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A $(His)_6$-tagged recombinant barley (*Hordeum vulgare* L.) endosperm ADP-glucose pyrophosphorylase expressed in the baculovirus-insect cell system is insensitive to allosteric regulation by 3-phosphoglycerate and inorganic phosphate

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Abstract ADP-glucose pyrophosphorylase from photosynthetic tissue is allosterically regulated by 3-phosphoglycerate and inorganic phosphate. In contrast, data from our laboratory indicated that the major AGPase from barley seeds is insensitive to these regulators. Verification of this conclusion has, however, been hindered by the proteolytic degradation of the enzyme from seeds. This report characterizes the barley seed AGPase expressed in the baculovirus-insect cell system, confirming that lack of allosteric regulation by 3-PGA/P_i is an intrinsic property of the enzyme. Purification of the enzyme was by Ni^{2+}-NTA agarose chromatography using a $(His)_6$ tag attached to the N-terminus of the small AGPase subunit.

Key words: ADP-glucose pyrophosphorylase; Expression; Baculovirus; Insect cell; Purification

1. Introduction

ADP-glucose pyrophosphorylase (ATP:α-glucose 1-phosphate adenylyltransferase, EC 2.7.7.27) catalyzes the conversion of Glc-1-P and ATP to ADP-glucose and PP_i [1]. ADP-glucose serves as a glucosyl precursor in the synthesis of glycogen in bacteria and starch synthesis in cyanobacteria, green algae and higher plants [1]. With the exception of enteric bacteria in which the enzyme is a homotetramer, all other ADP-glucose pyrophosphorylases (referred to as AGPases) are heterotetramers consisting of two subunits, the small subunit, SS (50–55 kDa), and the large subunit, LS (51–60 kDa) [1–6]. Over the past years, published cDNA sequences have demonstrated that the SS is highly conserved between different isoforms from the same plant, as well as among plant species. In contrast, the sequences of the LS are much more heterogeneous [7]. All AGPases from photosynthetic tissues are allosterically activated by 3-phosphoglycerate (3-PGA), and inhibited by inorganic phosphate (P_i), with the ratio of 3-PGA/P_i playing an important role in the regulation of starch biosynthesis [1,8,9].

Putative binding sites for 3-PGA have been mapped to the lysine residue 440 on the SS, and to lysine residues 134, 464 and 426 on the LS of the spinach leaf AGPase [10,11]. Furthermore, it has been generally accepted that AGPases were located exclusively in plastids (i.e. chloroplasts in photosynthetic tissues and amyloplasts in storage tissues), making ADP-glucose directly available for starch synthesis within these compartments [12–14]. Recently, however, AGPases from non-photosynthetic tissues such as barley endosperm ([15], Doan et al., in preparation), pea embryo [16] and wheat endosperm [17] have been reported to display low sensitivity to 3-PGA regulation. Further evidence supporting the possibility that at least some storage organ AGPases may be fundamentally different from those in leaves [18–20] comes from the finding that the major barley endosperm [21] and maize endosperm [22] AGPases are located in the cytosol.

Recent interest in the ADP-glucose pyrophosphorylase has been evoked by the demonstration of an increase in potato tuber starch in transgenic plants carrying the *Escherichia coli* gene *glgC16* encoding a mutant AGPase, which is less sensitive to allosteric regulation by 3-PGA and P_i [23]. Motivated by the instability of the cytosolic AGPase in extracts from barley endosperm [15] we expressed the two cDNAs encoding the cytosolic AGPase isoform in the baculovirus-insect cell system. Because of the instability of the protein and difficulties in purifying an active enzyme complex, we used the histidine tag system for rapid purification. Here we report the expression and purification of a recombinant AGPase, which contains a 6×histidine tag in the N-terminus. This partially purified $(His)_6$-tagged heterotetrameric AGPase displays the same insensitivity to the effectors 3-PGA and P_i as the partially purified enzyme from immature endosperms [15]. These data strongly suggest that insensitivity to 3-PGA/P_i regulation is an intrinsic property of the barley endosperm AGPase.

