Functional characterization of α-glucan,water dikinase, the starch phosphorylating enzyme

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GWD (α-glucan,water dikinase) is the enzyme that catalyses the phosphorylation of starch by a dikinase-type reaction in which the β-phosphate of ATP is transferred to either the C-6 or the C-3 position of the glycosyl residue of amylopectin. GWD shows similarity in both sequence and reaction mechanism to bacterial PPS (pyruvate,water dikinase) and PPDK (pyruvate,phosphate dikinase). Amino acid sequence alignments identified a conserved histidine residue located in the putative phosphohistidine domain of potato GWD. Site-directed mutagenesis of this histidine residue resulted in an inactive enzyme and loss of autophosphorylation. Native GWD is a homodimer and shows a strict requirement for the presence of α-1,6 branch points in its polyglucan substrate, and exhibits a sharp 20-fold increase in activity when the degree of polymerization is increased from 27.8 to 29.5. In spite of the high variability in the degree of starch phosphorylation, GWD proteins are ubiquitous in plants. The overall reaction mechanism of GWD is similar to that of PPS and PPDK, but the GWD family appears to have arisen after divergence of the plant kingdom. The nucleotide-binding domain of GWD exhibits a closer phylogenetic relationship to prokaryotic PPSs than to PPDKs.

Key words: dikinase, phosphorylation, site-directed mutagenesis, starch.

INTRODUCTION

The starch granule contains two distinct polysaccharides: amylose and amylopectin. While amylose is a linear chain of α-1,4-glucan with occasional α-1,6 branches, amylopectin is a much larger polyglucan with more frequent branch points introduced into the glucan structure by branching enzyme (EC 2.4.1.18). A fundamental property of tuberous starch is the phosphate found in the amylopectin fraction, where it is monoesterified at either the C-3 (~30 %) or the C-6 (~70 %) position of the glucosyl unit [1,2]. In potato starch, approx. 0.5 % of the glucose residues are phosphorylated, i.e. one in 200–300 units, whereas cereal starches contain much less covalently linked phosphate (< 0.01 %) [3,4]. The phosphate content of starches is a determinant of different physico-chemical properties, such as pasting properties, gel strength and stickiness. Starch phosphorylation is an integral part of *de novo* starch synthesis, as documented by ^{32}P-radiolabelling studies [5] and by *in vitro* studies using isolated intact potato amyloplasts [6].

A major breakthrough in the elucidation of the enzymic mechanism responsible for starch phosphorylation has been the discovery of a novel 157 kDa starch-granule-bound protein, designated GWD (α-glucan,water dikinase; formerly termed R1) [7]. Suppression of GWD synthesis in potato using antisense technology resulted in a 90 % decrease in starch-bound phosphate [7,8]. A mutation in a related gene in *Arabidopsis*, termed *sex1*, leads to a decrease in the phosphate content of leaf starch [9]. Interestingly, transgenic potato plants as well as the *sex1* mutant show a starch excess (*sex*) phenotype, suggested to be a response to impaired starch degradation [7]. This was corroborated by the fact that antisense GWD potato tubers stored at low temperatures show decreased cold sweetening [7]. The development of the starch excess phenotype demands several light periods, and no sign of an increase in starch turnover on a diurnal basis is detected. This indicates that the starch excess phenotype is the result of an accumulative process. The precise relationship between starch phosphorylation and starch degradation remains to be resolved.

GWD homologues recognized by antibodies against potato GWD have been reported in a variety of plants, e.g. in tubers of sweet potato and yam, seeds of maize and barley, and banana fruits [10]. This suggests that GWD is ubiquitous and exerts a general function throughout the plant kingdom, even though some plants (e.g. maize and barley) synthesize storage starch with a low or undetectable phosphate content.

Sequence analyses of GWD have revealed that the C-terminus of GWD shows identity with the nucleotide-binding domain and phosphohistidine domain of bacterial PPS (pyruvate,water dikinase; EC 2.7.9.2), which transfers phosphate from ATP to pyruvate and water in a dikinase-type reaction. PPDK (pyruvate, phosphate dikinase; EC 2.7.9.1) constitutes a third group of related enzymes. Recently it has been shown that GWD catalyses the phosphorylation of starch following a similar reaction mechanism [11]. Hence GWD is an α-glucan,water dikinase (EC 2.7.9.4), catalysing the transfer of the β-phosphate of ATP to either the C-6 or the C-3 position of the glycosyl residue. In this reaction, the γ-phosphate of ATP is concomitantly transferred to water to produce stoichiometric amounts of P_i and AMP in the overall reaction. GWD shows high affinity for the nucleotide substrate ATP (K_m 0.23 μM) [11], and follows a mechanism similar to that of PPS, generating an autophosphorylated enzyme intermediate prior to phosphotransfer to glucosyl residues [12]. Our

Abbreviations used: AP 150 (etc.), potato amylopectin elongated with 150 mg of glucose 1-phosphate (etc.); DP, degree of polymerization; DTT, dithiothreitol; EST, expressed sequence tag; Glc-1-*P*, glucose 1-phosphate; GWD, α-glucan,water dikinase; HPAEC, high-performance anion-exchange chromatography; PEP, phosphoenolpyruvate; PPDK, pyruvate,phosphate dikinase; PPS, pyruvate,water dikinase; SBE, starch branching enzyme; WT, wild type.

