Enhanced Heterotetrameric Assembly of Potato ADP-Glucose Pyrophosphorylase Using Reverse Genetics

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ADP-glucose pyrophosphorylase (AGPase) is a key allosteric enzyme in plant starch biosynthesis. Plant AGPase is a heterotetrameric enzyme that consists of large (LS) and small subunits (SS), which are encoded by two different genes. Computational and experimental studies have revealed that the heterotetrameric assembly of AGPase is thermodynamically weak. Modeling studies followed by characterization of revertants revealed that the LS I90VSSWT mutant exhibited altered allosteric properties of being less responsive and more sensitive to 3-phosphoglyceric acid activation and inorganic phosphate inhibition. This study not only enhances our understanding of the interaction between the SS and the LS of AGPase but also enables protein engineering to obtain enhanced assembled heat-stable variants of AGPase, which can be used for the improvement of plant yields.

Keywords: ADP-glucose pyrophosphorylase • Allosteric regulation • In vitro Mutagenesis • Protein assembly.

Abbreviations: AGPase, ADP-glucose pyrophosphorylase; BSA, bovine serum albumin; DTT, dithiothreitol; G1P, glucose-1-phosphate; IPTG, isopropyl-β-D-thiogalactopyranoside; LS, large subunit; 3PGA, 3-phosphoglyceric acid; Pi, inorganic phosphate; SS, small subunit; TBS, Tris-buffered saline; WT, wild type.

Introduction

Starch is the predominant carbohydrate reserve in many plants and is observed in both photosynthetic and non-photosynthetic tissues. Starch is a staple in the diet of much of the world's population and is also widely used in different industries as a raw material (Okita et al. 1998, Slattery et al. 2000). ADP-glucose pyrophosphorylase (AGPase) is one of the key enzymes for plant starch biosynthesis. The enzyme controls the carbon flux in the α-glucan pathway by converting glucose-1-phosphate (G1P) and ATP to ADP-glucose and pyrophosphate (Preiss et al. 1991, Kavakli et al. 2000, Ballicora et al. 2004, Hannah and James 2008). The activity of AGPase is allosterically regulated by small effectors. Plant AGPase is activated by 3-phosphoglycerate (3PGA) and inhibited by inorganic phosphate (Pi). Plant AGPases are heterotetrameric (α2β2) enzymes consisting of two subunits called the large subunit (LS) and small subunit (SS), which are encoded by two different genes (Okita et al. 1990, Iglesias et al. 1993). The molecular weights of tetrameric AGPases range from 200 to 240 kDa, depending on the tissue and plant species. Specifically, the molecular weights of the LS and SS in the potato tuber AGPase are 51 and 50 kDa, respectively (Okita et al. 1990). Studies have demonstrated that both subunits contribute to the activity and allosteric regulation of the heterotetrameric enzyme (Ballicora et al. 1998, Kavakli et al. 2001a, Kavakli et al. 2002, Cross et al. 2005, Hwang et al. 2005, Kim et al. 2007, Ventriglia et al. 2008, Georgelis et al. 2009). Evidence indicates that the LS may bind to the substrates G1P and ATP. The binding of the LS to its substrates may allow the LS to interact cooperatively with the SS for binding substrates and effectors, which, in turn, influences net catalysis (Kavakli et al. 2001a, Hwang et al. 2006, Hwang et al. 2007, Hwang et al. 2008). Furthermore, specific regions from both the LS and SS were found to be important for subunit association and enzyme stability (Green and Hannah 1999a, Green and Hannah 1999b, Tuncel et al. 2008, Baris et al. 2009). Using chimeric maize/potato small subunits, Cross et al. (2005) found a SS amino acid polymorphic motif in the SS that directly interacts with the LS and significantly contributes to the overall enzyme stability.
The heat stability properties of AGPase have also been shown to be considerably related to the interaction between subunits and the allosteric properties of the enzyme (Greene and Hannah 1998a, Linebarger et al. 2005, Boehlein et al. 2008). It is known that the heat stability of AGPase is because of strong interactions between subunits. In a study by Greene and Hannah (1998a), yeast two-hybrid experiments revealed heat-stable mutants displaying enhanced interaction between the LS and SS of maize endosperm AGPase. Most plant AGPases are stable because of the cysteine residues in the SS, which form disulfide bridges with the corresponding SS (Sowokinos and Preiss 1982, Fu et al. 1998). The placement of cysteine, including the N-terminal motif of the potato AGPase in the maize endosperm AGPase, significantly increased the heat stability of the enzyme without affecting its affinity for substrates and allosteric regulators (Linebarger et al. 2005). Later, it was found that in maize AGPase, heat stability increases when the active and allosteric sites are occupied by substrates and activators/inhibitors, respectively, indicating a relationship between allosteric regulation and the heat stability of the enzyme (Boehlein et al. 2008). Current knowledge about AGPase suggests that there is a strong relationship between subunit interaction, allosteric regulation and heat stability of the enzyme.

