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Allosteric regulation of the higher plant ADP-glucose pyrophosphorylase is a product of synergy between the two subunits

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Abstract The higher plant ADP-glucose pyrophosphorylase (AGPase) is a heterotetramer consisting of two regulatory large subunits (LSs) and two catalytic small subunits (SSs). To further characterize the roles of these subunits in determining enzyme function, different combinations of wildtype LS (L_{WT}) and variant forms (L_{UpReg1}, L_{M345}) were co-expressed with wildtype SS (S_{WT}) and variant forms (S_{TG-15} and $S_{devo330}$) and their enzyme properties compared to those measured for the heterotetrameric wildtype enzyme and SS homotetrameric enzymes. Analysis of the allosteric regulatory properties of the various enzymes indicates that although the LS is required for optimal activation by 3-phosphoglyceric acid and resistance to Pi, the overall allosteric regulatory and kinetic properties are specified by both subunits. Our results show that the regulatory and kinetic properties of AGPase are not simply due to the LS modulating the properties of the SS but, instead, are a product of synergistic interaction between the two subunits.

Keywords: ADP-glucose pyrophosphorylase; Allosteric regulation; Kinetics; Subunit; Synergistic effect

1. Introduction

ADP-glucose pyrophosphorylase (AGPase; EC 2.7.7.27) catalyzes the rate-limiting step in starch/glycogen synthesis in prokaryotes and plants and is considered one of the dominant regulatory enzymes in this biosynthetic process [1–5]. The enzyme activity from bacteria and from many plant species is allosterically regulated by small effector molecules whose nature reflects the primary carbon energy-generating pathways in these organisms. In higher plants, the enzyme is mainly activated by 3-phosphoglyceric acid (3-PGA), the product of CO_2 fixation, and inhibited by inorganic phosphate (Pi). In leaves, it has been suggested that the ratio of 3-PGA to Pi controls the catalytic activity of this enzyme and the extent of starch synthesis [6].

Although the bacteria and higher plant enzyme share similar regulatory and catalytic properties, they possess different oligomeric structures [7]. The bacterial AGPases are homotetramers containing a single subunit type (α_4). In contrast, the higher plant AGPase is a heterotetramer containing two large subunits (LSs) and two small subunits (SSs) which show considerable variation in size (LS, 51–60 kDa; SS, 50–54 kDa) depending on the tissue and plant species examined [1–5,8]. The primary sequences of the LS and SS share considerable sequence homology suggesting that they evolved from a common ancestral gene [8]. Results from previous studies [9–14] have demonstrated that the two subunit types play different roles in enzyme function with the LS being regulatory and the SS possessing both catalytic and regulatory roles. When expressed in the absence of the LS, the SS is capable of forming a catalytically active homotetrameric AGPase, whereas the LS is unable to assemble efficiently to form an oligomeric structure [14–17]. The homotetrameric SS (S_{WT}) enzyme [14], however, displays defective allosteric properties in requiring more than 15-fold greater levels of 3-PGA for activation than the heterotetrameric wildtype enzyme ($L_{WT}S_{WT}$). Moreover, the S_{WT} homotetramer shows less resistance to Pi inhibition than the wildtype enzyme. Hence, the LS is required to restore the normal allosteric regulatory properties of the native enzyme [11].

This functional assignment of AGPase subunit properties is supported by site-directed mutagenesis studies of residues conserved between the bacterial and plant AGPases. Lys-195 in the homotetrameric enzyme from *Escherichia coli* is involved in binding of the substrate glucose 1-phosphate [18]. Mutagenesis of this conserved Lys residue in the SS of the potato AGPase but not the LS drastically decreased the apparent affinity for this substrate [19]. Similar mutagenesis studies with Asp residues homologous with the bacterial Asp-142, previously demonstrated to be involved in enzyme catalysis, showed that changes in the SS were 100-fold more effective in abolishing enzyme activity than changes in the corresponding residue in the LS [20]. Collectively, these biochemical studies indicate that the SS is catalytic possessing defective allosteric regulatory properties, whereas the LS is regulatory in elevating the sensitivity of the heterotetramer to the activator 3-PGA.

These suggested roles for the AGPase subunits are also supported by genetic studies using random mutagenesis. Several mutations in the LS of the potato AGPase resulted in a change in the normal allosteric regulatory properties of the heterotetrameric enzyme without significant impact on substrate binding properties [9]. Replacement of lysine for glutamic acid ($E_{38}K$) in the LS gave rise to an upregulatory phenotype conferring reduced sensitivity to phosphate inhibition and increased activation by 3-PGA [21], while $E_{38}A$ [21] and $P_{52}L$

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Abbreviations: AGPase, ADP-glucose pyrophosphorylase; DTT, dithiothreitol; Glc-1-P, glucose-1-phosphate; IPTG, isopropyl- β-D-thiogalactopyranoside; L or LS, large subunit; 3-PGA, 3-phosphoglycerate; S or SS, small subunit; SDS, sodium dodecyl sulfate; PCR, polymerase chain reaction; Pi, orthophosphate

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[13] mutations resulted in opposing negative effects on allosteric regulatory properties (termed downregulation).

Laughlin et al. [10] identified several SS mutants through chemical mutagenesis techniques, which had impaired affinity for the substrates. $A_{106}T$ substitution generated a heterotetramer with lowered affinity for ATP and Mg^{2+}. $D_{252}N$ and $D_{121}N$ mutations severely lowered the affinity for Glc-1-P, while $P_{43}S$ substitution gave rise to decreased sensitivity to 3-PGA and phosphate. Recently, in vitro mutagenesis of the potato SS yielded several homotetrameric mutants that showed significantly higher sensitivity to 3-PGA activation and higher resistance to Pi, when compared to the S_{WT} homotetramer. For example, TG-15 (S_{TG-15}) homotetramer [16] containing $L_{46}F$ and $V_{57}I$ substitutions showed 3-PGA activation and phosphate inhibitory properties comparable to $L_{WT}S_{WT}$ but at the expense of reduced ATP binding affinity. A subsequent study [17] employing DNA shuffling method generated SS (devo) mutants, which displayed regulatory behaviors ranging from wildtype-like to upregulatory, and kinetic properties nearly comparable to $L_{WT}S_{WT}$. One mutant, devo330 ($S_{devo330}$), with $L_{46}F$, $V_{57}I$, $Y_{317}C$, and $L_{380}S$ substitutions showed the most dramatic changes in allosteric properties: i.e., highest sensitivity to 3-PGA and resistance to Pi.

