ADP-glucose pyrophosphorylase catalyzes the synthesis of ADP-glucose (ADP-Glc) from Glc-1-phosphate (G-1-P) and ATP. Kinetic studies were performed to define the nature of the reaction, both in the presence and absence of allosteric effector molecules. When 3-phosphoglycerate (3-PGA), the putative physiological activator, was present at a saturating level, initial velocity studies were consistent with a Theorell-Chance BiBi mechanism and product inhibition data supported sequential binding of ATP and G-1-P, followed by ordered release of pyrophosphate and ADP-Glc. A sequential mechanism was also followed when 3-PGA was absent, but product inhibition patterns changed dramatically. In the presence of 3-PGA, ADP-Glc is a competitive inhibitor with respect to ATP. In the absence of 3-PGA—with or without 5.0 mM inorganic phosphate—ADP-Glc actually stimulated catalytic activity, acting as a feedback product activator. By contrast, the other product, pyrophosphate, is a potent inhibitor in the absence of 3-PGA. In the presence of subsaturating levels of allosteric effectors, G-1-P serves not only as a substrate but also as an activator. Finally, in the absence of 3-PGA, inorganic phosphate, a classic inhibitor or antiactivator of the enzyme, stimulates enzyme activity at low substrate by lowering the $K_M$ values for both substrates.

Plant ADP-Glc pyrophosphorylase (AGPase) catalyzes an important, rate-limiting step in starch biosynthesis: the reversible formation of ADP-Glc from ATP and Glc-1-P (G-1-P). Most AGPases are regulated by effector molecules derived from the prevalent carbon metabolism pathway, with inorganic phosphate ($P_i$) and 3-phosphoglycerate (3-PGA) being the most studied effectors of higher plants. Interestingly, the barley (Hordeum vulgare) endosperm form of AGPase is unique among higher plant homologs in its insensitivity to both 3-PGA and $P_i$ (Kleczkowski et al., 1993a). Heat lability (as often found for endosperm AGPases) and reductive activation (for those AGPases harboring an N-terminal Cys residue in the small subunit) are also important mechanisms by which AGPases are regulated (Fu et al., 1998; Tiessen et al., 2002).

Transgenic plant studies emphasize the importance of allosteric effectors in controlling enzyme activity and, in turn, starch yield. For example, expressing an allosterically enhanced Escherichia coli AGPase resulted in a 35% increase in potato (Solanum tuberosum) tuber starch (Stark et al., 1992) and a 22% to 25% increase in maize (Zea mays) seed starch (Wang et al., 2007). Rice (Oryza sativa) seed weight was increased up to 11% by expression of a second E. coli-derived AGPase mutant with altered allosteric properties (Sakulsingharoj et al., 2004). In another example, expressing a maize AGPase variant with less sensitivity to $P_i$ and enhanced heat stability led to a 38% increase in wheat (Triticum aestivum) yield (Smidansky et al., 2002), a 23% increase in rice yield (Smidansky et al., 2003), and up to a 68% increase in maize yield (L.C. Hannah, unpublished data). Increases in these cases were due to enhanced seed number. Finally, transgenic expression of an allosterically altered potato tuber AGPase enhanced Arabidopsis (Arabidopsis thaliana) leaf transitory starch turnover and improved growth characteristics (Obana et al., 2006) and enhanced the fresh weight of aerial parts of lettuce (Lactuca sativa) plants (Lee et al., 2009).

In higher plants, AGPase is a heterotetramer, consisting of two large and two small subunits; by contrast, most bacterial AGPases are homotetramers. Crystal structures of a bacterial AGPase and a non-native, small subunit homotetramer derived from the potato tuber enzyme have been described recently (Jin et al., 2005; Cupp-Vickery et al., 2008). Unfortunately, since both structures were determined in the presence of high sulfate concentrations, both enzymes are in inactive forms.

