Characterization of an Autonomously Activated Plant
ADP-Glucose Pyrophosphorylase$^{[OA]}$

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ADP-glucose pyrophosphorylase (AGPase) catalyzes the rate-limiting step in starch biosynthesis in plants and changes in its catalytic and/or allosteric properties can lead to increased starch production. Recently, a maize (Zea mays)/potato (Solanum tuberosum) small subunit mosaic, MP [Mos(1–198)], containing the first 198 amino acids of the small subunit of the maize endosperm enzyme and the last 277 amino acids from the potato tuber enzyme, was expressed with the maize endosperm large subunit and was reported to have favorable kinetic and allosteric properties. Here, we show that this mosaic, in the absence of activator, performs like a wild-type AGPase that is partially activated with 3-phosphoglyceric acid (3-PGA). In the presence of 3-PGA, enzyme properties of Mos(1–198)/SH2 are quite similar to those of the wild-type maize enzyme. In the absence of 3-PGA, however, the mosaic enzyme exhibits greater activity, higher affinity for the substrates, and partial inactivation by inorganic phosphate. The Mos(1–198)/SH2 enzyme is also more stable to heat inactivation. The different properties of this protein were mapped using various mosaics containing smaller portions of the potato small subunit. Enhanced heat stability of Mos(1–198) was shown to originate from five potato-derived amino acids between 322 and 377. These amino acids were shown previously to be important in small subunit/large subunit interactions. These five potato-derived amino acids plus other potato-derived amino acids distributed throughout the carboxyl-terminal portion of the protein are required for the enhanced catalytic and allosteric properties exhibited by Mos(1–198)/SH2.

ADP-glucose pyrophosphorylase (AGPase) catalyzes the first committed step in starch biosynthesis in plants and glycogen production in bacteria, the conversion of Glc-1-P + ATP to ADP-Glc + pyrophosphate (PPi; Stark et al., 1992, Ballicora et al., 2004, Preiss 1973, 1978). ADP-Glc is the Glc donor for plant starch biosynthesis and bacterial glycogen synthesis. AGPases are tetramers composed of approximately 50,000–kD subunits. Bacterial isoforms are homotetramers, whereas two distinct subunits make up the α2β2 heterotetrameric plant enzymes. AGPase is located in the plastids of spinach (Spinacia oleracea) leaves (Okita et al., 1979) and potato (Solanum tuberosum) tubers (Kim et al., 1989); however, assorted studies identified a cytoplasmic location for endosperm AGPases (Giroux and Hannah, 1994; Villand and Kleczkowski; 1994, Cao et al., 1995; Denyer et al., 1996; Shannon et al., 1996; Thorbjørnsen et al., 1996; Brangeon et al., 1997; Beckles et al., 2001).

Evidence now available from six separate transgenic studies points to the rate-limiting role AGPase plays in starch biosynthesis. Allosteric properties and heat stability appear to be paramount. Activity of plant AGPases is altered by several cellular metabolites, and 3-phosphoglyceric acid (3-PGA) activation and inorganic phosphate (Pi) inhibition have been extensively investigated. For example, a mutant Escherichia coli AGPase (glgC-16) with altered allosteric properties was expressed in potato and the rate of starch synthesis increased 35% (Stark et al., 1992). The mutant E. coli enzyme was recently shown to increase maize seed weight 22% to 25% (Wang et al., 2007). AGPase of the transgenic maize seed was shown to be less sensitive to Pi inhibition compared to wild type. Expression in rice (Oryza sativa) seed of another E. coli-derived AGPase mutant with altered allosteric properties also

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enhanced seed weight up to 11% (Sakulsingharoja et al., 2004).

It was shown previously that reduced AGPase sensitivity to Pi causes increased maize seed weight (Giroux et al., 1996). A site-specific, in vivo transposon mutagenesis system was employed to cause mutations within a region of the maize large subunit thought important in allosteric regulation. One variant reduced Pi sensitivity and increased seed weight 11% to 18%.

Expression of an allosterically altered potato tuber AGPase (Greene et al., 1998) in Arabidopsis (Arabidopsis thaliana) leaf chloroplasts increased transient starch levels and improved various growth properties (Obana et al., 2006). Compared to wild type, this variant was more sensitive to activators and less sensitive to inhibitors.

Heat stability of AGPase is another important parameter in altering starch content (Hannah, 2007). Greene and Hannah (1998a, 1998b) isolated a heat-stable variant of the maize endosperm AGPase large subunit via use of an E. coli expression system. This variant was combined with the reduced Pi sensitivity mutation. Expression in various plants increased wheat (Triticum aestivum) yield 38% (Smidansky et al., 2002), rice yield 23% (Smidansky et al., 2003), and maize yield up to 68% (L.C. Hannah, T. Greene, and B. Futch, unpublished data). Unexpectedly, yield increases were due to increased seed number in all three cereals.

Here, we report a modified small subunit variant that conditions some interesting allosteric properties and heat stability characteristics. This variant, termed MP in previous studies (Cross et al., 2004, 2005; Boehlein et al., 2005) and termed mosaic [Mos(1–198)] here, is composed of the amino-terminal 198 amino acids from the maize endosperm small subunit (BT2) and the carboxyl-terminal 277 amino acids from the potato tuber small subunit. When expressed with the wild-type maize endosperm large subunit (SH2), Mos(1–198)/SH2, in the absence of activator, behaves like the wild-type maize endosperm AGPase (BT2/SH2) that is partially activated with 3-PGA. In the absence of 3-PGA, Mos(1–198)/SH2 exhibits greater activity, higher affinity for the substrates, and partial inactivation by Pi and is more stable to heat inactivation. In the presence of 3-PGA, however, Mos(1–198)/SH2 has virtually identical properties to those of the wild-type enzyme. The different properties were mapped to amino acid motifs using various mosaics containing smaller portions of the potato small subunit. Enhanced heat stability of Mos(1–198) is caused by five potato-derived amino acids between 322 and 377. These amino acids are also important in small subunit/large subunit interactions. These five potato-derived amino acids plus other potato-derived amino acids mapping throughout the carboxyl-terminal portion of the protein are required for the enhanced catalytic and allosteric properties exhibited by Mos (1–198).

