Running title: Heat Stability and Allosteric Properties of the Maize AGPase

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Heat Stability and Allosteric Properties of the Maize Endosperm ADP-glucose Pyrophosphorylase are intimately intertwined

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Abstract

ADP-glucose pyrophosphorylase (AGPase), a key regulatory enzyme in starch biosynthesis, is highly regulated. Transgenic approaches in four plant species showed that alterations in either thermal stability or allosteric modulation increase starch synthesis. Here we show that the classic regulators, 3-PGA and Pi stabilize maize endosperm AGPase to thermal inactivation. In addition we show that glycerol phosphate and ribose-5-phosphate increase the catalytic activity of maize AGPase to the same extent as the activator, 3-PGA, albeit with higher Ka values. Activation by fructose-6-phosphate and glucose-6-phosphate is comparable to that of 3-PGA. The reactants ATP and ADP-glucose, but not glucose-1-phosphate and PPI, protect AGPase from thermal inactivation, a result consistent with the ordered kinetic mechanism reported for other AGPases. 3-PGA acts synergistically with both ATP and ADP-glucose in heat protection, decreasing the substrate concentration needed for protection and increasing the extent of protection. Characterization of a series of activators and inhibitors suggests that they all bind at the same site or at mutually exclusive sites. Pi, the classic “inhibitor” of AGPase, binds to the enzyme in the absence of other metabolites, as determined by thermal protections experiments, but does not inhibit activity. Rather, Pi acts by displacing bound activators and returning the enzyme to its activity in their absence. Finally, we show from thermal inactivation studies that the enzyme exists in two forms that have significantly different stabilities and do not interconvert rapidly.
Introduction

ADP-glucose pyrophosphorylase (AGPase) represents a rate-limiting step in starch synthesis in plants as well as glycogen biosynthesis in bacteria (for reviews, see Preiss and Romeo, 1994; Preiss and Sivak, 1996; Hannah, 2005). AGPase catalyzes the first committed step in the starch biosynthetic pathway, the conversion of ATP and α-glucose-1-P (G-1-P) to ADP-glucose and pyrophosphate (for review, see Hannah, 1997). Definitive evidence for the rate-limiting role of AGPase in starch synthesis has been derived through transgenic studies in potato (*Solanum tuberosum*) tubers (Stark et al., 1992) and seeds of wheat (*Triticum aestivum*; Smidansky et al., 2002), rice (*Oryza sativa*; Smidansky et al., 2003; Sakulsingharoja et al., 2004), and maize (*Zea mays*; Greene and L.C. Hannah, unpublished data; Wang et al. 2007). Variants incorporated into plants giving rise to enhanced starch biosynthesis harbor alterations in allosteric properties and/or alterations in heat stability. Accordingly, the thermal stability and regulatory properties of AGPase have received considerable attention.

While bacterial AGPases are homotetramers, plant and unicellular green algae AGPases are composed of two identical small subunits and two identical large subunits (reviewed in Georgelis et al., 2007). Prokaryotic and eukaryotic AGPases have different quaternary structures and regulatory properties (Ballicora et al., 2003), yet their overall kinetic mechanisms appear to be similar (Paule and Preiss, 1971; Kleczkowski et al., 1993). The small and large subunits share much sequence identity and a gene duplication giving rise to the two subunits apparently occurred early in plant evolution (Bae et al., 1990; Bhave et al., 1990; Georgelis et al., 2007). The present-day small and large subunits are not fully functionally redundant although bacterial expression of each subunit alone does yield traces of enzyme activity (Iglesias et al., 1993; Burger et al., 2003). Mutations affecting catalytic and allosteric properties of AGPase map to both subunits (Cross et al., 2004, 2005; Hwang et al., 2005). While the small subunit is evolutionarily more conserved compared to the large subunit, this results from fewer genes encoding small subunits and their need to interact with multiple large subunit
partners, rather than an inherent inability to successfully accommodate amino acid changes (Georgelis et al., 2007).

At least three mechanisms have been proposed to modulate plant AGPase activity: allosteric regulation, thermal inactivation, and reductive activation. Plant AGPases are activated by several allosteric effectors with 3-phosphoglyceric acid (3-PGA) activation receiving the greatest attention. Inorganic phosphate (Pi) inhibits activity in many tissues. In the case of the maize endosperm AGPases, Pi negates 3-PGA activation and returns the enzyme’s catalytic activity to its basal level observed in the absence of 3-PGA.

The thermal stability of AGPase is also an important characteristic since exposure to high temperatures during plant growth causes reduced grain yield in many internationally-important cereal crops, including maize, wheat, and rice (reviewed in Singletary et al., 1994). Investigations by others have shown that AGPase is one of the enzymes most affected by elevated temperature (Singletary et al., 1993, 1994). Most plant AGPases, for example the potato tuber enzyme (Sowokinos and Preiss, 1982; Okita et al., 1990), are heat stable whereas the cytosolic endosperm isoforms are quite labile. Incubating the maize endosperm AGPase at 57°C for 5 min destroys 96% of the activity (Hannah et al., 1980) whereas the potato tuber enzyme is fully stable at 70°C. Expression of a heat stable AGPase in the seed of wheat (Smidansky et al., 2002), rice (Smidansky et al., 2003), and maize (Zea mays; Giroux et al., 1996; T.W. Greene and L.C. Hannah, unpublished data) increases grain yield.

A cysteine residue near the N-terminus of the potato tuber small subunit (Fu et al., 1998) plays an important role in heat stability. While most plant AGPases contain this residue, the heat labile endosperm enzymes lack this amino acid. Removal of the cysteine from the potato tuber enzyme reduces heat stability (Fu et al., 1998) whereas incorporation of this cysteine into the maize endosperm enzyme enhances heat stability (Linebarger et al. 2005). The presence of the small subunit cysteine also allows for reductive activation of AGPases (Fu et al., 1998; Ballicora et al., 1999; Linebarger et al. 2005). The presence of the cysteine can lead to disulfide bridge formation between the two small subunits (Fu et al., 1998; Ballicora et al., 1999; Linebarger et al. 2005). Such activation is impossible for wildtype endosperm AGPases, a phenomenon possibly
related to the cytosolic location of the major endosperm AGPases, in contrast to all other AGPases, which are thought to be plastid localized (reviewed in Hannah 2007).