Recent studies have suggested that subunit interaction is not only required for an active heterotetramer but also has a significant role in the allosteric regulation of the enzyme (Hwang et al. 2005, Boehlein et al. 2008, Georgelis et al. 2009). It has been demonstrated that by comparing the allosteric properties of the combinations of up-regulated variants of the SS and LS, the enzyme requires both subunits for allosteric regulation (Hwang et al. 2005). In addition, a phylogenetic analysis revealed the importance of the subunit interface residues for allosteric regulation of the enzyme (Georgelis et al. 2009). Although the crystal structure of heterotetrameric AGPase is not currently available, the subunit interaction dynamics and residues contributing to the interaction were mainly identified through a heterotetrameric model of potato AGPase by Tuncel et al. (2008) and confirmed using computational and experimental studies (Fig. 1A) (Baris et al. 2009). According to the model, energetically, the most favorable heterotetramer occurs via longitudinal and lateral interaction between the LS and SS. Because the lateral interaction is more stable because of its rich hydrophobic interactions, it was presumed that the lateral dimers form first, and then the longitudinal dimers combine with the lateral dimers to form the heterotetrameric enzyme (Tuncel et al. 2008). The model indicates that the interaction between longitudinal LS–SS dimers is thermodynamically much weaker than the interaction between lateral LS–SS dimers. In fact, experimental observation supports the model because the co-expression of the LS and SS AGPase in Escherichia coli glgC− followed by native gel analysis indicated that there are free monomers (SS and LS) and dimers (LS–SS) in addition to the heterotetrameric AGPase in the cell-free extract.

In this study, we enhanced heterotetrameric assembly between dimers and monomers of AGPase using the reverse genetics approach. The AGPase LS<sup>R88A</sup> hotspot mutant for the interaction of longitudinal LS–SS dimers (Baris et al. 2009) was subjected to error-prone PCR mutagenesis to identify second-site revertants. Revertants were co-expressed with WT SS, whereupon the cells were then screened for their capacity to form an enzyme restoring glycogen production in glycogen-deficient E. coli glgC− with iodine vapor. Several second-site revertants were identified. Characterization of the mutants enabled the identification of two groups of mutants: mutants (LS<sup>90V</sup> and LS<sup>378C</sup>) displaying a significant increase in heterotetramer formation due to both enhanced monomer–monomer and dimer–dimer interaction and a mutant (LS<sup>410G</sup>) with increased heterotetramer formation because of enhanced dimer–dimer interaction with modified allosteric properties. Our study not only clearly demonstrates the increased heterotetramer formation on AGPase but also highlights the importance of subunit interaction for the proper functioning and stability of the enzyme.

**Results**

**Identification of second-site revertants of the potato LS AGPase**

Previous work identified that several interface amino acid residues in the LS AGPase play a role in the interaction with the SS AGPase (Baris et al. 2009). Among those interface amino acids, it has been demonstrated that arginine at position 88 in the LS plays an important role in the heterotetrameric formation of the AGPase. Further characterization of the LS<sup>R88A</sup> mutant indicated that it is unable to interact with the SS to form heterotetrameric AGPase as assessed by yeast two-hybrid assays, bacterial complementation and native gel analyses (Baris et al. 2009). To gain a better understanding of subunit–subunit interaction between the LS and SS and also to identify the LS mutant(s) with enhanced heterotetramer assembly, we employed an error-prone PCR random mutagenesis approach using the LS<sup>R88A</sup> AGPase cDNA as template to select second-site LS suppressors that could reverse the iodine staining deficiency of the E. coli glgC− cells containing WT SS AGPase cDNA. Any colonies stained by iodine vapor were assumed to contain second-site mutation(s) in the LS<sup>R88A</sup> that restored AGPase activity and, in turn, substantial quantities of glycogen. Approximately 2 × 10<sup>4</sup> colonies were examined by iodine staining and, of these colonies, 14 colonies that restored glycogen production were identified. Fourteen plasmids containing mutant LS cDNAs were then purified and subjected to site-directed mutagenesis by PCR to convert the primary mutation Ala88 back to the corresponding WT amino acid residue (arginine). The LS cDNAs containing only the secondary mutations were then co-expressed with the WT SS in E. coli glgC−, and their activity was assessed by iodine staining. The results indicated that only the cells containing RM2 LS and WT SS accumulated more glycogen with respect to the cells containing the WT LS and the SS (Supplementary Fig. S1). Cells that
contain eight revertant LSs (RM6, RM7, RM10, RM11, RM13, RM20, RM22 and RM28) together with WT SS stained as dark as cells that contained WT cDNAs of the LS and SS, whereas the cells with five revertant LSs (RM4, RM5, RM25, RM27 and RM29) displayed low levels of the iodine staining phenotype compared with cells containing the WT LS and SS AGPase (Supplementary Fig. S1).