2. Materials and methods

2.1. Reagents

Spodoptera frugiperda (Sf9) cell culture was purchased from Invitrogen (R&D Systems Europe Ltd., UK). BaculoGold transfection kit, pAcSG-His-NT and pAcUW51 plasmids were purchased from PharMingen (AMS Biotechnology Ltd., Sweden). TNM-FH medium (powder) was from Sigma (Sigma Chemical Co., Poole, UK), fetal calf serum and antibiotics were from Gibco (Gibco BRL, Life Technologies Ltd, Renfrewshire, UK). All DNA restriction enzymes were from New England Biolabs (New England Biolabs, Ltd., Hertfordshire, UK) and Promega (Promega, Madison, WI, USA). Other chemicals, reagents and coupling enzymes, unless otherwise stated, were obtained from Sigma.

2.2. Construction of recombinant viruses

Manipulations in molecular biology were mainly performed according to Sambrook et al. [24]. The cDNA encoding the LS (bepL10) [25]

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was PCR amplified (forward primer, GGATCCATATGCAGTTCAGCA and reverse primer in the pBluescript II SK vector, T3, ATTAACCCTCACTAAAG) and cut with *Bam*HI and *Kpn*I. After making blunt ends the PCR product was subcloned into the transfer plasmid pAcUW51 in the *Eco*RI site (also made blunt ended). Full-length cDNA encoding the SS (bepSF2) [26] was amplified by PCR (forward primer, CCAGGACTCGAGGATGTACCT and reverse primer in the pBluescript II SK vector, T3, ATTAACCCTCACTAAAG) and inserted into the pAcSG His-NTA plasmid in the *Xho*I site such that the 5′ end was fused in frame to the sequence encoding 29 amino acids including a stretch of 6×histidine residues (Fig. 1). The integrity of the recombinant sequence was checked by sequencing. Subsequently, the sequence was excised from the recombinant plasmid by cutting with *Bam*HI and *Kpn*I, and after making blunt ends, subcloned into pUW-bepl in the *Bam*HI site (also made blunt ended), placing the SS cDNA under the control of the polyhedrin promoter and the cDNA for the LS under the control of the p10 promoter. The resulting plasmid is designated pUW-His-BEP. Recombinant baculovirus was produced by homologous recombination in vivo during cotransfection of Sf9 monolayers with a modified *Autographa californica* nuclear polyhedrosis virus DNA [27] and transfer vector pUW-His-BEP. Recombinant viruses were collected, serially diluted, and purified by one round of viral amplification and two rounds of plaque assays [28].

2.3. Expression of recombinant proteins in Sf9 cell culture

Spodoptera frugiperda Sf9 cells (Invitrogen R&D Systems Europe Ltd) were grown as monolayer cultures seeded in a 100-mm petri dish (Corning Costar, USA) at 27°C in TNM-FH complete insect medium (Sigma Chemical Co.) supplemented with 10% (v/v) fetal calf serum, 5 μg/ml fungizone, 10 μg/ml penicillin, and 10 μg/ml streptomycin (GibCo BRL Life Technologies Ltd). Sf9 cells were infected (3.0×10^6 cells/10 ml) with the purified recombinant virus at a multiplicity of infection (MOI) of at least 10 [27] and harvested 2.5 days after infection ($200\times g$, 5 min), washed with TNM-FH medium (without fetal calf serum) and resuspended in 0.8 ml/dish of buffer A (20 mM HEPES pH 7.5 containing 5 mM $MgCl_2$, 10% sucrose, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml pepstatin and 1 mM imidazole). The cell suspension underwent three cycles of freezing and thawing, and was subsequently vortexed for 30 s and centrifuged ($15000\times g$, 10 min). Insoluble material was washed with 0.2 ml of buffer A. Soluble fractions were combined and stored at −80°C.

