Both Subunits of ADP-Glucose Pyrophosphorylase Are Regulatory[1]

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The allosteric enzyme ADP-Glc pyrophosphorylase (AGPase) catalyzes the synthesis of ADP-Glc, a rate-limiting step in starch synthesis. Plant AGPases are heterotetramers, most of which are activated by 3-phosphoglyceric acid (3-PGA) and inhibited by phosphate. The objectives of these studies were to test a hypothesis concerning the relative roles of the two subunits and to identify regions in the subunits important in allosteric regulation. We exploited an *Escherichia coli* expression system and mosaic AGPases composed of potato (*Solanum tuberosum*) tuber and maize (*Zea mays*) endosperm subunit fragments to pursue this objective. Whereas potato and maize subunits have long been separated by speciation and evolution, they are sufficiently similar to form active mosaic enzymes. Potato tuber and maize endosperm AGPases exhibit radically different allosteric properties. Hence, comparing the kinetic properties of the mosaics to those of the maize endosperm and potato tuber AGPases has enabled us to identify regions important in regulation. The data herein conclusively show that both subunits are involved in the allosteric regulation of AGPase. Alterations in the small subunit condition drastically different allosteric properties. In addition, extent of 3-PGA activation and extent of 3-PGA affinity were found to be separate entities, mapping to different regions in both subunits.

Starch has many applications in industry as well as serving as an energy source in animal and human nutrition. ADP-Glc pyrophosphorylase (AGPase) catalyzes a rate-limiting step in starch synthesis. Modification of the regulatory properties of this enzyme increases starch yields in potato (*Solanum tuberosum*) tubers, and maize (*Zea mays*), wheat (*Triticum aestivum*), and rice (*Oryza sativa*) seeds (Stark et al., 1992; Giroux et al., 1996; Smidansky et al., 2002, 2003). Seed yield and plant biomass increases are conferred by deregulation of endosperm AGPase. Accordingly, AGPase has attracted wide interest for potential crop improvements.

Plant AGPases are tissue specific. They are heterotetramers of two small and two large subunits. Though forms of each subunit share significant sequence homology, the small subunit is generally more conserved than is the large subunit (Smith-White and Preiss, 1992). Sequence comparisons of the large subunits divide AGPases into four groups: stem/tuber, leaf, fruit/root, and endosperm AGPases. Further differences among AGPases are discernable in regulatory properties. While most plant AGPases are activated by 3-phosphoglyceric acid (3-PGA) and inhibited by phosphate (Ghosh and Preiss, 1966; Dickinson and Preiss, 1969; Sowokinos and Preiss, 1982; Kleczkowski et al., 1993a, 1993b; Sikka et al., 2001), extent of regulation varies from organ to organ. Leaf, potato tuber, and tomato (*Lycopersicon esculentum*) fruit AGPases are sensitive to 3-PGA and phosphate, while endosperm AGPases, except for that of rice (Sikka et al., 2001), are less sensitive to the regulators. AGPases also differ in subcellular localization. Leaf and potato tuber AGPases are found in the chloroplasts or amyloplasts (Okita et al., 1979; Kim et al., 1989). By contrast, the major activity of endosperm AGPases is localized in the cytosol, with a residual activity in the amyloplast (Denyer et al., 1996; Thorbjornsen et al., 1996; Sikka et al., 2001). The cytosolic and plastidial activities are encoded by different genes (Giroux and Hannah, 1994; Burton et al., 2002; Johnson et al., 2003). Surprisingly, the sequences of the proteins identified as barley (*Hordeum vulgare*) and wheat plastidial small subunits do not match any currently identified gene sequences (Burton et al., 2002; Johnson et al., 2003).

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Mutagenesis experiments to pinpoint regions of the two subunits involved in catalysis and regulation have been greatly simplified by the expression of plant-derived AGPase genes in *Escherichia coli* (Iglesias et al., 1993; Ballicora et al., 1995; Giroux et al., 1996). These mutagenic studies (for example, Giroux et al., 1996; Greene et al., 1996a, 1996b, 1998; Laughlin et al., 1998; Kavakli et al., 2001; Salamone et al., 2002) as well as others reviewed recently elsewhere (Ballicora et al., 2003), coupled with binding assays of various substrate and effector analogs (for example, Morell et al., 1988; Ball and Preiss, 1994), have greatly aided the identification of important amino acids.

Here, we present a complementary approach to the mutagenic studies mentioned above. We used the *E. coli* expression system to map genuine regulatory regions in AGPase that distinguish maize endosperm and potato tuber AGPases. Whereas potato and maize subunits have long been separated by speciation and evolution, they are sufficiently similar to form active mosaic enzymes. Our approach then consisted of constructing mosaic subunits composed of regions from potato tuber and maize endosperm AGPases. These comparisons enabled us to identify regions important for regulation and that differ in potato tuber and maize endosperm AGPases. Our results conclusively show that the small subunit plays a vital role in allosteric regulation. These data, in conjunction with others recently published, are discussed in terms of the proposed delineation of the roles of the small and large subunit.