DNA sequence analysis of the 14 LS mutants indicated that they fell into different mutation classes (Table 1). To classify these mutants based on their location in the homology-modeled heterotetrameric AGPase (Tuncel et al. 2008), the position of each mutation was determined on the modeled structure. The result indicated that several mutations (in RM2, RM7, RM10 and RM27) were located on or near both the lateral and longitudinal subunit interfaces (Fig. 1B). We previously reported that lateral interaction of the LS–SS is much stronger than the longitudinal interaction, which is mainly mediated by hydrophobic interactions (Fig. 1A) (Tuncel et al. 2008, Baris et al. 2009). Considering that the longitudinal LS–SS interaction is critical, as revealed in the LSR88A mutant, we focused on the mutations located in the region of the longitudinal LS–SS interface. Therefore, RM2, RM7, RM10 and RM27 were selected for further analysis (Table 1, Fig. 1C).

Sequence analysis of these selected LS revertants indicated that both RM2 and RM7 contained two amino acids changes. Ile90 and Tyr378 were changed into valine and cysteine in RM2, whereas Tyr378 and Asp410 were replaced with histidine and glycine in RM7, respectively (Table 1). Unlike RM2 and RM7, RM10 contained a single amino acid substitution, where Asp410 is replaced with glycine in the LS of AGPase. Observed common mutations, such as Tyr378 and Asp410, in different revertants indicated the importance of these residues in longitudinal LS–SS interactions. Another mutant, RM27, contained seven mutations (Table 1). Among these mutations, only two amino acid changes, A91T and F101L, were located on the longitudinal LS–SS interfaces (Fig. 1C). To assess the effect of the individual amino acid changes in RM2, RM7 and RM27 on heterotetrameric AGPase assembly, Ile90, Ala91 and Phe101 were changed into threonine, valine and leucine in the WT LS AGPases by site-directed mutagenesis. Similarly, tyrosine was

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**Fig. 1** (A) Order of tetramer formation in AGPase. First LS and SS form a lateral dimer, and then longitudinal tetramerization takes place. The lateral interaction of subunits is much stronger than the longitudinal interaction due to hydrophobic interactions. (B) Location of the mutated interface residues on homology-modeled AGPase in which the LS and the SS are depicted by violet and yellow colors, respectively. Residues (red) located at both the longitudinal and lateral interface. Coordinates of the model were set to define the lateral and longitudinal interaction regions in which strong hydrophobic interactions between LS and SS (A–B and C–D) are designated as lateral interactions, and the relatively weaker interaction of the dimers (AB–CD) are designated as longitudinal interactions. (C) Location of the residues (green) selected for further characterization in the region of the longitudinal interface (I90, A91, F101, Y378 and D410).
replaced with both cysteine and histidine at position 378 in WT LS AGPase for further analysis.

**Native-PAGE analysis of the mutants for the formation of heterotetramers**

The heterotetrameric assembly properties of the LS mutant AGPases (RM2, RM7, RM10, LS<sup>90V</sup>, LS<sup>378C</sup>, LS<sup>101I</sup>, LS<sup>378H</sup> and LS<sup>378C</sup>) with WT SS were investigated using 3–13% gradient native polyacrylamide gels followed by Western blot using potato anti-LS and anti-SS. First, these mutants were co-expressed with the WT SS in *E. coli* glgC– in the presence of isopropyl-β-D-thiogalactopyranoside (IPTG) and nalidixic acid. Then, the integrity and expression level of both subunits in all samples were assessed by 10% SDS–PAGE followed by Western blot analysis using anti-LS and anti-SS antibodies. As expected, the LS mutants and SS proteins in all cell-free extract were detected with a molecular weight of approximately 50 kDa, and their expression levels were found to be comparable with that of the WT AGPase (data not shown). Then, variable amounts of the samples were subjected to gradient native gel electrophoresis (Fig. 2A). The WT AGPase Western blot results revealed three major bands. One band corresponded to approximately 200 kDa, which indicated the presence of tetramers. The second band was detected at approximately 100 kDa, which indicated dimers. The third band was identified at approximately 50 kDa, demonstrating the presence of monomers (Fig. 2A). To ensure that an equal amount of AGPase in the total protein was loaded, the intensity of each band was calculated, and the total band intensities were found to be comparable (data not shown). In the WT AGPase, heterotetramers comprised 35% of the total band intensity, whereas dimers and monomers comprised 24% and 41% of the total band intensity, respectively (Fig. 2B). On the other hand, the band intensities of heterotetramers, dimers and monomers in the RM2 AGPase corresponded to 83, 3 and 17%, respectively (Fig. 2B). The disappearance of monomers and dimers in RM2 AGPase indicates that they were most probably assembled with the WT SS and formed more heterotetrameric AGPase as evidenced by the >2-fold increased heterotetramer amount compared with the WT AGPase (Fig. 2A). To see whether the WT SS was assembled with the RM2 LS, we decided to probe the same blot with anti-SS and then quantify the amount of the SS in monomers, dimers and heterotetramers. The results indicated that, in fact, the amount of the SS is the limiting factor for the heterotetrameric assembly of the RM2 LS AGPase. As seen in Fig. 2A, there were no detectable free monomers and dimers of the WT SS in RM2 samples, and they were all detected in heterotetramer form with RM2 LS compared with WT AGPase where 48% of the SSs were in the form of dimer and monomers.