2.4. Affinity purification of the His-tagged fusion protein

Ten 100-mm petri dishes with Sf9 cells (3×10^7 cells per dish) were infected with the recombinant baculovirus pUW-His-BEP, and harvested 2.5 days after infection. The cell lysate was resuspended in extraction buffer (buffer A) and crude extract was prepared as described above. All purification steps were carried out at 4°C unless otherwise indicated. Ammonium sulfate (powder) was added to the crude extract to a concentration of 30% and the precipitant was collected by centrifugation ($15000\times g$, 15 min). A small volume of extraction buffer was added and insoluble material was further removed by centrifugation. The supernatant was diluted to 10 ml with buffer A and run on an Econo-Pac high-Q column (5 ml cartridge, Bio-Rad) and was eluted with a linear gradient of 0–1.0 M NaCl (total volume 60 ml). Active fractions were pooled, equilibrated to 0.5 M NaCl and 5 mM imidazole and incubated with an aliquot of Ni^{2+}-NTA agarose (approximately 0.5 ml/2×10^8 cells; Qiagen) for 1 h with continuous agitation. After centrifugation ($200\times g$, 7 min, in a 'swing-out' rotor) the agarose pellet was washed five times with 5 volumes 5 mM imidazole in buffer A and five times with 5 volumes 20 mM imidazole in buffer A. In the last wash with 20 mM imidazole, the Ni^{2+}-NTA agarose was packed in a column and the AGPase was eluted with 5 volumes 250 mM imidazole in buffer A. Fractions with purified AGPase were combined and imidazole was removed by dialyzing against buffer B (20 mM HEPES pH 7.5 containing 5 mM $MgCl_2$, 20% sucrose, 2 mM DTT) for 12 h at 4°C. The purified AGPase was stored in small aliquots and stored at −80°C and/or checked directly for enzyme activity.

2.5. Enzyme assay

Activity of AGPase was assayed in the pyrophosphorolysis direction [15] by spectrophotometrically monitoring the formation of NADH at 340 nm and 25°C. All samples were assayed in triplicate.

A standard assay mixture (1 ml) containing 100 mM MOPS (pH 7.4), 0.1 mg/ml BSA, 1 mM ADP-Glc, 7 mM $MgCl_2$, 0.6 mM NAD, 1 mM sodium pyrophosphate, and 2 units each of glucose 6-phosphate dehydrogenase and phosphoglucomutase. The reaction was initiated by addition of ADP-glucose and allowed to proceed for 10 min. One unit of activity is defined as the amount of enzyme required to reduce 1 μmol of NAD per min at 25°C.

2.6. Protein determination

Protein concentration was measured using the Micro BCA Protein Assay kit (Pierce, Illinois, USA) with bovine serum albumin as standard.

2.7. Immunoblot analysis

Purified AGPase was separated in 10% SDS-polyacrylamide gels according to Laemmli [29] in a Hoefer SE 600 vertical apparatus (18×16 cm), and proteins were transferred to Hybond-P membrane (Amersham, UK). The membrane was probed with antibodies raised either against synthetic peptide of the LS [15] or against the *E. coli* expressed SS [26]. The apparent molecular weight on SDS gels was determined using the Rainbow molecular marker (Amersham, UK). Immunodetection was performed using the anti-rabbit secondary antibody conjugated with horseradish peroxidase (diluted 1:6000) and the ECL detection system (Amersham). After each immunoblotting, the membrane was stripped for reprobing by incubating at 50°C for 30 min in 62.5 mM Tris-HCl (pH 6.7) containing 2% (w/v) SDS and 100 mM 2-mercaptoethanol.

