Isolation and characterization of a higher plant ADP-glucose pyrophosphorylase small subunit homotetramer

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Abstract ADP-glucose pyrophosphorylase (AGPase) is the allosterically regulated gateway for carbon entry into transient and storage starch in plants as well as glycogen in bacteria. This enzyme plays a key role in the modulation of photosynthetic efficiency in source tissues and directly determines the level of storage starch in sink tissues, thus influencing overall crop yield potential. AGPase is a tetrameric enzyme; in higher plants it consists of two regulatory large subunits (LS) and two catalytic small subunits (SS), while in cyanobacteria and prokaryotes the enzyme is homotetrameric. The potato SS gene in pML10 was mutated by hydroxylamine and mutants were screened for elevated homotetrameric activity by iodine vapor staining. This search strategy led to the isolation of SS mutants (SUP-1, TG-15) that had pyrophosphorylase activity in the absence of the LS. TG-15 has a leucine to phenylalanine change at position 48 ($L_{48}F$) that corresponds to a phenylalanine residue at the analogous position in the *Escherichia coli* homotetrameric AGPase as well as a valine to isoleucine change at position 59 ($V_{59}I$). TG-15 was partially purified and kinetic analysis revealed substrate and effector affinities equal to wild type heterotetrameric enzyme with the exception of ATP binding.

Key words: ADP-glucose pyrophosphorylase; Mutagenesis; Starch; Homotetramer; Allosteric regulation

1. Introduction

Starch synthesis in higher plants and glycogen formation in bacteria proceed through similar biosynthetic pathways. The first step is the formation of adenosine 5′-diphosphate (ADP)-glucose from glucose 1-P and ATP, a reaction catalyzed by ADP-glucose pyrophosphorylase (AGPase, EC 2.7.7.27). The glucosyl moiety of ADP-glucose is then transferred to an existing α-glucan chain through an α-1,4 glucosidic bond by glycogen/starch synthase (EC 2.4.1.21). Branching enzyme (EC 2.4.1.18) then catalyzes the formation of α-1,6 glucosidic branches in the growing glucan chain.

The flow of carbon into storage reserves as glycogen in bacteria and starch in plants is regulated mainly at formation of ADP-glucose by AGPase. AGPase is regulated at the genetic level [1–4] as well as at the biochemical level [1,5,6]. AGPase exists as a tetramer of approximately 200 kDa in the active form and is allosterically regulated by metabolites that are indicative of the major carbon assimilatory pathway utilized in plant tissue or microorganisms [7]. Both activating and inhibiting metabolic effectors regulate AGPase with the in vivo physiological ratio of the effector molecules determining the overall rate of AGPase catalysis [8]. For example, the AGPase from *Escherichia coli* is activated by fructose 1,6-diphosphate and inhibited by AMP. Other prokaryotic AGPases utilize various metabolic intermediates that are indicators of the major catabolic pathway in these organisms as activators and indicators of low cellular energy charge as inhibitors [7]. While the cyanobacterial enzyme is homotetrameric like the bacterial enzyme, it has evolved allosteric sensitivity to the same effectors as the higher plant enzyme, 3-phosphoglycerate (3-PGA) as activator and inorganic phosphate (P_i) as inhibitor [9]. This adaptation in allosteric effectors reflects the main carbon assimilation pathway, i.e. photosynthesis, in cyanobacteria.

AGPase structure and regulation has been investigated from photosynthetic (source) and non-photosynthetic (sink) tissues in higher plants. All sources of this enzyme studied to this point have shown the higher plant AGPase to be a heterotetramer of two large subunits (LS) and two small subunits (SS) [10–12]. The size of the individual subunits and the relative difference in size between subunits varies between species. Conservation of sequence between SS across species is greater than sequence conservation between LS and SS within a species [13]. LS sequences show less conservation than SS sequences across species [13,14].