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current knowledge of the dikinases divides them into three main groups:

Group A (GWD): ATP + α-glucan + H_2O $\leftrightarrow$ AMP + α-glucan-P + P_i

Group B (PPS): ATP + pyruvate + H_2O $\leftrightarrow$ AMP + PEP + P_i

Group C (PPDK): ATP + pyruvate + P_i $\leftrightarrow$ AMP + PEP + PP_i

where PEP is phosphoenolpyruvate.

In the present study, in order to characterize the enzymic properties of GWD, we employed site-directed mutagenesis to investigate the function of the conserved histidine residue, His-992, in the putative catalytic phosphohistidine domain of GWD. Furthermore, we report on the general enzymic properties of GWD, such as the dimeric state, glucan branch length and α-1,6 branch point requirement for recombinant potato GWD purified to homogeneity. The specific phylogenetic relationship of the conserved dikinase domain with other dikinases is addressed.

MATERIALS AND METHODS

Escherichia coli GWD expression vector

A pBluescript plasmid containing the full-length GWD cDNA (GenBank accession no. AAK11735) was used as a PCR template with the following primers: 5′-CTCCTCCCATGGTACTTACCACTGATACC-3′ (*Nco*I site underlined) and 5′-ATGGCCACGTCGACTTCACATCTGTGGTCTTGT-3′ (*Sal*I site underlined). The resulting DNA fragment encoding GWD devoid of transit peptide was digested with *Nco*I and *Sal*I restriction enzymes (New England Biolabs), and subsequently ligated into a pBAD/Myc-His C vector (Invitrogen) linearized with *Nco*I and *Xho*I. A stop codon was placed upstream of the tag sequence, and as a result the plasmid pGWD was devoid of the Myc/His tag. Restriction analyses and DNA sequencing of the entire coding region validated the construct.

Sequence manipulations and phylogenetic analyses

Sequence searches of the non-redundant and unfinished genome or EST (expressed sequence tag) databases at the NCBI were conducted using the tBLASTN program with the entire potato GWD coding sequence as query sequence. In addition, the overall conserved C-terminal nucleotide-binding domain was submitted to the PSI-BLAST program [13]. Iterative profile searches using the PSI-BLAST program were done using a profile inclusion cut-off of 0.01. Multiple sequence alignments of the nucleotide-binding domain were generated using the ClustalX program [14], and the phylogenetic tree was displayed using the Treeview program [15].

Construction of site-directed mutant

The desired His-to-Ala mutation at residue 992 of GWD was introduced using the QuickChange site-directed mutagenesis kit from Stratagene with the pGWD plasmid as template using two complementary oligonucleotides: 5′-GCCAGATGTTCTTTCA-**GCG**GTTTCTGTTCGAGCTAGAAATGGG-3′ and 5′-CCCATTTCTAGCTCGAACAGAAAC**CGC**TGAAAGAACATCTGGC-3′ (new codons indicated in bold). DNA sequencing of the entire coding region confirmed the introduced mutation.

Expression and purification of WT (wild-type) GWD and the H992A mutant

Overnight cultures of *E. coli* TOP10 (Invitrogen) harbouring pGWD plasmids were diluted 1:20 in fresh LB medium containing 50 μg/ml carbenicillin. Cells were grown to $D_{600} = 0.6$ at 37 °C (250 rev./min), and protein expression was induced with 0.002 % (w/v) arabinose. After 18 h induction at 25 °C (250 rev./min), the cells were harvested in a refrigerated centrifuge, frozen in liquid nitrogen and stored at − 80 °C.

Cells were resuspended and sonicated on ice in 50 mM Tris/HCl, pH 7.5, 3 mM EDTA, 2.5 mM DTT (dithiothreitol), 10 % (v/v) glycerol and Complete Protease Inhibitor Cocktail (Roche). After centrifugation to remove debris, proteins in the supernatant were precipitated with 70 %-satd ammonium sulphate. The pellet was dissolved in buffer A [50 mM Tris/HCl, pH 7.0, 3 mM EDTA, 2.5 mM DTT, 10 % (v/v) glycerol, 0.5 mM PMSF, 1 mM benzamidine, 1 μg/ml antipain and 1 μg/ml leupeptin]. After desalting using a HiPrep 26/10 desalting column (Amersham Biosciences), the sample was applied to a 6 ml RESOURCE Q (Amersham Biosciences) anion-exchange column and the proteins were eluted with a linear gradient of 0–50 % (v/v) buffer B (buffer A containing 1 M KCl). Fractions containing GWD were combined and concentrated 10–15-fold using a 15 ml Millipore protein concentrator (50 kDa molecular mass cut-off). Proteins were then size fractionated by gel filtration on a Superdex 200 HR 10/30 column (Amersham Biosciences) equilibrated in 50 mM Tris/HCl pH 7.0, 1 mM EDTA, 2.5 mM DTT, 10 % (v/v) glycerol, 150 mM KCl, 0.5 mM PMSF, 1 mM benzamidine, 1 μg/ml antipain and 1 μg/ml leupeptin. Fractions containing GWD were combined and aliquots were stored at − 80 °C until use.