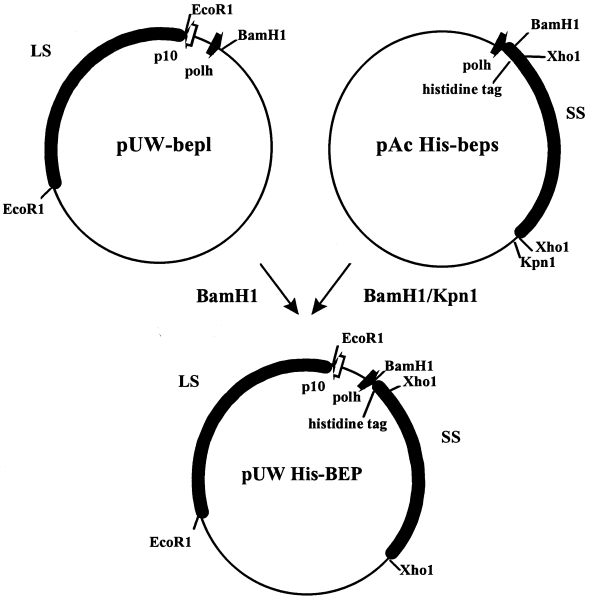

Fig. 1. Construction of the pUW-His-BEP baculovirus expression vector. The cDNA encoding the SS with truncated 5′ end (bepSF2′) was PCR amplified (for primers, see Section 2) and inserted into the *Xho*I site of the pAcSG His-NTA plasmid to give plasmid pAC His-beps. Subsequently, beps containing the $(His)_6$ tag at the 5′ end was excised from pAC His-beps with *Bam*HI and *Kpn*I, blunt ended and subcloned into pUW-bepl in the blunt ended *Bam*HI site. The pUW-bepl construct was made after PCR amplification (primers, see Section 2) of the cDNA encoding the large truncated 5′ end (bepL10′) subunit. After cutting with *Bam*HI and *Kpn*I and blunt ending, the fragment was subcloned into the baculovirus transfer plasmid pAcUW51 in the *Eco*RI site. The resulting baculovirus expression vector with the cDNA encoding the SS under the control of the polyhedrin promoter and the cDNA encoding the LS under the control of the p10 promoter is designated pUW-His-BEP.

3. Results

3.1. Construction of the baculovirus transfer vector for expression of the cytosolic barley endosperm AGPase in insect cells

The recombinant baculovirus pUW-His-BEP, containing the small and the large barley cytosolic endosperm AGPase subunit cDNAs bepSF2 [26] and bepL10 [25], respectively, was constructed as outlined in Fig. 1 and in Section 2. In this construct, the cDNA for the SS is under the control of the polyhedrin promoter and the LS is under the control of the p10 promoter. The two transcriptional entities are inserted in opposite directions to prevent spurious readthrough. In order to increase the translational efficacy of the barley AGPase in the insect cells [28], 57 nucleotides of the untranslated leader were removed from the small (bepSF2) subunit cDNA. To the 5′ end of this sequence, we subsequently fused the sequence encoding an amino-terminal $(His)_6$ tag as outlined in Fig. 2. To ensure initiation of translation at the methionine immediately in front of the $(His)_6$ tag sequence, the initiator methionine codon of the SS AGPase was deleted (Fig. 2). In addition to the $(His)_6$ tag, the SS fusion protein also contains a site for thrombin cleavage, making proteolytic removal of the $(His)_6$ tag possible (Fig. 2). For the bepL10 cDNA encoding the large cytosolic AGPase subunit [25], 190 nt of the 5′ leader sequence were removed before insertion in the pUW-bepl plasmid (Fig. 1). This cDNA is expected to encode the complete LS of the cytosolic barley AGPase (Fig. 2). Thus, except for the addition of the extra 29 amino acid residues carrying the $(His)_6$ tag, and the accompanying deletion of the initial methionine of the SS, the pUW-His-BEP vector encodes the complete small and large cytosolic AGPase from barley endosperm.

A.

SS AGPase:

6 Histidine Tag

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ATG GGA CAT CAT CAT CAT CAT CAC GGA AGG AGA AGG GCC
>M   G   H   H   H   H   H   H   G   R   R   R   A
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Thrombine Cleavage Site

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AGT GTT GCG GCG GGA ATT TTG GTC CCT CGT GGA AGC CCA
 S   V   A   A   G   I   L   V   P   R   G   S   P

GGA CTC GAG GAT GTA CCT TTG GCA....
 G   L   G   D   V   P   L   A
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B.

LS AGPase:

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ATG TCA TCG ATG CAG TTC AGC AGC GTG CTG CCC CTG....
>M   S   S   M   Q   F   S   S   V   L   P   L
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Fig. 2. Nucleotide sequence and deduced amino acid sequence of the 5′ ends of the cytosolic small (A) and large (B) AGPase subunits of the plasmid pUW-His-BEP. A: Nucleotide sequence and deduced amino acid sequence of the 5′ end of the cDNA (57 nt removed from the 5′ untranslated region) encoding the small subunit (SS) in frame to the sequence encoding 29 amino acids, including a stretch of 6 histidine residues and a thrombine cleavage site. B: Nucleotide sequence and deduced amino acid sequence of the 5′ end of the cDNA (190 nt removed from the 5′ untranslated region) encoding the large subunit (LS).