As RM2 has two amino acid changes, isoleucine and tyrosine at positions 90 and 378 were separately replaced with valine and cysteine in the WT LS using site-directed mutagenesis to determine the effects of these two mutations independently on heterotetrameric assembly (Fig. 2A). The results of Western blots of the native gel indicated that both mutants had enhanced heterotetrameric assembly, in which 75% and 80% of the LS<sup>90V</sup>SS<sup>WT</sup> and the LS<sup>378C</sup>SS<sup>WT</sup> AGPases were in the form of heterotetramers, respectively (Fig. 2B). As expected, the majority of the WT SSs in mutant AGPases (>94%) were also detected in the heterotetramers (Fig. 2B). These results indicated that the individual or combined effect of the LS<sup>90V</sup>
and the LS Y378C mutations had a similar effect on the heterotetrameric assembly of the potato AGPase with WT SS (Fig. 2A, B).

A similar analysis was performed with RM7 (Y378H and D410G) and RM10 (D410G) to assess the effect of the mutations on heterotetrameric AGPase assembly. Western blot analyses of the native gel indicated that mutations in the revertants caused the disappearance of the dimers rather than the monomers (Fig. 2A). The amount of dimers that disappeared correlated with the amount of increased tetramers (Fig. 2B). The amount of dimers decreased by approximately 40–60% in both RM7 and RM10, whereas the amounts of tetramers increased by similar ratios. The detection of a decrease only in dimers indicated an enhanced interaction between dimers but not monomers. Next, the LS Y378H SS WT AGPase was examined to determine the independent contribution of this mutation to the formation of heterotetramers. The tyrosine change into histidine at position 378 in LS did not cause any changes in the heterotetramer formation with WT SS of the AGPase (Fig. 2B). Comparison of the LS Y378H SS WT and LS Y378C SS WT mutants for the heterotetrameric assembly indicated the importance of the hydrophobic residue, cysteine, for the subunit interaction of the enzyme at this identical position. Hydrophobic amino acid residues possibly increase the rigidity of the interface and enable better interaction of the subunits.

The effects of longitudinal interface mutations of RM27, LS A91T and LS F101L, were analyzed using native-PAGE. However, the results indicated that both mutants had a very similar ratio of the heterotetramer, dimer and monomer compared with WT AGPase (Supplementary Fig. S2).

**Heat stability of the mutants**

The native potato tuber and recombinant AGPase retained major activity after 5 min of incubation at between 60 and 70°C, depending on the presence of P, (Iglesias et al. 1994). It has been demonstrated that the heat stability of AGPase is dependent on the intermolecular disulfide bridge located between Cys12 of the SSs (Ballicora et al. 1999). However, other studies have demonstrated that mutations in the subunit–subunit interfaces or mutations in the allosteric sites increased the temperature stability of the AGPase (Boehlein et al. 2008). Thus, we investigated the heat stability of the mutant AGPases to determine whether the mutation(s) had an effect on the enzyme's structural stability. The heat stability of the mutants was examined by incubating the enzymes at 60°C for 5 min. The remaining activity of each sample was measured at saturated concentrations of substrates and 3PGA with the reverse direction assay. At 60°C, there were no significant differences in heat stability of the RM7, LS I90V SS WT, LS D410G SS WT and LS Y378H SS WT AGPases compared with WT AGPase, in which they had 65% and 75% of the remaining activities (Fig. 3). However, RM2 and LS Y378C SS WT AGPases were heat stable under the identical assay conditions compared with the activity of the WT AGPases. The remaining
activities of RM2 and LS$^{Y378CSSWT}$ AGPases were 92% and 85%, respectively (Fig. 3). It is interesting to note that cysteine at position 378 in the LS played a significant role in the heat stability properties of the LS$^{Y378CSSWT}$ AGPase.