Biochemical analysis of GWD

Protein was quantified by the method of Bradford [16] using BSA as a standard. SDS/PAGE was performed using 8–25 % (w/v) linear gradient polyacrylamide gels according to Laemmli [17], and protein was visualized with Coomassie Brilliant Blue. Western blotting was carried out as described by Burnette [18]. GWD was detected immunologically using rabbit anti-GWD IgG (1:1000 dilution) and swine anti-rabbit IgG conjugated with alkaline phosphatase (1:1000 dilution; DAKO 0306) using a chromogenic substrate (5-bromo-4-chloro-3-indolyl phosphate toluidinium and NitroBlue Tetrazolium).

Production of glucan substrates for GWD

Chain elongation of potato amylopectin

Potato amylopectin (200 mg; Sigma) dissolved in 40 ml of 50 mM sodium citrate, pH 7.0, various amounts of Glc-1-*P* (glucose 1-phosphate) and 5 mM AMP were incubated with 10 mg of phosphorylase *a* (Sigma) for 18 h at 37 °C. The reaction was inactivated by boiling for 5 min, and the polyglucan was precipitated by 80 % (v/v) ethanol. Following centrifugation, the polyglucan was washed twice with 30 ml of water and precipitated as above. Finally, the polyglucan was resuspended in 30 ml of water and precipitated with 150 ml of acetone.

Branching of potato amylose

Potato amylose type III (20 mg; Sigma) was dissolved in 2 ml of a buffer containing 50 mM potassium phosphate, pH 8.0, 0.5 mg/ml BSA and 5 mM DTT. The branching reaction was initiated

by adding 15 μg of potato SBE I (starch branching enzyme I) purified as described previously [19]. Following incubation at 30 °C for 18 h, the reaction was terminated by boiling for 5 min, and the product was precipitated by 80 % (v/v) ethanol. After centrifugation, the product was washed twice with 2 ml of water and precipitated as above. Finally, the polyglucan was resuspended in 2 ml of water and precipitated with 12 ml of acetone.

Assay of GWD activity

The assay was carried out in 2 ml Eppendorf tubes and contained, in a final volume of 100 μl, 25 mM Hepes/KOH, pH 7.0, 10 mM NH_4Cl, 10 mM $MgCl_2$, 0.5 mM DTT, 0.2 mg/ml BSA, 10 μM [β-^{33}P]ATP (150 000 d.p.m.; Perkin-Elmer Life Sciences) and 5 mg/ml gelatinized polyglucan. Reactions were initiated by adding the appropriate amount of GWD. Unless specified otherwise, reactions were incubated for 1 h at 30 °C and terminated by boiling for 2 min. The polyglucan was precipitated with 1.8 ml of 75 % (v/v) methanol/1 % (w/v) KCl. Following centrifugation, the supernatant was discarded, and the pellet was resuspended in 200 μl of water, mixed briefly and then reprecipitated as before. This procedure was repeated four times and the final pellet was dissolved in 400 μl of water. Incorporated radioactivity was measured using a MicroBeta Trilux liquid scintillation counter (Wallac). One unit of GWD activity is defined as 1 μmol of phosphate incorporated into α-glucan per min at 30 °C.

Analysis of chain length distribution by HPAEC (high-performance anion-exchange chromotography)

The synthesized polyglucans were debranched by isoamylase (Megazyme) and analysed for chain length distribution by HPAEC using a Dionex DX 500 system (Dionex Corp., Sunnyvale, CA, U.S.A.) equipped with an S-3500 autosampler and a CarboPac PA-100 column, as described previously [20]. The distribution of chains phosphorylated by GWD was analysed following labelling according to the method described above, with a few modifications. We used 10 mg/ml polyglucan and 0.02 μM [β-^{33}P]ATP (7×10^6 d.p.m./ml), reactions were incubated for 3 h, and a total of seven washes was performed to reduce the background. After debranching, phosphorylated chains were separated by HPAEC as described in [20], and 1.6 ml fractions were collected. Radioactivity in the samples was determined with a MicroBeta Trilux liquid scintillation counter (Wallac).

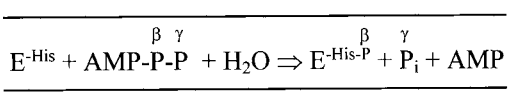
Autocatalytic phosphorylation of GWD

A 10 μg sample of WT GWD or the H992A mutant was incubated in 25 mM Hepes/KOH, pH 7.0, 5 μCi of [β-^{33}P]ATP, 10 mM $MgCl_2$ and 0.5 mM DTT for 25 min at 30 °C. Following denaturation by adding SDS sample buffer and incubation for 25 min at 30 °C, the proteins were subjected to SDS/PAGE. The gel was stained with Coomassie Brilliant Blue for 5 min and destained for only 5 min to avoid acidic hydrolysis of the phosphohistidine linkage. The gel was washed thoroughly in water to reduce the radioactive background. Incorporated radioactivity was analysed by autoradiography.

Determination of the native molecular mass of GWD

The apparent molecular mass of GWD was determined from gel filtration using an ÄKTA FPLC system (Amersham Biosciences) and a Superdex 200 HR 10/30 column (Amersham Biosciences). Protein samples of 25 μl were eluted with a flow rate of 0.4 ml/min in a buffer consisting of 50 mM Tris/HCl, pH 7.0, 1 mM EDTA, 10 % (v/v) glycerol, 2.5 mM DTT and 150 mM KCl. High- range molecular mass markers (660, 440, 215 and 158 kDa) from Amersham Biosciences were used as molecular mass standards.