In the studies described below, we show that a diverse series of metabolites affects maize endosperm AGPase in two, fundamentally different ways. Metabolites that serve as reactants, activators or inhibitors of AGPase also stabilize activity to heat inactivation. This includes the reactants ADP-glucose and ATP as well as all known activators and inhibitors. We also describe two previously unknown activators of the maize endosperm AGPase. While the activators and inhibitors differ significantly in structure, all data are consistent with a common binding site (or mutually exclusive sites) for these effector molecules. Finally we note that the enzyme can apparently exist in two states that differ in their rates of thermal denaturation. This observation has also been noted in apple leaf AGPase (Zhou and Cheng 2005).
Results

Pi binds tightly to maize endosperm AGPase as seen from protection, but does not inhibit catalytic activity. Allosteric regulation of AGPase is a physiologically important phenomenon. 3-PGA is the most characterized activator and its activation is reduced by the presence of Pi. While this is a common feature of virtually all plant AGPases, interesting differences distinguish specific isoforms of the enzyme. In the case of the maize endosperm, Pi does not appreciably reduce activity in the absence of 3-PGA (Figure 1). Activity was measured in the ADP-glucose synthesis direction either in the presence or absence of 2.5 mM 3-PGA and at various concentrations of Pi. Very little Pi inhibition occurs if 3-PGA is omitted from the reaction mixture. This is in direct contrast to the closely-related wheat endosperm AGPase (Gomez-Casati and Iglesias 2002), whereby Pi interacts with the enzyme to inhibit activity, and 3-PGA then acts as a deinhibitor and the barley endosperm which is insensitive to both 3-PGA activation and Pi inhibition (Kleczkowski et al., 1993, Doan et al., 1999).

Two mutually exclusive hypotheses explain Pi deactivation of the 3-PGA stimulated maize endosperm AGPase. 1) Pi does not bind to this isoform in the absence of 3-PGA or 2) Pi binding to AGPase occurs in the absence of 3-PGA but binding has no appreciable effect on activity. To distinguish between these possibilities, we tested whether Pi, in the absence of 3-PGA, might affect other properties of maize endosperm AGPase. Heat stability was chosen since previous reports (Zhou and Cheng 2005 and Gomez-Casati et al., 2000) have shown that allosteric effectors and enzyme reactants enhance enzyme stability in the presence of elevated temperatures.

In the first series of experiments, we investigated whether the allosteric activator, 3-PGA (3-phosphoglyceric acid), could reduce or negate heat-induced loss of maize endosperm AGPase activity (Figure 2). Purified maize endosperm AGPase was mixed with various concentrations of 3-PGA and one aliquot of each mixture was incubated for 10 minutes at 42°C and then immediately placed on ice until assay. Heated and unheated aliquots of each mixture were then assayed for AGPase activity and the percentage of remaining activity is presented in Figure 2. Clearly, 3-PGA reduces the heat lability of maize endosperm AGPase. This is consistent with reports of 3-PGA stabilization of other
AGPases (Zhou and Cheng 2005 and Gomez-Casati et al., 2000). It is noteworthy that although 3-PGA protects the enzyme, protection is not complete; in fact, only about 35% of activity is protected at the highest concentration of 3-PGA (50mM) employed.

Pi, in the absence of 3-PGA, also stabilizes maize endosperm AGPase (Figure 2). In fact, Pi provides substantially more protection than does 3-PGA at the concentrations shown (55% at 50mM). Hence, the maize endosperm AGPase binds Pi in the absence of 3-PGA; however, this binding does not substantially reduce catalytic activity. To characterize Pi protection of maize endosperm AGPase in more detail, enzyme activity was measured following exposure to temperatures at 42°C for various times in the presence of several Pi concentrations (Figure 3A & B). Activity loss follows first order kinetics with respect to time. Values of $t_{1/2}$ (the time required for loss of one-half of the activity) at each Pi concentration were calculated from the slope of the plot of log activity versus time. $t_{1/2}$ for inactivation increases with increasing amounts of Pi. A Kd of 65uM +/- 7 uM for Pi was then determined from a re-plot of the data, using the method of Scrutton and Utter (1965).

The activators and inhibitors of AGPase are intimately intertwined. We next asked whether metabolites known or suspected (Ghosh and Preiss 1966, Gomez-Casati and Iglesias 2002, Plaxton and Preiss 1987) of activating AGPase might also stabilize activity. Prior to these experiments, we monitored activation of AGPase catalytic activity and determined the $K_a$ values in the forward direction (Table I). While activation by 3-PGA has received considerable attention, other metabolites, such as fructose-6-phosphate, glucose-6-phosphate, ribose-5-phosphate and especially glycerol phosphate condition activities that are comparable to that produced by 3-PGA. Although the activators F-6-P and G-6 P have activation constants higher than that of 3-PGA, these constants are in the range of their cellular concentration of approximately 0.5 and 3.5mM respectively in the maize endosperm, see Table I (Liu and Shannon 1981). This is in contrast to the potato tuber AGPase where 3-PGA is a highly specific activator, with F-6-P stimulating the activity to only 4% of that seen with 3-PGA (Iglesias et al 1993). Glycerol phosphate activates AGPase to a similar extent as 3-PGA, but the amount needed for ½ activation is approximately 15 fold higher than that of 3-PGA. While many phosphate monoesters activated the maize AGPase, not all did. We found no activation
with erythrose 4-P or ribulose 1,5- diphosphate. In addition neither methyl phosphate nor the reducing agent DTT activated or protected the maize endosperm AGPase. These observations from the maize enzyme also stand in contrast to those for the spinach leaf chloroplast AGPase, where ribose 5-P and F-6-P activate the enzyme to 15 and 40% of the activity seen with 3-PGA. (Ghosh and Preiss 1966).