While AGPase allosteric regulation has received a great deal of attention, the kinetic mechanism has been defined completely only for two cases: the helmotetra-
meric form from the bacterium *Rhodospirillum rubrum* and the plant heterotetrameric enzyme from barley leaf (Paule and Preiss, 1971; Kleczkowski et al., 1993b). The kinetic mechanism is sequential in both cases, with ATP the first substrate bound and ADP-Glc the final product released. Despite this similarity, there are important differences, most notably the existence of an isomerization step following ADP-Glc release, so that this product and ATP bind to different forms of the barley enzyme. This isomerization step is absent from the bacterial enzyme. Interestingly, isoforms of the closely related nucleoside diphospho-Glc family exhibit fundamentally different kinetic mechanisms. Some UDP-Glc pyrophosphorylases catalyze a sequential BiBi mechanism (Elling, 1996), while others, such as dTDP-Glc and CDP-Glc pyrophosphorylases from *Salmonella*, employ a ping-pong mechanism (Lindqvist et al., 1993, 1994).

Because of the mechanistic diversity exhibited by pyrophosphorylases in general and by the two well-characterized AGPases in particular, we investigated the kinetic mechanism of the recombinant maize endosperm AGPase. We were particularly interested in the roles played by allosteric effectors that appear to be critically important in catalytic efficiency and, thus, starch content. Surprisingly, patterns of initial velocity at varying substrate concentrations as well as product inhibition behavior were identical to those observed for the homotetrameric bacterial enzyme (Paule and Preiss, 1971) and differed significantly from the heterotetrameric barley leaf enzyme (Kleczkowski et al., 1993b). Moreover, we found that both G-1-P and ADP-Glc could stimulate AGPase catalytic activity beyond that expected for simple substrate effects. We also found that the classic inhibitor, Pi, actually enhanced AGPase activity at low substrate concentrations but inhibited activity at high substrate levels. A model is presented to account for this observation. Finally, we determined that 3-PGA only stimulates AGPase activity by 2.5-fold if care is taken to saturate with substrates during assays.

**RESULTS**

Initial Velocity Studies of Maize Endosperm AGPase in the Presence of 3-PGA

Initial velocity studies in the forward direction (ADP-Glc synthesis) were performed in which one substrate was varied and the other was held constant at several nonsaturating concentrations. All data were obtained using a coupled pyrophosphate (PPi) assay, unless otherwise stated, and enzyme samples were doubly desalted to remove trace amounts of Pi. Initial velocity patterns for the AGPase were determined in the presence and absence of the activator, 3-PGA, and the effector molecule, Pi.

In the presence of 5.0 mM 3-PGA, plots of 1/v versus 1/[ATP] at fixed concentrations of G-1-P intersected to the left of the y axis (Fig. 1A). The reciprocal plot of 1/v versus 1/[G-1-P] at fixed concentrations of ATP also intersected to the left of the y axis (Fig. 1B). These patterns are characteristic of a sequential mechanism in which both substrates are added to the enzyme before a product is released. Values of K_M for both substrates were determined from replots of 1/V_{max,app} versus 1/[substrate] of the double reciprocal plots, which were linear (Fig. 1, A and B, insets). The K_M,G-1-P value was 0.04 ± 0.002 mM while K_M,ATP was 0.18 ± 0.02 mM, which are consistent with previously reported data (Boehlein et al., 2005). The calculated maximum velocity was 15.0 μmol min^{-1} mg^{-1}. Initial velocity studies in the back direction of ADP-Glc pyrophosphorylation yielded nonhyperbolic patterns and were not pursued further.