A



B

PPDK (Maize)	GGMTS**H**AAVVAR
PPDK (*E. coli*)	GGRTS**H**TSIMAR
PPS (*E. coli*)	GGRTS**H**TSIMAR
GWD (*C. reticulata*)	PDVLS**H**VSVRAR
SEX1 (*Arabidopsis*)	PDVLS**H**VSVRAR
GWD (Potato)	PDVLS**H**VSVRAR

Figure 1 Putative phosphohistidine domains of GWD

(**A**) During catalysis, the β-phosphate of ATP is transferred to a histidine residue of GWD, generating a stable phosphohistidine intermediate. Subsequently this phosphate is transferred to the glucan molecule. The γ-phosphate of ATP is transferred to water to generate P_i. (**B**) The regions surrounding the putative phosphohistidines of GWDs from *Solanum tuberosum* (potato) and *Citrus reticulata* (tangerine) and that of SEX1 from *Arabidopsis thaliana* are aligned with the phosphohistidine sites of PPDKs from *Zea mays* (maize) and *E. coli* and of PPS from *E. coli*. The putative phosphohistidine, residue 992 of potato GWD, is printed in boldface.

RESULTS

Identification of the putative catalytic histidine of GWD

GWD follows a dikinase reaction mechanism, whereby the formation of an autophosphorylated intermediate, possibly a phosphohistidine, precedes phosphotransfer to the glucan [11], as illustrated in Figure 1(A). This mechanism is analogous to the phosphotransfer seen with PPDK [21] and with PPS from *E. coli* [12]. In order to identify the particular histidine residue in GWD putatively involved in formation of the phosphohistidine intermediate, we aligned the known phosphohistidine domains of PPDK and PPS with the putative domain of GWD. Figure 1(B) shows the partial sequence alignment of the regions surrounding the putative phosphohistidine of GWD, identifying histidine residue 992 in potato GWD as the residue possibly involved in autophosphorylation. This His-992 residue was targeted for site-directed mutagenesis, and a H992A mutant was generated (see the Materials and methods section).

Purification and characterization of WT and mutant GWD

WT GWD and the H992A mutant were heterologously expressed in *E. coli* and purified to apparent homogeneity using a two-step purification procedure involving anion-exchange and size-exclusion chromatography. The anion-exchange chromatography was employed as a first step, and produced GWD of approx. 50 % purity (results not shown). The concentrated proteins obtained by anion-exchange chromatography were size fractionated using a Superdex 200 HR 10/30 column (Amersham Biosciences), taking

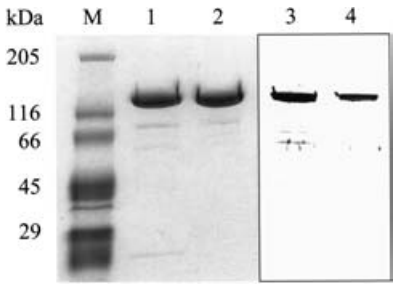


Figure 2 Purification of WT GWD and the GWD mutant H992A

Proteins were analysed by SDS/PAGE and stained with Coomassie Brilliant Blue (lanes 1 and 2), or subsequently transferred to a nitrocellulose membrane and probed with an antibody raised against GWD (lanes 3 and 4). Lanes: M, molecular mass markers; 1, purified WT GWD (5 μg); 2, purified H992A GWD (5 μg); 3, Western blot analysis of WT GWD (0.5 μg); 4, Western blot analysis of H992A GWD (0.5 μg).

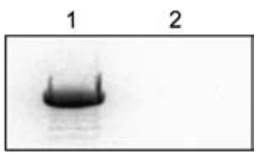


Figure 3 Autocatalytic phosphorylation of WT GWD and the GWD mutant H992A

Purified WT GWD (lane 1) and H992A (lane 2) (5 μg) were incubated with [β-^{33}P]ATP (5 μCi). Following SDS/PAGE, radioactivity was visualized using autoradiography (see the Materials and methods section).

advantage of the relatively large size of GWD (157 kDa). GWD was the first protein to be eluted from the column, and exhibited $>95\%$ purity, as evaluated by SDS/PAGE (Figure 2, lanes 1 and 2). WT and H992A GWD had identical purification profiles, and both proteins reacted with antibodies raised against WT GWD (Figure 2, lanes 3 and 4). The total yield of GWD from a 3-litre batch was typically 1.5–2 mg.

The GWD-catalysed ATP-dependent phosphorylation of glucans was determined using potato amylopectin chain-elongated by phosphorylase *a* as glucan acceptor substrates. Chain elongation afforded a substrate which proved to be much more efficient than regular amylopectin. Different degrees of elongation of the outer side chains of the amylopectin molecule were obtained using different amounts of Glc-1-*P* in the phosphorylase *a*-catalysed reaction (see the Materials and methods section). The molecular structure of these elongated amylopectin molecules is described in more detail below. In the GWD assay, 60 min was the standard incubation time, and potato amylopectin elongated with 150 mg of Glc-1-*P* (AP 150) was used as the acceptor substrate. Using this substrate, the reaction was linear with time for up to 4 h. Following inactivation of the enzyme, a simple wash procedure was employed to remove excess radioactive ATP before measuring radioactive phosphate incorporated into glucan. Using this assay with a 200-fold excess of mutant protein as compared with WT protein, the specific activity of the WT enzyme was 51.7 m-units/mg, whereas H992A showed background levels of activity (<0.01 m-unit/mg). This demonstrates that the conserved histidine residue is essential for the phosphorylating activity.