Differences in the evolutionary conservation of the LS and SS likely reflect their roles in enzyme function. Indeed, while the LS when expressed by itself lacks enzymatic activity, the SS does exhibit catalytic activity. However, the wild type (WT) SS homotetramer requires elevated levels of 3-PGA for activation and is more sensitive to P_i inhibition than WT heterotetrameric AGPase [15]. An in vivo example of SS homotetramer activity exists in the *Arabidopsis thaliana* mutant TL-46 [16] which is deficient in the LS due to a lesion at the adg2-1 locus [17]. This mutant contains only 5% of WT AGPase activity, yet accumulates 40% as much starch as WT plants [16,18]. Characterization in vitro showed that the TL-46 AGPase was a SS homotetramer that was less sensitive to 3-PGA activation and more sensitive to P_i inhibition as well as exhibiting increased K_m values for ATP, Glc-1-P and Mg^{2+}

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Abbreviations: AGPase, adenosine 5′-diphosphate glucose pyrophosphorylase; 3-PGA, 3-phosphoglycerate; P_i, inorganic phosphate; PP_i, inorganic pyrophosphate; WT, wild type; SS, small subunit; LS, large subunit; PMSF, phenylmethylsulfonyl fluoride

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[18]. These observations together with results from structure–function studies of AGPase utilizing random and site-directed mutagenesis suggest that the LS is mainly involved in modulating the allosteric sensitivity of the heterotetramer, while the SS plays a role in allosteric sensitivity as well as being solely responsible for catalysis [15,19–24]).

As part of a structure–function analysis of the higher plant AGPase a random mutagenesis approach combined with a novel bacterial complementation system [15,25] was utilized to identify a LS which when combined with WT SS, formed an allosteric-defective enzyme. Mutagenesis of the SS and expression with the LS allosteric mutant, M-345, led to the isolation of SS suppressor mutants as well as a SS mutant that had a positive iodine staining phenotype when expressed by itself. The latter observation suggested a mutagenesis approach to isolate plant homotetrameric enzyme with WT kinetic and allosteric responses. In this report we describe the isolation and kinetic characterization of a potato SS homotetrameric AGPase that exhibits an enzyme activity similar to WT heterotetrameric AGPase.

2. Materials and methods

2.1. Mutagenesis and screening

Random chemical mutagenesis using hydroxylamine of vector pML10 containing the coding region of the potato SS AGPase gene [15] was done as previously described [21]. The mutated SS gene was expressed in *E. coli* AC70R1-504, (a *glg* $C^{-/-}$ strain), containing the potato LS mutant M-345 [21] and was screened by iodine vapor staining [26] to identify SS suppressor mutations of M-345. One of the suppressor mutants isolated, SUP-1, was subjected to subsequent hydroxylamine mutagenesis, expressed by itself, and screened as described above. Homotetrameric SS mutants were screened for more rapid staining in comparison to SUP-1 by growth on low glucose (0.1%) medium and iodine staining. Mutations were identified by sequencing the entire coding region of the SS in the sense and anti-sense directions by cycle sequencing with fluorescently labeled dideoxy nucleotides and detection with an ABI automated sequencer. Secondary structural predictions and physicochemical profiles were done at the Pole Bio-Informatique Lyonnais website (http://pbil.ibcp.fr) via Network Protein Sequence @nalysis by multivariate linear regression combination [27] of the SOPMA-GOR4-SIMPA prediction programs.

2.2. Assay of ADP-glucose pyrophosphorylase

Assay A: The pyrophosphorolysis of ADP-glucose was followed by the formation of [^{32}P]ATP from ^{32}inorganic pyrophosphate (PP_i) [10,11]. The reaction mixture contained 80 mM HEPES pH 7.5, 5 mM DTT, 5 mM $MgCl_2$, 10 mM NaF, 1 mM ADP-glucose, 10 mM 3-PGA, 0.4 mg/ml BSA, 3.0×10^6 cpm/ml $^{32}PP_i$, 1.5 mM $NaPP_i$, and enzyme sample in a final volume of 0.25 ml. The reaction was initiated by addition of enzyme and incubated at 37°C for 10 min.