We then tested the same series of metabolites for their ability to stabilize the enzyme to heat inactivation (Figure 4). In addition to the activators listed, sulfate was also tested, since sulfate inhibits the potato tuber homotetramer (Jin et al 2005) and saturating levels of sulfate negate the activating effect of 3-PGA. The maize endosperm AGPase is also inhibited by SO$_4$- with a Ki of ~ 20 mM with a [3-PGA] = 2.5 mM; (unpublished). As shown in Figure 4, all of the metabolites that activate or inhibit activation of the maize endosperm AGPase also stabilize the enzyme against thermal inactivation. While it is evident that all metabolites can stabilize enzyme activity, major differences are seen in terms of the extent of protection and the concentrations of metabolites required for maximal stabilization. For example, 3-PGA and SO4- protect the enzyme to the same extent and also require a similar concentration for maximal protection, while the amount of fructose-6-phosphate has not reached saturation under these conditions. Pi provides the best protection at saturation against heat inactivation (Figure 4). The reducing reagent, DTT, and a non-effector ion, KCl, had no effect on the heat stability of the enzyme (data not shown).

We next measured the ability of the substrates to protect AGPase activity. Purified AGPase was mixed with the substrates (5mM final concentration) listed in Table II and exposed to 42°C for 7.5 min prior to assay in the forward (ADP-glucose synthesis) or back (ADP-glucose degradation) direction. Activities are presented as a percentage of activity remaining from a control maintained on ice prior to assays. Several effectors were also included in these studies. Various kinetic studies have shown that ATP is the first substrate bound to the enzyme and ADP-glucose is the last product released in other AGPase homologs(Kleczkowski et al., 1993, Paule and Preiss, 1971). Hence, it was expected that these kinetically productive complexes formed with reactants might prevent heat-induced activity loss. Under the conditions employed, ADP-glucose provided the greatest amount of protection against thermal inactivation (~60%), regardless of
direction. This protection was enhanced by adding either 3-PGA or Pi and virtually complete protection under these experimental conditions was achieved by the addition of both ADP-glucose and 3-PGA (Figure 5). It is also interesting that 3-PGA greatly decreased the amount of ADP-glucose required for ½ maximal protection (Figure 5). The synergistic effect was not surprising, since 3-PGA decreases the Km for the substrates as well as increasing Vmax (Boehlein et al 2005).

Although ATP alone provided some protection (~15%), the addition of Pi or 3-PGA to the ATP/enzyme mixture greatly enhanced enzyme stability at 42°C (Table II). Increasing MgCl₂ and ATP concentration to 50 mM allowed 46% of the initial activity to be maintained after heat treatment. Pyrophosphate, (PPi) did not protect the enzyme against thermal inactivation and actually reduced the amount of stabilization elicited by 3-PGA and Pi. G-1-P also was unable to stabilize the enzyme at concentrations up to 50mM. Additional experiments were performed in which 3-PGA, ATP, and G-1-P were added to the enzyme in the presence of Ca²⁺. Ca²⁺ was used as a cofactor, so ATP would bind, but the reaction would not proceed to an appreciable extent during the incubation period (Fu et al 1998). This set of conditions produced no additional protection compared to experiments containing only ATP and 3-PGA. Also, the addition of ADP-glucose and PPi in the presence of Ca²⁺, did not condition additional protection compared to addition of only ADP-glucose (Table II).

Taken together, these data are consistent with an ordered kinetic mechanism, with reactants binding productively to free enzyme. In turn, this conditions protection against heat-induced activity loss. 3-PGA and Pi protect against thermal inactivation independently, and each acts synergistically with ADP-glucose and ATP to protect the enzyme. Substrates that do not bind productively to free enzyme (PPi and G-1-P) do not enhance thermal stability.

To further characterize binding of these effector molecules to AGPase, the method of Scrutton and Utter (1965) was used to determine the Kd values at 37°C (Table III). The dissociation constant for Pi is the lowest, at 70μM while those for 3-PGA, ATP and ADP-glucose (1.84 mM, 1 mM and 0.2 mM respectively) are higher than their corresponding Ka, and Km values. However, heat stability experiments were performed on kinetically quiescent enzyme whereas Ka, Ki and Km values were determined with
catalytically active enzyme. The Kd’s reported here reflect binding to enzyme in the absence of other ligands. In addition to obtaining the Kd values, the ratios of inactivation constants (k2/k1) were also determined. These reflect the degree of protection provided by the stabilizing metabolites, a smaller ratio indicating a higher degree of protection. All ligands investigated here provide comparable levels protection except for ribose-5-phosphate, suggesting a common binding mode.

We then probed the possibility that all the ligands responsible for modulating catalytic activity and thermal stability might bind to the site(s) occupied by 3-PGA. If so, several conditions should be met. First, the addition of a non-saturating concentration of a second activator should enhance the activation caused by non-saturating amounts of 3-PGA. This effect was observed when 0.1mM 3-PGA (1/2 Ka value) was added to the enzyme, followed by a second activator equal to ½ its Ka value. Significant additional stimulation was seen for most activators (Table IV). A second prediction of this hypothesis is that a metabolite that activates to a lesser extent than 3-PGA, should, at increasing concentrations, inhibit the activation produced by 3-PGA. PEP was chosen for this experiment since the activity in the presence of PEP was only about 40% of its activity in the presence of 3-PGA. 3-PGA was used at a concentration of 0.75mM ~(3 x Ka) and PEP was varied from 0-100mM (0-20x Ka). Inhibition by PEP begins at about 25mM and increases up to about 100mM (Figure 6). The decrease in activity caused by the competition of activators indicates that PEP and 3-PGA are binding to the same site, or to mutually exclusive sites.