Affinity labeling studies with pyridoxal phosphate and protection with effectors identified several lysine residues, conserved in all higher plant AGPases, which may lie in the 3-PGA and Pi binding sites of the LSs and SSs [12]. Site-directed mutagenesis of two lysine residues (Lys-404 and Lys-441) in the SS resulted in a substantial decrease in the apparent affinity for 3-PGA and Pi, whereas corresponding replacements at the two equivalent Lys residues in the LS resulted only a moderate reduction in the apparent affinity for the activator and no effect on Pi binding [11]. These results indicate that the regulatory sites of the SS are more important than those in the LS.

Despite recent advances in the identification of the regulatory and catalytic residues in the higher plant AGPase, much more information is required to fully understand the roles of the AGPase subunits in enzyme function. To gain further insight on the roles of the AGPase subunits in allosteric regulation and catalysis, nine combinations of three LS types (L_{WT}, L_{UpReg1} with $E_{38}K$ [21], and L_{M345} with $P_{52}L$ [13] substitutions) were co-expressed together with three SS types (S_{WT}, S_{TG-15} [16], and $S_{devo330}$ [17]). The results of the study of these enzymes showed that allosterism and catalysis are the products of an interplay between the LSs and the SSs that result in a synergistic response to the allosteric effectors and substrates.

2. Materials and methods

2.1. Reagents

[^{32}P]PPi and [^{14}C]glucose-1-phosphate were purchased from Perkin-Elmer (NEN) and ICN Pharmaceuticals, respectively. All other compounds were from Sigma and of the highest commercial grade.

2.2. Plasmids and protein expression

Glycogen deficient *E. coli* mutant AC70R1-504 (*glgC*$^-$) was used for protein expression. As results from a previous study [22] have shown that addition of a polyhistidine-tag to the amino terminus of the LS did not significantly affect the enzymatic properties of the wild type potato AGPase, the 6× His-tag was attached to three other LS variant genes (L_{UpReg1} with $E_{38}K$, $L_{DownReg}$with $E_{38}A$, and L_{M345} with $P_{52}L$ substitutions) in order to facilitate the purification of the expressed enzymes. The His-tag sequences were incorporated in the LSs by PCR amplification using two primers, 5′ GAA*CCATGG*GACACCATCACCATCACCATGCTTACTCTGTGATCACTACTG 3′ (*Nco*I site is italicized and 6× His sequence is underlined) and 5′CCC*GAGCTC*ACTATATGACTGTTCCATCTCTAATTGTGGC 3′ (*Sac*I site is italicized) by using *Pfu Turbo* DNA polymerase (Stratagene) under the conditions as described in [22]. The subsequent procedures for cloning into pML7 [10,15] are also described in [22]. These His-tagged LSs were co-expressed with pML10-based plasmids harboring the recombinant wildtype SS (S_{WT}), mutant TG-15 (S_{TG-15}), and mutant devo 330 ($S_{devo330}$) [16,17]. *E. coli* AC70R1-504 cells were individually transformed with the three different SSs and then each SS-containing line was transformed with each of the four LSs, which generated twelve enzyme combinations. All combinations were tested on Kornberg agar media (1.1% K_2HPO_4, 0.8% KH_2PO_4, 0.6% yeast extract, 0.2% glucose, 1.5% agar) supplemented with 100 μg/ml spectinomycin and 50 μg/ml kanamycin to monitor glycogen production and, in turn, AGPase activities. Selected bacterial cells containing the different combinations were transferred to 1-L fresh media and induction of the protein was carried out as described in [22].

2.3. Purification of the AGPase heterotetramers

All purification steps were performed at 4 °C. Induced cells were harvested by centrifugation and resuspended in 30 ml of cold lysis buffer (50 mM HEPES–NaOH, pH 8.0, 3 mM DTT, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM ATP, and 10% glycerol) supplemented with 1 μg/ml each of leupeptin, benzamidine, pepstatin A, and chymostatin, and 1 mM phenylmethylsulphonyl fluoride. Following incubation on ice with 200 μg/ml lysozyme for 30 min, the cells were disrupted by sonication. Crude cell extracts were obtained by centrifugation at 20000*g* for 20 min at 4 °C and then passed through a 1 μm membrane filter (Whatman). Cleared cell extracts were then loaded on a DEAE–Sepharose Fast Flow (Amersham) column pre-equilibrated with buffer A (50 mM HEPES–NaOH, pH 8.0, 5 mM $MgCl_2$, 20 mM imidazole, pH 8.0, and 10% v/v glycerol) and washed with the same buffer. Proteins were eluted with a linear gradient of 0–0.5 M KCl in buffer A. Fractions containing activity were pooled, passed through a 1 μm filter, and then directly loaded on a Ni–NTA affinity column pre-equilibrated with buffer B (buffer A plus 0.3 M KCl). After extensive washing with 10 column volumes of buffer B, the His-tagged AGPases were eluted with buffer C (buffer B plus 200 mM imidazole). The eluted fractions containing AGPase activity were pooled and dialyzed for 24 h at 4 °C against buffer D (25 mM HEPES–NaOH, pH 7.5, 3 mM $MgCl_2$, 2 mM DTT, 1 mM EDTA, and 10% v/v glycerol). Dialysates were then concentrated using a 30 kDa ultrafiltration membrane (Millipore), cleared by centrifugation, aliquoted and stored at −80 °C.

2.4. Enzyme assay

For monitoring purification of the enzyme, AGPases activities were measured in the reverse pyrophosphorylase (Assay A) direction. For kinetic characterization the activities were measured in the forward ADP-glucose synthesis (Assay B) direction. One unit (U) is defined as the amount of enzyme that produces 1 μmol of ATP or ADP-glucose in 1 min.

Assay A. [^{32}P]ATP formation was measured from [^{32}P]PPi and ADP-glucose. Reaction was performed at 37 °C in 0.2 ml of 100 mM HEPES–NaOH, pH 7.5, 7 mM $MgCl_2$, 5 mM DTT, 5 mM 3-PGA, 10 mM NaF, 0.4 mg/ml bovine serum albumin, 1 mM ADP-glucose, 1.5 mM PPi, 3×10^6 cpm/ml [^{32}P]PPi as described in [22].

Assay B. [^{14}C]Glc-1-P incorporation into ADP-glucose was determined for all kinetic measurements with saturating substrates and/or effectors. Reaction was performed at 37 °C in 0.1 ml of 100 mM HEPES–NaOH, pH 7.5, 7 mM $MgCl_2$, 3 mM DTT, 5 mM 3-PGA, 0.4 mg/ml bovine serum albumin, 0.15 U inorganic pyrophosphatase (Sigma), 1.5 mM ATP, 1 mM Glc-1-P, and [^{14}C]Glc-1-P (1000–1200 dpm/nmol) as described in [22].