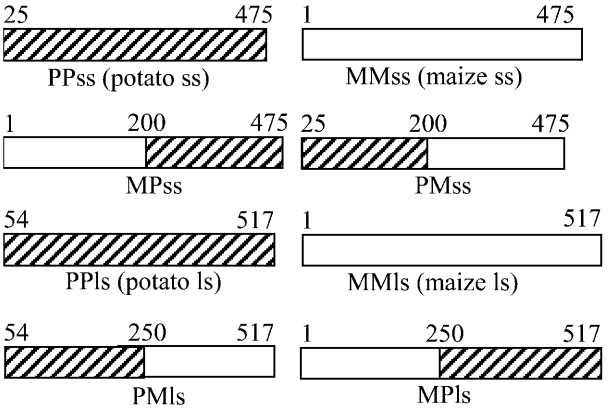

Figure 1. Summary of AGPase mosaics. White and hatched rectangles represent fragments originating from the maize endosperm (M) and potato tuber (P) small (ss) and large (ls) subunits, respectively. Numbers on top of the rectangles identify junctions. Numbers match amino acid positions in the maize endosperm subunit. Hence, potato tuber subunits start at residue 25 for the small subunit and 54 for the large subunit.

RESULTS

The Mosaics Display Variable Heterotetrameric AGPase Activity

The mosaic subunits constructed for this study are shown in Figure 1. Each of the large and small subunits was first equally divided at conserved regions of the proteins, resulting in the mosaic subunits MPss, PMss, MPls, and PMls (where ss is small subunit and ls is large subunit). Heterotetramers involving all mosaics were then expressed in *E. coli* strain AC70R1-504. This strain is deficient in AGPase and consequently is unable to synthesize glycogen. If an active AGPase is expressed in AC70R1-504, the strain regains the ability to produce glycogen. Glycogen synthesis can be easily scored by growing the *E. coli* strain on Glc and staining resulting colonies with iodine. Colonies producing glycogen stain brown. Several mosaic AGPases did not stain (Table I). The reason for this lack of staining is under examination.

Active mosaics, as detected by staining, were assayed for 3-PGA activation and phosphate inhibition. To quantify any AGPase activity arising from homotetramers rather than heterotetramers, each of the wild-type potato and maize small and large subunits was expressed individually. Activity detected ranged from 2.5% to 10% of that obtained from expression of the recombinant wild-type maize or potato enzymes (Burger et al., 2003). In a similar manner, we expressed the mosaic small subunits of interest here alone or with a mutant large subunit (Table II). The mutant large subunit consisted of a maize large subunit protein with alterations at residues 512, 513, and 514. Expression of each small subunit alone led to low activity compared to activity arising from wild-type heterotetramers. Furthermore, activity decreased in the presence of the mutant large subunit. Hence, the vast majority of activity produced by coexpression of large and small subunits arises from heterotetramers.

Mosaics PPss/MMls, MPss/MMls, MPss/PMls, PPss/PMls, and PMss/MMls and the recombinant AGPases from potato tuber (PPss/PPls) and maize endosperm (MMss/MMls) were then partially purified for kinetic studies. Mosaic MMss/PPls could not be purified because of low activity and instability. Instability is inferred from the significant loss of activity during initial purification steps. The last purification step consisted of size separation by gel filtration chromatography. The active fraction eluted around 220 kD for all mosaics purified, a size compatible with a tetrameric structure. Purification varied from 15-fold for maize endosperm AGPase to 160-fold for potato AGPase. The variation in purification fold results mostly from differences in the stability of the various AGPases during purification. Indeed, activity lost during purification varied from enzyme to enzyme, with maize endosperm and potato tuber AGPase retaining 5% and 25% of their starting activity, respectively. Activity lost and final specific activities for the mosaic AGPases ranged between the values obtained for maize endosperm and potato tuber AGPases.

Table I. *Summary of iodine staining for detection of AGPase activity in the mosaic AGPases*

Each of the 16 mosaics examined contained the small subunit identified in the left column and the large subunit in the heading above the mosaic. AGPase staining is symbolized + for a dark brown stain, +−− for a very light stain, and – for no visible stain.