Several observations suggest that Pi and 3-PGA sharing a common binding site. Heat protection studies were employed to further investigate this possibility. If 3-PGA and Pi bind to a common site, the presence of one will not have a synergistic effect on the other during thermal protection studies. If, on the other hand, binding of the activator and binding of the inhibitor are independent or coupled, a cumulative or synergistic pattern would be seen. To determine the relationship between 3-PGA and Pi, several activator/inhibitor combinations were used in thermal protection studies. (Table II). First, when assayed in the reverse direction, 10mM Pi conditions 64% protection under the conditions specified. Similarly, 3-PGA conditions 39% protection. Significantly, the combination of 3-PGA and Pi gives 67% protection, a value comparable to the protection
observed with only Pi. This suggests that 3-PGA and Pi are binding to the same site or mutually exclusive binding sites. In contrast, we note that the addition of 3-PGA greatly increases the protection caused by the substrates ADP-glucose and ATP (Table II). This is indicative that 3-PGA (and Pi) and the substrates do not share common binding sites.

Thus far, it has been shown that Pi can bind to the enzyme with a fairly high affinity, however binding does not itself inhibit catalytic activity. A common binding site for Pi and 3-PGA should also be manifest by a Ki value for Pi that is dependent on 3-PGA concentration. This was observed (Figure 7). As the 3-PGA concentration increases, the amount of Pi needed to inhibit activity rises. Thus, the role of Pi is not to inhibit the enzyme per se, but rather to deactivate the activated state by competing with the activator for binding to the enzyme. To determine whether Pi interacts with the effectors fructose-6-P and glucose-6-P in a similar manner, the Ki for Pi in the presence of 3-PGA, F-6-P and G-6-P was determined. Enzymes were incubated with activators (3-PGA, F-6-P or G-6-P) at 10X their corresponding Ka values. Pi inhibition studies were then performed to determine the Ki value of Pi in the presence of these activators. If phosphate is able to compete with these activators in a comparable manner, then all three of their phosphate Ki values should be the same. This was the case (Table V).

Heat denaturation curves for AGPase are bi-phasic. While all of the thermal protection experiments described above employed a temperature of 42°C and exposure times ranging up to 10 minutes, longer incubations at 42°C showed that catalytic activity remaining after the initial exposure to 7-10 minute exhibited greater heat stability than the activity lost at the beginning of the experiment. Representative data are shown in Figure 8. Catalytic activity was monitored over 40 minutes in the forward (Figure 8A) and back (Figure 8B) directions in the presence of 10 mM Pi. All plots exhibited a clear biphasic pattern of thermal inactivation with a break around 10 minutes. To determine whether the transition between the two states of the enzyme was a fast or slow process, AGPase plus Pi was placed at 42°C for 10 minutes and then cooled on ice for 5 minutes. The sample was then re-incubated at 42°C and catalytic activity was monitored over time. If the transition back to the unstable form were a fast process, a rapid loss in enzyme activity should occur during the first 5-10 minutes following re-exposure to heat treatment. Conversely, if the transition is a slow process, and the enzyme is locked into a
more stable conformation, activity of the preheated enzyme should not exhibit a biphasic decay curve and a lower initial activity would be seen. As can be seen in Figure 8C, the preheated AGPase retains the properties of the more stable configuration of the enzyme. This experiment demonstrates that the enzyme form, which is rapidly heat inactivated, cannot be restored by cooling, and that the remaining catalytic activity is due to a more heat-stable form to the enzyme. We then probed whether this inter-conversion of heat labile to heat stable form required the presence of Pi. At 42°C, the wild type AGPase activity inactivates rapidly, in the absence of Pi, and a clear distinction between enzyme conformations was not obvious. The temperature of inactivation was therefore lowered to 37°C (Figure 8D) and 30°C (data not shown). These data showed that the biphasic nature of decay is still present, thereby indicating that although Pi protects the enzyme from heat inactivation, its presence is not obligatory for the biphasic loss of catalytic activity. A similar experiment was performed in the presence of DTT and no discernable effect on the biphasic nature of inactivation was noted.
Discussion.

Plant transgenic approaches have shown that AGPases harboring alterations in allosteric properties as well as enhanced thermal stabilities can enhance starch synthesis in agriculturally important tissues and organs such as the potato tuber and seeds of maize, wheat and rice. Accordingly, we have focused our investigations on these two properties. The results reported here show that allostery and heat stability are intimately connected. Metabolites that affect catalytic activity also affect heat stability. Several other features of the maize endosperm AGPase, as outlined below, are noteworthy.

First, while 3-PGA has received the greatest attention as an activator of AGPase activity, we show that the maize endosperm AGPase can also be activated by a variety of other phosphate monoesters. Metabolites ranging from three carbon 3-PGA to the 12 carbon sucrose-6-phosphate cause comparable levels of activation. Activating metabolites appear to bind at the same or mutually exclusive sites in stimulating maize endosperm AGPase, since the addition of metabolites to AGPase partially activated by 3-PGA result in further activity enhancements. This has been directly demonstrated for 3-PGA and PEP and it is a distinct possibility that all activating metabolites bind to the same site.

While fructose-6-phosphate and glucose-6-phosphate both stimulate the activity of several AGPases (Gomez-Casati and Iglesias 2002, Ghosh and Preiss 1965, Frueauf et al 2002), the reported activation levels are less than those observed with the maize endosperm AGPase where fructose-6-phosphate and glucose-6-phosphate at physiological concentrations both stimulate the activity to approximately 70% of the maximum observed with 3-PGA (Plaxton and Preiss 1987 and Boehlein et al 2005). On the other hand, the extent of activation caused by ribose-5-phosphate for the maize endosperm AGPase is much higher, relative to 3-PGA-induced activation, than the level noted for the spinach chloroplast AGPase (Ghosh and Preiss 1965).