To test whether the mutant enzyme was still capable of autocatalytic phosphorylation, WT and H992A GWD were incubated with [β-^{33}P]ATP, and incorporated radioactivity was visualized by autoradiography following SDS/PAGE. As shown in Figure 3, the WT enzyme was autophosphorylated (lane 1), whereas the mutant enzyme H992A showed no histidine phosphorylation (lane 2). These results demonstrate that the conserved target histidine residue at position 992 is transiently autophosphorylated during catalysis, and that it mediates phosphotransfer to the glucosyl residue.

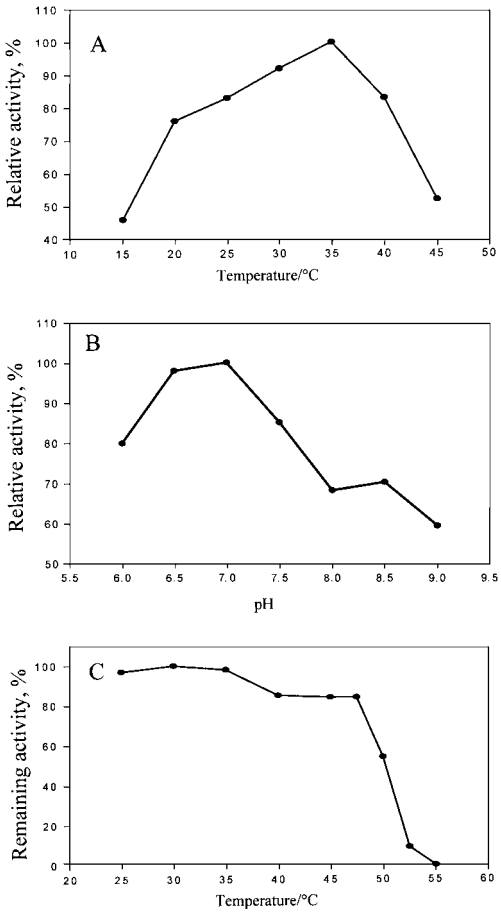


Figure 4 Characterization of potato GWD

Amylopectin elongated by phosphorylase *a* (AP 150) was used as a substrate. Optimum temperature (**A**) and pH (**B**) were analysed. For the pH optimum, the enzyme was assayed in 0.1 M Hepes buffer. Thermal stability (**C**) was evaluated by heating protein samples at the indicated temperature for 5 min, followed by cooling on ice for 5 min before assaying the residual activity. The activity after heat treatment at 30 °C is set to 100 %.

Temperature and pH optima of GWD

More detailed biochemical analysis of GWD was carried out using the GWD assay with the AP 150 as the glucan acceptor substrate. Enzyme activity was optimal at 35 °C (Figure 4A). However, the temperature curve was exceptionally wide. The pH optimum for GWD activity was 7.0 (Figure 4B), which agrees with earlier studies [11]. The large size of the GWD enzyme (157 kDa) could potentially render the protein unstable at elevated temperatures. Hence thermal stability was investigated by heating protein samples at various temperatures for 5 min following analysis of residual GWD activity at 30 °C (Figure 4C). These results showed that the enzyme is fairly stable up to approx. 50 °C, but incubation at 55 °C completely inactivates the enzyme. Hence, despite its molecular mass, GWD is surprisingly heat stable.

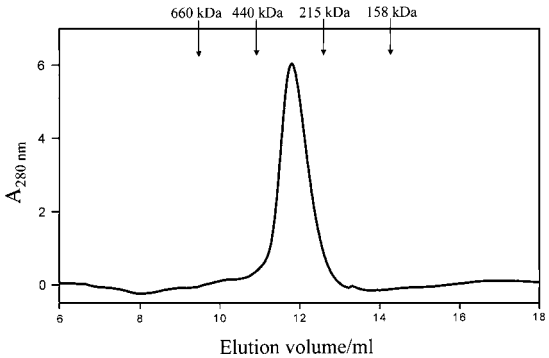


Figure 5 Quaternary structure of purified potato GWD

Purified enzyme (25 μg) was eluted from a Superdex 200 HR 10/30 column (Amersham Biosciences) in a buffer, as described in the Materials and methods section. Elution volumes of the molecular mass standards are indicated with arrows.

Quaternary structure of GWD

Large proteins have a tendency to exist in a multisubunit state. Gel filtration chromatography was employed to determine the apparent molecular mass of GWD in its native and active state. Figure 5 shows the elution profile of GWD from a Superdex 200 HR 10/30 column. From the elution volumes of the four molecular mass standards, the molecular mass of GWD was estimated to be 330 kDa. These results demonstrate that the active form of GWD exists as a homodimer. Similar experiments with the H992A mutant and with the autophosphorylated form of WT GWD showed that these also exist in a dimeric state (results not shown). This demonstrates that phosphorylation of the catalytic His or its substitution with Ala do not result in a change in the oligomerization state of the enzyme.