Assay B: Kinetic parameters were determined by the synthesis (forward) assay. Synthesis of ADP-glucose was followed by the formation of [^{14}C]ADP-glucose from ATP and [^{14}C]glucose 1-phosphate [28]. The reaction mixture contained 100 mM HEPES pH 7.5, 3 mM DTT, 5 mM $MgCl_2$, 0.3 U inorganic pyrophosphatase, 1.5 mM ATP, 5 mM 3-PGA, 0.4 mg/ml BSA, 5.5×10^5 cpm/ml [^{14}C]glucose 1-phosphate, 0.5 mM glucose 1-phosphate and enzyme sample in a final volume of 0.2 ml. Reactions were initiated by addition of enzyme and incubated for 10 min at 37°C and then terminated by boiling for 2 min. K_m, K_a and K_i values, corresponding to the concentration giving 50% maximal activity, activation and inhibition, respectively, are the mean of at least two determinations and were calculated using the EnzymeKinetics software program. Protein concentrations were determined by the standard assay method of Bradford [29] with BSA as the standard.

2.3. Expression of TG-15 in E. coli

Modified LB liquid medium for heterologous protein expression contained: 1.0 g/l NaH_2PO_4, 20.0 g/l NZ case plus, 5.0 g/l yeast extract and 6.5 ml 1 N NaOH. *E. coli* strain AC70R1-504 containing the pML10 vector with SS homotetrameric mutant TG-15 insert was grown at 37°C, 250 rpm, to an OD_{600} of 1.0–1.2, then transferred to a room temperature (~25°C) incubator for 30 min before addition of β-mercaptoethanol to a final concentration of 20 mM and induction with IPTG (isopropyl-β-D-thiogalactopyranoside) at a final concentration of 0.2 mM. After 44 h the culture was placed on ice for 30 min and then harvested by centrifugation at $8000\times g$ for 5 min at 4°C.

2.4. Partial purification

All purification steps were carried out at 4°C unless otherwise stated. Assay A was used to follow enzyme activity during the partial purification. Cell paste (approximately 15 g) was resuspended in 30 ml lysis buffer containing 50 mM HEPES pH 7.5, 5% (v/v) glycerol, 1 mM $MgCl_2$, 0.5 mM DTT, 0.1 mM ATP, 5 mM EDTA, 1 mM benzamidine, 2 mM Pefabloc®, 5 μg/ml aprotinin, 1 μg/ml leupeptin and 50 μg/ml lysozyme. The cell suspension was incubated on ice for 30 min, disrupted by sonication immediately after the addition of 1 mM phenylmethylsulfonyl fluoride (PMSF) and then clarified by centrifugation at $22\,500\times g$ at 4°C for 15 min. The clarified crude extract was heated in a water bath at 55°C with continuous agitation for 7 min, then cooled rapidly in an ice water bath. The sample was then centrifuged at $22\,500\times g$ at 4°C for 15 min and the supernatant collected and passed through a 0.2 μm cellulose acetate filter. The crude filtrate pH was adjusted to 8.0 and then loaded onto a PerSeptive Biosystems POROS 20 HQ anion exchange column (bed volume 8 ml) equilibrated with buffer A (50 mM HEPES pH 8.0, 5% (v/v) glycerol, 0.5 mM DTT, 1 mM $MgCl_2$, 5 mM P_i, 5 mM EDTA). The column was washed with five bed volumes of buffer A and eluted with a 0–40% gradient of buffer B (50 mM HEPES pH 7.0, 5% (v/v) glycerol, 0.5 mM DTT, 1 mM $MgCl_2$, 5 mM P_i, 5 mM EDTA, 1.0 M KCl) at 8.0 ml/min over 20 min. Fractions containing AGPase activity were pooled, concentrated to 1 ml in an Amicon concentrator with a PM 30 membrane and loaded onto a Pharmacia 10×30 (mm×cm) Superdex® 200 prep grade gel filtration column equilibrated with buffer C (50 mM HEPES pH 7.5, 5% (v/v) glycerol, 1 mM $MgCl_2$, 5 mM EDTA, 1 mM DTT). The column was eluted with buffer C at 0.25 ml/min over 2 h. Fractions containing AGPase activity were pooled, concentrated to 2 ml and aliquots of 50 μl were frozen in liquid nitrogen and stored at −80°C.