	MMls (Maize ls)	PPls (Potato ls)	MP ls	PM ls
MMss (Maize ss)	+	+−−	−	−
PPss (Potato ss)	+	+	+	+
MP ss	+	+	−	+
PM ss	+	−	−	−

3-PGA Fold Activation and 3-PGA K_a Are Separate Entities

Mosaics were separated into different groups based on their K_a (concentration giving half-maximal activation) for 3-PGA, their fold activation by 3-PGA, or their cooperativity (Table III). Each parameter results in a different classification. For instance, PPss/MMls and PMss/MMls exhibit indistinguishable K_a values for 3-PGA but vastly different extents of 3-PGA activation. Likewise, MMss/MMls and PMss/MMls belong to the same activation group but reside in different 3-PGA affinity groups.

Both Subunits Are Equally Important in 3-PGA Activation

Mosaics were separated into three groups based on 3-PGA fold activation (Table III). Group 3 is insensitive to 3-PGA. Compared to the other mosaics, those of group 3 exhibit one common feature: a small subunit C terminus from potato tuber AGPase and a large subunit N terminus from maize endosperm AGPase. In all other mosaics, the small subunit C terminus and the large subunit N terminus originate from the same source. Interestingly, mosaics composed of the C terminus of the maize small subunit and the N terminus of the potato tuber large subunit exhibited very little activity as measured by staining (mosaics MMss/PPls, PMss/PMls, PMss/PPls, and MMss/PMls in Table I). These observations point to an interaction between the small subunit C terminus and the large subunit N terminus.

Group 1, composed of only the potato wild-type heterotetramer, is the most sensitive to 3-PGA activation. In contrast to the other mosaics, this enzyme contains the N terminus of the small subunit and the C terminus of the large subunit from potato tuber AGPase. Group 2 mosaics, which exhibit intermediate 3-PGA activation compared to other enzymes, are composed of either the N terminus of the maize endosperm small subunit or the C terminus of the maize endosperm large subunit. Since group 2 mosaics are less sensitive to 3-PGA activation compared to the potato tuber AGPase, we conclude that both the C terminus of the large subunit and the N terminus of the small subunit influence 3-PGA fold activation.

Nonadditive effects are also apparent. Replacement of the C terminus small subunit of the relative 3-PGA insensitive maize enzyme with its counterpart from the 3-PGA sensitive potato enzyme (compare PMss/MMls with PPss/MMls) reduces rather than increases 3-PGA activation.

Both Subunits Are Equally Important for 3-PGA Affinity

Mosaics were separated into five groups based on 3-PGA affinity (Table III). Potato tuber and maize endosperm AGPase displayed hyperbolic curves as noted previously (Dickinson and Preiss, 1969; Sowokinos and Preiss, 1982). Mosaics MPss/MMls and PPss/PMls exhibited sigmoidal 3-PGA saturation kinetics in contrast to the potato tuber and maize endosperm AGPases. This implies modified subunit interactions compared with the potato tuber and maize endosperm AGPases. Hence, the protein conformation may be altered and may account for the high 3-PGA K_a value of these mosaics. Consequently, these mosaics were not used for mapping 3-PGA affinity.

Comparison of mosaics exhibiting hyperbolic 3-PGA saturation kinetics led to the following conclusions. PMss/MMls has a lower K_a for 3-PGA than does MMss/MMls. Hence, the N terminus of the small subunit is important in 3-PGA affinity. PPss/MMls and PMss/MMls exhibit similar K_a values for 3-PGA. We conclude that the 3-PGA affinity difference(s) between potato tuber and maize endosperm does not map to the C terminus of the small subunit. MPss/PMls belongs to the same 3-PGA affinity group as do PPss/MMls and PMss/MMls. In addition, MPss/PMls displays a lower K_a than does MMss/MMls. Since we eliminated the C terminus of the small subunit as a determinant of 3-PGA affinity differences, we conclude that the N terminus of the large subunit is important for 3-PGA affinity. Furthermore, both subunits equally influence 3-PGA affinity differences. No mosaic exhibits a K_a as low as that of potato tuber AGPase. Hence, at least both N termini from potato tuber AGPase are required for a potato tuber-type K_a for 3-PGA.

Table II. *Activity arising from expression of each small subunit alone or with a mutant large subunit*

The mutant large subunit consisted of a virtually inactive MMls subunit altered at three residues. Assays were performed in the direction of ADP-Glc synthesis on crude extracts in the presence of 10 mM 3-PGA. Activity is expressed as the percentage of average activity detected in maize and potato heterotetramers.

Small Subunit	Homotetrameric Activity	Activity with a Mutant SH2
None		2.1%
PPss	6.6%	2.1%
MMss	3.4%	2.0%
MPss	9.6%	6.9%
PMss	1.9%	0.8%

Table III. *Regulatory properties of the mosaic AGPases*

Proteins were partially purified through hydrophobic and gel filtration columns and assayed in the direction of ADP-Glc synthesis. Parameters of 3-PGA activation were calculated by nonlinear regression with the Prism program using a $1/y^2$ weighting and considering each replicated point as a separate datum. Each parameter was determined from two to three experiments with duplicate measurements. Numbers in parenthesis in the K_a and fold activation columns represent SD. Numbers in parentheses in the cooperativity column represent Hill coefficients. H, hyperbolic; S, sigmoidal; Gp, classification group based on the criterion of the left column.