2.5. Kinetics studies

$S_{0.5}$ for ATP, Mg^{2+}, and Glc-1-P and $A_{0.5}$ for 3-PGA corresponding to the levels required for 50% maximal activity or activation, were determined by fitting the experimental data from *Assay B* to the Hill equation [23], $v = V_{max}[L]^h/(K^h + [L]^h)$, with the aid of Kaleidagraph (Synergy Software, Reading, PA). In this equation v represents the reaction rate, V_{max} the maximal reaction rate, [L] the concentration of the ligand (substrate or effector), K the half saturation constant

($S_{0.5}$, $A_{0.5}$, or $I_{0.5}$), and h the Hill coefficient (nH). For inhibition analysis, $I_{0.5}$ for Pi corresponding to the level required for 50% inhibition was calculated from the fractional inhibition of enzyme activities plotted vs. Pi concentrations (0–10 mM) by fitting the data to the Hill equation as above.

2.6. SDS–PAGE and immunoblot analysis

Measurement of protein concentration, SDS–PAGE, immunoblot analysis, and image analysis were done as described in [22].

3. Results

3.1. Expression and purification of the LS/SS heterotetramers

Because the AGPase SS alone can form a functional homotetrameric enzyme in the absence of the LS [14,16,17], conditions were sought to minimize formation of the SS homotetramer and its possible contamination with the heterotetrameric enzyme. The most optimum procedure developed was to attach a polyhistidine peptide on the LS and use this tag to facilitate purification by IMAC chromatography [22]. Attachment of the polyhistidine tag to the N terminus of the AGPase LS not only facilitated purification of the enzyme, but also caused no significant changes in the kinetic properties in terms of $A_{0.5}$, $I_{0.5}$, $S_{0.5}$ for 3-PGA, Pi, and substrates (ATP, Mg^{2+}, and Glc-1-P), respectively. Our preliminary experiment also revealed that attachment of the His_6 instead of the original His_5 did not significantly alter catalytic and allosteric parameters of the AGPase (see also Tables 1 and 2). This strategy enabled one to directly purify the various heterotetrameric enzyme types even in the presence of any potentially formed SS homotetramer.

Each of the His_6 tagged LSs (L_{WT}, L_{UpReg1}, L_{M345}, and L_{E38A}) were co-expressed with the various SSs (S_{WT}, S_{TG-15}, and $S_{devo330}$) in transformed *glgC*$^-$ AC70R1-504 bacteria. These cells were exposed to iodine to assess the extent of glycogen accumulation using the intensity of iodine staining as a measure. Compared to cells expressing the wildtype enzyme ($L_{WT}S_{WT}$) as a reference, the various enzyme subunit combinations showed varying levels of iodine staining and, in turn, glycogen accumulation (Fig. 1). Expression of S_{WT} with L_{UpReg1} yielded cells with darker staining and enhanced capacity for glycogen production, whereas S_{WT} co-expression with L_{M345} yielded cells exhibiting no significant iodine staining and little, if any, glycogen accumulation. These iodine-staining phenotypes are consistent with the in vitro upregulatory and downregulatory properties of these enzymes [13,21]. When the LS variants were co-expressed with the upregulatory S_{TG-15} or $S_{devo330}$, the cells accumulated greater amounts of glycogen compared to those co-expressing S_{WT}. This effect was clearly evident when these upregulatory SS variants were co-expressed with the downregulatory L_{M345}. These observations indicate that these SS mutants contribute significantly to the regulation of AGPase activity.

Co-expression of the downregulatory L_{E38A} [21] with the various SSs showed an iodine staining pattern identical to that for L_{M345} with no significant staining with S_{WT} and enhanced staining with S_{TG-15} and $S_{devo330}$. However, immunoblotting of crude extracts from these cells with anti-LS and anti-SS antibodies revealed very low levels of L_{E38A} as compared to the readily detectable SS levels (Fig. 1A, B, and C). Hence, elevated glycogen accumulation evident in cells expressing $L_{E38A}$and S_{TG-15} or L_{E38A} and $S_{devo330}$ were derived not from the catalytic activity of the AGPase heterotetrameric forms but from the upregulatory SS homotetrameric forms. This result re-emphasizes the importance of the use of purified His-tagged LS protein containing heterotetrameric enzyme for reliable kinetic characterization.

The AGPase heterotetramers were purified by ion exchange and Ni–NTA affinity column chromatography steps. The resulting enzyme preparations were near homogeneous when viewed by SDS–PAGE and Coomassie brilliant blue staining (Fig. 1C). Final purification levels of the various AGPases

Table 1
3-PGA and Pi inhibition profiles of the recombinant wildtype and mutant heterotetrameric AGPases

Enzyme	$A_{0.5}$			$I_{0.5}$@$A_{0.5}$		$I_{0.5}/A_{0.5}$		$I_{0.5}$ @ 0.05 mM 3-PGA			$I_{0.5}$ @ 0.5 mM 3-PGA		
	mM	nH[a]	(Fold[b])	mM	nH		(Fold)	mM	nH	(Fold)	mM	nH	(Fold)
S_{wt}[c]	2.38	–[d]	(1)	0.11[e]	–	0.05	–	–	–	–	–	–	–
S_{TG-15}[c]	0.14	–	(17)	0.40[f]	–	2.9	–	–	–	–	–	–	–
$S_{devo330}$[c]	0.012	–	(198)	7.5[f]	–	625	–	–	–	–	–	–	–
$L_{WT}S_{WT}$	0.10	0.9	(1)	0.10	1.1	1	(1)	0.04	1.3	(1)	0.27	1.1	(1)
$L_{WT}S_{TG-15}$	0.003	0.8	(33)	0.09	1.3	29.7	(30)	0.41	1.2	(10)	2.08	1.1	(8)
$L_{WT}S_{devo330}$	0.003	1.1	(33)	0.12	1.4	40	(40)	0.39	1.6	(10)	2.91	1.1	(11)
$L_{UpReg1}S_{WT}$	0.03	1.2	(3)	0.07	1.0	2.2	(2.2)	0.08	1.4	(2)	0.98	1.4	(4)
$L_{UpReg1}S_{TG-15}$	0.001	1.0	(100)	0.19	1.0	190	(190)	1.75	1.2	(44)	7.81	n.d.[e]	(29)
$L_{UpReg1}S_{devo330}$	0.001	0.9	(100)	0.28	0.9	282	(282)	2.92	1.1	(73)	10.39	n.d.	(38)
$L_{M345}S_{WT}$	0.70	0.9	(0.14)	0.29	1.3	0.4	(0.4)	0.30	0.9	(8)	0.29	1.3	(1)
$L_{M345}S_{TG-15}$	0.04	0.9	(2.5)	0.25	1.2	6.3	(6)	0.40	1.2	(10)	1.50	1.0	(5.6)
$L_{M345}S_{devo330}$	0.05	1.1	(2)	0.40	1.0	8	(8)	0.39	1.0	(10)	1.75	1.4	(6.5)