2.5. SDS–PAGE and Western blotting

SDS–PAGE was performed according to the method of Laemmli [30] through a 10% separating gel. Duplicate gels were run at 125 V (continuous voltage) for 1.5 h. One gel was stained for protein with Coomassie blue R-250 and the other gel was blotted onto nitrocellulose membrane at 90 V (constant voltage) for 1 h. After electroblotting, the nitrocellulose membranes were blocked with 5% skim milk/Tris-buffered saline (TBS), rinsed and treated with rabbit anti-potato AGPase SS immune serum. The antibody–antigen complex was visualized using goat anti-rabbit IgG secondary antibody conjugated to horseradish peroxidase and Pierce SuperSignal® chemiluminescent substrate. Chemiluminescence was detected by exposure of photograph film and subsequent development.

2.6. Native molecular mass determination

Molecular mass estimation of TG-15 was done on a column of Pharmacia Superdex® 200 (10×30 cm) using 0.1 ml sample volumes and buffer C as eluent. Fractions (2 ml) were collected and assayed for AGPase activity. The native molecular mass of TG-15 was estimated from a plot of K_{av} (partition coefficient) versus log molecular mass for the standard proteins from a Pharmacia HMW gel filtration calibration kit: thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa) and aldolase (158 kDa). Void volume was determined using a 1 mg/ml solution of blue dextran 2000 (2000 kDa).

3. Results and discussion

3.1. Mutagenesis and screening

A random mutagenesis approach in the structure–function study of the potato AGPase led to the isolation of M345, a LS mutant AGPase defective in allosteric regulation. The isolation of LS AGPase mutant M345 [21], that was defective in

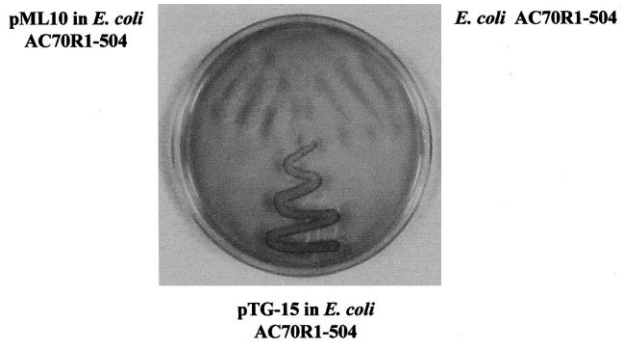

Fig. 1. Iodine vapor staining of *E. coli* AC70R1-504 and AC70R1-504 containing TG-15 (pTG-15) or WT potato SS AGPase (pML10). The plate was streaked from a single colony of each strain onto a Kornberg's 2% glucose enriched plate and incubated overnight at 37°C. Iodine staining was done as described in Section 2 for a duration of 60 s.