Substrate requirements for GWD, and analysis of glucans phosphorylated by GWD

Structural data for native starch have demonstrated previously that amylopectin, and not amylose, is phosphorylated, and that longer chains of amylopectin are more highly phosphorylated than shorter chains [20]. These data could reflect a specific substrate requirement of GWD. The substrate specificity of GWD was characterized further using a series of enzymically generated branched glucan substrates with known molecular structures. The branched glucans were obtained by elongation of amylopectin with phosphorylase *a* as well as by modification of amylose with potato SBE I. The chain length distribution of the substrates was determined using HPAEC. Figures 6(A) and 6(B) show the chain length distribution of potato amylopectin before and after chain elongation. The elongated amylopectin (AP 150; Figure 6B) showed a clear shift towards higher DPs (degrees of polymerization). Chains shorter than DP 6 were also generated in this phosphorylase *a* reaction, due to the hydrolytic properties of the enzyme, although the experimental conditions clearly favour chain elongation. Hence elongated amylopectin shows a much broader chain distribution than amylopectin. Figure 6(C) shows the chain length distribution of the final product obtained from branching of essentially linear potato amylose with potato SBE I.

The mean DPs of the various glucan substrates calculated for chains of between DP 3 and DP 60, as well as the peak DP, are given in Table 1, together with the specific activity of GWD using each of these glucan substrates. Elongation of amylopectin resulted in a higher phosphorylating activity. Interestingly, the AP 50 substrate, with a mean DP of 27.8, showed a specific activity of 2.7 m-units/mg, whereas AP 150, which was on average only DP 1.7 longer (DP 29.5), resulted in an almost 20-fold increase in specific activity (51.7 m-units/mg). Further elongation did not result in more efficient phosphorylation. Potato amylose proved to be a poor substrate for the GWD enzyme (< 0.2 m-unit/mg). However, by introducing α-1,6 branch points in the molecule by using SBE I, phosphorylating activity was enhanced to levels similar to that with native or slightly elongated amylopectin (Table 1).

To analyse the distribution of GWD-catalysed phosphorylation, GWD was incubated with the glucan substrates and [β-^{33}P]ATP, as described in the Materials and methods section. Figures 6(D)–6(F) show the distributions of labelled chains, as analysed by HPAEC. Based on previous data [20], the phosphorylated chains shown in Figures 6(D)–6(F) all range between DP 30 and DP 100, with only minor variations between the three substrates. These results indicate that GWD phosphorylates the same range of specific chains in the three glucan substrates tested.

Phylogenetic analysis of the conserved dikinase domain

A single dikinase belonging to the GWD family of enzymes has been identified in potato. Three GWD genes have been found in the *Arabidopsis* genome sequence, one has been identified in the genomic sequencing project of *Citrus reticulata* (tangerine), and three partial GWD sequences were found by combining the newly released preliminary version of the *Chlamydomonas reinhardtii* genome with short EST sequences for this alga. ESTs for GWDs are found in the GenBank database for soybean, barley, maize, grape, rape, tomato, potato and wheat. The GWD family members show regional sequence similarities to the dikinases in the so-called PEP family, which includes PPDK, bacterial PPS and enzyme I (EI) of the PEP-dependent phosphotransferase system [22]. In alignments, the nucleotide-binding domains positioned C-terminally to the active-site His in the GWD family display the highest sequence similarities and constitute an across-phylae conserved region. In the PEP family, this region is positioned in the extreme N-terminus.

In order to improve our understanding of the evolutionary relationships of GWD with other dikinases, a partial phylogenetic tree was constructed using these respective regions of the GWD family and representatives of the PEP family enzymes (Figure 7). The C-terminal conserved domain of potato GWD covers residues 1158–1464. Because it is expected that sequences with similar lengths align more accurately, the sequences covering the N-terminal 300 residues of PPS and PPDK were chosen for alignment, although the active-site histidine is positioned in another, shorter, conserved region. As shown in the tree (Figure 7), one subgroup or clade is formed by the selected representative group of bacterial PPSs. The second clade includes the PPDKs of plants, a representative protist PPDK and a representative prokaryotic PPDK. The third clade is formed exclusively by plant GWDs. Hence these three clades each represent the different types of reactions catalysed by enzymes currently described in the dikinase family.

DISCUSSION

In the present study, the essential catalytic function and importance of the conserved histidine residue located in the putative catalytic phosphohistidine domain of GWD have been determined experimentally. PPS and PPDK catalyse the transfer of the β-phosphate of ATP to pyruvate and the transfer of γ-phosphate