We next investigated the importance of cysteine at position 378 of the AGPase in terms of heat stability in a time-dependent manner at 65°C. The LS$^{Y378CSSWT}$ AGPase was subjected to 65°C along with WT and LS$^{Y378HSSWT}$ AGPases in a time-dependent manner. The remaining activities of the WT and LS$^{Y378HSSWT}$ AGPases were measured as 30% and 20% at 65°C for 5 min, respectively. On the other hand, under identical conditions, the remaining activity of the LS$^{Y378CSSWT}$ AGPase was measured as 50%, which is 2-fold higher than the WT AGPase (Fig. 4A). A very similar heat stability trend was observed with the experiment performed at 65°C for 15 min (Fig. 4A). Both WT and LS$^{Y378HSSWT}$ AGPases had approximately 10% remaining enzymatic activities, whereas LS$^{Y378CSSWT}$ AGPase had 22% remaining activity, which is again 2-fold higher than the WT AGPase (Fig. 4A). To see how such activity correlates with the amount of the heterotetrameric AGPase, the samples were subjected to native-PAGE analysis. The results clearly indicated that the LS$^{Y378CSSWT}$ mutant contained more heterotetrameric enzyme compared with the WT and LS$^{Y378HSSWT}$ AGPases at the end of 5 and 15 min (Fig. 4B). To explore the possibility that the Cys378 residue in the AGPase makes an additional disulfide bond in the interface region, LS$^{Y378CSSWT}$ AGPase was subjected to non-reducing SDS–PAGE followed by Western blot. The results indicated that both WT and LS$^{Y378CSSWT}$ AGPases had all LS subunits as monomers, which indicates that there was no disulfide bond formation through the cysteine residue (Supplementary Fig. S3). In addition, heat stability analysis was performed with and without dithiothreitol (DTT) to see if Cys378 is involved in a stabilizing disulfide bridge. LS$^{C378CSSWT}$ and the WT AGPase had similar responses (data not shown).

All these results indicate that the presence of cysteine at position 378 in the LS not only enhances the heterotetrameric assembly but also enhances the heat stability of the AGPase.

**Fig. 3** Comparing the heat stability of mutants with the wild type. The remaining AGPase activity was demonstrated after heat treatment at 50 and 60°C for 5 min. Percentage heat stability is the activity remaining after heat treatment divided by the activity before heating. Duplicate samples were left on ice and their activity was taken to be 100% which was calculated at around 0.3–0.4 nmol min$^{-1}$ for each sample. Activity measurements were performed by the reverse direction assay in the presence of saturated substrate and 3PGA concentrations. Results are the average of 4–6 independent sample preparations with the standard error compared with Student’s t-test with regard to the wild type: **P < 0.01; *P < 0.05.

**Fig. 4** Heat stability profiles of the LS$^{WTSSWT}$, LS$^{Y378CSSWT}$ and LS$^{Y378HSSWT}$ at 65°C. (A) Remaining activities of the samples were measured after 5 and 15 min heat treatment at 65°C. Duplicate samples were left on ice, and their activity was taken to be 100%. Activity measurements were accomplished using the reverse direction assay in the presence of saturated substrate and 3PGA concentrations. (B) Native-PAGE analysis of heat-treated samples for 5 and 15 min at 65°C. The same amount and the same volume of total protein was loaded onto a 3–13% native gradient gel and proteins were detected with anti-LS and anti-SS antibodies.
Kinetic and allosteric properties of the mutants

To determine the catalytic and allosteric properties of the mutant AGPases displaying enhanced heterotetrameric assembly, each mutant enzyme was partially purified using ammonium sulfate fractionation and Macro-Prep DEAE weak anion exchange chromatography with a final purity of 20–30%. Because degraded AGPase exhibits altered kinetic behavior (Salamone et al. 2000), all mutant enzymes were subjected to SDS–PAGE and a subsequent Western blot analysis using potato anti-LS and anti-SS antibodies. No apparent degradation was observed for any of the mutant enzymes that were analyzed (Supplementary Fig. S4). The $K_m$ values of all mutants for the substrates (ATP and G1P) and the cofactor (Mg$^{2+}$) were determined in the presence of 3PGA and were comparable with WT values (Table 2). Allosteric analysis revealed that RM2, LS$^{Y378C}$$^{SSWT}$, LS$^{Y378C}$$^{WWT}$ and LS$^{Y378H}$$^{SSWT}$ AGPases did not display a significant change in sensitivity towards 3PGA/P, compared with the WT AGPase (Table 2). However, RM7 and RM10 AGPases exhibited altered allosteric behavior compared with the WT. $A_{0.5}$ values were calculated as 0.84 and 1.9 mM for RM7 and RM10, respectively (Table 2; Supplementary Fig. S5). Both RM7 and RM10 required 4- to 8-fold more 3PGA than the WT enzyme AGPase, which required 0.2 mM 3PGA to reach $A_{0.5}$. Both revertants contained an aspartate change at position 410 into glycine. This residue was previously identified as an important residue for the allosteric function of the AGPase (Greene et al. 1996), as replacing Asp410 by alanine generated an AGPase mutant that requires 6- to 10-fold higher levels of Pi than the WT enzyme. A bacterial expression system has facilitated the understanding of plant AGPase sequences were performed using CLUSTALW2 (Fig. 5). Conservation analysis revealed that the isoleucine residue at position 90 in the LS is highly conserved in different plant species of the LS AGPases, but Arabidopsis LS and potato SS have leucine instead of isoleucine. Tyrosine at position 378 in the LS is conserved in Arabidopsis and lentils, and in the potato SS. However, E. coli AGPase lacks this residue, rice LS has isoleucine and maize LS has an uncharged polar threonine residue at this location. Our conservation analysis revealed that the LS$^{25410}$ residue is highly conserved among different species, including E. coli.