All values were determined from ADP-glucose synthesis assay (*Assay B*) data of at least two iterations, and the S.E. was <10% in all cases. When the S.E. value was greater than 10%, further two reactions were performed to obtain reliable data. The mean values are presented here. The $I_{0.5}/A_{0.5}$ ratios were obtained by measuring the $I_{0.5}$ value in the presence of known concentration ($A_{0.5}$) of 3-PGA for each heterotetramer. The $I_{0.5}$ value for each heteroteffamer was also obtained by performing the reactions in the presence of varied 3-PGA concentrations (0.05 and 0.5 mM) for the L_{WT}, L_{UpReg1} and L_{M345} heterotetramers.

[a]Hill coefficient.

[b]Fold decrease or increase in the $A_{0.5}$, $I_{0.5}$ or $I_{0.5}/A_{0.5}$ values of the homotetramers, respectively, with respect to S_{WT} and $L_{WT}S_{WT}$.

[c]Homotetrameric AGPases; S_{WT} [14], S_{TG-15} [16], and $S_{devo330}$ [17].

[d]–, Not available; n.d., not determined.

[e]Values were determined at 3 mM 3-PGA.

[f]Values were determined at 0.1 mM 3-PGA.

Table 2
Kinetic parameters for ATP, Mg^{2+}, and Glc-l-P of the recombinant wildtype and the mutant heterotetrameric AGPases

Enzyme	@ 1 or 5 mM 3-PGA (optimal condition)										No 3-PGA			
	ATP			Mg^{2+}			Glc-1-P			Apparent V_{max}[c]	Glc-1-P			V_{max}
	$S_{0.5}$ (mM)	nH[a]	C.E.[b]	$S_{0.5}$ (mM)	nH	C.E.	$S_{0.5}$ (mM)	nH	C.E.		$S_{0.5}$ (mM)	nH	C.E.	
S_{wt}[d,e]	0.20	–[f]	–	2.2	–	–	0.29	–	–	–	–	–	–	–
S_{TG-15}[d,e]	1.20	–	–	1.4	–	–	0.25	–	–	–	–	–	–	–
$S_{devo330}$[d,e]	0.50	–	–	1.4	–	–	0.20	–	–	–	–	–	–	–
$L_{WT}S_{WT}$[e]	0.12	1.4	765	2.1	4.1	44	0.09	1.0	1020	27	0.87	1.0	3	0.8
$L_{WT}S_{TG-15}$	0.15	1.6	635	2.5	3.1	38	0.14	0.9	680	28	0.88	0.9	7	1.8
$L_{WT}S_{devo330}$	0.26	1.8	392	3.0	3.0	34	0.14	1.1	729	30	1.28	1.1	9	3.2
$L_{UpReg1}S_{WT}$[e]	0.10	1.3	1054	1.9	3.8	55	0.07	1.1	1506	31	0.44	0.9	6	0.7
$L_{UpReg1}S_{TG-15}$	0.16	1.1	595	2.4	2.7	40	0.10	1.0	952	28	0.51	1.0	24	3.6
$L_{UpReg1}S_{devo330}$	0.25	1.2	381	2.8	2.8	34	0.14	1.0	680	28	0.78	1.0	25	5.8
$L_{M345}S_{WT}$[e]	0.57	1.1	48	1.8	4.2	15	0.10	1.0	272	8	1.01	1.0	3	1.0
$L_{M345}S_{TG-15}$	0.53	1.3	96	2.2	3.4	23	0.23	0.3	222	15	0.66	1.0	13	2.5
$L_{M345}S_{devo330}$	0.49	1.4	208	3.1	2.8	33	0.21	0.9	486	30	0.43	1.1	28	3.5

The statistical explanation is same as in Table 1. The apparent $S_{0.5}$ V_{max}, k_{cat}, and catalytic efficiencies for ATP, Mg^{2+}, and Glc-l-P were obtained under the saturating (optimal) condition where 5 mM 3-PGA for all of the LSs assembled with S_{WT} and 1 mM 3-PGA for all of the LSs assembled with S_{TG-15} and $S_{devo330}$ were added, and under the condition where no 3-PGA was added.
[a]Hill coefficient.
[b]Catalytic efficiency, $K_{cat}/S_{0.5}$ (s^{-1} M^{-1}); $\times 10^{-3}$ values are presented.
[c]V_{max}, μmol/min/mg.
[d]Homotetrameric AGPases; S_{WT} [14], S_{TG-15} [16] and Sdevo 330 [17].
[e]Assays were perofmed at 5 mM 3-PGA, but the rest were assayed at 1 mM 3-PGA.
[f]Not available.

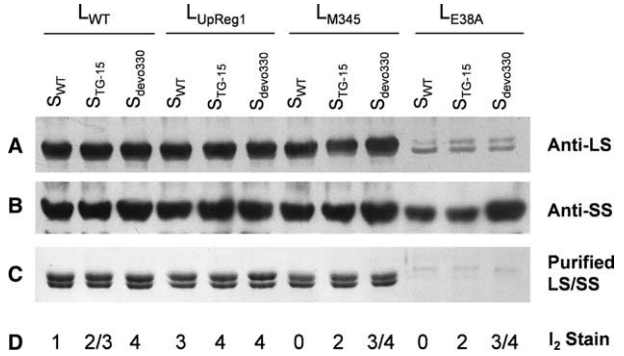

Fig. 1. Expression and purification of the heterotetrameric AGPases. Immunoblot analyses of the AGPase LS proteins (A) and SS proteins (B) in crude extracts using anti-potato AGPase LS and SS antibodies, respectively. (C) SDS–PAGE analysis of purified heterotetrameric AGPases (0.8 μg for each lane) used in this study. (D) The extent of iodine vapor staining of *E. coli* cells expressing the various LS/SS heterotetramers. The relative intensities of the stained cells were measured using arbitrary units from 0 (null), 1 (wildtype) and 2–4 (glycogen levels higher than wildtype).

were in the range of 26–35-fold over the activities determined in crude extracts (data not shown). These purified enzyme preparations were used to determine the kinetic and allosteric regulatory parameters of the heterotetrameric AGPase variants.