Reaction rates were also measured by varying [G-1-P] in the presence of 1.0 mM and 25 mM 3-PGA. At the lower level of 3-PGA, G-1-P saturation was reached between 25 and 50 mM, but the velocity data did not fit a simple hyperbolic curve; instead, the data were consistent with two-site kinetics. Negative cooperation

![Figure 1](https://www.plantphysiol.org/content/152/2/1057/F1.large.jpg)

**Figure 1.** A, Double reciprocal plot of 1/v versus 1/[ATP] in the presence of 5.0 mM 3-PGA at different [G-1-P]: 0.030 mM (▲), 0.060 mM (●), 0.090 mM (▼), 0.15 mM (◇), 0.30 mM (●), and 0.75 mM (□). Inset, Replot of 1/v versus 1/[G-1-P]. B, Double reciprocal plot of 1/v versus 1/[G-1-P] in the presence of 5.0 mM 3-PGA at different [ATP]: 0.060 mM (▲), 0.090 mM (▼), 0.15 mM (◇), 0.30 mM (●), and 0.75 mM (□). Inset, Replot of 1/v versus 1/[ATP].
between the G-1-P binding sites (Hill coefficient of 0.55 ± 0.07) was detected at the higher 3-PGA concentration. Analogous studies were performed in the presence of another known allosteric activator (Fru-6-P [F-6-P]), and comparable results were obtained (Fig. 2). In the presence of F-6-P, two distinct G-1-P binding sites were observed and a Hill coefficient of 0.64 ± 0.17 was apparent at the higher F-6-P concentration. In sum, these data show that G-1-P can activate AGPase in the presence of nonsaturating levels of allosteric activators.

**Product Inhibition Studies in the Presence of 3-PGA**

Product inhibition studies were undertaken to determine the order of substrate binding and product release and to gain insight into the regulation of the enzyme activity by product levels. All reactions were carried out in the presence of 5.0 mM 3-PGA. The ATP concentration was varied in the presence of nonsaturating levels of G-1-P (0.20 mM), at several fixed concentrations of ADP-Glc. In addition, velocities were measured at varying G-1-P concentrations at a nonsaturating level of ATP (0.40 mM) at several fixed levels of ADP-Glc. ADP-Glc was found to be competitive with ATP (see Supplemental Fig. S1). A replot yielded a value for \( K_{ii} \) of 0.12 ± 0.03 mM for ADP-Glc. ADP-Glc was noncompetitive with respect to G-1-P with values for both \( K_{ii} \) and \( K_{is} \) of approximately 1.1 ± 0.28 mM (Table I). The maximum velocity of the reaction was calculated to be 18.5 ± 0.5 μmol min\(^{-1}\) mg\(^{-1}\). These data are consistent with ATP and ADP-Glc being substrate-product pairs that compete for the same enzyme form.

In a similar manner, product inhibition studies were performed in the presence of varying concentrations of PP\(_i\). These data were obtained using a radioactive assay in which \(^{14}\)C G-1-P was converted to \(^{14}\)C ADP-Glc since the normal, coupled assay measures PP\(_i\) synthesis. PP\(_i\) was noncompetitive with respect to ATP (Fig. 3A), with a \( K_{is} \) value of 70 ± 24 mM and a \( K_{ii} \) value of 0.28 ± 0.03 mM (Fig. 3A, inset). By contrast, PP\(_i\) was a competitive inhibitor of G-1-P (Fig. 3B), with a \( K_{is} \) of 72 ± 13 mM (Fig. 3B, inset). Such behavior is consistent with a sequential mechanism whereby G-1-P and PP\(_i\) are substrate-product pairs, competing for the same enzyme form. Taken together, the initial velocity and product inhibition patterns are consistent with a Theorell-Chance BiBi mechanism, in which the concentration of the central complex is extremely low compared to other enzyme forms.

**Initial Velocity Studies in the Presence of P\(_i\)**

We next determined the initial velocity patterns in the presence of 5.0 mM P\(_i\) to determine whether the
The kinetic mechanism differed from that observed in the presence of 3-PGA. While Pi has previously been believed to be a strong inhibitor of several AGPases, we (Boehlein et al., 2008) and others (Plaxton and Preiss, 1987) have shown that this is not the case for the maize endosperm enzyme. Phosphate exhibits a $K_{i,app}$ of only approximately 3.0 mM in the presence of 5.0 mM 3-PGA. We have also shown that concentrations of Pi near the $K_{i,app}$ value inhibit activity only weakly in the absence of 3-PGA; in fact, Pi actually activates the enzyme at low substrate concentrations (Boehlein et al., 2009).