RESULTS

Characterization of Mos(1–198) in the Absence of 3-PGA

Previously, we (Cross et al., 2004, 2005; Boehlein et al., 2005) showed that the mosaic AGPase Mos(1–198), termed MP in previous publications, when expressed with the maize endosperm large subunit, had several characteristics that may give rise to increased starch synthesis in plants. Mos(1–198) is a maize/potato mosaic small subunit in which the first 198 amino acids come from the maize endosperm small subunit, whereas the carboxyl-terminal 277 amino acids are from the potato tuber AGPase small subunit. Although the potato and maize small subunits are highly conserved, 79 amino acids distinguish the subunits, with 33 amino acid differences residing in the C-terminal 277 amino acids. In the studies reported below, Mos(1–198) and its derivatives are expressed exclusively with the wild-type maize large subunit, SH2.

An intriguing characteristic of Mos(1–198) is that enzymatic activity in the absence of 3-PGA is substantially greater than that of wild type. Typically, the Mos (1–198) enzyme has an activity 2- to 5-fold higher than the wild-type enzyme in the absence of 3-PGA (Table I). Activity was measured in the presence of 2 mM ATP and 2 mM Glc-1-P. Even though Mos(1–198) activity is much greater in the absence of 3-PGA, its extent of activation in the presence of 3-PGA is less. In the presence of 3-PGA, Mos(1–198) AGPase activity is actually less than that of wild type. In other words, Mos(1–198) is less sensitive to 3-PGA and the enzyme appears as if a baseline amount of activator is present at all times.

Three experiments were initially performed to determine whether residual activator resided in the Mos (1–198) preparations. In the first experiment, the Mos (1–198) enzyme was washed through a desalting column (Zeba micro column; Pierce) and activity was measured after each passage. If trace amounts of activator were loosely bound, it would have been removed and activity would have been reduced after desalting. Neither passage reduced 3-PGA-independent activity. In the second experiment, a saturating amount of 3-PGA was added to Mos(1–198) to possibly replace any activator bound to the enzyme. The enzyme was incubated for 10 min with saturating concentrations of 3-PGA, desalted, and the activity was measured. 3-PGA addition and removal did not affect 3-PGA-independent activity. In the third experiment, the Mos(1–198) enzyme was placed in 0.4 M phosphate, desalted, and assayed. This did not affect the high level of 3-PGA-independent activity exhibited by Mos(1–198). Therefore, if there is an activator bound to the Mos(1–198) enzyme, it is extremely tightly bound and cannot be removed by repetitive desalting columns or by first flushing the preparation with concentrated 3-PGA or phosphate and subsequent desalting.
To distinguish between the possibilities that the Mos (1–198) enzyme contains a tightly bound activator that cannot be displaced by 3-PGA or Pi or whether Mos(1–198) naturally exists in a state or conformation induced by an activator in the wild-type enzyme, we monitored Pi inhibition of Mos(1–198). Morell et al. (1988) showed that binding of the activator 3-PGA and the inhibitor Pi are coordinated and that one site on the spinach leaf small subunit is important for binding of both molecules. Binding of pyridoxal phosphate to this single site reduces 3-PGA activation and Pi inhibition. If Mos(1–198) tenaciously binds an activator to this site, greater levels of Pi would be required for inhibition, relative to wild type. In contrast, if Mos(1–198) naturally exists in a semiactivated state in the absence of an activator, then Mos(1–198) should be more sensitive to Pi relative to wild type because the wild-type enzyme is recalcitrant to Pi in the absence of 3-PGA (Boehlein et al., 2008).

The Pi $K_i$ values were determined for the wild-type and the Mos(1–198) enzymes using a Dixon plot ($1/V$ versus $[I]$) in the presence of 2.5 mM 3-PGA. Values for wild type and Mos(1–198) were 1.4 and 8.7 mM, respectively. In the absence of 3-PGA, wild-type AGPase exhibits little inhibition (Fig. 1; Boehlein et al., 2008). In contrast, Mos(1–198) is significantly inhibited in the absence of 3-PGA even at low Pi concentrations (Fig. 1). Because Mos(1–198) exhibits enhanced Pi inhibition, we conclude that Mos(1–198) does not contain a tightly bound activator; rather, Mos(1–198) naturally occurs in a semiactivated state in the absence of an activator.

The Pi inhibition pattern of Mos(1–198) is complex and biphasic. Inhibition at relatively high Pi concentrations is less than predicted at low Pi concentrations (Fig. 1). The calculated $K_i$ for Pi is approximately 1 mM before 50% inhibition and 36 mM after 50% inhibition. Interestingly, the $K_i$ for Pi of the wild-type enzyme in the presence of 3-PGA is approximately 1 mM, whereas in its absence it is almost 70 mM.

Previous studies (Boehlein et al., 2008) showed that the binding of substrates and binding of 3-PGA to the wild-type maize endosperm enzyme are intertwined. The presence of saturating concentrations of 3-PGA reduced the $S_{0.5}$s for ATP and Glc-1-P by a factor of 10- and 20-fold, respectively, and the V versus S curves became hyperbolic in the presence of 3-PGA. Because Mos(1–198) has activity resembling the wild-type enzyme in a semiactivated state, we asked whether Mos (1–198) had an ATP $K_m$ lower than that of the wild-type AGPase in the absence of 3-PGA (Table II). $K_m$ values of the two enzymes are nearly indistinguishable in the presence of 3-PGA (Table II; Boehlein et al., 2005). In the experiments reported here, a relatively high concentration of Glc-1-P (100 mM) was used to obtain hyperbolic curves for the wild-type enzyme in the absence of 3-PGA because, in previous work, nonhyperbolic kinetics in the absence of 3-PGA were observed at low Glc-1-P (2 mM) concentrations (Boehlein et al., 2005). Substitution of 100 mM KCl for 100 mM Glc-1-P did not affect the ATP saturation profile; hence, this effect is not due to simply ionic strength. Also, the concentrated Glc-1-P was adjusted to the pH of the assay before addition to the reaction mixture.