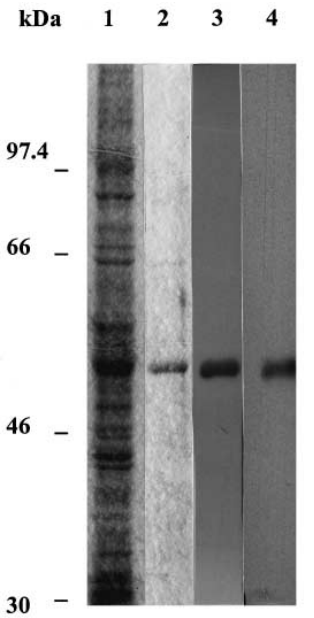

Fig. 3. SDS-PAGE and immunoblot analysis of expressed AGPase purified from insect cells. Crude extract and purified AGPase were denatured before separation on 10% SDS-PAGE and visualization with Coomassie brilliant blue R. Lane 1, crude extract (10 μg); lane 2, partially purified AGPase eluated from Ni^{2+}-NTA column (5 μg). Immunoblot of expressed and purified AGPase probed with antibodies against the small and the large subunit. Lane 3, antibody against the SS (5 μg); lane 4, antibody against the LS (5 μg). Molecular weights are in kDa.

3.2. Expression of the barley AGPase in Spodoptera frugiperda cells

Recombinant viruses containing the AGPase cDNAs were obtained by cotransfecting *Spodoptera frugiperda* (Sf9) cells with linearized BaculoGold virus DNA and the pUV-His-BEP vector, and subsequent identification by plaque assays (described in Section 2). Initial infection experiments of Sf9 cells using this virus stock were carried out in order to assess the level of barley endosperm AGPase expression. Immunoblot analysis with antibodies against the small [26] and the large LS [15] endosperm AGPase subunits on cell lysate from infected Sf9 cells confirmed the expression of both AGPase subunits (Fig. 3). In this analysis, estimates of the size of the two subunit proteins are in accordance with the molecular weight predicted by the ORFs of the cDNAs, 57 kDa for the SS containing the $(His)_6$ tag peptide and 58 kDa for the untagged LS protein (Fig. 3). Due to the addition of the $(His)_6$ tag in the N-terminus of the SS, we expect the protein to have a molecular weight at least 2–3 kDa higher than predicted by the ORF of the SS AGPase cDNA alone. Therefore, the resulting SS with the extra $(His)_6$ tag is expected to have a molecular weight of approximately 54 kDa. The observable discrepancy of an additional 3 kDa between the expected and observed molecular weight of the SS may be explained by retardation of the $(His)_6$ tag by several kDa in the SDS-PAGE, reported by many authors [30]. For both protein subunits, loading of 5 μg on the gel resulted in single bands, demonstrating a much higher stability of the AGPase subunits in extracts from insect cells than in barley endosperm extracts [15,31]. Doubling the amount of protein loaded, however, produced an additional faint band for the LS. No background bands were recognizable by the antibodies in lysate from in-

Table 1
Affinity purification of the $(His)_6$-tagged cytosolic barley endosperm AGPase from insect cells (from 100 ml culture)

Step	Protein (mg)	Activity (units[a])	Specific activity (units/mg)	Yield (%)	Purification (-fold)
Crude extract	61	82	1.30	100	1.0
$(NH_4)_2SO_4$	11.34	65.60	5.78	80	4.4
High Q	6.48	39.40	6.08	47	4.6
Ni^{2+}-NTA	0.70	20	28.60	24	22

Activity was measured in the pyrophosphorolysis direction (assay A).
[a]One unit of activity is defined as the amount of enzyme required to reduce 1 μmol of NAD per min at 25°C. Enzyme activities were assayed in triplicate. Protein concentrations were determined with the Micro BCA Protein Assay kit (Pierce).

sect cells infected by non-recombinant baculovirus particles, nor from uninfected insect cells (data not shown). Assay for AGPase activity carried out using the same extract as in the Western analysis demonstrated that the enzyme was active in the absence of 3-PGA. Furthermore, the same experiments showed that the recombinant AGPase was not stimulated by adding 3-PGA, and not inhibited by inorganic phosphate (Table 2).

From these studies we conclude that the barley endosperm AGPase subunits were expressed in the insect cells after infection with the purified recombinant virus, and that the active heterotetrameric form of the enzyme assembles either spontaneously within the insect cells, or in the lysate.