3.2. The influence of subunit types on specifying allosteric regulation of the AGPase heterotetramers

To determine the contribution of each subunit type in terms of allosterism, $A_{0.5}$ for 3-PGA, $I_{0.5}$ for Pi, and $I_{0.5}/A_{0.5}$ were measured for each enzyme (Table 1) using the standard Assay B. The recombinant wildtype enzyme $L_{WT}S_{WT}$ showed an $A_{0.5}$ of 0.1 mM, a value similar to that reported in the earlier studies [10,14] and a 24-fold increase in 3-PGA affinity compared to the S_{WT} homotetramer. The 3-PGA sensitivity of the upregulatory $L_{UpReg1}S_{WT}$ was higher than that measured for $L_{WT}S_{WT}$, while the $L_{M345}S_{WT}$ enzyme showed reduced affinity to 3-PGA than $L_{WT}S_{WT}$. However, the $A_{0.5}$ value for $L_{M345}S_{WT}$ was still 3.4-fold lower than that evident for the S_{WT} homotetramer. This increase in 3-PGA activation response was also evident for heterotetramers containing the L_{WT} and the upregulatory SS mutants, S_{TG-15} and $S_{devo330}$. $L_{WT}S_{TG-15}$ and $L_{WT}S_{devo330}$ showed a 47-fold and 4-fold increase in 3-PGA sensitivity compared to the homotetrameric SS forms (Table 1). These results indicate that the LS, irrespective of whether it is upregulatory or downregulatory in nature, is essential for optimal 3-PGA activation by the S_{WT}.

Analysis of the heterotetramers containing the variant LSs and SSs provide further insights into the interaction of these subunits in specifying 3-PGA activation. $L_{UpReg1}S_{TG-15}$ shows about a 100-fold increase in 3-PGA affinity compared to $L_{WT}S_{WT}$. When each of the mutant subunits are assembled with the wildtype subunit, the increase in 3-PGA is smaller with $L_{UpReg1}S_{WT}$ showing a 3.3-fold enhancement over $L_{WT}S_{WT}$ while $L_{WT}S_{TG-15}$ exhibiting a 33-fold increase. Collectively, the enhancement in 3-PGA affinity is specified by each mutant subunit and accounts for the dramatic increase in 3-PGA affinity for $L_{UpReg1}S_{TG-15}$ than that evident for $L_{WT}S_{WT}$. A similar cooperative interaction between the subunits in specifying the degree of 3-PGA activation is also evident with $L_{UpReg1}$and $S_{devo330}$, with each subunit contributing to the increase in 3-PGA affinity (100-fold over $L_{WT}S_{WT}$). Interestingly, this interaction can also be seen with heterotetramers containing the downregulatory L_{M345} and upregulatory S_{TG-15} and $S_{devo330}$ subunits. In these instances, the $A_{0.5}$ values measured for $L_{M345}S_{TG-15}$ and $L_{M345}S_{devo330}$ can be accounted by the individual contributions from each respective subunit with L_{M345} decreasing the apparent 3-PGA affinity whereas S_{TG-15} and $S_{devo330}$ increasing the apparent 3-PGA affinity. Overall, these results indicate that the sensitivity of these

heterotetramers to the activator 3-PGA is due to a cooperative interaction between the LSs and SSs.

To determine the effect of these subunit types on susceptibility of the heterotetramers to Pi inhibition, the $I_{0.5}$ values for Pi were measured at the corresponding $A_{0.5}$ concentrations of 3-PGA (Table 1). The observed $I_{0.5}$ values for L_{WT} and L_{M345} heterotetramers were not significantly altered by either S_{TG-15} or $S_{devo330}$ compared to the wildtype enzyme, although those values for L_{UpReg1} heterotetramers were elevated as high as 4-fold. The corresponding $I_{0.5}/A_{0.5}$ ratios for the heterotetramers containing S_{TG-15} or $S_{devo330}$, however, were approximately 30–40-fold (L_{WT}), 86- to 128-fold (L_{UpReg1}) and 16- or 20-fold (L_{M345}) higher, respectively, due to the increased affinity for 3-PGA by these mutant SSs. When measured at increasingly higher levels of 3-PGA, these enzymes required much more Pi for inhibition than the wildtype. The $L_{WT}S_{TG-15}$ and $L_{WT}S_{devo330}$ enzymes required nearly 10-fold higher levels of Pi for 50% inhibition compared to the wildtype enzyme at 0.05 mM 3-PGA and this antagonistic effect was similar at 10-fold higher 3-PGA levels. A similar Pi inhibition pattern was also evident for the $L_{UpReg1}$and L_{M345} heterotetrameric enzymes. Among the variant LSs the reversal of Pi inhibition by 3-PGA was most evident for the L_{UpReg1} enzymes.

Comparison of the $I_{0.5}/A_{0.5}$ values for the various heterotetramers again shows that the degree of Pi resistance is specified by both subunits. For example, $L_{UpReg1}S_{devo330}$ displays an $I_{0.5}/A_{0.5}$ value of 282, whereas $L_{UpReg1}S_{WT}$ and $L_{WT}S_{devo330}$ have values of about 2.2 and 40, respectively. The pronounced Pi resistance exhibited by $L_{UpReg1}S_{devo330}$ is much greater than the sum of the properties conferred by each individual subunit type. Hence, the cooperative interaction of $L_{UpReg1}$and $S_{devo330}$ subunits results in synergistically increasing Pi resistance of the heterotetramer.