### Table 1. 3-PGA activation of recombinant, purified AGPases from wild-type and various mosaic AGPases

| Border amino acid | Specific Activity (+3PGA) | Specific Activity (+3PGA) | Activation Fold | $K_a$ |
|-------------------|---------------------------|---------------------------|----------------|-------|
| wt                | 10 5 8 7                  | 10 5 8 7                  | 10 5 8 7       |       |
| Mos(1–198)        | 2.45 ± 0.32               | 21.81 ± 1.85              | 8.9            | 0.26 ± 0.07 |
| Mos(1–176)        | 4.87 ± 1.16               | 10.87 ± 1.63              | 2.2            | 1.18 ± 0.54 |
| Mos(1–198, 377–475) | 1.47 ± 0.09              | 9.38 ± 0.24              | 6.4            | 0.13 ± 0.04 |
| Mos(1–198, 430–475) | 0.72 ± 0.02              | 8.59 ± 0.24              | 11.9           | 0.78 ± 0.18 |
| Mos(1–198, 377–429) | 1.68 ± 0.18              | 6.14 ± 0.95              | 3.6            | 3.45 ± 1.4 |
| Mos(1–277)        | 1.78 ± 0.08               | 6.81 ± 0.37              | 3.8            | 1.22 ± 0.46 |
| Mos(1–321)        | 1.80 ± 0.07               | 7.29 ± 0.69              | 4.1            | 0.70 ± 0.18 |

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Figure 1. Pi inhibition of wild type (●) and Mos(1–198) (●) in the absence of 3-PGA. Data are presented as a Dixon plot ($1/V$ versus $[Pi]$), and the apparent $K_i$ for Pi are indicated on the graph.
shown in Table II, the ATP \( K_m \) of Mos(1–198) (0.18 mM) is approximately 4-fold lower than that of wild-type AGPase (0.75 mM) in the absence of 3-PGA and is approaching the wild-type \( K_m \) of ATP in the presence of 3-PGA (0.074 mM). Whereas the ratio of the ATP \( K_m \) (+3-PGA/−3-PGA) is approximately 10 for the wild-type enzyme, it is only 3.5 for Mos(1–198). Therefore, in the absence of 3-PGA, the ATP \( K_m \) of Mos(1–198) resembles that of the wild-type enzyme with a sub-saturating amount of 3-PGA.

One further prediction we made is that an enzyme that is autonomously in a partially activated state would be more resistant to heat denaturation. We recently showed that the thermal stability of the maize endosperm enzyme is greatly influenced by many allosteric effectors (Boehlein et al., 2008). Here, we show that Mos(1–198) is more heat stable than wild type in the absence of 3-PGA. Wild type in the absence of 3-PGA has a half-life of 5.5 min. When 3-PGA is added to wild type, the half-life increases to 9.4 min. Addition of 3-PGA enhances the half-life of Mos(1–198) to 9.4 min. These data are consistent with the idea that Mos (1–198) behaves like a partially activated wild-type enzyme.

Mapping the Polymorphic Sites Important in Altered Allosteric Properties and Heat Stability

To map polymorphic sites conditioning the interesting properties of Mos(1–198), we constructed a series of mosaics having smaller portions of the potato subunit substituted into the maize endosperm small subunit (Table I).

Location of Polymorphic Amino Acids Important in Determining Activity in the Absence of 3-PGA

Because Mos(1–198) had high activity in the absence of 3-PGA, we assayed the new mosaics in the absence and presence of 10 mM 3-PGA to map the site conditioning high, 3-PGA-independent activity. We began by replacing the carboxyl-terminal 15 polymorphic amino acids of Mos(1–198) with the maize counterparts to synthesize Mos(1–198, 377–475) (Table I). This substitution resulted in 3-PGA-independent activity that was approximately 7-fold lower than that of Mos(1–198). We then divided this carboxyl-terminal region containing 15 polymorphic amino acids from 377 to 475 to create Mos(1–198, 430–475) and Mos(1–198, 377–429), to determine which amino acids in the potato enzyme were necessary to maintain the high 3-PGA-independent activity of Mos(1–198). Activity of each construct was lower than that of wild type. We conclude that, at a minimum, at least one polymorphic amino acid lying between 377 and 429 and at least one polymorphic amino acid lying between 430 and 475 are required for high 3-PGA-independent activity. Next, we determined whether the C-terminal potato-derived fragment containing amino acids from 377 to 475 was sufficient for high 3-PGA-independent activity. We created Mos(1–376), composed mostly of maize sequence, with the extreme carboxyl terminus (the last 15 nonconserved amino acids) of potato origin (Table I). This mosaic also resulted in low activity in the absence of 3-PGA. Therefore, not only are at least two of the terminal 15 polymorphic amino acids required for high 3-PGA-independent activity, sequences N-terminal to this region are also required.

To identify those amino-terminal amino acids, we synthesized two mosaics, Mos(1–277) and Mos(1–321). Mos(1–277) exhibited 3-PGA-independent activity that was less than Mos(1–198), and wild type and Mos(1–321) had no detectible activity in the absence of 3-PGA. In the presence of 3-PGA, however, Mos(1–321) activity in the reverse direction (Glc-1-P formation) was identical to that of Mos(1–277) (data not shown). Several conditions were employed in the presence of 3-PGA in an attempt to decipher the cause of no activity in the absence of 3-PGA. Activity was tested, in the forward direction, with various concentrations of substrates and activators. Standard conditions (2 mM ATP, Glc-1-P, and 10 mM 3-PGA), as well as assay in the presence of high ATP (5 mM), high Glc-1-P (4 mM), and high 3-PGA (25 mM) all yielded the same specific activity of approximately 5 nmol min\(^{-1}\) mg\(^{-1}\). In the absence of 3-PGA, no activity could be detected under all permutations of substrate/enzyme concentrations we tested.