3.3. *Purification of the recombinant AGPase fusion protein is facilitated by the histidine tag of the small subunit*

In order to facilitate fast purification and to avoid protein degradation of the recombinant AGPase from insect cell lysate, crude lysate from infected insect cells was precipitated by ammonium sulfate, eluted from an Econo-Pac high-Q column and subjected to affinity chromatography with nickel chelating resins. Based on the observation that the active AGPase was assembled in crude extract from insect cells, we expected that the amino-terminal $(His)_6$ tag on the SS AGPase would retain the heterotetrameric enzyme in affinity chromatography. As demonstrated below this expectation was confirmed. Enzyme assays were carried out in the pyrophosphorolysis direction on each fraction. A typical example of an SDS gel showing the pooled fractions displaying AGPase activity is shown in Fig. 3 (lane 2), demonstrating the presence of a strong protein band with an estimated molecular mass of around 58 kDa. As explained above, this molecular weight corresponds to the mass of both the small AGPase fusion protein (57 kDa) and the LS (58 kDa). The interpretation of the protein band seen in the Coomassie-stained gel of Fig. 3, lane 2, is confirmed by the Western blot of the same gel, detecting single bands of similar molecular masses using antibodies directed against the small and the large barley endosperm AGPase subunits (Fig. 3, lanes 3 and 4, respectively). As inferred from the single bands on the Western blot, very little protein degradation occurred during the affinity purification procedure.

From these experiments, we conclude that rapid purification of the recombinant barley cytosolic AGPase can be achieved using the $(His)_6$ tag only on the small AGPase subunit. To our knowledge, this is the first report of purification of a multimeric plant enzyme after the attachment of a histidine tag on only one of the subunits of a multimeric enzyme.

3.4. *Kinetic properties of the affinity-purified cytosolic His-tagged barley endosperm AGPase*

Using 3×10^8 cells grown in a volume of 100 ml medium, we typically obtained 82 units of recombinant $(His)_6$-tagged AGPase activity in the crude extract as measured in the pyrophosphorolysis direction (Table 1). After affinity chromatography, the $(His)_6$-tagged AGPase was purified approximately 22-fold to a specific activity of around 29 units/mg, with a recovery of 24% of the enzyme activity in the crude extract.

As reported before, the leaf AGPase requires the presence of 1 mM 3-PGA to display measurable activity and is activated up to 13-fold in the presence of 3-PGA and inhibited by low concentrations of P_i (1.5 mM) [31]. In contrast, the AGPase in crude extract from barley endosperm does not require presence of 3-PGA to be active, and was observed to be relatively insensitive to 3-PGA activation and P_i inhibition [15]. Using the purified $(His)_6$-tagged cytosolic AGPase described here, the effect of these two regulators on the activity of the enzyme was measured in the pyrophosphorolysis direction. As described above, the partially purified AGPase from the present experiments is characterized by high activity in the absence of 3-PGA. Furthermore, neither 3-PGA or P_i alone nor the two in combination had significant effects on the enzyme activity (Table 2). Kinetic measurements for the substrates ADP-glucose and PP_i resulted in K_m values of 0.13 and 0.043, respectively (Table 3). These values are similar to the analogous partially purified barley endosperm AGPase values of 0.13 and 0.027. On the other hand, the K_m values for the barley leaf AGPase are much higher (0.71 and 0.059) [31], but

Table 2
Effects of 3-PGA and P_i on the activity of expressed, affinity-purified AGPase

Effector	Pyrophosphorolysis[a] (%)
None	100
3-PGA (10 mM)	103 ± 2
P_i (20 mM)	114 ± 1
3-PGA (10 mM)+P_i (20 mM)	117 ± 5

[a]Determined at 25°C.

Table 3
Kinetic constants of expressed affinity-purified AGPase

Substrate	Kinetic constant K_m (mM)	
	Native[a] AGPase	Expressed $(His)_6$-AGPase
ADP-Glc	0.13	0.13 ± 0.01
PP_i	0.027	0.043 ± 0.01

[a][13].

