] |
|        |                | (mM)    | (mM)    | (mM)    |
|        |                | $K_{i,\text{app}}$ | $K_{i,\text{app}}$ | $K_{i,\text{app}}$ |
| wild type | ---       | 2.5     | 5.0     | 25      |
| R104A   | large       | 40      | 10      | 40      |
| R77K    | small       | 15      | 100     | 100     |
| R116A   | large       | 8.0     | 14      | 60      |
| R107A   | small       | 12      | 3.0     | 20      |
| R146A   | large       | 15      | 30      | 60      |
| R340A   | small       | 35      | 70      | 100     |
| R381A   | large       | 20      | 30      | 100     |
Table IV. Phosphate binding and thermodynamic stability of wild type and mutant maize endosperm AGPases.

| Enzyme  | Mutant Subunit | \( K_0 \) (mM) | \( k_1 \) (s\(^{-1}\)) | \( k_2 \) (s\(^{-1}\)) | \( k_1 / k_2 \) | \( t_{1/2} \) (min) |
|---------|----------------|----------------|-----------------|-----------------|-----------------|----------------|
|         |                | no P\(_i\)     | 2.5 mM P\(_i\)  |                 |                 |                 |
| wild type                      | ---            | 0.11           | 0.19            | 0.066           | 2.9             | 1.5            | 3.1            |
| R104A  | large          | 0.05           | 0.21            | 0.032           | 6.7             | 1.45           | 7.5            |
| R77K   | small          | 0.23           | 0.29            | 0.075           | 3.8             | 1.03           | 2.8            |
| R116A  | large          | 0.18           | 0.14            | 0.043           | 3.2             | 2.2            | 8.8            |
| R107A  | small          | 0.10           | 0.11            | 0.047           | 2.3             | 2.8            | 6.9            |
| R146A  | large          | 0.19           | 0.30            | 0.072           | 4.2             | 1.0            | 3.8            |
| R340A  | small          | 0.33           | 0.14            | 0.045           | 3.0             | 2.2            | 5.6            |
| R381A  | large          | 0.15           | 0.25            | 0.05            | 4.5             | 1.17           | 4.7            |
Table V. $K_{\text{M},\text{ATP}}$ values in the presence of activators. Concentrations of 3-PGA were 10 mM for the wild-type and 20 mM for R104A. The F-6-P concentration was 10 mM for both enzymes while 50 mM G-6-P was employed for both enzymes.

| Enzyme   | Mutant Subunit | $K_{\text{M},\text{ATP}}$ (mM) with [3-PGA] | $K_{\text{M},\text{ATP}}$ (mM) with F-6-P | $K_{\text{M},\text{ATP}}$ (mM) with G-6-P |
|----------|----------------|---------------------------------------------|------------------------------------------|------------------------------------------|
| wild type| ---            | 0.09 ± 0.01                                  | 0.057 ± 0.008                            | 0.10 ± 0.02                              |
| R104A    | large         | 1.4 ± 0.2                                    | 0.15 ± 0.07                              | 0.17 ± 0.02                              |
Figure 1. Close-up views of polar contacts with sulfate ions in the potato small subunit homotetramer. Carbon atoms are colored by subunit and dotted lines indicate calculated polar interactions. Figures were rendered with PyMOL (DeLano 2002). **A. Sulfate ion binding sites 1 and 2.** Black labels correspond to residues in the potato small subunit; those in yellow refer to maize small (ss) or large (ls) subunits.
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