3.3. PGA: allosterically a dual effector at saturating concentration

To gain more insight into the role of these subunits in AGPase allosteric regulatory behaviors we analyzed the catalytic responses of these enzymes to varying levels of 3-PGA activator (Fig. 2A). Enzymes containing S_{WT} exhibited hyperbolic activation curves irrespective of the nature of the LS. Heterotetramers containing S_{TG-15} or $S_{devo330}$, however exhibited a biphasic response to 3-PGA. AGPase activities of heterotetramers containing the mutant SSs were maximal at ca. 1 mM 3-PGA (termed optimal concentration) but, thereafter, declined significantly at higher 3-PGA concentrations. These observations suggest that 3-PGA can serve as a partial inhibitor at high concentrations. To determine whether the inhibitory effects by high 3-PGA levels alter the Pi inhibition profile, the activation responses of $L_{WT}S_{WT}$, $L_{WT}S_{TG-15}$, and

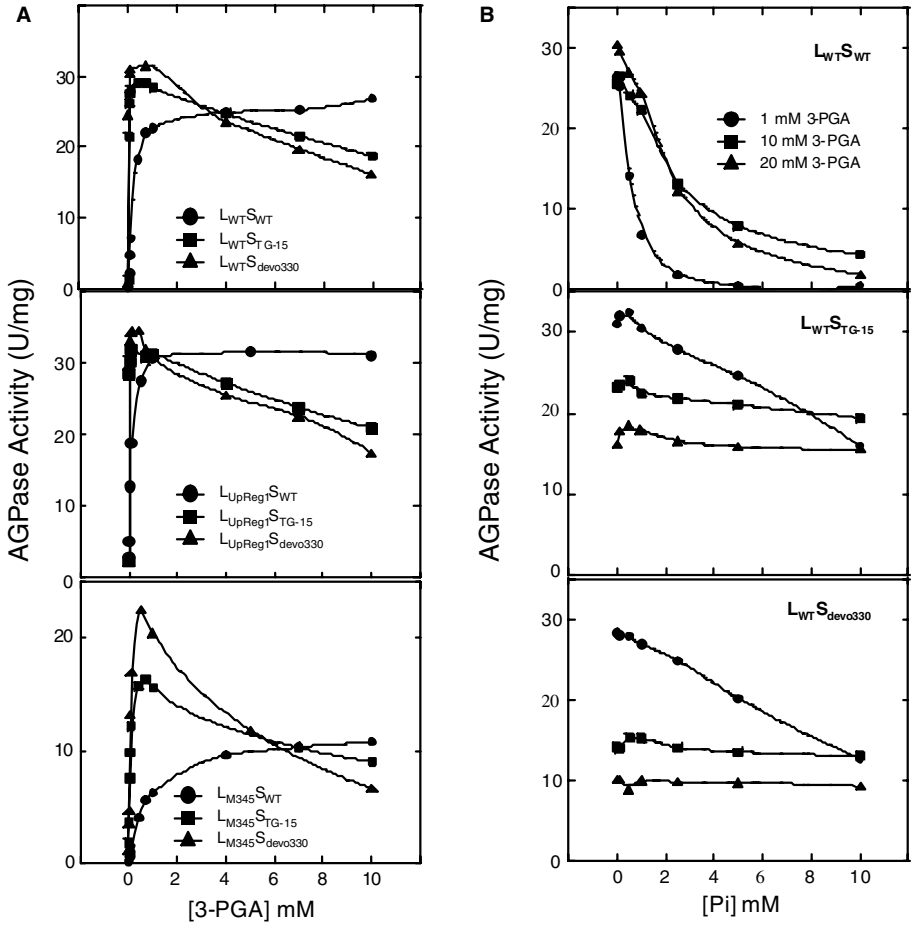

Fig. 2. 3-PGA activation profiles and the interplay of effector molecules on the various heterotetramers. (A) Specific activities of the AGPase heterotetramers were measured at various concentrations of 3-PGA from 0 to 10 mM. (B) Specific activities of L_{WT} assembled with S_{WT} S_{TG-15} and $S_{devo330}$ were measured at 1, 10, and 20 mM 3-PGA with concentrations of Pi ranging from 0 to 10 mM.

$L_{WT}S_{devo330}$ were measured at 1, 10, or 20 mM 3-PGA over a wide range of Pi concentration (Fig. 2B). The wildtype enzyme, $L_{WT}S_{WT}$, showed the typical Pi inhibition response curves, where Pi inhibition is reversed to a large extent by 3-PGA. However, while $L_{WT}S_{TG-15}$ and $L_{WT}S_{devo330}$ exhibited less inhibition by Pi at 1 mM 3-PGA compared to $L_{WT}S_{WT}$, both heterotetramers were not significantly impaired by Pi inhibition at 10 and 20 mM 3-PGA. These results indicate that Pi exerted very little effect on the activities of the two enzymes under conditions where 3-PGA suppresses activity.

3.4. Influence of subunit types on catalysis of the AGPase heterotetramers

The substrate binding affinities ($S_{0.5}$), Hill coefficients (nH), maximum velocities (V_{max}), and catalytic efficiencies ($k_{cat}/S_{0.5}$) were determined for the various heterotetramers (Table 2). These parameters were measured in the presence of optimal (5 mM 3-PGA except for heterotetramers containing the upregulatory SS variants where 1 mM was used) and far-optimal concentration (10 mM) of 3-PGA, the latter concentration at which enzymes containing S_{TG-15} and $S_{devo330}$ were significantly impaired (Fig. 2). For comparison, the kinetic parameters for Glc-1-P were also measured in the absence of 3-PGA. Compared to the S_{WT} homotetramer [14], $L_{WT}S_{WT}$ and $L_{UpReg1}S_{WT}$ had lower apparent $S_{0.5}$ for ATP, whereas $L_{M345}S_{WT}$ had a higher value. These results indicate that the LS modulates ATP binding of the heterotetramer and that the nature of the LS either stimulates (L_{WT} and L_{UpReg1}) or diminishes (L_{M345}) ATP binding properties.

Assembly of L_{WT} and L_{UpReg1} with the SS mutants also showed enhancement in ATP affinity compared to the S_{TG-15} and $S_{devo330}$ homotetramers. When compared to $L_{WT}S_{WT}$, however, these enzymes showed reduced ATP affinity. The downregulatory L_{M345} had different effects on ATP binding depending on the nature of the mutant SS. $L_{M345}S_{TG-15}$showed more than a 2-fold reduction in $S_{0.5}$, while $L_{M345}S_{devo330}$ ATP binding properties were no different than the homotetramer counterparts. However, the apparent V_{max}(or k_{cat}) values for the L_{M345}-containing heterotetramers at optimal 3-PGA concentrations changed dramatically depending on the SS type ($S_{devo33} > S_{TG-15} > S_{WT}$).