allosteric response led to the development of a reverse genetics approach to isolate reversion mutants in the LS that ultimately yielded up-regulated forms of heterotetrameric AGPase [31]. To investigate whether the SS could also accept mutations that might lead to up-regulated heterotetrameric AGPase, a similar strategy utilizing hydroxylamine mutagenesis and iodine vapor staining to isolate suppressor mutations of M345 in the SS was developed [21,32]. Mutagenesis was carried out on pML10 containing the potato AGPase SS gene which was then transformed into *E. coli* AC70R1-504 harboring pML7-M345. The transformants were dilution plated onto Kornberg's 2% glucose enriched medium, grown at 37°C overnight and iodine vapor-stained [26]. The positive clones (SUP) which exhibited staining were cultured and the pML10-based SS plasmid DNA was isolated so the coding region could be transferred into non-mutagenized ML10 vector as described above to eliminate vector-encoded regulatory mutations that might yield a positive phenotype. As a routine control step, the SUP clones were expressed alone and also with pML7-M345 in *E. coli* AC70R1-504. Screening of the SUP clones expressed alone showed that one clone, SUP-1, exhibited weak iodine staining when expressed by itself. The plasmid DNA was isolated and subsequently subjected to a second iteration of hydroxylamine mutagenesis. The transformants were screened on Kornberg's 0.1% glucose-enriched medium for faster and more intense iodine staining mutants. One such clone, TG-15, was isolated, the coding region cloned into non-mutagenized pML10 vector and the iodine staining phenotype verified (Fig. 1).

3.2. Molecular characterization

DNA sequence analysis of SUP-1 and TG-15 revealed two single nucleotide mutations that resulted in two amino acid changes, a C-to-T transition resulting in a leucine to phenylalanine change at position 48 ($L_{48}F$), which occurred in SUP-1 and was retained in TG-15, and a G-to-A transition resulting in a valine to isoleucine change at position 59 ($V_{59}I$) in TG-15 only. Interestingly, the TG-15 $L_{48}F$ analogous position in *E. coli* homotetrameric AGPase is also phenylalanine, but the significance of this amino acid at position 48 for homotetramer formation may be minimal since the analogous amino acids in the homotetrameric AGPase from the cyanobacteria *Anabaena* and *Synechocystis* are valine and leucine,

			+	*	+	*	*	*	*					*	*	*	*	+
E. coli	39	K	R	A	K	P	A	V	H	F	G	G	K	F	R	I	I	D
Anabaena	23	L	R	A	K	P	A	V	P	V	A	G	K	Y	R	L	I	D
Synechocystis	23	L	R	A	K	P	A	V	P	L	A	G	K	Y	R	L	I	D
maize SS	62	K	R	A	K	P	A	V	P	L	G	A	N	Y	R	L	I	D
potato SS	40	K	R	A	K	P	A	V	P	L	G	A	N	Y	R	L	I	D
SUP-1	40	K	R	A	K	P	A	V	P	**F**	G	A	N	Y	R	L	I	D
TG-15	40	K	R	A	K	P	A	V	P	**F**	G	A	N	Y	R	L	I	D
2nd structure		c	c	c	c	c	c	c	c	c	c	c	c	**e**	**e**	**e**	**e**	**e**

Fig. 2. Primary amino acid sequence alignment of an AGPase polypeptide region from *E. coli glg*C (GenBank, S58224), *Anabaena sp.* AnaAGP (GenBank, Z11539), *Synechocystis sp.* SynAGP (GenBank, AAA27275), *Zea mays* bt2 [42], *Solanum tuberosum* SS, (GenBank, X61186) and mutants, SUP-1 and TG-15 from this study. Mutated amino acid residues are labeled in boldface and boxed. Conserved residues are labeled by an (*) and conserved charged residues are labeled by a (+). Individual and consensus secondary structures determined as listed in Section 2. Consensus secondary structure labeled as random coil (**c**, 77%) or extended strand (**e**, 23%).

respectively. Similarly, the analogous amino acids to the TG-15 $V_{59}I$ mutation in *E. coli*, *Anabaena* and *Synechocystis* are leucine, valine and valine, respectively, all of which retain a general hydrophobic character (Fig. 2).