We were surprised to find that glycerol phosphate stimulates the maize endosperm AGPase. This has not been reported for other AGPases, to the best of our knowledge. Whether this provides a mechanism to coordinate starch synthesis and glycolysis and/or lipid synthesis is presently unknown. Alternatively, this activation may simply reflect the structural similarity of glycerol phosphate and 3-PGA.
Metabolites that activate or inhibit AGPase also stabilize the enzyme to thermal inactivation. While Zhou and Cheng (2005) reported that 3-PGA stabilized the apple leaf AGPase and stabilization of the cyanobacterial homolog by 3-PGA and Pi has also been observed (Gomez-Casati et al. 2000), the data reported here show that the range of suitable ligands for the maize endosperm AGPase is much more diverse. Interestingly, while the dissociation constants of ligands that protect against thermal inactivation differ markedly, the degree of protection (as judged by k2/k1 ratios) is quite similar for all metabolites except ribose-5-phosphate. For example, both 3-PGA and glycerol phosphate provide similar levels of protection but differ markedly in their affinity for the enzyme, perhaps as a result of the additional negative charge in 3-PGA. Given the physiological concentrations of these ligands (Table I) and their binding affinities for the enzyme (Table III), maize endosperm AGPase likely enjoys nearly maximal thermal stabilization in its cellular environment.

Protection by ATP and ADP-glucose but not glucose-1-phosphate and pyrophosphate against thermal inactivation is consistent with the well-precedented ordered kinetic mechanism in which ATP is the first substrate bound and ADP-glucose is the last product released. In this regard, the maize endosperm AGPase exhibits a striking dissimilarity with a cyanobacterial AGPase. This bacterial enzyme is protected from thermal inactivation only by ATP (Gomez-Casati et al., 2000). It is possible that a conformational change occurs after ADP-glucose release in the cyanobacterial enzyme that disfavors rebinding of ADP-glucose. Such an iso-step is unlikely to be present in the maize homolog. The observation that 3-PGA and ADP-glucose acts synergistically to protect the maize endosperm AGPase suggests cooperative binding of these ligands. If a similar situation holds for the cyanobacterial enzyme, ADP-glucose may afford thermal protection if 3-PGA were also present. In addition we would predict that ATP would not protect the potato tuber enzyme in the absence of activator, since no apparent activity is seen in the absence of 3-PGA, but in the presence of 3-PGA, ATP would protect synergistically. Whether these predictions will be borne out awaits experimental test.

The synergistic interaction of substrates and activators/inhibitors in protecting the maize endosperm AGPase against thermal inactivation suggests that enhanced heat protection occurs when both the active and allosteric sites are occupied. On the other
hand, synergy was not observed in studies involving saturating levels of activators and Pi, suggesting that these species bind to the same or mutually exclusive sites. The data obtained from crystallization studies of the potato tuber small subunit homotetramer suggests that Pi has to 3 or 4 separate binding sites on this particular enzyme, and that 3-PGA binds at or near one of these inhibitor binding sites (Jin et al, 2005). This notion is consistent with the work of Morell et al, (1988) who showed that both Pi and 3-PGA blocked binding of pyridoxal phosphate to the spinach leaf AGPase. Comparing the Ka values for the activators with their physiological concentrations (Table II) and the Ki values for Pi (Table V) indicates that the maize endosperm AGPase probably functions at about 50% of its maximal activity, and relatively small changes in [Pi] would have significant impacts on this value. Such data are consistent with our experience in generating mutant forms that increase starch production.

Several explanations can account for the biphasic nature of the thermal stability exhibited by the maize endosperm AGPase. In the simplest case, two forms of enzyme exist at all times. The major form of activity, Form 1, denatures rapidly, while the initially minor form 2 inactivates slowly. Therefore, during the first few minutes of heat treatment, large decreases in activity are seen from form 1 inactivation. This is then followed by a second, slow decrease in activity as form 2 is denatured. A second model assumes that before heat treatment, only one form of the enzyme exists. This form is heat labile. Exposure to heat causes the highly active, heat labile form to convert to a more stable, less active form. Inter-conversion between the two forms is slow, giving rise to the observed bi-phasic pattern. A third, hybrid model can also be envisaged. Here, the enzyme exists as one heat labile form. In the presence of heat, a proportion of molecules are denatured whereas other molecules are slowly converted into a more heat stable form. According to this model both forms have comparable activity. The proposed inter-conversion of one form to another is reminiscent of the activation process of the potato tuber AGPase (Fu et al, 1998). A several minute exposure to DTT and ADP-glucose at 37 C. gives rise to a 10-fold increase in potato tuber AGPase activity. Subsequent characterization showed that a disulfide bridge between two cysteine residues in the N-termini of the small subunits must be reduced for activation to occur. ADP-glucose then activates the enzyme. While the conversion of the heat labile to heat stable form of
maize endosperm AGPase requires a several minute exposure to elevated temperatures, the presence of DTT neither blocks nor enhances the conversion process. In addition, the maize endosperm AGPase small subunit lacks the cysteine involved in the DTT/ADP-glucose activation process of the potato tuber AGPase.
Materials and Methods

Growth and assay conditions for expression of maize wild type AGPase in E.coli. 

*E. coli* AC70R1-504 (Iglesias et al., 1993), which lacks the functional AGPase gene and cannot synthesize glycogen, was transformed with both pMOncSh2 and pMOncBt2, (plasmids containing the large and small subunit of wild type-AGP respectively) (Giroux et al., 1996) allowed to recover in SOC for 1 hour and directly grown overnight at 37°C in LB media containing 75 ug/ml spectinomycin and 50ug/ml kanamycin. When the cultures reached an OD of 0.7-1.0 (16-20 hours), they were cooled to room temperature and protein expression was induced by the addition of 0.2mM isopropyl-beta-D-thiogalactoside (IPTG) and 0.02mg/ml nalidixic acid. Expression continued for 3 hours at room temperature with constant shaking. Cells were harvested by centrifugation at 8000 x g and the pellets were stored at –80°C.