Initial velocity studies of $1/v$ versus $1/[ATP]$ with varying [G-1-P] concentrations and $1/v$ versus $1/[G-1-P]$ at varying ATP concentrations both yielded intersecting lines (Supplemental Fig. S2). The $K_M$ values for G-1-P and ATP, as determined from intercept replots, were 1.1 ± 0.17 mM and 0.75 ± 0.15 mM, respectively. The former value is considerably higher (approximately 28-fold) than $K_M,G-1-P$ determined in the presence of 5.0 mM 3-PGA. By contrast, the value of $K_M,ATP$ was only slightly elevated (approximately 4-fold). The value of $V_{max}$ in the presence of 5.0 mM Pi was 6.3 ± 0.2 µmol min$^{-2}$ mg$^{-1}$, approximately 2.4-fold lower than that determined in the presence of 5.0 mM 3-PGA.

Product Inhibition Studies in the Presence of Pi

The product inhibition patterns in the presence of 5.0 mM Pi were determined using the methods described above. First, velocity versus [ATP] curves were obtained in the presence of increasing concentrations of ADP-Glc, with [G-1-P] held constant at 5.0 mM (approximately $5 \times K_M$). Interestingly and unexpectedly, ADP-Glc did not inhibit the reaction but actually activated the enzyme in the absence of 3-PGA (Fig. 4). Increasing concentrations of ADP-Glc increased the $V_{max}$ by approximately 1.5-fold while the $K_{M,ATP}$ remained approximately constant (Supplemental Fig. S3). We also determined the effect of ADP-Glc when varying the concentration of G-1-P at constant [ATP] (2.5 mM or $3.5 \times K_{M,ATP}$). Again, ADP-Glc activated the enzymatic activity by approximately 2-fold, and did not significantly affect $K_{M,G-1-P}$ (Supplemental Fig. S4).

Product inhibition studies were then carried out in the presence of PP$_i$. PP$_i$ was found to be a strong inhibitor with respect to ATP when G-1-P was held constant at 0.75 mM. A Dixon plot revealed that at 0.5 mM ATP, the $K_{i,app}$ for PP$_i$ was 0.31 ± 0.02 mM. Experiments with varying G-1-P concentrations could not be performed due to the extremely high concentrations of radioactive G-1-P necessary to perform the experiments.

Initial Velocity Measurements in the Absence of Effector Molecules

We next measured initial velocities in the absence of both 3-PGA and Pi. While many AGPases have very low activity levels in the absence of allosteric activators, the maize endosperm enzyme exhibits substantial catalysis without 3-PGA. To determine the level of G-1-P necessary to achieve saturation, the [ATP] was held constant at 2.0 mM and the concentration of G-1-P was varied up to 100 mM. To our surprise, no evidence for saturation was obtained (Fig. 2). Plots of initial velocity versus $1/[ATP]$ with varying [G-1-P] concentrations and $1/v$ versus $1/[G-1-P]$ at varying ATP concentrations both yielded intersecting lines (Supplemental Fig. S2). The $K_M$ values for G-1-P and ATP, as determined from intercept replots, were 1.1 ± 0.17 mM and 0.75 ± 0.15 mM, respectively. The former value is considerably higher (approximately 28-fold) than $K_M,G-1-P$ determined in the presence of 5.0 mM 3-PGA. By contrast, the value of $K_M,ATP$ was only slightly elevated (approximately 4-fold). The value of $V_{max}$ in the presence of 5.0 mM Pi was 6.3 ± 0.2 µmol min$^{-2}$ mg$^{-1}$, approximately 2.4-fold lower than that determined in the presence of 5.0 mM 3-PGA.