Computer-generated secondary structure predictions for this region (amino acids 40–65) from WT potato SS, SUP-1, TG-15, *E. coli*, *Anabaena* and *Synechocystis* by multivariate linear regression combination (SOPMA-GOR4-SIMPA) [27] show the region to be mostly random coil with a short (6 amino acid) region exhibiting extended strand characteristics (Fig. 2). Physicochemical profiles for this region also reveal no significant alterations in hydrophilicity, hydropathy, flexibility or accessibility (data not shown), although hydrophobicity scales for individual amino acids [33] indicate that $L_{48}F$ and $V_{59}I$ amino acid changes should result in a net increase in hydrophobicity which could increase subunit interaction and stability. This particular region of AGPase has previously been implicated in subunit interaction through analysis of the adg2-1 mutant in *Arabidopsis* [17], and activator binding through the M345 mutant [21] so mutations that alter the physical properties of this region could account for the altered phenotype exhibited by TG-15.

3.3. Expression and purification

Initial attempts to overexpress TG-15 under conditions set forth previously [15] resulted in low (>0.02 U/mg) specific

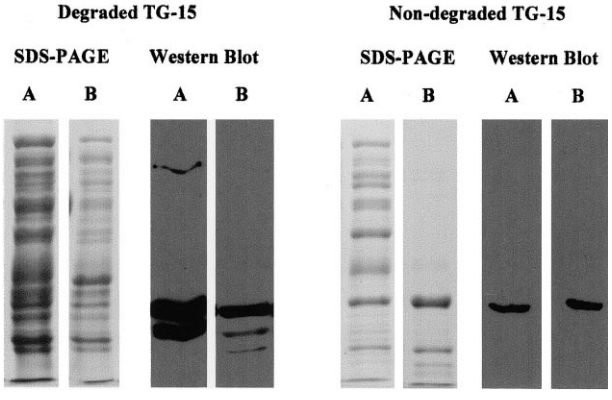

Fig. 3. SDS–PAGE and Western blot analysis of degraded preparation of TG-15: crude lysate A (10 μg), partially purified preparation B (5 μg), and non-degraded preparation of TG-15: crude lysate A (10 μg), partially purified preparation B (5 μg).

Table 1
Kinetic parameters of selected ADP-glucose pyrophosphorylases

Substrate/effector		*E. coli*[a]	rWT[b]	pML10[c]	TL-46[d]	Degraded TG-15[e]	TG-15[e]
ATP	K_m	0.26	0.30	0.20	0.30	0.64	1.20
Glucose 1-phosphate	K_m	0.03	0.26	0.29	0.19	1.57	0.25
Mg^{2+}	K_m	1.40	2.53	2.20	3.65	3.22	1.40
3-PGA	K_a	ND	0.10	2.38	0.87	0.71	0.14
Fructose 1,6-diphosphate	K_a	0.04	ND	ND	ND	ND	ND
Fructose 6-phosphate	K_a	ND	ND	ND	ND	1.43	0.35
Inhib. @ act. concentration[f]	K_i	0.07 @ 0.04 mM	0.65 @ 0.1 mM	0.11 @ 3 mM	0.13 @ 1 mM	0.95 @ 1 mM	0.61 @ 1 mM
Inhib./act. ratio		1.50	6.50	0.04	0.13	0.95	0.61

[a]*E. coli* homotetrameric AGPase from [2].
[b]Recombinant WT heterotetrameric AGPase from Greene et al. (1996).
[c]Recombinant homotetrameric SS AGPase from [15].
[d]Homotetrameric *Arabidopsis* SS AGPase from [18].
[e]Homotetrameric SS AGPase from this study.
[f]K_i of AMP[a] or P_i [bcde] at the given concentration of fructose 1,6-diphosphate[a] or 3-PGA[bcde].

activity in the crude lysate. Despite the positive iodine staining phenotype after growth at 37°C on solid media, *E. coli* AC70R1-504 harboring plasmid pTG-15 grown in liquid medium did not produce significant levels of soluble, active AGPase. Alteration of the concentration of IPTG from 10–200 μM, post-induction incubation for 44 h and addition of β-mercaptoethanol to a final concentration of 20 mM resulted in a specific activity increase of 15 fold to 0.30 U/mg. This is greater than the 0.20 U/mg specific activity reported for expression of recombinant WT SS from pML10 [15] and while still significantly lower than the 1.8 U/mg specific activity reported for the recombinant WT heterotetramer this was high enough to proceed with purification of TG-15.