Taken in total, our analysis identified a distributed number of at least four polymorphic amino acids that are important in conditioning high 3-PGA-independent activity.

### Table II. \( K_m \) determinations in the presence and absence of 3-PGA of purified recombinant wild-type and Mos(1–198) AGPases

| Enzyme         | +3-PGA | −3-PGA |
|----------------|--------|--------|
|                | ATP \( K_m \) | Glc-1-P \( K_m \) | \( V_{max} \) | ATP \( K_m \) |
| Wild type      | 0.074 ± 0.007 | 0.062 ± 0.004 | 23.4 ± 0.48 | 0.75 ± 0.006 |
| Mos(1–198)     | 0.051 ± 0.005 | 0.047 ± 0.010 | 15.2 ± 0.70 | 0.18 ± 0.026 |

To obtain hyperbolic saturation curves and to avoid substrate inhibition, the nonvariable substrate concentrations were 2 mM in the presence of 10 mM 3-PGA and 100 mM in the absence of 3-PGA. All reactions were performed in triplicate and the averages presented.
activity. At least one amino acid between positions 199 to 277, at least one amino acid between positions 278 and 321, at least one amino acid between positions 322 and 377, and at least one amino acid between positions 378 and 475 must be of potato origin to obtain high activity. Furthermore, because some mosaics exhibited specific activities outside the range defined by the two parents, wild type and Mos(1–198), we conclude that the motifs identified through mapping likely interact in concert and some of these effects are global in nature.

**Location of Polymorphic Amino Acids Important in ATP Km Values**

Because Mos(1–198) had kinetic properties resembling a partially activated wild-type enzyme (Table II), the new mosaics were assayed to determine whether any had properties similar to Mos(1–198). As can be seen in Table III, ATP Km values of each of the dissecting mosaics fall between that of the wild type and that of Mos(1–198). None of the ATP Km’s were as low as the Mos(1–198) ATP Km. We conclude that at least one amino acid between positions 199 to 277, at least one amino acid between positions 278 and 321, at least one amino acid between positions 322 and 377, and at least one amino acid between positions 378 and 475 must be of potato origin to obtain the relatively low ATP Km value in the absence of 3-PGA.

**Location of Polymorphic Amino Acids Important in Pi Inhibition**

As shown above, 2.5 mM Pi causes a 40% reduction in the activity of Mos(1–198), whereas the wild-type enzyme is actually activated by this level of Pi. This distinction was used with the dissecting mosaics to map the site of Pi inhibition. None of the these mosaics exhibited the inhibition pattern of Mos(1–198) (Table IV). These data then exhibit the pattern observed with 3-PGA-independent activity and ATP Km. Hence, like

| Enzyme ATP % Activity +Pi/−Pi | Wild type | Mos(1–198) | Mos(1–376) | Mos(1–198, 377–475) | Mos(1–198, 430–475) | Mos(1–198, 377–429) | Mos(1–277) | Mos(1–321) |
|-------------------------------|----------|------------|------------|-------------------|-------------------|-------------------|------------|------------|
| Wild type                     | 199      | 64         | 182        | 381               | 332               | 239               | 150        |            |

3-PGA-independent activity and ATP Km in the absence of 3-PGA, we conclude that, at least one amino acid between positions 199 to 277, at least one amino acid between positions 278 and 321, at least one amino acid between positions 322 and 377, and at least one amino acid between positions 378 and 475 must be of potato origin to obtain the Pi inhibition pattern exhibited by Mos(1–198).

**Location of Polymorphic Amino Acids Important in 3-PGA Kα**

We attempted to map the polymorphic sites important in determining the Kα for 3-PGA (Table I). Whereas Mos(1–198) possesses an elevated 3-PGA Kα relative to wild type, dissecting mosaics exhibited complex patterns. For example, substitution of the potato-derived terminal 377 to 475 fragment containing 15 polymorphic amino acids into the maize subunit did not increase the 3-PGA Kα, suggesting that these amino acid differences are relatively unimportant in determining the difference in 3-PGA Kα. However, subdivisions of this fragment gave rise to significantly elevated 3-PGA Kα values when expressed with variants containing potato-derived amino acids from 199 to 377 (Table I, entries 4, 5, and 6). A more striking example of the complex and global nature of these amino acid substitutions is seen in the region from 278 to 377. When the amino acids in this region are of maize endosperm or potato tuber origin, the AGPases have activity in the absence of 3-PGA and exhibit relatively low 3-PGA Kα values. However, when this region contains polymorphic amino acids derived from both potato and maize, Mos(1–321), the resulting enzyme, lacks activity in the absence of 3-PGA and exhibits an extremely high 3-PGA Kα. These data point to interactions occurring among several amino acids in controlling 3-PGA Kα values. The data also suggest that different types of interactions important in the determining 3-PGA Kα have evolved since the separation of the genes encoding the maize endosperm and the potato tuber AGPases.
**Location of Polymorphic Amino Acids Important in Heat Stability**

Previously we showed that Mos(1–198) increased heat stability (Boehlein et al., 2005), a characteristic that appears to be associated with enhanced yield in cereal seeds (Smidansky et al., 2002, 2003; L.C. Hannah, unpublished data). Various studies have shown that heat stress decreases yield in many cereal crops. Because the cereal endosperm AGPase is extremely heat labile, increasing heat stability apparently leads to increased starch production in cereal seeds. Therefore, Mos(1–198) was dissected to identify the potato amino acids necessary for increased heat stability.