Table 1. Inhibition patterns in the presence of 5.0 mM 3-PGA

<table>
<thead>
<tr>
<th>Substrate</th>
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<th>Inhibitor</th>
<th>Pattern</th>
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<th>$K_s$</th>
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<tr>
<td>G-1-P</td>
<td>**ATP</td>
<td>ADP-Glc</td>
<td>NC</td>
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<tr>
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<td>G-1-P</td>
<td>PP$_i$</td>
<td>NC</td>
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</tr>
<tr>
<td>G-1-P</td>
<td>ATP</td>
<td>PP$_i$</td>
<td>C</td>
<td>0.07</td>
<td></td>
</tr>
</tbody>
</table>

Figure 3. A, Double reciprocal plot of $1/v$ versus $1/[ATP]$ in the presence of 5.0 mM 3-PGA at different [PP$_i$]: 0 mM ( ), 0.10 mM ( ▲ ), 0.25 mM ( ▼ ), 0.50 mM ( ● ), and 0.75 mM ( ○ ). The [G-1-P] was held constant at 0.20 mM. Inset, Replot of slope ( ) or intercept ( ▲ ) versus [PP$_i$]. B, Double reciprocal plot of $1/v$ versus $1/[G-1-P]$ in the presence of 5.0 mM 3-PGA at different [PP$_i$]: 0 mM ( ), 0.10 mM ( ▲ ), 0.25 mM ( ▼ ), 0.50 mM ( ● ), and 0.75 mM ( ○ ). The [ATP] was held constant at 0.40 mM. Inset, Replot of slope versus [PP$_i$].
velocity versus [ATP] at nonsaturating G-1-P concentrations (4.0–15 mM) yielded hyperbolic curves, and double reciprocal plots were linear. Both the apparent $K_{\text{M,ATP}}$ and $V_{\text{max,app}}$ values decreased with increasing concentrations of G-1-P. Double reciprocal plots intersected to the right of the $y$ axis (Fig. 5) and the replot of these data yielded a descending curve. We then plotted velocity versus [G-1-P] at constant ATP concentrations. Here, hyperbolic kinetics were exhibited only at the higher ATP levels. When [ATP] was <5 mM, velocities increased linearly with increasing ATP concentrations, suggesting that the enzyme was not near saturation, even at 20 mM G-1-P (Supplemental Fig. S5).

**Product Inhibition Studies in the Absence of Effector Molecules**

We first measured the effect of ADP-Glu on the saturation curves for ATP. At ADP-Glc levels up to 0.25 mM, the enzyme activity was stimulated by approximately 1.5-fold, and $K_{\text{M,ATP}}$ remained constant at approximately 1.0 mM in the presence of 7.5 mM G-1-P. In addition, velocities measured by varying the [G-1-P] at a constant ATP level (2.5 mM) also revealed stimulation by ADP-Glc. G-1-P saturation was not observed, even at concentrations up to 20 mM. Double reciprocal plots indicate that the value $K_{\text{M,G-1-P}}$ is >35 mM when [ATP] is 2.5 mM. We were unable to investigate inhibition caused by PPi because of the high concentration/low specific activity of the radioactive G-1-P necessary to perform them.

**Inhibition Studies with Varying $P_i$**

We were intrigued by the discovery that the maize endosperm AGPase could not be saturated with G-1-P in the absence of 3-PGA, but saturation could be achieved in the presence of $P_i$. To determine a threshold for this effect, we measured velocities at several [P$_i$] in the face of varying concentrations of ATP or G-1-P. In the first study, the [ATP] was held constant at 2.5 mM and the [G-1-P] was varied from 0 to 20 mM in the presence of several fixed $P_i$ levels. Under these conditions, saturation with G-1-P could be reached in the presence of $P_i$ (Fig. 6A). Interestingly, both the $K_{\text{M,G-1-P}}$ and $V_{\text{max}}$ values decreased with increasing $P_i$ concentrations. In fact, velocities at low [G-1-P] actually increased with increasing [P$_i$], providing further evidence that $P_i$ is not simply an inhibitor of maize endosperm AGPase. Double reciprocal plots of 1/v versus 1/[G-1-P] at different $P_i$ levels intersected to the right of the $y$ axis (Fig. 6A), showing that $P_i$ is both an activator and an inhibitor, more specifically a hyperbolic mixed-type inhibitor. This ion increases catalytic efficiencies at low [G-1-P] but inhibits at high [G-1-P].