	Fold Activation	Gp	K_a	Gp	Cooperativity	Gp
			mM			
PPss/PPls	28 (12)	1	0.02 (0.008)	1	H	1
MMss/MMls	5 (1.7)	2	0.40 (0.06)	3	H	1
PPss/PMls	4.9 (1.2)	2	0.66 (0.03)	4	S (n = 1.3)	2
MPss/PMls	4.7 (0.02)	2	0.07 (0.01)	2	H	1
PMss/MMls	4.8 (1.6)	2	0.05 (0.01)	2	H	1
PPss/MMls	1.3 (0.15)	3	0.11 (0.05)	2	H	1
MPss/MMls	1.2 (0.07)	3	3.72 (0.34)	5	S (n = 0.7)	2

Both Subunits Equally Influence Phosphate Inhibition

Phosphate inhibition studies were performed in the presence of 3 mM 3-PGA. All AGPases displayed significant activity in the absence of phosphate at this 3-PGA level. While this concentration is saturating for all mosaics except for MPss/MMls, this mosaic is the least sensitive to phosphate inhibition at 3 mM 3-PGA (Fig. 2) as well as at 10 mM (data not shown).

Several mosaics as well as the maize endosperm AGPases are stimulated by low phosphate concentrations. This phenomenon has been well described in barley (Kleczkowski et al., 1993a; Kleczkowski, 1999) and can be seen in data published previously for the maize endosperm AGPase (Dickinson and Preiss, 1969).

Phosphate inhibition divided the AGPases into three groups. MPss/MMls and PPss/MMls were relatively insensitive to phosphate (Fig. 2). MPss/PMls and PMss/MMls displayed phosphate inhibition curves intermediate between those of maize endosperm and potato tuber AGPases (Fig. 3), while PPss/PMls was inhibited comparatively to potato tuber AGPase (Fig. 3). Since PPss/PMls belongs to the same inhibition group as potato tuber AGPase (Fig. 3), phosphate inhibition differences between potato tuber and maize endosperm do not map to the C terminus of the large subunit. PMss/MMls is more sensitive to phosphate than is MMss/MMls (Fig. 2). Hence, phosphate inhibition differences map to the N terminus of the small subunit. PPss/MMls is less sensitive to phosphate inhibition than is PMss/MMls (Fig. 3). This may result from PPss/MMls containing a C terminus of the small subunit and an N terminus of the large subunit from different origins. Indeed, MPss/MMls is also insensitive to phosphate inhibition, while MPss/PMls is relatively sensitive to phosphate inhibition (Figs. 2 and 3). In fact, MPss/PMls and PMss/MMls belong to the same group of intermediate phosphate inhibition. MPss/PMls and PMss/MMls both contain a C terminus of the small subunit and an N terminus of the large subunit from identical origins. However, in MPss/PMls, those two fragments originate from potato tuber, while in PMss/MMls they come from maize endosperm. Hence, sensitivity to phosphate inhibition is possible when the C terminus of the small subunit and the N terminus of the large subunit originate from the same parental AGPase. Whether both fragments come from potato tuber or maize endosperm does not seem important. Finally, MPss/PMls is more sensitive to phosphate inhibition than is MMss/MMls. Hence, phosphate inhibition differences between potato tuber and maize endosperm AGPases map to the C terminus of the small subunit or the N terminus of the large subunit. Since the C terminus of the small subunit and the N terminus of the large subunit must originate from the same AGPase for phosphate inhibition to occur, one cannot distinguish which of these fragments influences the phosphate inhibition differences distinguishing potato tuber and maize endosperm AGPases.

Extent of 3-PGA Activation, K_a for 3-PGA, and Phosphate Inhibition Map to Different Motifs

While the maize endosperm AGPase is less sensitive to 3-PGA activation and phosphate inhibition compared to the potato tuber AGPase, the data presented here show that these parameters map to different motifs in the enzyme. A particularly striking example of this is the mosaic PP/PM. This enzyme exhibits the phosphate sensitivity of potato, is activated by 3-PGA to the extent noted for maize, but has a K_a for 3-PGA outside of the range defined by potato and maize.