Accordingly, several alterations were made in the potato-derived portion of Mos(1–198). First, the C-terminal 98 amino acids of Mos(1–198) were swapped for their maize counterparts to create Mos(1–198, 377–475). This new mosaic retained the increased heat stability of Mos(1–198) (Table V). We also made the reciprocal substitution in which the amino-terminal 178 potato-derived amino acids of Mos(1–198) were switched with the maize counterparts to produce Mos(1–376). This mosaic did not retain enhanced heat stability. Two additional mosaics were then synthesized to identify the amino acids critical for heat stability. Mos(1–277, 377–475) contained potato sequence from amino acids 278 to 377 and Mos(1–321, 377–475) harbored the potato sequence from amino acids 322 to 377. Both constructs yielded heat-stable enzymes, identifying the region from 322 to 377 as important for heat stability. This region differs from maize at only five amino acids. In an effort to determine whether a single amino acid residue could confer the heat-stable phenotype, each maize amino acid was individually changed to its potato counterpart. The following mutants were individually constructed (Bt2I323V, Bt2F332S, Bt2H341Y, Bt2V347M, and Bt2N369H). Resulting heat stability of each single amino change was only slightly greater or equal to that of maize. Therefore, no single amino acid change confers the heat-stable phenotype.

**Table V. Heat stability of purified recombinant AGPases from wild type, Mos(1–198), and various dissecting mosaics**

| # amino acid differences | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | 29 | 30 | 31 | 32 | 33 | 34 | 35 | 36 | 37 | 38 | 39 | 40 | 41 | 42 | 43 | 44 | 45 | 46 |
|--------------------------|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Mos(1–198)               | ++|   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
| Mos(1–198, 377–475)      | ++|   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
| Mos(1–321)               | ++|   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
| Mos(1–321, 377–475)      | ++|   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
| Mos(1–376)               | ++|   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |

| border amino acid        | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | 29 | 30 | 31 | 32 | 33 | 34 | 35 | 36 | 37 | 38 | 39 | 40 | 41 | 42 | 43 | 44 | 45 | 46 |
|--------------------------|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|
| I323V                    | ++ |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
| F332S                    | ++ |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
| H341Y                    | ++ |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
| V347M                    | ++ |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
| N369H                    | ++ |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |

**DISCUSSION**

The plant AGPase containing the maize endosperm large subunit and a mosaic small subunit having sequences derived from the maize endosperm and potato tuber small subunits and termed Mos(1–198) has been extensively characterized and a variety of properties of the enzyme are noteworthy. As judged by various criteria, this mosaic AGPase in the absence of an added allosteric activator appears to be virtually identical to a partially activated wild-type maize endosperm AGPase. In the absence of 3-PGA, but not in its presence, Mos(1–198) exhibits a greater \( K_{m} \) for Pi, greater heat stability, lower ATP \( K_{m} \), and greater sensitivity to Pi inhibition in comparison to the wild-type maize endosperm AGPase.

We initially asked whether purified Mos(1–198) enzyme preparations might contain an activator not found in wild-type preparations. However, repeated passages through desalting columns in the presence or absence of saturating levels of 3-PGA or Pi did not reduce the 3-PGA-independent activity. If Mos(1–198) contains a bound activator, binding must occur with an affinity not exhibited by the wild-type AGPase. Pi can, however, bind to the Mos(1–198) enzyme as evidenced by the Pi-mediated reduction in catalytic activity. Taken in total, all extant data strongly suggest that, in the absence of an activator, the Mos(1–198) mosaic enzyme naturally exists in a conformation resembling or mimicking the conformation of wild-type AGPase in the presence of suboptimal levels of an activator.

We next asked whether we could identify the specific amino acid polymorphisms responsible for the altered parameters of Mos(1–198). Through analysis of a series of constructs containing divided segments of the potato portion of Mos(1–198), we identified at least one amino acid between positions 199 to 277, at least one amino acid between positions 278 and 321, at least one amino acid between positions 322 and 377, and at least one amino acid between positions 378 and 475 that must be of potato origin to obtain the Pi inhibition pattern, the high 3-PGA-independent activity, and the low ATP \( K_{m} \) of Mos(1–198). It is interesting to note that when the entire potato small subunit is combined with the wild-type maize large subunit, the activity in the absence of 3-PGA is comparable to wild-type AGPase (Boehlein et al., 2005). Thus, some of the N-terminal maize amino acids are required for the unusual properties of Mos(1–198). We also attempted to map polymorphic sites important in creating 3-PGA \( K_{m} \) values distinguishing wild-type and Mos(1–198). Resulting data point to interactions occurring among polymorphic amino acids distributed throughout the subunit. This global pattern of interactions strongly suggests that different compensating amino acid changes have been selected over evolutionary time since the duplication and separation of the genes encoding the small subunits of the maize endosperm and the potato tuber.
Interestingly, and in contrast to the results from mapping high 3-PGA-independent activity, low ATP \( K_m \), and Pi inhibition, heat stability mapped to a single region, identifying five important amino acids between residues 322 to 327. This shows that, whereas enhanced heat stability may be important in conditioning the other changes, it is not sufficient for these changes to occur. Hence, we envisage at least two fundamental differences distinguishing wild type and Mos(1–198).

Other interesting findings are also noteworthy. Previously, we (Boehlein et al., 2008) showed that activator and substrate binding are intertwined; the binding of one greatly influences the binding of the other. We also found that, in the absence of 3-PGA, velocity versus substrate concentration plots are not hyperbolic and the \( S_{0.5} \) values were much higher than the \( K_m \)s for substrates in the presence of 3-PGA (Boehlein et al., 2005). Here, we show that velocity versus [ATP] plots in the absence of 3-PGA become hyperbolic if the Glc-1-P concentrations is increased.