Purification of AGPase. Maize endosperm AGPase was purified as described elsewhere, (Boehlein et al., 2005) with the following modifications. All steps were performed at 4°C and centrifugations were at 30000 x g unless otherwise stated. Cell pellets from 4 L of *E. coli* cultures were extracted in 20 mL buffer A (50 mM KH$_2$PO$_4$ pH 7.0, 5 mM MgCl$_2$, 0.5 mM EDTA), lysozyme, 50 µg/mL and protease inhibitors (1 µg/mL pepstatin, 0.1 mM PMSF, 10 µg/mL chymostatin, and 1 mM benzamidine). Cells were thawed on ice and broken in a French press and the extract was clarified by centrifugation for 20 minutes. The protein concentration was then adjusted to 30mg/ml. Three tenths volume of a 1% protamine sulfate solution was then added and the mixture was stirred on ice for 20 minutes followed by centrifugation for 20 minutes. The extract was then brought to 45% saturation with solid ammonium sulfate, stirred on ice for 20 minutes and centrifuged for 20 minutes. The resulting pellet was re-suspended in buffer A and stored at -80°C. Further purification using column chromatography (ion exchange and hydroxyapatite) is described in Boehlein et al. 2005. Concentrated, purified proteins were stored at –80°C for many months without appreciable loss of activity.

Prior to kinetic analysis, proteins were desalted using Zeba Micro Desalt Spin Columns according to manufactures instructions (Pierce). Proteins were routinely
exchanged into 50mM HEPES, 5mM MgCl$_2$, 0.5mM EDTA, assayed for protein concentration and then BSA (0.5mg/ml) was added for stability.

**Assay A (reverse direction):** 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 and Preiss 1982) Specific details of the reaction are given in (Boehlein et al., 2005). Purification of the wild type enzyme was monitored using assay A.

**Assay B (forward reaction):** A non-radioactive endpoint assay was used to determine the amount of pyrophosphate (PPi) produced by coupling it to a decrease in NADH concentration. Standard reaction mixtures contained 50 mM HEPES pH 7.4, 15 mM MgCl$_2$, 1.0 mM ATP, and 2.0 mM G-1-P in a total volume of 200 uL. When activators were added to the reaction, their concentration is specified in tables or figure legends. Reaction tubes were pre-warmed to 37$^\circ$C and assays were initiated by enzyme addition. Reactions were performed at 37$^\circ$C and were terminated by boiling for 1.5 minutes. The reactions were developed by adding 300 uL of coupling reagent (described below) to each tube and the absorbance at 340 nm was determined. Blank samples contained complete reaction mixtures without enzyme. The amount of pyrophosphate (PPi) produced was determined from a standard curve using PPi in complete reaction mixtures and omitting enzyme. The change in absorbance between the blank and the reaction was used to calculate the amount of PPi. Reactions were linear with time and enzyme concentration. All kinetic constants were obtained using the forward reaction, unless otherwise stated.

**Preparation of coupling reagent:** The gene encoding the 62kd pyrophosphate dependent phosphofructokinase (PPi-PFK) from *Borrelia burgdorferi* was obtained via PCR from genomic DNA purchased from the ATCC. After insertion into a pET duet expression vector, the protein was expressed in BL21DE3 cells and purified according to Deng et al, (1999). The enzyme was concentrated to 0.8mg/ml and stored in aliquots at -80$^\circ$C. All other enzymes were purchased from Sigma.

The coupling reagent contained a final concentration of 25mM imidazole pH7.4, 4.0 mM MgCl$_2$, 1.0 mM EDTA, 0.2 mM NADH, 0.725 U aldolase, 0.4 U triose
phosphate isomerase, 0.6 U glycerophosphate dehydrogenase, 1.0 mM fructose 6-phosphate and 0.8 ug purified PPI-PFK per reaction. The coupling reagent was made fresh daily.

**Determination of the kinetic constants.** Activation constants ($K_a$) for various AGPase effectors were determined by incubating purified AGPase (0.3ug) in reaction mixtures with the following components: 50 mM HEPES, pH 7.4, 15 mM MgCl$_2$, 1mM ATP and 2mM G-1-P and varying effector concentrations (0.05mM to 25mM). Assays were initiated with purified enzyme, incubated 10 minutes at 37°C and inactivated by boiling for 1.5 minutes. Kinetic constants were obtained by nonlinear regression using equations derived from the full kinetic expression using the software program Prism (Graph Pad, San Diego CA).

**Heat stability of purified maize AGPase.** Heat stability was determined using desalted enzyme at concentration of 2.6 ug protein/mL with or without stabilizing molecules (5mM). Preparations were placed in a water bath at either 42°C or 37°C for varying times, and then cooled on ice. All preparations were subsequently assayed at 37°C for 10 minutes in the forward direction in the presence of 10 mM 3-PGA. Reactions were started with 1.3 ug enzyme. Data were plotted as log % activity versus time, and the inactivation constant $t_{1/2}$ was calculated as follows: slope = $-k/(2.3)$. $t_{1/2}$ is calculated from the equation $k = 0.693/t_{1/2}$. $K_d$ was determined by the method of Scrutton and Utter (1965), using the following equations:

$$K_d = \frac{[E][A]}{[EA]}$$

$$k_1 [E] \rightarrow D$$

$$k_2 [EA] \rightarrow D$$

Where E = free enzyme, D = denatured enzyme, A = stabilizing molecule, $K_d$ = dissociation constant of EA, $k_1$ = rate constant for denaturation of E, $k_2$ = rate constant for
denaturation or EA. If the equilibrium between E, A and EA is rapid compared with

denaturation, then the following relationship applies

\[
\frac{V_a}{V_o} = \frac{k_2}{k_1} + \frac{K_d}{1} \left[ \left( 1 - \frac{V_a}{V_o} \right) \frac{A}{V_o} \right]
\]