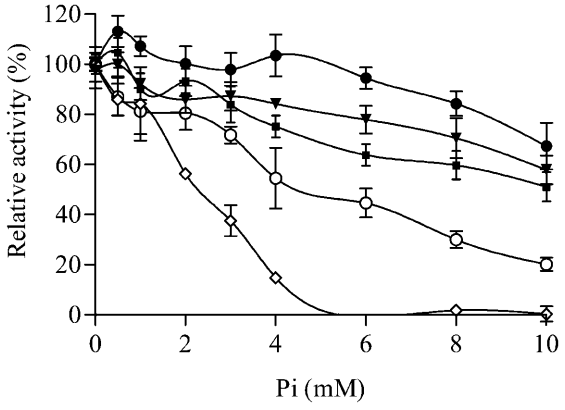

Figure 2. Phosphate inhibition of potato tuber, maize endosperm, MPss/MMls, PPss/MMls, and PMss/MMls AGPases. Proteins were partially purified through hydrophobic and gel filtration columns. Inhibition studies were performed in the presence of 3 mM 3-PGA. AGPase activity was plotted as a percentage of activity in the absence of phosphate. Each point is the result of two experiments with duplicate measures. Error bars represent SD. ●, MPss/MMls; ▼, maize endosperm; ■, PPss/MMls; ○, PMss/MMls; ◇, potato tuber.

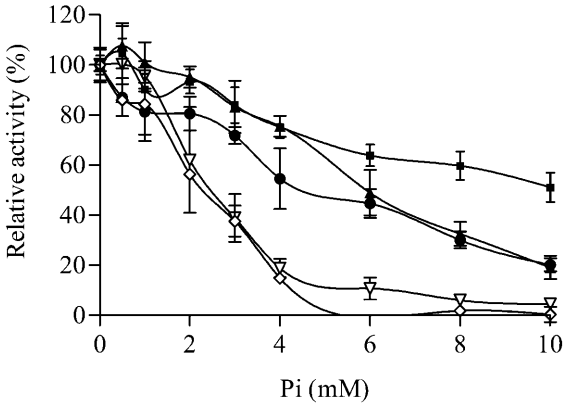

Figure 3. Phosphate inhibition of PPss/MMls, PMss/MMls, MPss/PMls, PPss/PMls, and potato tuber AGPases. Experimental approach was identical to that of Figure 2. ■, PPss/MMls; ▲, MPss/PMls; ●, PMss/MMls; ∇, PPss/PMls; and ◇, potato tuber.

DISCUSSION

Experiments were done to map sites in the small and large subunits of AGPase that are important in conditioning the allosteric properties of the enzyme. Sites mapped are ones that differ in the allosterically distinguishable forms of the maize endosperm and the potato tuber. Here, we switched regions of maize endosperm and the potato tuber AGPase and monitored alterations in allosteric properties. Changes in both subunits have substantial effects on allosteric properties. For example, replacement of the potato large subunit with that from maize effectively abolishes 3-PGA activation of the potato tuber AGPase and increases the K_a for 3-PGA by a factor of 5. Substitution of the C terminus of the potato small subunit for the maize counterpart decreases 3-PGA activation of the maize endosperm AGPase by 70% and increases the K_a for 3-PGA by an order of magnitude. Because of the conserved nature of the small subunit, swapping of the small subunit C termini involves substitutions of only 27 amino acids. These data are summarized in Figure 4.

The data presented show that there are multiple important sites as well as interactions occurring among the various motifs for each of the three traits studied (K_a for 3-PGA, degree of activation by 3-PGA, and degree of phosphate inhibition). The large differences seen in 3-PGA activation and phosphate inhibition are controlled to a great extent by the C terminus of the small subunit apparently interacting with the N terminus of the large subunit. The N terminus of the small subunit, in conjunction with the C terminus of the large subunit, greatly modulates 3-PGA activation. Also, differences distinguishing maize endosperm and potato tuber in the N termini of both subunits are important for 3-PGA affinity but not those in the C terminus of the small subunit.

It is also interesting to note that some of the mosaic AGPases described above exhibit a K_a for 3-PGA, degree of 3-PGA activation, or extent of phosphate inhibition that falls outside the range defined by the two parental AGPases. These variants