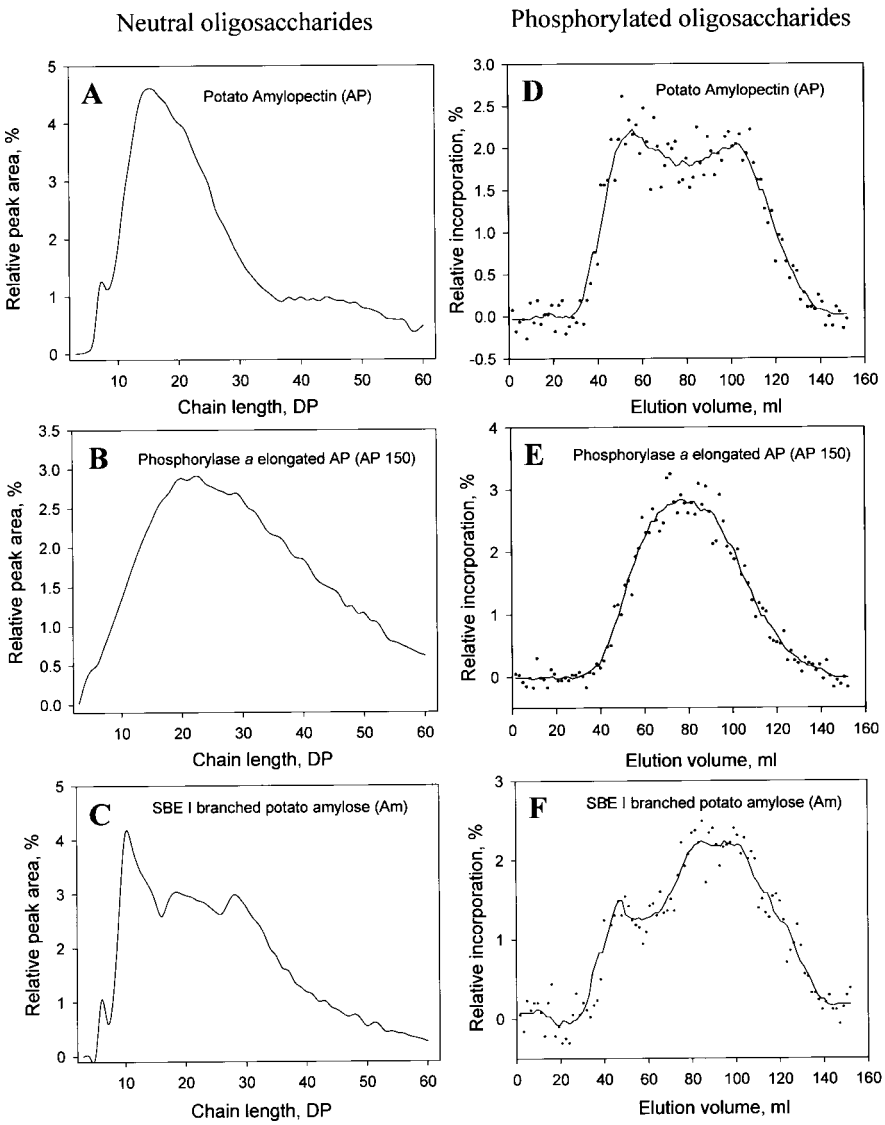


Figure 6 Distribution of linear neutral (A–C) and phosphorylated (D–F) α-glucan chains of debranched amylopectin and SBE I-treated, debranched amylose analysed by HPAEC

The α-glucan substrates were generated as described in the Materials and methods section. Relative peak areas of the neutral chains were obtained by taking the sum of peak areas with DP 3–60 as 100 %. ^{33}P-radiolabelled phosphate groups were introduced into the α-glucans by GWD, and the distribution of the labelled chains was analysed by HPAEC. The distribution of label is indicated by the smoothed line.

Table 1 Specific GWD activity with various glucan substrates

Mean DP and peak DP were determined from HPAEC analysis.

Substrate	Mean DP	Peak DP	Specific activity (m-units/mg)
Potato amylopectin	24.9	15	3.4
AP 50	27.8	20	2.7
AP 150	29.5	22	51.7
AP 200	31.0	24	49.6
Potato amylose	–	–	<0.2
SBE I-branched potato amylose	25.2	10	5.1

to water and P_i respectively, with the generation of a phosphohistidine intermediate containing the β-phosphate. Based on amino acid sequence alignments, a putative phosphohistidine domain of GWD located in the C-terminus of the protein was identified. Replacement of the conserved His residue at position 992 with Ala resulted in a mutant enzyme devoid of phosphorylating activity. Furthermore, we showed that during the enzymic reaction, a phosphorylated intermediate was not formed using the H992A mutant enzyme, whereas WT GWD exhibited autocatalytic phosphorylation. These results document that it is the conserved histidine residue at position 992 of GWD that undergoes autophosphorylation during catalysis and that mediates phosphotransfer to the glucosyl residue. Accordingly, the overall reaction mechanism of GWD is very similar to that of PPS and PPDK.

Alignments of regions containing the catalytic histidine residue and phylogenetic analyses of the relationship between the GWD, PPS and PPDK nucleotide-binding domains indicate a common origin. Phylogenetically, the functionally different dikinases group into separate clades. The GWD family has arisen after the divergence of the plant kingdom. The PPS family is found only