\( V_a \) and \( V_o \) are the rates of inactivation of E and the presence and absence of stabilizing
molecule A.
Literature cited


pyrophosphorylase modulates interactions between the small and large subunits. Plant J. **41**: 501–511


Hwang SK, Salamone PR, Okita TW. (2005) Allosteric regulation of the higher plant ADP-glucose pyrophosphorylase is a product of synergy between the two subunits. FEBS Lett. 579:983-90


**Figure legends**

Figure 1. Phosphate effects in the presence or absence of 3-PGA. The forward reaction was used to measure the velocity at various concentrations of phosphate using standard reaction conditions. Velocity in the absence (■) of 3-PGA, or in the presence (▲) of 2.5mM 3-PGA.

Figure 2. Phosphate and 3-PGA protection from thermal inactivation. Desalted enzymes containing 0.5mg/ml BSA were heated at 42°C for 10 minutes in the presence of varying concentrations (■)Pi or (▲)3-PGA. Duplicate samples were left on ice and their activity was taken to be 100%. All preparations were assayed for 10 minutes in the forward direction in the presence of 10 mM 3-PGA. The percentage of activity remaining was then calculated by dividing the heated versus non-heated samples and plotted against the effector concentration.

Figure 3. (A) Thermal stability of AGPase in the presence of varying concentrations of Pi, (■)0, (▲)0.25, (▼) 0.5, (♦)1.0, (●)2.5, (△)5, (□)10,. Enzyme preparations were desalted and contained 0.5mg/ml BSA. Various concentrations of Pi were then added prior to incubation on ice for 10 minutes. The samples were then heated at 42°C for various times. Duplicate samples were left on ice and their activity was taken to be 100%. All assays and calculations were determined as in figure 2. Data were plotted as log % activity versus time, and the inactivation constant t1/2 was calculated as described.
in Materials and Methods. (B) Determination of Phosphate Kd by the method of Scrutton and Utter (1965).

Figure 4. Thermal stability of AGPase in the presence of sulfate, F-6-P and G-6-P. Desalted enzymes containing 0.5mg/ml BSA were heated at 42°C for 10 minutes in the presence of varying concentrations of (●) PO4-, (▼) 3-PGA (◆) sulfate, (■)F-6-P or (▲) G-6-P. Duplicate samples were left on ice and their activity was taken to be 100%. All preparations were assayed for 10 minutes in the forward direction in the presence of 10 mM 3-PGA. The percentage of activity remaining was then calculated by dividing the heated versus non-heated samples and plotted against the effector.

Figure 5. Thermal stability of AGPase in the presence of ADP-glucose and/or 3-PGA. Desalted enzymes containing 0.5mg/ml BSA were heated at 42°C for 10 minutes in the presence of varying concentrations of (▲)ADP-glucose or (■) ADP-glucose with 10mM 3-PGA. Duplicate samples were left on ice and their activity was taken to be 100%. All preparations were assayed for 10 minutes in the forward direction in the presence of 10 mM 3-PGA. The percentage of activity remaining was then calculated by dividing the heated versus non-heated samples and plotted against the effector concentration.

Figure 6. The effect of 3-PGA and PEP on the AGPase activity. The activity of AGPase was measured in the presence of 0.75mM 3-PGA at varying concentrations of PEP. The percentage of activity remaining was calculated by dividing the amount of activity in the
presence of PEP divided by the amount of activity in the absence of PEP (0.75mM 3-PGA) and plotted against the PEP concentration.

Figure 7. (A) Inhibition of AGPase activity by phosphate. The activity of AGPase was measured using the forward reaction in the presence of varying concentrations of Pi at several fixed concentrations of 3-PGA. (▲) 1mM, (●)2.5mM, (▼)5mM, (◆) 10mM. The Ki for Pi was determined from the X intercept at each 3-PGA concentration. (B) Determination of the binding constant of Pi in the absence of 3-PGA. A plot of Ki vs 3-PGA concentration was used to determine the binding constant of Pi in the absence of 3-PGA.

Figure 8 Biphasic nature of heat inactivation. (A) AGPase (0.02mg/ml) was mixed to a final concentration of 10mM phosphate, and incubated on ice for 5 min. Enzymes were then heated at 45°C for the specified amount of time and placed back on ice. When all of the time points were completed, the enzymes were assayed using standard protocols. (B) AGPase was prepared as above, and assayed in the reverse direction. (C) Enzymes were prepared as in panel A. The entire mixture was then heated at 45°C for 7 minutes, and then cooled on ice. The same experiment was then performed, as in panel A. (D) No phosphate was added to the enzyme. Heat treatment was performed at 37°C.
Table I: Ka for activation of various effectors

<table>
<thead>
<tr>
<th>Activator</th>
<th>Ka</th>
<th>^a% activity</th>
<th>bPhysiological concentration mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-PGA</td>
<td>0.22 +/- 0.034</td>
<td>100</td>
<td>1.75</td>
</tr>
<tr>
<td>Fructose 6-P</td>
<td>0.4 +/- 0.09</td>
<td>70</td>
<td>0.5</td>
</tr>
<tr>
<td>Glucose 6-P</td>
<td>2.7 +/- 0.79</td>
<td>67</td>
<td>3.23</td>
</tr>
<tr>
<td>Glycerophosphate</td>
<td>3.6 +/- 0.76</td>
<td>103</td>
<td></td>
</tr>
<tr>
<td>Phosphoenolpyruvate</td>
<td>5.0 +/- 1.7</td>
<td>37</td>
<td>0.27</td>
</tr>
<tr>
<td>Ribose 5-P</td>
<td>1.0 +/- 0.19</td>
<td>82</td>
<td></td>
</tr>
<tr>
<td>6-phosphogluconic acid</td>
<td>6.0 +/- 2.6</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>Sucrose- 6- phosphate</td>
<td>11.9 +/- 7.1</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>No activator</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pi</td>
<td></td>
<td></td>
<td>6.69</td>
</tr>
</tbody>
</table>