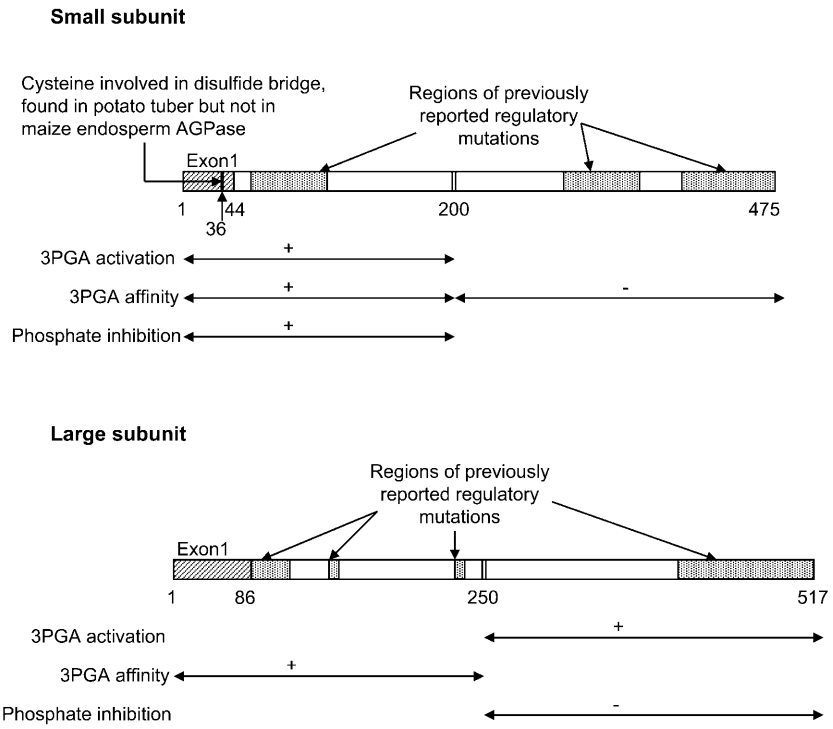

Figure 4. Regulatory sites within each subunit. Exon 1 and junction regions (double vertical bars) have been placed on the rectangles representing each subunit. Numbers underneath the rectangles identify the beginning and end of exon 1 as well as the junction regions. Numbers correspond to amino acid positions in the maize endosperm AGPase. The regions of previously reported mutations that modify regulatory properties were derived from the data of Giroux et al. (1996), Greene et al. (1996a, 1996b, 1998), Laughlin et al. (1998), Kavakli et al. (2001), and Salamone et al. (2002). Information from this study has been placed beneath the rectangles representing each subunit. For each parameter, i.e. phosphate inhibition, 3-PGA activation fold, and 3-PGA affinity, the symbols are the following: ↔, region of the subunit delineated; +, region conditioning differences between potato tuber and maize endosperm AGPase; −, region not conditioning differences between potato tuber and maize endosperm AGPase.

likely may have agricultural importance in light of the role played by the enzyme's allosteric properties in controlling the amount of starch (Stark et al., 1992; Giroux et al., 1996) in starch-rich plant organs. These are under test.

The experiments presented here were designed and initiated in part to test a hypothesis arising from two important but unrelated facts concerning plant AGPases: (1) the small subunits of plant AGPases are, in general, much more conserved compared to the large subunits and (2) the enzymes from different plant organs exhibit dramatic differences in allosteric properties. One reasonable explanation for these two facts is the idea that the small subunit is catalytic, whereas the large subunit is allosteric. This hypothesis and its rationale have been reviewed in detail elsewhere (Smith-White and Preiss, 1992; Hannah, 1997; Ballicora et al., 2003).

In contrast to the prediction of the hypothesis above, resultant allosteric data from mosaic subunits derived from these two isoforms of AGPase show that both the small and the large subunits participate equally in controlling the regulatory properties of AGPase.

Of relevance to the hypothesis above, we note the recent data from expression of the Arabidopsis small and large subunits in *E. coli*. Crevillen et al. (2003) expressed the lone Arabidopsis small subunit with the various Arabidopsis large subunits. Resultant constructs exhibited different substrate affinities, implying a catalytic role for the large subunit.

We also note expression of either small or large AGPase subunits from potato tuber or maize endosperm in *E. coli* gave rise to detectable AGPase activity (Burger et al., 2003). Comparable activity levels, equaling only a small percentage of wild-type heterotetrameric activity, were detected when each of the four subunits was expressed separately in *E. coli*. Taken together, the combined data clearly show that both subunits are important for catalysis and for regulation.

Finally, we note that the structure of the tetrameric TDP-Glc pyrophosphorylase, an enzyme with significant sequence similarity to AGPase, was solved recently (Blankenfeldt et al., 2000). This enzyme contains four catalytic sites.

Since there is not a simple delineation of function distinguishing the small and large subunits of AGPase, the discrepancy in the rate of sequence divergence exhibited by the two plant AGPase genes deserves attention. Two facts are relevant.