Discussion

AGPase is one of the key regulatory enzymes of the starch biosynthetic pathway. Plant AGPases are structurally complex and consist of two large and two small subunits. Because of the lack of a crystal structure of the native heterotetrameric AGPase, mutagenesis studies have come to prominence to describe the structure–function relationship of the enzyme. A bacterial expression system has facilitated the understanding of plant AGPase function because random mutagenesis and the rapid screening of activity in E. coli are feasible. Detailed analyses have identified sites important for the kinetic and allosteric properties and heat stability of the enzyme (Greene and Hannah 1998a, Greene et al. 1998, Kavakli et al. 2001a, Kavakli et al. 2001b, Kim et al. 2007, Boehlein et al. 2008).

Although there is evidence suggesting that the involvement of the interface amino acids contributes to both the lateral and

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<tr>
<th>$K_m$ (mM)</th>
<th>$A_{0.5}$ (mM)</th>
<th>$I_{0.5}$ (mM) for P$i$ at 1 mM 3PGA</th>
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<tr>
<td>ATP</td>
<td>G1P</td>
<td>Mg$^{2+}$</td>
</tr>
<tr>
<td>LS$^{WWTSW}$</td>
<td>0.11 ± 0.05</td>
<td>0.073 ± 0.018</td>
</tr>
<tr>
<td>RM2</td>
<td>0.14 ± 0.017</td>
<td>0.11 ± 0.035</td>
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<tr>
<td>RM7</td>
<td>0.14 ± 0.026</td>
<td>0.029 ± 0.013</td>
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<tr>
<td>RM10</td>
<td>0.34 ± 0.1</td>
<td>0.26 ± 0.12</td>
</tr>
<tr>
<td>LS$^{MDIVSSW}$</td>
<td>0.38 ± 0.013</td>
<td>0.071 ± 0.03</td>
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<tr>
<td>LS$^{Y378CSSW}$</td>
<td>0.20 ± 0.0058</td>
<td>0.22 ± 0.018</td>
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<tr>
<td>LS$^{Y378HSSW}$</td>
<td>0.17 ± 0.095</td>
<td>0.081 ± 0.019</td>
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Kinetic and regulatory properties were determined in the ADP-glucose synthesis direction. All values are in mM. $K_m$ and $A_{0.5}$, for substrates/cofactor and 3PGA, respectively, correspond to the concentration of these molecules required for the enzyme activity to attain 50% of maximal activity. $I_{0.5}$ is the amount of P$i$ required to inhibit the enzyme activity by 50% of maximal activity. Depicted are the mean values with standard error of at least two independent experiments.
longitudinal interaction between the LS and SS, there is currently no information pertaining to specific amino acid changes that may enhance the longitudinal LS–SS interaction and, in turn, increase heterotetrameric assembly of AGPase. A previous study of maize endosperm AGPase revealed heat-stable mutants that enhance the interaction between the LS and SS (Greene and Hanhah 1998a). In this study, we applied a second-site genetic reversion approach to identify peptide region(s) and amino acids that may comprise a portion of the longitudinal LS–SS interaction in AGPase. Using this approach, the LS

\[ \text{Y378CSSWT} \]