In a similar manner, we measured the effect of variable $P_i$ concentrations on the saturation curves for G-1-P at constant levels of both ATP (0.40 mM) and 3-PGA (5.0 mM; Fig. 6B). Under these conditions, the $K_{\text{M,G-1-P}}$ value was not affected by $P_i$ and the double reciprocal plots intersected to the left of the $y$ axis. This behavior is consistent with that of a pure noncompetitive inhibitor. On the other hand, replots of both the slopes and intercepts versus [P$_i$] yielded hyperbolic curves, indicating that $P_i$ is a slope-hyperbolic, intercept-hyperbolic noncompetitive inhibitor of G-1-P (Segel, 1975). In the presence of the activator 3-PGA, $P_i$ does not affect the affinity of the enzyme for either substrate but it does decrease the reaction rate. In the absence of 3-PGA, however, $P_i$ increases the affinity of the enzyme for each substrate (Fig. 6A).

We determined the effect of varying $P_i$ levels on ATP saturation behavior in the presence of constant [G-1-P] (7.5 mM; Fig. 7A). We were unable to achieve saturation with ATP at concentrations up to 15 mM in the absence of $P_i$, although the affinity for ATP increased in the presence of 3-PGA.
over 10-fold with increasing concentrations of P_i. Increasing [P_i] decreased \( V_{\text{max, app}} \), although the decrease was 2-fold. As observed in our G-1-P studies, the double reciprocal plot intersected to the right of the \( y \) axis, and the replot obtained from the slope versus [P_i] was a hyperbolic descending curve, consistent with a hyperbolic mixed-type inhibitor.

We next investigated the effect of varying [P_i] on the ATP saturation behavior when both [3-PGA] and [G-1-P] were held constant (5.0 mM and 0.20 mM, respectively). In contrast to the patterns observed in the absence of 3-PGA, P_i behaved as a partial competitive inhibitor of ATP under these conditions (Segel, 1975), as evidenced by the nonlinear slope replot (Fig. 7B). The maximum velocity of the reaction was essentially independent of [P_i], while \( K_{M, \text{ATP}} \) increased with increasing [P_i]. The double reciprocal plot intersected on the \( y \) axis, but the replot of slope versus [P_i] was hyperbolic.

We considered the possibility that the G-1-P used in these studies was contaminated with an effector molecule, and the observed increase in activity was not due to G-1-P, but rather to a contaminant. G-6-P and P_i were the most logical possibilities. A spectrophotometric assay revealed \( <10 \mu M \) G-6-P in our 100 mM stock of G-1-P. Free phosphate analysis yielded a value of 0.3% P_i in our G-1-P stock. In both cases, the levels of contaminating allosteric effectors were too low to account for the stimulatory effects described above.
Our ordered kinetic scheme for maize endosperm AGPase is consistent with the patterns proposed for the two AGPases that have been examined in detail in previous studies (Paule and Preiss, 1971; Kleczkowski et al., 1993b). In all cases, ATP is the first substrate bound and ADP-Glc is the last product released. Unexpectedly, the kinetic mechanism of the maize endosperm heterotetrameric AGPase appears identical to the homotetrameric bacterial enzyme, and it differs from the heterotetrameric barley leaf enzyme in two important aspects. In the barley enzyme, ADP-Glc was a noncompetitive inhibitor with respect to ATP, implying that two distinguishable free isoforms of the barley leaf enzyme exist. In addition, PP_i was not a competitive inhibitor with respect to G-1-P for the barley enzyme, suggesting that the central ternary complexes (E·ATP·G-1-P and E·PP_i·ADP-Glc) accumulate. This means that the barley enzyme follows an ordered BiBi pattern rather than the Theorell-Chance BiBi mechanism observed in the R. rubrum and maize endosperm AGPases. The data presented here are inconsistent with an earlier speculation (Kleczkowski et al., 1993b) that the more complex kinetic mechanism followed by the barley enzyme reflected the greater structural complexities of heterotetrameric plant AGPases.