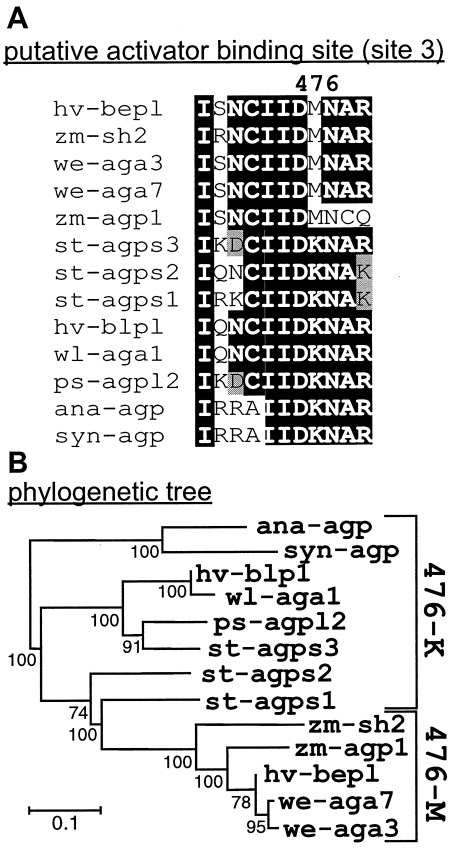

Fig. 4. Comparison of the deduced amino acid sequences (A) and phylogenetic analysis (B) of higher plant and cyanobacterial LS AGPases. The analyzed sequences are from barley endosperm [25] and barley leaf [37], wheat endosperm and wheat leaf [38], maize endosperm [39] and maize embryo [40], pea leaf [41], potato tuber [42] and potato leaf [43], *Anabaena* [44] and *Synechocystis* (Kakefuda, from the EMBL data bank, accession number M83556). A: Alignment of the amino acid sequence of the 3-PGA binding site of the AGPase LSs from cyanobacteria, cereals and other plant species. Amino acid residue 476 corresponds to the methionine in barley endosperm LS AGPase (site 3). B: The phylogenetic distance tree was constructed by the neighbor-joining method [45], using the Dayhoff PAM matrix [46] for distance estimation and bootstrap analysis [47] for testing statistical significance of the branching pattern (from the software package PHYLIP, version 3.5; J. Felsenstein, Department of Genetics, University of Washington). Numbers at the nodes indicate the percentage of 500 bootstrap trees in which the cluster descending from the node was found. Relative sequence distances are indicated according to the Dayhoff PAM matrix. Isoforms of the AGPase LSs: barley endosperm (hv-bepl), barley leaf (hv-blpl), wheat endosperm (ta-we-aga7/aga3), wheat leaf (ta-we-aga1), maize endosperm (zm-sh2), maize embryo (zm-agp1), pea leaf (ps-agpl2), potato tuber (st-agpS3), potato tuber (st-agpsl) and potato leaf (st-agps2), *Anabaena* (ana-agp), *Synechocystis* (syn-agp).

decreased on adding the activator 3-PGA (0.07 and 0.025) [31]. The K_m values for the activated leaf AGPase are then comparable with the endosperm AGPase, assayed in the absence of 3-PGA. Based on these data, we conclude that the regulation of the recombinant AGPase studied here is fundamentally different from the plastidial AGPase from leaves.

4. Discussion

In this paper we describe the expression of a $(His)_6$-tagged recombinant cytosolic isoform of the barley endosperm AGPase in the baculovirus-insect cell system. Using this heterologous system as a source for the enzyme has several advantages over the study of the enzyme from barley endosperm. First, the insect cell system circumvents the problem of the presence of two AGPase isoforms in the barley endosperm. According to Thorbjørnsen et al. [21], the cytosolic form represents 85% and the plastidial form 15% of the total AGPase activity. Assuming, therefore, that the plastidial isoform is activated by 3-PGA, even if AGPase could successfully be isolated from barley endosperm, an accurate measurement of the kinetic properties of the cytosolic isoform would not be possible unless the two isoforms could be separated. Second, we achieved purification of undegraded active enzyme using the histidine tag method. As reported previously, the native enzyme could only be partially purified from barley endosperm, due to rapid proteolytic degradation [15]. This proteolysis, in particular of the large subunit, occurred within minutes even in the presence of protease inhibitors. As shown in Fig. 3, the problem of proteolysis can be circumvented by the use of the baculovirus-insect cell system. Third, the heterologous expression system permits the addition of a $(His)_6$ tag, facilitating purification of the active heterotetrameric enzyme complex using affinity chromatography.