SDS–PAGE and Western blotting of crude lysate samples revealed that TG-15 was experiencing significant proteolysis (Fig. 3). Whole cell samples showed no degradation, so proteolysis was occurring after cell lysis which indicates that the proteolytic agent is not located in the host cytosol. Alteration of the lysis buffer by increasing the concentration of EDTA from 1 to 5 mM and lowering the $MgCl_2$ concentration to 1 mM abated the degradation in the crude lysate and subsequent purification steps.

A purification scheme that included a heat shock step at 55°C followed by anion exchange and gel filtration chromatography steps yielded a partially purified (20 fold) preparation with a specific activity of 6.0 U/mg which was utilized for subsequent kinetic characterization. Previously purified and characterized SS homotetrameric AGPases from potato [15] and *Arabidopsis* [18] have similar purification profiles. An initial TG-15 preparation was partially purified and although it had considerable degradation products present (Fig. 3), it was characterized kinetically. This partially degraded sample had significant alterations in effector sensitivities as exemplified by decreased sensitivity to the activators 3-PGA and fructose-6-P (Tables 1 and 2). Maize endosperm AGPase, when assayed as a partially degraded sample and a non-degraded sample showed clear differences in kinetic behavior including enhanced enzyme activity in the absence of added activator as well as decreased sensitivity to the effectors 3-PGA and P_i in the degraded sample [34,35]. Deletion of small N- and C-terminal peptides of either AGPase subunit [36] also clearly shows the importance of intact N- and C-termini of AGPase for native function. A 19 amino acid C-terminal deletion in either the LS or SS results in abolishment of enzyme activity through lack of assembly into an oligomeric structure. In addition, truncation of the SS N-terminus by 10 amino acids results in alteration of the holoenzymes substrate and allosteric effector binding affinity as well as heat stability. An LS N-terminal 17 amino acid truncation resulted in a 10 fold increase in 3-PGA sensitivity and substantial reduction of P_i inhibition, while a 28 amino acid N-terminal truncation reversed the effects of the 17 amino acid deletion [36].

SDS–PAGE and Western blot analysis with anti-potato SS immune serum revealed TG-15 to be composed of a single polypeptide chain with a molecular mass of ~50 kDa (Fig. 3). Subsequent investigation of the native state of active TG-15 by gel filtration chromatography indicated a molecular

Table 2
Effect of metabolites on the relative activity of selected ADP-glucose pyrophosphorylases[a]

Metabolite	Maize AGPase[b]	WT AGPase[c]	rWT AGPase[d]	TL-46[e]	Degraded TG-15[f]	TG-15[f]
None	1.0	1.0	1.0	1.0	1.0	1.0
3-PGA	25.3	32.7	36.0	25.0	12.0	24.7
Fructose 1,6-diphosphate	ND	3.3	3.3	3.9	0.5	5.7
Fructose 6-phosphate	17.0	ND	1.5	3.0	7.2	16.2
Glucose 1,6-diphosphate	ND	ND	1.3	2.8	0.4	1.6
Glucose 6-phosphate	14.0	ND	ND	3.0	2.0	3.3
PEP	4.0	2.6	4.0	9.8	3.3	8.7
2-PGA	ND	ND	1.7	2.9	1.6	1.0

[a]Data generated with the synthesis (forward) assay B.
[b]Non-proteolysed heterotetrameric AGPase from [35].
[c]WT AGPase purified from potato tissue from [6].
[d]Recombinant truncated heterotetrameric AGPase from [25].
[e]Homotetrameric *Arabidopsis* SS AGPase from [18].
[f]Homotetrameric SS AGPase from this study.

mass of ~200 kDa suggesting that TG-15 is active as a homotetramer.