Pi inhibition in the absence of 3-PGA is also noteworthy. The wild-type AGPase shows very little inhibition in the absence of 3-PGA and it has been proposed that Pi acts as a deactivator or antiactivator (Boehlein et al., 2005) because Pi inhibition occurs only in the presence of an activator. Pi competes for binding at the activator site to remove the activator. However, at lower concentrations, Pi can bind to the enzyme without reducing activity, as shown by its ability to greatly enhance heat stability in the absence of an activator (Boehlein et al., 2008). As expected, if Mos(1–198) exists in an autonomously semiactivated state, Pi can inhibit Mos(1–198) activity in the absence of activator; however, inhibition exhibits a biphasic 1/V versus [Pi] pattern. This type of inhibition has not been seen with any other AGPases. Mos(1–198) activity is quite sensitive to Pi inhibition at low Pi concentrations (\( K_i 1.35 \text{mM} \)). Thereafter, activity is much less sensitive and is inhibited with a \( K_i \) of 36 \text{mM}.

We envisage three possibilities to explain the biphasic inhibition of Mos(1–198) in the absence of 3-PGA. All are compatible with extant data. (1) Pi binds to Mos(1–198), which is in an autonomously semiactivated conformation. This Pi binding places the enzyme in a conformation resembling that of wild-type AGPase in its low activity state. (2) Pi binds to Mos(1–198) at low concentrations and totally abolishes activity arising from the Mos(1–198) small subunit. Only the large subunit is catalytically active and it accounts for the 50% activity observed at low Pi concentrations. Large subunit activity is then inhibited at the higher Pi concentrations. (3) Pi only inhibits the activated activity. Because Mos(1–198) in the absence of 3-PGA exhibits only approximately 50% of the activity observed in the presence of 3-PGA (Table I), only the activated form is inhibited at low Pi concentrations.

Interestingly, the five amino acid polymorphisms between amino acids 322 to 327 identified here as important in heat stability were found previously (Cross et al., 2005) to be important in small subunit/large subunit interactions. We (Cross et al., 2005) showed previously that for the maize endosperm small subunit to interact and function with the potato tuber large subunit, the small subunit must contain the five potato-derived amino acids lying between position 322 and 327. And, as we noted here for heat stability, Cross et al. (2005) found that activity with the potato large subunit was not dependent on any single

| Mosaic Template | 5′ Primer, First PCR | 3′ Primer, First PCR |
|-----------------|----------------------|----------------------|
| Mos(1–198, 377–475) | GAAGGAGATATATCCATGG | GCACCTTCAGATATGCAAGATCTGAGTCCAAC-TACGAGAAGGC |
| pMOnCpBn2 | ATCCCGGTGTGGAGCTCAGATCTGAGTCCAAC-TACGAGAAGGC |
| Mos(1–376) | GAAGGAGATATATCCATGG | GCACCTTCAGATATGCAAGATCTGAGTCCAAC-TACGAGAAGGC |
| pMOnCpBn2 | ATCCCGGTGTGGAGCTCAGATCTGAGTCCAAC-TACGAGAAGGC |
| Pss | ATTCTGTAGTTGGACCTCCGATCATA-TACGAGAAGGC |
| Mos(1–198, 430–475) | CCTGAAGCCCAATGACTTGTAGTAGTGAACTTCTCTGGTCAACATCCGAGC |
| Pss | GCACCTTCAGATATGCAAGATCTGAGTCCAAC-TACGAGAAGGC |
| Mos(1–376) | CCTGAAGCCCAATGACTTGTAGTAGTGAACTTCTCTGGTCAACATCCGAGC |
| Pss | GCACCTTCAGATATGCAAGATCTGAGTCCAAC-TACGAGAAGGC |
| Mos(1–198, 377–429) | CCTGAAGCCCAATGACTTGTAGTAGTGAACTTCTCTGGTCAACATCCGAGC |
| Pss | GCACCTTCAGATATGCAAGATCTGAGTCCAAC-TACGAGAAGGC |
| Mos(1–198, 377–429) | CCTGAAGCCCAATGACTTGTAGTAGTGAACTTCTCTGGTCAACATCCGAGC |
| Pss | GCACCTTCAGATATGCAAGATCTGAGTCCAAC-TACGAGAAGGC |
Amino acid change. Furthermore, structural data for the potato tuber homotetramer (Jin et al., 2005) suggest that this motif interacts with the equivalent region of another subunit and is composed of a long loop that connects the N- and C-terminal domains. Taking all observations into consideration, we suspect that the greater heat stability we note here is due to alteration in the interaction of the large and small subunit. Greene and Hannah (1998a) found that heat stability of the large subunit also involved altered large subunit/small subunit interactions. Thus, it appears that these subunit-subunit interactions are critical for the thermal stability of the enzyme.

MATERIALS AND METHODS

Construction of Mosaics

The mosaics were constructed following method 2 of Cross et al. (2005) with some variation in the cloning of the final amplified PCR product. Templates and primers used in the first PCR amplification are listed in Table VI. The 5' primer for all second PCR amplifications was GTTGAATCTGGA-ATTGAACCTC. The 5' primer in these reactions was GAAGCAGATATAATCCATC-CCATGC for Mos(1–198, 377–425), Mos(1–376), Mos(1–277), and Mos(1–321). The 5' primer was CCTGAAGCCATATGCTTGTAGTGAAGTTACTCT-GGTGCG for Mos(1–198, 430–475) and Mos(1–198, 377–429). Mos(1–198, 377–475) and Mos(1–376) final products were digested with Ncol and Sfil and cloned directly into the expression vector. Mos(1–198, 377–475) and Mos(1–321) products were cloned directly into pCR-2.1-TOPO using Invitrogen’s TOPO-TA cloning kit. The Ncol/Sfil fragment was then excised from the shuttle plasmid and subcloned into the expression vector.