Classically, 3-PGA is viewed as an activator of AGPases while P_i is considered an inhibitor. This simple picture is not applicable to the maize endosperm AGPase. Instead, our data clearly demonstrate that P_i activates the enzyme at low substrate concentrations but inhibits catalysis at high substrate concentrations. This notion is supported by double reciprocal plots that intersect to the right of the y axis and replots of slope and intercept versus [P_i] that are hyperbolic, descending curves.

Our data show that each substrate has a greater affinity for the enzyme in the presence of P_i (most likely binding to an E-[P_i]_k complex containing one or more bound phosphate ions), but the reaction rate is reduced by the presence of phosphate (Segel, 1975). When the substrate concentrations are low, catalysis via the E-(P)_k complex dominates because the substrates have a greater affinity for this complex as compared to the free enzyme. The greater substrate affinity more than compensates for the reduced catalytic rate of the E-(P)_k complex as compared to free enzyme under the same conditions. The overall result is P_i-mediated activation. By contrast, under conditions where substrate concentrations approach saturation, both the free enzyme and the E-(P)_k complex become loaded with substrates. Overall, a reduced rate is observed when comparing the E-(P)_k complex to the free enzyme-mediated reaction because the former is a less efficient catalyst. On the other hand, the addition of 3-PGA to the reaction changes the role of P_i. The effect of 3-PGA is to enhance the enzyme’s affinity for both substrates. Thus, at both low and high substrate concentrations, P_i acts as a partial noncompetitive inhibitor of G-1-P and a partial competitive inhibitor of ATP in the presence of 3-PGA.

The unexpected finding that P_i can activate AGPase by lowering K_M values explains the conflicting reports in the literature concerning the extent of 3-PGA activation on the maize endosperm AGPase. Reported values range from 2- up to 20-fold (Dickinson and Preiss, 1969; Hannah and Nelson, 1975; Plaxton and Preiss, 1987; Burger et al., 2003). These differences have been ascribed to partial proteolytic cleavage of the enzyme (Plaxton and Preiss, 1987) and to polymorphic differences in protein sequence (Burger et al., 2003). This study demonstrated that in the absence of allosteric effectors, G-1-P saturation cannot be attained; hence, measured velocities in the absence of effectors underestimate the true velocity and, in turn, overestimate the extent of 3-PGA activation. Previous studies that reported low extents of 3-PGA-mediated activation were carried out with enzyme preparations that contained significant amounts of P_i. Under these conditions, the true extent of 3-PGA activation could not be measured since the enzyme was already partially activated by P_i.

We were also surprised to discover that G-1-P and ADP-Glc are not only reaction substrates but also allosteric effectors. In the absence of 3-PGA and P_i, increasing concentrations of G-1-P decreased K_M,ATP and also decreased V_max. This was evident in the double reciprocal plots that intersected to the right of the y axis, the same pattern observed when P_i was the varied reaction component. Similarly, in the presence of subsaturating levels of the activators 3-PGA and F-6-P, G-1-P binding is best described by a two-site kinetic model. At high levels of 3-PGA and F-6-P, Hill coefficients less than 1 are noted for G-1-P saturation. This suggests that, like ATP, G-1-P serves not only as a substrate but also as an inhibitor of the reaction.