AGPase also exhibited increased stability, for example, in LSY378CSSWT AGPase may be the location of this residue, which is notably close to both SSs within a range of 4–5 Å according to the heterotetrameric model (Tuncel et al. 2008). Tyr378 on the D chain of the LS, for example, is at a distance of 4.6 Å from Glu124, 4.9 Å from the Thr126 residue in the B chain of the SS and 4.6 Å from the Arg298 residue in the C chain of the SS (Supplementary Fig. S6A). Moreover, substitution of Tyr378 by cysteine in the LS reduced the 4.6 Å distance to 3.2 Å between LS

\[ \text{D} \]

and SS

\[ \text{E}^{124} \]

and it appears to generate a new hydrogen bond between the side chains of LS

\[ \text{C}^{378} \]

and SS

\[ \text{E}^{124} \]

residues (Supplementary Fig. S6B). In addition, it is possible that mutations in the LS (at positions 90 and 378) may increase the rigidity of the interface and therefore enable better interactions between the subunits. However, to elucidate the exact role of these residues in subunit–subunit interactions, the crystal structure of the heterotetrameric plant AGPase is essential. We next investigated the effect of the mutations obtained in this study on heat stability properties of the AGPase. Heat stability analysis indicated that only LS

\[ \text{Y378CSSWT} \]

AGPase was heat stable compared with the other mutant and WT AGPases. Tyr378 of the LS possibly generates new hydrogen bonds, which is the result not only of enhanced heterotetrameric interaction between the subunits but also of the increasing heat tolerance of the AGPase. Another possibility is that having a specific hydrophobic amino acid residue at the indicated position in the LS subunit interface results in increasing the rigidity and, in turn, enhancing subunit–subunit interaction and heat stability.

Kinetic analysis revealed that only LS

\[ \text{D}^{378} \]

SS

\[ \text{WT} \]

AGPase has distinct allosteric properties (Table 2; Supplementary Fig. S5). These results are consistent with an aspartate residue at position 410 of the LS, which has been previously demonstrated to be crucial for the normal allosteric behavior of the potato AGPase and spinach AGPase (Ball and Preiss 1994, Greene et al. 1996). Although the aspartate at position 410 in the LS was not demonstrated to be positioned directly at the subunit interface, its location on the model was close to the subunit interface (Fig. 1C). Previous studies have indicated the relationship between allosteric properties and subunit interaction of the AGPase (Georgeilis et al. 2009, Boehlein et al. 2010). The homologous residue of potato LS

\[ \text{R88} \]

in maize SH2 (R146) was demonstrated to be involved in P binding. SH2

\[ \text{R166A} \]

mutation altered the 3PGA and P affinity of the enzyme in a down-regulatory manner (Boehlein et al. 2010). In addition, a potato LS

\[ \text{R88} \]

mutant has been demonstrated to be defective in heterotetramer formation, as stated previously (Baris et al. 2009). These studies suggest that subunit interaction and allosteric properties of the AGPase are closely related to each other.

In this study, we identified a novel amino acid on the LS AGPase interface important for the heterotetrameric assembly and heat stability of the AGPase. The novel mutations, LS

\[ \text{D}^{378} \]

and LS

\[ \text{Y378C} \]

were identified and characterized as significantly enhancing the LS–SS interaction and, in turn, increasing
heterotetrameric assembly of the enzyme without affecting the kinetic and allosteric properties. In addition, we also identified the amino acid residue that moderately enhanced heterotetrameric assembly with altered kinetic properties.

**Materials and Methods**

**PCR mutagenesis, screening and selection**

Potato AGPase LS[**R**88[**A**] (30 fmol) in the pML7 plasmid was amplified using the primers LSDS1 and LSDS2 (Supplementary Table S1). The PCR mixture contained 1 × mutagenic buffer (3 mM MgCl2, 0.5 mM MgSO4, 50 mM KCl and 10 mM Tris–HCL, pH 8.3), 1 mM dGTP, 1 mM dATP, 0.2 mM dCTP, 0.2 mM dTTP, 30 pmol of each primer, and 5 U of Taq polynucleotide kinase. PCR was performed using a Biorad T-100 robocycler for 15 cycles under the following conditions: 30 s at 94°C, 30 s at 50°C and 2 min at 72°C. The PCR products were purified using a PCR clean-up kit. The amplified products were digested with Ncol and HindIII, cloned into the corresponding sites of the pML7 vector, and transformed into E. coli DH5α, carrying the SS expression plasmid pML10. Revertants were identified by their ability to complement the ΔglgE phenotype, thereby restoring glycogen production, which was readily scored using iodine staining of the bacterial colonies on Kornberg medium enriched with 2% glucose.

**Site-directed mutagenesis**

Mutations were introduced into potato AGPase LS using PCR, as described in Kavakli et al. (2001b). The PCR was performed in a total volume of 50 μl containing 50–100 ng of template plasmid pML7, 20 pmol of each appropriate primer (Supplementary Table S1), 0.2 mM dNTPs and 2.5 U of PfuTurbo DNA polymerase. The PCR was performed for 14 cycles under the following conditions: 95°C for 30 s, 50°C for 30 s and 68°C for 14 min. The PCR products were digested with DpnI to remove template plasmid DNA and transformed into E. coli DH5α. The presence of the site-directed mutations was confirmed by DNA sequencing using the Macrogen sequencing facility (The Netherlands).