^a Relative activation of AGPase by the indicated metabolite compared to that of activation by 3-PGA

^bData taken from Liu and Shannon 1981

No activation is seen in the presence of ribulose 1,5 di-P, erythrose -4-P or methyl phosphate using concentrations up to 20mM
Table II: Effect of substrates and effectors on heat denaturation as assayed in both the forward and back directions

<table>
<thead>
<tr>
<th>Effector/substrate</th>
<th>Forward</th>
<th>Back</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% Activity Remaining</td>
<td>% Activity Remaining</td>
</tr>
<tr>
<td>none</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>ATP</td>
<td>14</td>
<td>19</td>
</tr>
<tr>
<td>ATP + Pi</td>
<td>58</td>
<td>71</td>
</tr>
<tr>
<td>ATP + 3PGA</td>
<td>62</td>
<td>85</td>
</tr>
<tr>
<td>ATP + G-1-P + 3-PGA + Ca</td>
<td>63</td>
<td>aND</td>
</tr>
<tr>
<td>G-1-P</td>
<td>0</td>
<td>ND</td>
</tr>
<tr>
<td>G-1-P + Pi</td>
<td>51</td>
<td>ND</td>
</tr>
<tr>
<td>G-1-P + 3PGA</td>
<td>21</td>
<td>ND</td>
</tr>
<tr>
<td>ADPG</td>
<td>59</td>
<td>60</td>
</tr>
<tr>
<td>ADPG + Ca</td>
<td>ND</td>
<td>54</td>
</tr>
<tr>
<td>ADPG + Pi</td>
<td>73</td>
<td>75</td>
</tr>
<tr>
<td>ADPG + 3PGA</td>
<td>100</td>
<td>ND</td>
</tr>
<tr>
<td>ADPG + PPi + Ca</td>
<td>ND</td>
<td>47</td>
</tr>
<tr>
<td>Pi</td>
<td>47</td>
<td>64</td>
</tr>
<tr>
<td>3PGA</td>
<td>30</td>
<td>39</td>
</tr>
<tr>
<td>Pi + 3PGA</td>
<td>ND</td>
<td>67</td>
</tr>
<tr>
<td>PPi + 3PGA</td>
<td>ND</td>
<td>12</td>
</tr>
<tr>
<td>PPi</td>
<td>ND</td>
<td>0</td>
</tr>
<tr>
<td>PPi + Pi</td>
<td>ND</td>
<td>45</td>
</tr>
</tbody>
</table>

*aND—not determined

Substrates and effectors were added to a final concentration of 5mM, with the exception of PPi which was added to a final concentration of 10mM. All substrate/effector mixes contain 10mM MgCl₂, except where CaCl₂ is specified. Enzymes were incubated for 10 min at 42°C, placed on ice, then assayed in either the forward or reverse direction.
Table III: Kd for various effector molecules

<table>
<thead>
<tr>
<th>Effector molecule</th>
<th>Kd mM</th>
<th>k_2/k_1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pi</td>
<td>0.07</td>
<td>0.14</td>
</tr>
<tr>
<td>3-PGA</td>
<td>0.84</td>
<td>0.19</td>
</tr>
<tr>
<td>ATP</td>
<td>1.0</td>
<td>0.13</td>
</tr>
<tr>
<td>ADP-Glc</td>
<td>0.2</td>
<td>0.13</td>
</tr>
<tr>
<td>F-6-P</td>
<td>1.7</td>
<td>0.23</td>
</tr>
<tr>
<td>Glycerophosphate</td>
<td>8.7</td>
<td>0.17</td>
</tr>
<tr>
<td>Ribose 5-P</td>
<td>7.0</td>
<td>0.48</td>
</tr>
</tbody>
</table>
### Table IV: Stimulatory effect of the addition of a secondary activator

<table>
<thead>
<tr>
<th>Activator</th>
<th>Fold stimulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>No secondary activator (0.1mM 3-PGA)</td>
<td>1</td>
</tr>
<tr>
<td>Fructose 6-P</td>
<td>1.64</td>
</tr>
<tr>
<td>Glucose 6-P</td>
<td>2.19</td>
</tr>
<tr>
<td>Glycerophosphate</td>
<td>2.15</td>
</tr>
<tr>
<td>Phosphoenolpyruvate</td>
<td>1.55</td>
</tr>
<tr>
<td>Ribose 5-P</td>
<td>1.78</td>
</tr>
<tr>
<td>6-phosphogluconic acid</td>
<td>1.11</td>
</tr>
<tr>
<td>3-PGA</td>
<td>1.90</td>
</tr>
</tbody>
</table>

All samples contained 0.1mM 3-PGA (~1/2 Ka). Metabolites were then added to a concentration of ½ their Ka (see Table I) and fold stimulation was calculated as the amount of activity in the presence of activator divided by amount of activity in the presence of 0.1mM 3-PGA.
Table V: Phosphate Ki in the presence of various activators

<table>
<thead>
<tr>
<th>Effector</th>
<th>Ki (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-PGA</td>
<td>1.75 +/- 0.025</td>
</tr>
<tr>
<td>F-6-P</td>
<td>0.99 +/- 0.35</td>
</tr>
<tr>
<td>G-6-P</td>
<td>1.07 +/- 0.39</td>
</tr>
<tr>
<td>none</td>
<td>&gt;100mM</td>
</tr>
</tbody>
</table>

*Effectors were used at ~10X their corresponding Ka value (see Table I)