Finally, these studies also revealed that in the absence of 3-PGA (but not in its presence), ADP-Glc activates AGPase in the forward direction of ADP-Glc synthesis. This was totally unexpected and the physiological relevance of this observation is not clear. This situation would only arise in the absence of both an activator molecule and PP_i, since the latter is a potent inhibitor of catalysis in the forward direction. Experimental measurements of metabolite levels in the cytosol of developing endosperm cells should shed light on the possible relevance of this surprising finding.

**MATERIALS AND METHODS**

Purification and expression of recombinant AGPase is described elsewhere (Boehlein et al., 2005, 2008). Briefly, *Escherichia coli* AC70R1-504 (Iglesias et al., 1993) was transformed with both pMoncSh2 (large subunit of AGPase) and pMoncBt2 (small subunit of AGPase; Giroux et al., 1996) and grown and purified as described in Boehlein et al. (2008). Importantly, all preparations were twice desalted to remove P_i before assay. This was performed using Zeba micro desalt spin columns according to the manufacturer’s instructions (Fierce). The Ibo-Rad protein assay kit was used to determine protein concentration. A standard curve was made using a known concentration of AGPase determined from total amino acid analysis. Stock solutions of G-1-P were monitored for phosphate, using the EnzChek phosphate assay kit from...
Invitrogen. G-6-P contamination in these G-1-P solutions was monitored by use of the AGPase forward assay in the absence of phosphoglucomutase (Boehlein et al., 2008).

Initial Velocity Studies
Kinetic parameters were determined by incubating purified AGPase in reaction mixtures in which one substrate was varied and the other was held constant, at several different concentrations. In the presence of activator, assays contained 50 μm HEPES pH 7.4, 5.0 μm 3-PGA, 15 μm MgCl₂, and 0.05 μg of the enzyme in a total volume of 300 μL. When the [ATP] was varied, the concentration ranged from 0.06 to 0.75 μm, with G-1-P concentrations varied from 0.03 to 0.75 μm. In the absence of an activator, 0.20 μg of enzyme was used and the ATP concentration was varied from 0.75 to 20 μm with G-1-P concentrations varied from 2.5 to 20 μm. In the presence of 5.0 mM Pi, or the absence of effector, 0.2 μg of enzyme was used and the ATP concentration was varied from 0.25 to 10 μm with G-1-P concentrations varied from 2.0 to 15 μm. Initial velocities were determined spectrophotometrically by following the production of PPi, during ADP-Glc synthesis, as described previously (Boehlein et al., 2008). Assays were duplicated and averages are presented. Kinetic constants were obtained by nonlinear regression using equations derived from the full kinetic expression using the software program Prism (Graph Pad). All assays were linear with time and protein concentration.

Product Inhibition Studies
Velocities were measured spectrophotometrically as described above. Velocities are the average of two experiments. All assays contained 50 μm HEPES pH 7.4, 5.0 mM 3-PGA, 15 μm MgCl₂, and 0.05 μg of the enzyme in a total volume of 300 μL. When the [ATP] was varied, the concentration range was 0.050 to 3.0 μm with [G-1-P] held constant at 0.20 μm. When the [G-1-P] was varied, the concentration ranges were 0.050 to 3.0 μm with the [ATP] held constant at 0.40 μm. The inhibitor, ADP-Glc was added at 0.1 to 0.75 μm. When PPi was used as an inhibitor, a radioactive assay was used in which incorporation of [14C]G-1-P into ADP-Glc was measured. All reaction mixtures contained ATP, G-1-P, and ATP concentrations varied from 0.03 to 20 μm. In the absence of an activator, 0.20 μg of enzyme was used and the ATP concentration was varied from 0.03 to 2.0 μm. In the presence of 5.0 mM Pi or the absence of effector, 0.20 μg of enzyme was used and the ATP concentration was varied from 0.03 to 2.0 μm.

Supplemental Data
The following materials are available in the online version of this article. The following materials are available in the online version of this article.

LITERATURE CITED


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