The homotetramers had similar apparent $S_{0.5}$ values for Glc-1-P of between 0.2 and 0.3 mM (Table 2) [14,16,17]. All of the heterotetramers containing L_{WT} and L_{UpReg1} showed moderate to significant increases in the apparent affinity for this sugar phosphate. While $L_{M345}S_{WT}$ showed increased affinity for this substrate compared to the S_{WT} homotetramer, the heterotetramers with the SS mutants, however, showed no significant differences in Glc-1-P binding properties compared to the corresponding SS homotetramers. In the absence of 3-PGA (Table 2) the overall $S_{0.5}$ values for Glc-1-P were significantly higher than those under optimal 3-PGA. This trend in the changes in $S_{0.5}$ values exhibited by the SS mutants was very similar to those under 3-PGA activation. Interestingly, in the absence of 3-PGA, the SS had a more dominant role in catalytic capacity of the heterotetrameric enzyme forms. Irrespective of the nature of the LS type, all S_{WT}containing heterotetramers had similar V_{max} values. In contrast, moderate (2-fold) to pronounced (8-fold) increases in catalysis was observed for heterotetramers containing the mutant SSs compared to those containing S_{WT}. It is also evident that fold-activation (V_a/V_o) was significantly lowered by the SS mutants (34–16- or 9-fold for the L_{WT} heterotetramers; 44–8- or 5-fold for the L_{UpReg1} heterotetramers) while those values for the L_{M345} heterotetramers were roughly unchanged. Mg^{2+} binding constants were relatively stable for the heterotetramers containing S_{WT} although those containing the SS mutants tended to have higher $S_{0.5}$ values compared to the corresponding homotetramers.

The catalytic parameters of the L_{WT} heterotetramers were also determined at far-optimal 3-PGA concentration (10 mM) which suppresses the catalytic activities of the heterotetramers containing the SS mutants (Fig. 2). In addition to a significant reduction in the apparent V_{max} for $L_{WT}S_{TG-15}$ (1.6-fold) and $L_{WT}S_{devo330}$ (1.9-fold), these enzymes showed higher requirements for ATP and Mg^{2+} (1.4–2.2-fold) but not for Glc-1-P when compared to those at optimal 3-PGA concentration.

4. Discussion

AGPase is considered one of dominant enzymes in controlling carbon metabolism in plants via its allosteric regulatory properties and regulation by redox potential. Results from previous studies [14,24,25] have indicated that the two AGPase subunits play asymmetric roles in enzyme function: the SS is catalytic and is capable of forming an active enzyme, albeit with defective allosteric regulatory properties while the LS is regulatory and increases the sensitivity of the heterotetrameric enzyme to the activator 3-PGA and resistance to Pi inhibition. To further characterize the roles of these subunit types in enzyme function, wildtype and variant LSs and SSs were co-expressed and the resulting heterotetrameric enzymes were purified and studied kinetically. As the SS is capable of self-assembly to form a catalytically active enzyme, efforts were made to optimize expression and to purify the heterotetrameric enzyme forms. The latter was accomplished by adding a His_6 peptide to the N-terminus of the LS and using this ligand as a chelator for affinity purification of the heterotetramer. The addition of the His_6 tag on the LS had no discernible effect on the kinetic and allosteric regulatory properties of the wildtype $L_{WT}S_{WT}$ ([22], Tables 1 and 2). Significant differences, however, were noted between our $A_{0.5}$ values reported here for $L_{UpReg1}S_{WT}$ (30 μM in this study and 2 μM in [21]) and $L_{M345}S_{WT}$ (0.7 mM reported here and 5 mM reported in [13]). A direct comparison between the His_6-tagged $L_{WT}S_{WT}$ and unmodified $L_{WT}S_{WT}$ from the same purification step showed identical $A_{0.5}$ values (unpublished results), indicating that the differences in enzyme kinetic properties between those described here and those reported in previous studies [13,21] may be due to different degrees of enzyme purity and protein integrity.

Although both subunits have 3-PGA binding sites, only the ones located in the SS are sufficient for allosteric regulation by this effector. Hence, it has been suggested that the LS is simply required to modulate the regulatory properties of the SS [11]. Our results support and extend this hypothesis. In all instances, assembly of the LS with SS forms an enzyme with enhanced 3-PGA affinity as compared to the corresponding homotetrameric enzyme type. For example, when compared to the S_{WT} homotetramer, the $L_{WT}S_{WT}$ enzyme requires nearly 24-fold less 3-PGA for 50% activation (Table 1). This activating effect by the LS on enzyme regulatory properties is even more

pronounced with the upregulatory L_{UpReg1}, which requires nearly 3.3-fold less 3-PGA than the wildtype heterotetrameric enzyme. Although $L_{M345}S_{WT}$ has an $A_{0.5}$ more than 7-fold higher than the wildtype $L_{WT}S_{WT}$, its sensitivity to 3-PGA activation was still about 3-fold better than the S_{WT} homotetramer. Moreover, increases in 3-PGA affinity were evident for the upregulated S_{TG-15} and $S_{devo330}$ when combined with any LS form (Table 1), These results show that the LS is required for optimal 3-PGA activation of the heterotetrameric enzyme.

Although the LS is essential for optimal 3-PGA activation response, our results also show that the both subunits contribute to the overall allosteric regulatory properties of the heterotetramer. For example, $L_{UpReg1}S_{WT}$ and $L_{WT}S_{devo330}$ show about 3.3- and 33-fold, respectively, increases in 3-PGA sensitivity compared to $L_{WT}S_{WT}$ (Table 1, Fig. 3). When the two variant L_{UpReg1} and $S_{devo330}$ subunits are combined, the resulting enzyme $L_{UpReg1}S_{devo330}$ shows about 30- and 3-fold increases in 3-PGA sensitivity compared to $L_{UpReg1}S_{WT}$ and $L_{WT}S_{devo330}$ and 100-fold increase compared to the wildtype enzyme. A similar pattern is evident when S_{TG-15} is included in these comparisons. As the $A_{0.5}$ for $L_{UpReg1}S_{devo330}$ is significantly smaller than the $A_{0.5}$ values contributed by each mutant subunit containing the corresponding wildtype subunit, the contribution of each subunit is not simply additive. Instead, subunit interaction results in a cooperative effect which results in a synergistic activation ($30 \times 3.3 = 100$-fold) to 3-PGA. On the other hand, an antagonistic interaction with the various SSs is evident with the downregulatory L_{M345}. For example, $L_{M345}S_{WT}$ shows a 7-fold higher $A_{0.5}$ than the wildtype enzyme. When L_{M345} is combined with $S_{devo330}$, however, the $A_{0.5}$ values are 14-fold lower than $L_{M345}S_{WT}$ but still 2-fold lower than the wildtype ($1/7 \times 14 = 2$-fold; Table 1, Fig. 3).