3.4. Enzyme kinetic analysis – metabolite activation

Assay B was used to determine kinetic parameters for TG-15 with all reported values being the mean of at least two separate assays. Michaelis–Menten constants were determined from double reciprocal plots with R values greater than 0.95. Table 1 contains the various kinetic parameters determined for substrates and effectors of AGPases from this study as well as selected others as listed while Table 2 lists the metabolite activation profiles of selected AGPases. Comparison of the data for the TG-15 degraded and non-degraded samples from this study clearly show the importance of verifying the integrity of the enzyme in reporting accurate kinetic data. There is a fivefold difference in the K_a for the activator 3-PGA and there are sixfold differences in the K_m for the substrate glucose-1-phosphate and the K_a for fructose-6-phosphate. Metabolite activation in the degraded sample is approximately half of the value generated for the non-degraded sample for almost all of the metabolites tested, indicating that proteolysis reduces the sensitivity of AGPase to allosteric effectors and allows higher activity levels in the absence of activator molecules.

Comparison of TG-15 kinetic parameters with other reported homotetrameric higher plant AGPase data brings forth two significant differences. First, TG-15 has significantly higher affinity for 3-PGA than either TL-46 (sixfold) [18] or pML10 (20 fold) [15]. The TG-15 3-PGA K_a of 0.14 mM is almost equal in affinity to that of rWT heterotetrameric AGPase for 3-PGA. The second significant difference is the decreased affinity TG-15 has for the substrate ATP. TG-15 has a K_m of 1.20 mM for ATP, TL-46 and pML10 have four- and sixfold, respectively, greater affinity for this substrate. Kinetic comparison of TG-15 to the *E. coli* homotetrameric AGPase shows that despite the $L_{48}F$ mutation in TG-15 that mimics the *E. coli* sequence, the prokaryotic enzyme has greater affinity for all substrates and effectors with the exception of Mg^{2+}. Metabolite activation profiles for higher plant AGPase show that 3-PGA is the most highly activating effector followed by fructose-6-phosphate. Other than the maize endosperm AGPase no other AGPase characterized to date is capable of recognizing this sugar-phosphate as an effector. This must be mediated through enzyme topology determined by a separate amino acid region, perhaps in the LS sequence, since the potato SS has an identical sequence in this region with maize, yet is not sensitive to fructose-6-phosphate activation. The metabolic significance of the maize AGPase sensitivity is undefined at this time, and may be completely insignificant. Yet with storage tissue AGPase subcellular location is an important issue [37–41] and the seeming lack of physiological significance of 3-PGA sensitivity in storage tissues, sensitivity to effectors other than 3-PGA may bear investigation in the future. The enhanced affinity of TG-15 for fructose-6-phosphate as an activator implies that there is a degree of plasticity at the activator binding site of AGPase that the newly created subunit interface of this homotetrameric mutant has been able to exhibit. Evidence that slight alterations in amino acid sequence can mediate differential sensitivity to allosteric effectors may allow for directed change of sensitivity to specific effector molecules that could aid metabolic engineering efforts in future endeavors.

Current theory on the structure–function relationship of the LS and SS of AGPase holds that the SS is solely responsible for catalysis as indicated by a recent site-directed mutagenesis study [24]. An initial hypothesis on the role of the LS was that it modulated the sensitivity of the heterotetramer to allosteric effectors [15]. While this role has been substantiated through mutagenic studies, the SS has also been shown to play a concurrent role in allosterism of the enzyme [21,22,24]. The mutations present in TG-15, $L_{48}F$ and $V_{59}I$, are both situated in a region of the polypeptide that has previously been identified as influencing both allosteric behavior and subunit association in heterotetrameric AGPase. The increased hydrophobic nature at the mutation sites seems to indicate that this would lead to greater subunit association characteristics due to entropic considerations, and that may well be the reason for this mutant phenotype, since there has been no previous report of hydrophobic residues playing a role in binding of effectors. Phenotypically there are obvious characteristics that this mutant displays that indicate it may have utility as a higher plant single gene product that is able to provide physiological properties heretofore only associated with heterotetrameric AGPase.

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