Site-Directed Mutagenesis

Individual small subunit point mutations were prepared using Stratagene’s QuikChange site-directed mutagenesis protocol. The starting template for each mutagenesis was the maize (Zea mays) wild-type expression clone preMOnC82. Amplification of the plasmid was performed using two reverse complementary primers with the mutated codon near the center. The following are the sense primers for each mutation: B2/332F, GGAATATACCA-GAACCGATACAGTCACTTCTCATAGCC; B2/333S, GATTTCAG-CTTCTATGACCGTTCGGCCCAATTTATACACAACCTCGAC; B2H341Y, GCTTCCATATTACACACACCTGGAACCTCCTGAGGACG; B2V357F, GCTTCCATATTACACACACCTGGAACCTCCTGAGGACG; B2N369H, GGTGGAAGGATGTTATATAAAAACCTG-CAAGAATCCACCTTCTGAGTTACCC.

Escherichia coli Growth Conditions for AGPase

The growth conditions for wild-type AGPase and the AGPase mosaics were as described by Boehlein et al. (2008). Briefly, the wild-type AGPase small subunit or small subunit mosaics were transformed into E. coli AC7082-504 (Iglesias et al., 1993) that contained the plasmid encoding the maize endosperm wild-type large subunit. Transformation methods were diluted and directly grown overnight at 37°C in Luria-Bertani medium containing 75 µg/ml spectinomycin and 50 µg/ml kanamycin. After reaching an OD of 0.7 to 1.0 (16–20 h), protein expression was induced at room temperature for 3 h by the addition of 0.2 mM isopropyl β-D-thiogalactoside and 0.02 mg/mL nalidixic acid. Cells were harvested by centrifugation at 8,000 g and pellets stored at −80°C.

AGPase Purification

Purification of AGPase is described in Boehlein et al. (2008). AGPase was purified using protease sulfate and ammonium sulfate fractionation, fol-

lowed by ion-exchange and hydroxypapatite chromatography. Proteins were concentrated and remained stable for many months when stored at −80°C.

Prior to kinetic analysis, proteins were desalted using Zeba micro desalt spin columns according to the manufacturer’s instructions (Pierce). Proteins were routinely exchanged into 50 mM HEPES, pH 7.4, 5 mM MgCl2, 0.5 mM EDTA. Bovine serum albumin (0.5 mg/mL) was added for enzyme stability following measurement of AGPase concentration.

Standard Assays

All data were obtained using an enzymatic endpoint assay. The amount of PPI produced was coupled to a decrease in NADH concentration (Boehlein et al., 2008). Standard reaction mixtures contained 50 mM HEPES, pH 7.4, 15 mM MgCl2, 2.0 mM ATP, and 2.0 mM Glc-1-P in a total volume of 200 µL. When activators or inhibitors were added, their concentration is specified in tables or figure legends. Reactions were performed at 37°C in prewarmed tubes. Assays were initiated by enzyme addition and terminated by boiling for 1 min. The reactions were developed by adding 300 µL of coupling reagent (25 mM imidazole, pH 7.4, 4 mM MgCl2, 1 mM EDTA, 0.2 mM NADH, 0.725 units aldolase, 0.4 units triose phosphate isomerase, 0.6 units glyceraldehyde dehydrogenase, 1 mM Fru-6-P, and 0.8 µg purified PPI-PFK per reaction) to each tube and determining the A405. Blank samples contained complete reaction mixtures without enzyme. The amount of PPI produced was determined from a standard curve using PPI in complete reaction mixtures lacking AGPase. 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.

Heat Stability of Purified Maize AGPase and AGPase Mosaics

Heat stability was determined using desalted enzymes (0.01 µg/µL in 0.5 mg/mL bovine serum albumin) in a total of 10 µL. Enzymes were placed in a water bath at 42°C for 0 to 7.5 min and then cooled on ice. All preparations were assayed for 10 min using the standard assay in the presence of 10 mM 3-PGA. Reactions were started with 0.1 µg enzyme. Data were plotted as log % activity versus time and the inactivation constant (t1/2) was calculated as follows: slope = −k/(2.3). t1/2 is calculated from the equation k = 0.693/t1/2.

Determination of Kinetic Constants

The kinetic constant, K,i, and activation constant, k,a, were obtained by incubating purified AGPase with the following components: 50 mM HEPES, pH 7.4, 15 mM MgCl2. When held constant and saturating, ATP, Glc-1-P, and 3-PGA were 1.0, 2.0, and 10.0 mM, respectively, unless stated otherwise. Assays were performed as stated above, and the kinetic constants were obtained by nonlinear regression using equations derived from the full kinetic equation using the software program Prism (Graph Pad).

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers AF334959 (B12), M61603 (Sb2), X61186 (potato small subunit), and X61187 (potato large subunit).

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LITERATURE CITED

Balicora MA, Iglesias AA, Preiss J (2004) ADP-glucose pyrophosphorylase: a regulatory enzyme for plant starch synthesis. Photosynth Res 79:1–24

Beckles DM, Smith AM, ap Rees T (2001) A cytosolic ADP-glucose pyrophosphorylase is a feature of graminaceous endosperms, but not of other starch-storing organs. Plant Physiol 125:818–827

Boehlein SK, Sewell AK, Cross J, Stewart JD, Hannah LC (2005) Purification and characterization of adenosine diphosphate glucose pyrophosphorylase from maize/potato mosaics. Plant Physiol 138:1552–1562

Boehlein SK, Shaw JR, Stewart JD, Hannah LC (2008) Heat stability and allosteric properties of the maize endosperm ADP-glucose pyrophosphorylase are intimately intertwined. Plant Physiol 146:289–299
Boehlein et al.