In the analysis of the enzyme kinetics, we used the $(His)_6$-tagged form of the enzyme, although the $(His)_6$ tag could potentially be cleaved off at the thrombin cleavage site (Fig. 2). The reason for not cleaving off the $(His)_6$ tag sequence is the instability of the AGPase, incubation of the enzyme with thrombin under the conditions required (20°C for 2 h) leading to an almost complete degradation of the enzyme (data not shown). Based on the high number of $(His)_6$-tagged protein studies reported in the literature, however, it is unlikely that the $(His)_6$ tag and its surrounding sequence influence the regulatory properties of the AGPase. First, the histidine tag is non-immunogenic and uncharged at physiological pH. In over 150 proteins purified using this system, including enzymes [32], transcription factors [33] and antigens [34,35], the $(His)_6$ tag has never been found to interfere with the structure or function of the purified proteins. This conclusion is also supported by the present data, showing that the $(His)_6$ tag does not interfere with enzyme multimerization. As shown in Table 3, the expressed $(His)_6$-AGPase was highly active in the absence of the activator 3-PGA and its activity was not inhibited by P_i. In our interpretation, therefore, the $(His)_6$-AGPase has the same kinetic and allosteric properties as the enzyme purified from barley endosperm [15] (Table 3), as well as its insensitivity to 3-PGA and P_i (Table 2). We therefore conclude that the insensitivity of the barley endosperm AGPase is an intrinsic property of the enzyme, and not an artefact caused by proteolytic degradation of the enzyme [9].

Whether or not the insensitivity to 3-PGA/P_i regulation is functionally related to the location in the cytosol remains an open question. As proposed by Villand and Kleczkowski [19], Kleczkowski [20], Thorbjørnsen et al. [21] and Denyer et al. [22], one possibility is that AGPase in the cytosol serves to couple sucrose breakdown with ADP-glucose formation. The ADP-glucose produced in the cytosol can be exported to the amyloplast via the proposed ADP-glucose/adenylate translocator [36]. Studies of the subcellular location of these enzymes will shed light on the question of whether or not insensitivity to 3-PGA/P_i regulation is a common feature of cytosolic AGPases.

The finding that the regulatory properties of the cytosolic AGPase isoform from barley endosperm is different from that of the leaves identifies the barley system as an interesting model system to study the structural basis for AGPase allosteric regulation. In barley, the SS of the two AGPase isoforms are encoded by a single gene, giving proteins that are identical in over 90% of their length [26]. The only difference is in the amino-terminal part of the two proteins, consisting of 40 amino acid residues for the cytosolic form and 81 for the plastidial form. In the plastidial form, a transit peptide consisting of the first 24 amino acid residues is identifiable, which most likely is involved in amyloplast targeting. It is interesting to note that the site corresponding to putative 3-PGA regulatory binding site 1 of the spinach small subunit [10,11] is located in the identical region of the cytosolic and the plastidial barley small AGPase subunits. Based on the identity between the two small AGPase subunits from barley in this region of the peptide, however, site 1 in barley does not appear to be involved in the specification of the difference in 3-PGA sensitivity between the two barley AGPases.

In their studies, Morell et al. [10] and Ball and Preiss [11], identified three putative 3-PGA binding sites on the LS from spinach leaves. These sites correspond to Lys residues 513 (site 2), 185 (site 4) and 476 (site 3) in the barley cytosolic AGPase. Recently the cDNA clone encoding the LS from barley leaf was isolated (*blpl*14) and the deduced amino acid sequence showed 97% identity to the corresponding protein from wheat, but only 63% identity at the nucleotide level with the barley endosperm LS clone *bepl*10 [37]. Comparing the amino acid sequences of the AGPase subunits from algae, cyanobacteria and higher plants for these sites, a high degree of conservation is observed, supporting the possibility that these sites are important for AGPase function and regulation [11]. Interestingly, some cereal endosperm enzymes are reported to display decreased sensitivity or insensitivity to 3-PGA regulation, i.e. barley, pea and wheat. Alignment of the amino acid sequence around site 3 replaces the highly conserved lysine of the regulated AGPases with methionine (Met^{476} for the barley endosperm AGPase) (Fig. 4A). The phylogenetic distance tree of the large subunits generated from these data shows that the lysine residue may have changed to methionine at a relatively late stage in evolution (Fig. 4B). The significance of this substitution for the lack of 3-PGA regulation of the cytosolic AGPase is currently being investigated using site-directed mutagenesis on the recombinant enzyme. This, together with studies of barley cytosolic-plastidial hybrid AGPases, as well as domain swapping experiments between the subunits from the different subcellular compartments, will hopefully contribute to the clarification of the mechanism(s) causing the difference between the cytosolic and plastidial barley AGPases.

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