**Protein expression and blue native gel analysis**

The WT and various LS mutants were expressed in E. coli AC70R1-504 (ΔglgC−) cells containing pML7 grown in LB medium. Expression of both the LS and SS was induced with 10 mg l−1 nalidixic acid and 200 mM IPTG at room temperature for 20 h. Cells were harvested by centrifugation when the culture OD600 reached 1–1.2. The cells were disrupted by sonication in lysis buffer [Tris-buffered saline (TBS), 200 mg ml−1 lysozyme, 5 mg ml−1 protease inhibitor (Sigma) and 1 mM phe- nylmethylsulfonyl fluoride (PMSF) (Roche)]. The crude homogenate was centrifuged at 14,000 × g for 15 min. The resulting supernatant was used in PAGE and Western blotting analysis. Protein levels were determined using the Bradford assay according to the manufacturer’s (Bio-Rad) instructions. All native gel and Western blot analysis were carried out under identical conditions using identical reagents and solutions. Results are the average of at least three independent experiments.

Native-PAGE was performed using a Bio-Rad Mini-PROTEAN III electrophoresis cell. Cell lysates, containing the indicated amounts of total protein, were mixed with Laemmli’s sample loading buffer without any reducing or denaturing agent. Samples were then electrophoresed using a 3–13% polyacrylamide gradient gel with 1 × running buffer (192 mM glycine, 25 mM Tris, pH 7.0) containing 0.002% Coomassie Blue G at 100 V for 20 min at 4°C, and the blue buffer was then exchanged with a buffer that did not contain Coomassie Blue G. The gels were run at 180 V until the dye front migrated to the bottom of the gel. The observed position of the protein complexes was compared with the bovine serum albumin (BSA) oligomer running pattern. After native-PAGE, the gels were transferred to polyvinylidene difluoride membranes (Biotrace PVDF, Pall Corporation) using a semi-dry transfer unit. After blocking with 5% BSA dissolved in TBS–TWEEN-20 (0.15%), the membranes were incubated with anti-LS or anti-SS primary antibodies and a horseradish peroxidase (HRP)-conjugated secondary anti-rabbit IgG antibody. Proteins were detected with ECL reagents.

SDS–PAGE was performed using a Bio-Rad Mini-PROTEAN III electrophoresis unit. Cell lysates or partially purified proteins were electrophoresed on a 10% separating gel. Gels were run at 150 V for 1.5 h. The gels were subjected to Western blotting as previously described.

Quantification of the Western blots was performed using the ImageJ software.

**Heat stability**

The WT and mutant AGPases were expressed, and the cells were harvested as previously described (Greene et al. 1998). Cells were dissolved in 50 mM HEPEs pH 7.5, 5 mM KPO4, 5 mM MgCl2 and 10% glycerol. Aliquots of cell-free extracts of the WT and mutant AGPases were exposed to temperatures of 50 and 60°C for 5 min, cooled on ice and then harvested by centrifugation at 4°C at maximum speed for 15 min. The supernatants were transferred into Eppendorf tubes; the AGPase activity was determined using the reverse direction assay in the presence of saturated substrates, and the 3PGA concentration was determined by monitoring G1P production. Duplicate samples were left on ice and their activity was taken to be 100% which was calculated as around 0.3–0.4 nmol G1P min−1 for each sample. The heat exposure was applied at 65°C for 5, 10 and 15 min.

**Reverse direction assay**

A non-radioactive end-point assay was used to determine the amount of G1P produced by coupling its formation to NADPH production using phosphoglucomutase and glucose-6-phosphate dehydrogenase (Sowokinos 1976). The standard reaction mixture contained 100 mM HEPEs (pH 7.4), 4 mM DTT, 5 mM 3PGA, 5 mM MgCl2, 1 mM NAPPi, 1 mM
obtained by non-linear regression analysis using the Prism software (Graph Pad). The $I_{0.5}$ for $P_i$ was determined in the presence of 5 mM 3PGA by adding increasing amounts of $P_i$.

Reducing and non-reducing conditions for AGPase
Cell-free extracts were prepared as described previously. For reducing conditions, samples were heated at 95°C for 5 min with Laemmli sample buffer containing 4 mM DTT. For non-reducing conditions, Laemmli sample buffer without any reducing agent was used. The same 10% SDS–polyacrylamide gels were used for both conditions. Western blotting and detection were performed as described previously.

For activity measurements, samples were incubated in 100 mM HEPES, pH 8.0 and 3 mM DTT for 30 min at room temperature. This condition was referred to as the reducing condition. In the control, samples were incubated similarly without DTT.

**Supplementary data**
Supplementary data are available at PCP online.

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**Disclosures**
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