Brangeon J, Reys A, Prioul JL (1997) In situ detection of ADPglucose pyrophosphorylase expression during maize endosperm development. Plant Physiol Biochem 35:845–858

Cao H, Sullivan TD, Boyer CD, Shannon JC (1995) Bt1, a structural gene for the major 39-44 kD amyloplast membrane polypeptides. Physiol Plant 95:176–186

Cross JM, Clancy M, Shaw JR, Greene TW, Hannah LC (2004) Both subunits of ADP-glucose pyrophosphorylase are regulatory. Plant Physiol 135:137–144

Cross JM, Clancy M, Shaw J, Boehlein SK, Greene T, Schmidt R, Okita T, Hannah LC (2005) A polymorphic motif in the small subunit of ADP-glucose pyrophosphorylase modulates interactions between the small and large subunits. Plant J 41:501–511

Denyer K, Dunlap E, Thorbjornsen T, Keeling P, Smith AM (1996) The major form of ADP-glucose pyrophosphorylase in maize endosperm is extra-plastidial. Plant Physiol 112:779–785

Georgelis N, Braun EL, Shaw JR, Hannah LC (2007) The two AGPase subunits evolve at different rates in angiosperms, yet they are equally sensitive to activity altering amino acid changes when expressed in bacteria. Plant Cell 19:1458–1472

Giroux MJ, Hannah LC (1994) ADP-glucose pyrophosphorylase in shrunken-2 and brittle-2 mutants of maize. Mol Gen Genet 243:400–408

Giroux MJ, Shaw J, Barry G, Cobb BG, Greene T, Okita T, Hannah LC (1996) A single gene mutation that increases maize seed weight. Proc Natl Acad Sci USA 93:5824–5829

Greene TW, Kavakli IH, Kahn M, Okita TW (1998) Generation of up-regulated allosteric variants of potato ADP-glucose pyrophosphorylase by reversion genetics. Proc Natl Acad Sci USA 95:10322–10327

Greene TW, Hannah LC (1998a) Assembly of maize endosperm ADP-glucose pyrophosphorylase requires motifs located throughout the large and small subunit units. Plant Cell 10:1295–1306

Greene TW, Hannah LC (1998b) Enhanced stability of maize endosperm ADP-glucose pyrophosphorylase is gained through mutants that alter subunit interactions. Proc Natl Acad Sci USA 95:13342–13347

Hannah LC (2007) Starch formation in the maize endosperm. In O Olsen, ed. Endosperm, Developmental and Molecular Biology. Springer Books, New York, pp 179–194

Iglesias AA, Barry GF, Meyer C, Bloksberg L, Nakata PA, Greene T, Laughlin MJ, Okita TW, Kishore GM, Preiss J (1993) Expression of the potato tuber ADP-glucose pyrophosphorylase in Escherichia coli. J Biol Chem 268:1081–1086

Jin X, Ballicora MA, Preiss J, Geiger JH (2005) Crystal structure of potato tuber ADP-glucose pyrophosphorylase. EMBO J 24:694–704

Kim WT, Franceschi VR, Okita TW, Robinson NL, Morell M, Preiss J (1989) Immunocytochemical localization of ADPglucose pyrophosphorylase in developing potato tuber cells. Plant Physiol 91:217–220

Morell M, Bloom M, Preiss J (1988) Affinity labeling of the allosteric activator sites(s) of spinach leaf ADP-glucose pyrophosphorylase. J Biol Chem 263:633–637

Obana Y, Omoto D, Kato C, Matsumoto K, Nagai Y, Kavakli IH, Hamada S, Edwards GE, Okita TW, Matsu H, Ito H (2006) Enhanced turnover of transitory starch by expression of up-regulated ADP-glucose pyrophosphorylase in Arabidopsis thaliana. Plant Sci 170:1–11

Okita TW, Greenberg E, Kuhn DN, Preiss J (1979) Subcellular localization of the starch degradative and biosynthetic enzymes of spinach leaves. Plant Physiol 64:187–192

Preiss J (1973) Part A: Nucleotidyl Transfer, Nucleosidyl Transfer, Acyl and Large subunits. Plant Cell 4:1–11

Preiss J (1978) Regulation of adenosine diphosphate glucose pyrophosphorylase. Adv Enzymol Relat Areas Mol Biol 46:317–381

Sakulsingharoja C, Choi SB, Hwang SK, Edwards GE, Bork J, Meyer CR, Preiss J, Okita TW (2004) Engineering starch biosynthesis for increasing rice seed weight: the role of the cytoplasmic ADP-glucose pyrophosphorylase. Plant Sci 167:1323–1333

Shannon JC, Fang-Mei P, Kang-Hein L (1996) Nucleotides and nucleotide sugars in developing maize endosperms. Plant Physiol 112:835–843

Smidansky ED, Clancy M, Meyer FD, Lanning SP, Blake NK, Talbott LE, Giroux MJ (2002) Enhanced ADP-glucose pyrophosphorylase activity in wheat endosperm increases seed yield. Proc Natl Acad Sci USA 99:1724–1729

Smidansky ED, Martin JM, Hannah LC, Fischer AM, Giroux MJ (2003) Seed yield and plant biomass increases in rice are conferred by deregulation of endosperm ADP-glucose pyrophosphorylase. Planta 216:42–50

Stark DM, Timmerman KP, Barry G, Preiss J, Kishore GM (1992) Regulation of the amount of starch in plant tissues by ADP-glucose pyrophosphorylase. Science 258:287–291

Thorbjornsen T, Villand P, Denyer K, Olsen O, Smith AM (1996) Distinct isoforms of ADPglucose pyrophosphorylase occur inside and outside the amyloplast in barley endosperm. Plant J 10:243–250

Villand P, Kleczkowski LA (1994) Is there an alternative pathway for starch biosynthesis in cereal seeds? Z Naturforsch [C] 49:215–219

Wang Z, Chen X, Wang J, Liu T, Liu Y, Zhao L, Wang G (2007) Increasing maize seed weight by enhancing the cytoplasmic ADP-glucose pyrophosphorylase activity in transgenic plants. Plant Cell Tissue Organ Cult 88:83–92