First, regions of the small subunit are as variable as the large subunit. Amino acids encoded by exon 1 of the small subunit are more variable than those of the large subunit (Hannah et al., 2001). Interestingly, none of the bacterial AGPases contain sequences corresponding to the plant exon 1 sequences, and of the several structurally solved pyrophosphorylase domains (Brown et al., 1999; Blankenfeldt et al., 2000; Sulzenbacher et al., 2001; Zuccotti et al., 2001), sequence alignments show that the first residues of the pyrophosphorylase domains align with exon 2 of AGPase. Furthermore, a Cys residue involved in redox regulation of AGPase activity is encoded by exon 1 (Fu et al., 1998; Tiessen et al., 2002). This Cys is present in the potato tuber small subunit but is not found in the maize endosperm small subunit or in some other small subunits. Hence, exon 1 represents a viable candidate for determining the allosteric properties unique to plant AGPases. In view of this, attempts were made to examine mosaics containing exchanged exon 1 sequences. Resulting small subunit mosaics exhibited high levels of activity when expressed alone, negating analysis of heterotetrameric activities in these small subunit/large subunit combinations. These subunits are currently under further investigation.

Secondly, inherent in the argument of different roles for the two subunits is the assumption that both genes are equally subject to mutation, and the differences in sequence variation exhibited by the two subunits are caused by different evolutionary selection pressures on the proteins. An examination of the data shows that this assumption may not be correct. Sequences of 33 *Sh2* alleles and 33 *Bt2* alleles isolated from diverse maize inbreds and landraces and recently deposited in GenBank were examined. Consistent with the more conserved nature of the small subunit, three amino acid changes were found in the 1,693 sequenced coding bases of the 33 small subunit *Bt2* alleles, while eight amino acid changes were found in the 1,551 bases of coding information in the 33 *Sh2* alleles.

Significantly, an even larger bias was found in intron changes: 137 mutations were found in the 4,356 bases of sequenced *Sh2* introns, but only 30 alterations occurred in the 4,319 bases of sequenced *Bt2* introns. Intron sequences obviously have no bearing on the structure of the protein encoded by the gene. That intron sequences exhibit the same pattern of differential sequence divergence as noted in the coding regions has a number of explanations. Since there are now ample examples of genes with differing intrinsic mutation rates, we cannot exclude the possibility that the differential rate of divergence exhibited by genes for the large and small subunits simply reflects differing mutation rates of the two genes.

Finally, we note that while the approach used in these studies is powerful in identifying and mapping extant variation among different AGPases, conserved regions important in regulatory properties are not resolved by this methodology. For instance, whereas the C terminus of the large subunit was shown by mutagenic studies to be involved in phosphate inhibition, our data did not identify this region as important for this parameter. The approach used here and resultant data complement more conventional mutagenic studies reported previously. Taken together, data from mutagenic approaches and this approach can identify regions important in various parameters but that are evolutionarily conserved across the various AGPases. This is especially important in regions exhibiting significant sequence dissimilarity.

MATERIAL AND METHODS

Escherichia coli Strains and Plasmids

Mosaics were constructed from the plasmids pMONcSh2-377.1, pMONcBt2-375.6 (Giroux et al., 1996), pML10 (Ballicora et al., 1995), and pMON17336 (Iglesias et al., 1993) containing the genes for *Sh2*, *Bt2*, the potato (*Solanum tuberosum*) tuber complete small subunit, and the potato tuber large subunit, respectively. Cloning was performed in *Escherichia coli* strain DH5α, while enzyme expression was carried out in the AGPase-deficient *E. coli* strain AC70R1-504.

Construction of Mosaics

The mosaics are described in Figure 1. Two PCR reactions were performed on each small subunit plasmid, pMONcBt2-375.6 and pML10, to create mosaics MPss and PMss. One reaction amplified the vector and half of the AGPase gene, while the other reaction amplified the second half of the gene. The resulting products were then blunt-end ligated to form the mosaic plasmids. Primers used were CAG TTC GAT GTA ACC CAC TCG, P GAT CTC AAC AGC GGT AAG ATC C in addition to P AAA CGT GCA ACT GCA TTT GGC CTC, and CTC GTC CAT TGG CAG TGC GGC AAC for PMss, and P AAG CGT GCC ACT GCA TTT GGT CTC and CTC ATC CAT CGG TAG GGC AGC AAC for MPss. The same method was used to synthesize PMls with the primers CCA GCC CAA AAT CTG ATG CT, P TGA AGA TTG ATC ATA CTG GA, P ATC CCC GGG TAC CGA GCT, and TCT CCC TAT AGT GAG TCG TA, and MPls with the primers CTA GCC CAT TTT TAG AAG CT, P TCA AGA TTG ACA GCA GAG GC, P ATC CCC GGG TAC CGA GCT, and TCT CCC TAT AGT GAG TCG TA. Reaction conditions were the following: a heating step of 4 min at 94°C, followed by 25 cycles of 1 min denaturing at 94°C, 1 min annealing at 55°C, and 1 min/kb extension at 72°C. Amplification was performed with Vent DNA polymerase (New England Biolabs, Beverly, MA). Resulting clones were sequenced to verify proper junctions.