The synergistic interaction between the subunits is also evident for Pi inhibition. For example, the $I_{0.5}/A_{0.5}$ values of $L_{UpReg1}S_{WT}$ and $L_{WT}S_{devo330}$ exhibit 2.2- and 40-fold higher than $L_{WT}S_{WT}$, respectively. In contrast, the $L_{UpReg1}S_{devo330}$ enzyme shows 128- and 7-fold greater values than $L_{UpReg1}S_{WT}$ and $L_{WT}S_{devo330}$, respectively ($2.2 \times 128 = 282$-fold; Table 1, Fig. 3). A similar pattern is also evident when the contribution of S_{TG-15} is analyzed in the various enzyme combinations.

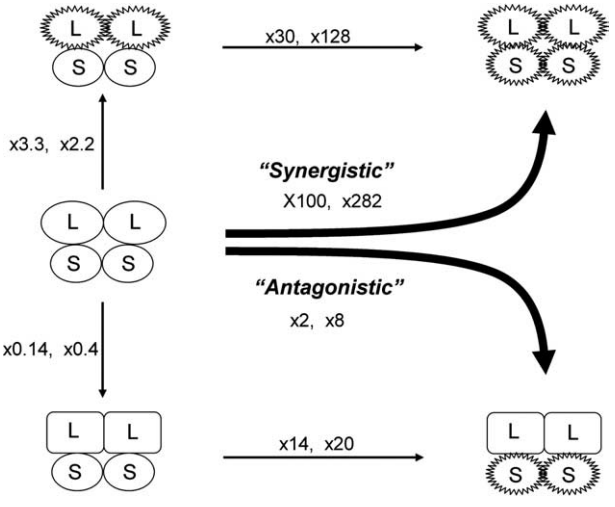

Fig. 3. The effect on allosteric regulation of AGPase by subunit interactions. The two numbers by the arrows indicate fold decrease (or increase) in the $A_{0.5}$ values and $I_{0.5}/A_{0.5}$ values, respectively. The smooth ovals represents the wildtype AGPase subunits (L_{WT} or S_{WT}), the jagged ovals representing the upregulatory subunits (L_{UpReg1}or $S_{devo330}$), while the rounded rectangle denotes the downregulatory L_{M345}.

The gain in apparent 3-PGA affinity by S_{TG-15} and $S_{devo330}$ heterotetramers (as compared to the homotetratmer forms) is not without some adverse consequences. Irrespective of the nature of the LS, heterotetramers containing these mutant SSs showed abnormal activation kinetics (Fig. 2A). AGPase activity increased as concentrations of 3-PGA were raised up to 1 mM (optimal concentration) but, thereafter, activity decreased at higher 3-PGA concentrations. This biphasic response by $L_{WT}S_{TG-15}$ and $L_{WT}S_{devo330}$ indicates that 3-PGA serves as a partial inhibitor at high concentrations and interacts with the Pi binding site. Consistent with this view is that Pi is able to inhibit $L_{WT}S_{TG-15}$ and $L_{WT}S_{devo330}$ at 1 mM 3-PGA but not at higher levels (=10 mM) (Fig. 2B). These results corroborate conclusions made from previous studies [9,26] that the 3-PGA and Pi binding sites are close to or overlapped each other. Alternatively, these results may indicate that 3-PGA at its high concentrations and phosphate bind to the same effector site in these variant enzyme forms.

In the absence of 3-PGA, the catalytic rates (V_{max} or k_{cat}) of the heterotetramers are determined by the nature of the SS, with S_{TG-15} and $S_{devo330}$ having V_{max} values up to 8-fold larger than enzymes containing S_{WT} (Table 2). However, under fully activated conditions all of the L_{WT} and L_{UpReg1} heterotetramers showed very similar apparent V_{max} values, in the range of 27–31 U/mg, and the values were unaffected by the SS mutants. In contrast, the V_{max} values of the L_{M345} heterotetramers were strongly influenced by the nature of the SS with $L_{M345}S_{devo330}$ showing a V_{max} equivalent to the other heterotetramers, $L_{M345}S_{TG-15}$ showing an intermediate value, and $L_{M345}S_{WT}$ having a low value. Hence, L_{M345} not only modulates the allosteric regulatory properties of the catalytic SS [13] but the catalytic rate as well. As a whole, the affinities of the enzymes for Glc-1-P are 2–10-fold higher compared to the values measured in the absence of 3-PGA. Under the same conditions the AGPases show 1.5–3-fold lower affinity for ATP (data not shown). Binding of 3-PGA also increases the catalytic rates of the wildtype and variant AGPases [20] (Table 2). These results indicate that AGPase is both a K-type and V-type regulatory enzyme.

In many instances, subunit interactions also influenced the kinetic properties of the various enzyme forms. This view is most evident for ATP binding. The $S_{0.5}$ for ATP ranges from 0.2 mM (for S_{WT} homotetramer) to 1.2 mM (for S_{TG-15} homotetramer). Assembly with L_{WT} resulted in an increase in affinity for this substrate ranging from 2-fold for $L_{WT}S_{WT}$ and $L_{WT}S_{devo330}$ to 8-fold for $L_{WT}S_{TG-15}$. In contrast, the binding properties for Mg^{2+} were only slightly diminished for the heterotetramers containing the SS mutants, while those containing S_{WT}were unchanged. Apparent affinities for Glc-1-P were improved by the assembly of LS for all of the enzyme forms except for the L_{M345} heterotetramer containing the SS mutants which showed similar affinities with the corresponding SSs. Interestingly, L_{WT} and L_{UpReg1} heterotetramers containing the SS mutants showed lower affinity to ATP, Mg^{2+}, and Glc-1-P compared to those containing the wildtype SS. Hence, the observed gain in upregulatory allosteric properties of heterotetramers containing the SS mutants was at the expense of reduced substrate binding properties. The exception to this

relationship was L_{M345}-containing heterotetramers. In the latter instances, $S_{0.5}$ values were not significantly affected by the nature of the SS.

This study provides detailed information of the allosteric and kinetic properties of various AGPase variant enzymes. In addition in providing additional insights into the role of each of the subunits in enzyme function, this information provides the basis for manipulation of starch synthesis in photosynthetic and sink organs of plants. On-going studies [27–30] have demonstrated that starch metabolism is a limiting process in developing seeds and can be manipulated to augment sink strength and, in turn, crop yields. Moreover, leaf starch is required not only to provide a source of carbon and energy during the heterotrophic phase of growth during the night but also to accommodate excess photosynthate and alleviate potential feedback effects on photosynthesis [31]. Based on the results in this study, these upregulatory subunit types are being introduced into rice and *Arabidopsis* to determine the relationship between the in vitro kinetic behaviors of this enzyme activity and its impact on carbon metabolism such as photosynthesis, carbon partitioning and starch accumulation.

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