Enzyme Expression and Purification

E. coli AC70R1-504 with the constructs of interest was inoculated into 10 mL of Luria-Bertani broth (LB) plus spectinomycin (75 μg/mL) and kanamycin (50 μg/mL) and grown overnight at 37°C. The culture was then transferred to 1 L of LB plus spectinomycin (75 μg/mL) and kanamycin (50 μg/mL) and grown at 37°C until an optical density at 600 nm of 0.5 to 0.6 was reached. Induction was then performed for 6.5 h at room temperature with the addition of 0.2 mM isopropyl β-D-thiogalactoside and 0.02 mg/mL of nalixidic acid. After induction, cells were pelleted and resuspended in extraction buffer (200 mM KCl, 50 mM HEPES, pH 7.5, 10 mM $MgCl_2$, 5 mM EDTA, 30% ammonium sulfate, 20% Suc, and freshly added 1 mM dithiothreitol, 50 μg/mL of lysozyme, 1 μg/mL of pepstatin, 1 μg/mL of leupeptin, 10 μg/mL of chymostatin, 1 mM phenylmethylsulfonyl fluoride, and 1 mM benzamidine). Cells were then sonicated to extract protein. The extract was filtered to 0.45 μm. For assaying crude extracts, the same induction and extraction procedures were used on a final LB volume of 100 mL.

After filtration, the extract was loaded onto a Pharmacia HR 10/10 column packed with a Pharmacia Fractogel EMD C_3 aminopropyl medium (Piscataway, NJ). Step gradients of 1 M, 0.8 M, 0.6 M, 0.4 M, 0.2 M, and 0 M ammonium sulfate were performed at 3 mL/min by combining different proportions of buffer A (50 mM HEPES, pH 7.5, 5 mM EDTA, 10 mM magnesium chloride, 20% Suc, 1 M ammonium sulfate, and freshly added 1 mM dithiothreitol) and buffer B (buffer A without ammonium sulfate). One fraction per gradient was collected. Each fraction was tested for activity in the direction of ATP synthesis as described previously (Greene et al., 1996b). The active fraction was concentrated before gel filtration chromatography. Gel filtration chromatography was performed at 0.5 mL/min on a prepacked HR 10/30 Pharmacia Superdex 200 column with equilibration buffer (50 mM HEPES, pH 7.5, 5 mM EDTA, 10 mM magnesium chloride, 200 mM potassium chloride, 5% Suc, and freshly added 1 mM dithiothreitol). Fractions of 250 μL were collected after elution of the void volume. Active fractions, as determined by the ATP synthesis assay, were pooled, supplemented with 2 mg of bovine serum albumin, concentrated to 0.6 mL, mixed with 0.4 mL of 100% glycerol, and stored in aliquots at −20°C. A subset of mosaic AGPases monitored on western blots did not show any signs of degradation (data not shown).

Kinetic Studies

Kinetic studies were performed in the direction of ADP-Glc synthesis as described previously (Dickinson and Preiss, 1969). Incorporation of [^{14}C]Glc-1-P into ADP-Glc was measured. The reaction mixture contained 80 mM HEPES, pH 7.5, 1 mM dithiothreitol, 1 mM Glc-1-P, 4 mM $MgCl_2$, 0.5 mg/mL of bovine serum albumin, 15,000 cpm of [^{14}C]Glc-1-P and, where appropriate, 3-PGA and/or inorganic phosphate. Reaction volume was 50 μL. Assays were initiated by addition of 1.5 mM ATP. Reaction mixtures were incubated for 30 min at 37°C and terminated by boiling for 2 min. Unincorporated Glc-1-P was cleaved by addition of 0.6 units of bacterial alkaline phosphatase (Worthington Biochemical, Lakewood, NJ) and incubation for 1 h at 37°C. A 30-μL aliquot of the reaction mixture was spotted on DEAE paper, washed with distilled water, dried, and quantified in a liquid scintillation counter. The K_a (concentration giving half-maximal activation) for 3-PGA was determined by nonlinear regression analysis with the Prism program version 3.00 for Windows 95 and NT (GraphPad Software, San Diego). The Michaelis and Menten equation [$v = V_m \times S/(K_m + S)$] in the Prism program was modified to $v = V_{min} + V_m \times S/(K_m + S)$ to account for significant activity levels in the absence of 3-PGA. The parameters for sigmoidal curves were determined by nonlinear regression analysis by use of the Prism program curve for a sigmoidal response with a variable slope. The equation was: $y = V_{min} + (V_{max} - V_{min})/(1 + 10^{(\log K_a - \log x) \times Hillcoeff})$, where V_{min} is the minimal velocity, V_{max} is the maximal velocity, and Hillcoeff the Hill coefficient. The Hill coefficients determined by the Prism equation were checked for accuracy by Hill plots.

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