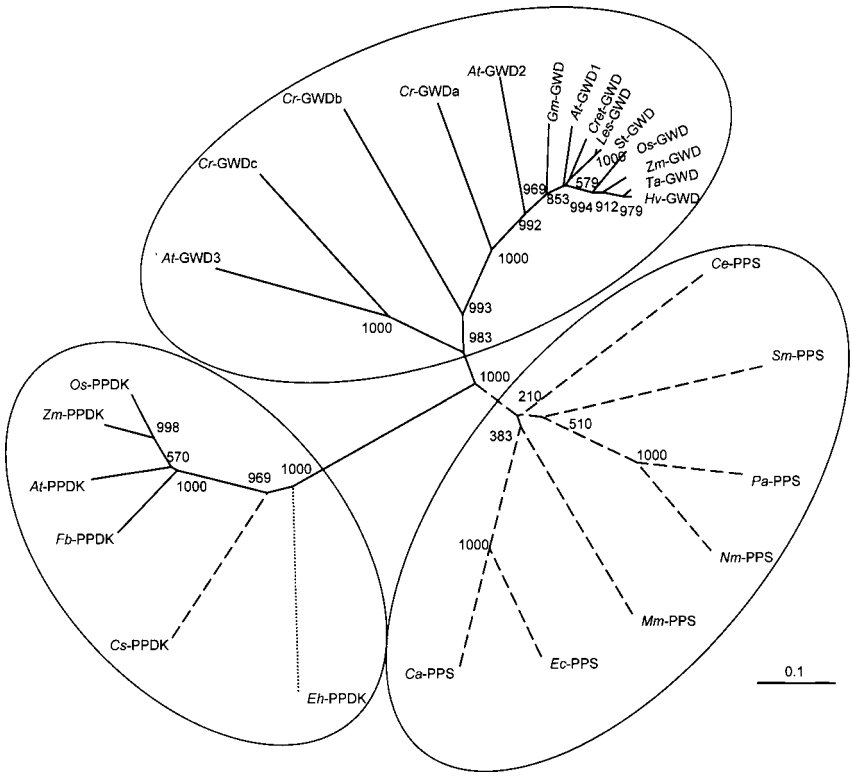


Figure 7 Phylogeny of GWD family and PEP family proteins/ORFs (open reading frames) in the nucleotide-binding region

The tree was created using the Clustal X software package. It is the consensus of the 1000 most likely trees created by the neighbour-joining method from bootstrapped data sets. The number of bootstrap replicates is indicated next to each branch. The three dikinase clades or subgroups are circled. The scale indicates the average substitutions per site. solid line, plant protein/ORF; dashed line, prokaryotic protein/ORF; dotted line, protist protein. The abbreviations and GenBank accession numbers of these dikinases are as follows. *At*-GWD1, *Arabidopsis thaliana* GWD1 (SEX1), accession no. AAG47821; *At*-GWD2, *A. thaliana* GWD homologue 2, accession no. AAO42141; *At*-GWD3, *A. thaliana* GWD homologue 3, accession no. NP198009; *Cr*-GWDa, *Chlamydomonas reinhardtii* GWD homologue a, accession no. BG857380; *Cr*-GWDb, *C. reinhardtii* GWD homologue b, accession no BF866967/AW661031; *Cr*-GWDc, *C. reinhardtii* GWD homologue c, genie 538.9 (*C. reinhardtii* Genome Release version 1.0, scaffold 538); *Cret*-GWD, *Citrus reticulata* (tangerine) GWD, accession no. AAM18228; *Gm*-GWD, *Glycine max* (soybean) GWD, accession no. AW133227/BI945390; *Hv*-GWD, *Hordeum vulgare* (barley) GWD, accession no. BU993123; *Les*-GWD, *Lysopersicon esculentum* (tomato) GWD, accession no. BE435569/AI489255; *Os*-GWD, *Oryza sativa* (rice) GWD, patent no. WO 00/28052A1; *St*-GWD, *Solanum tuberosum* (potato) GWD (R1), accession no. T07050; *Ta*-GWD, *Triticum aestivum* (wheat) GWD, accession no. CAC22583; *Zm*-GWD, *Zea mays* (maize) GWD, accession no. AY109804; *At*-PPDK, *A. thaliana* PPDK homologue, accession no. T01857; *Cs*-PPDK, *Clostridium symbiosum* PPDK, accession no. P22983; *Fb*-PPDK, *Flaveria bidentis* PPDK, accession no. S56650; *Os*-PPDK, *Oryza sativa* (rice) PPDK, accession no. BAA22420; *Eh*-PPDK, *Entamoeba histolytica* PPDK, accession no. AAA18944; *Zm*-PPDK, *Zea mays* (maize) PPDK homologue, accession no. P11155; *Ca*-PPS, *Clostridium acetobutylicum* PPS, accession no. AAK78513; *Ce*-PPS, *Corynebacterium efficiens* PPS, accession no. NP_737171; *Ec*-PPS, *Escherichia coli* PPS, accession no. S20554; *Mm*-PPS, *Methanococcus maripaludis* PPS, accession no. AAD28736; *Nm*-PPS, *Neisseria meningitidis* PPS, accession no. NP_273662; *Pa*-PPS, *Pseudomonas aeruginosa* PPS, accession no. AAG05159; *Sm*-PPS, *Staphylothermus marinus* PPS, accession no. S51006.

in bacteria and archaea, whereas the PPDK family is more widespread across phylae, including the eukaryotic kingdom (plants and protist parasites) and the prokaryotic kingdom (bacteria). It can be assumed that the conserved dikinase domain has been subject to domain shuffling in the plant genome after being acquired by the plant from an ancestral cyanobacterial protein via the endosymbiotic origin of the chloroplast. Three GWD-like sequences have been identified in the green alga *C. reinhardtii*. Only one of the algal GWD-like sequences carries a nucleotide-binding domain, which groups specifically with one of the three *Arabidopsis* GWDs and is thought to be an orthologue of AtGWD3. No putative orthologue in plants can be assigned for the two other *C. reinhardtii* sequences. Evolutionarily, the algal genes may reflect gene duplication that occurred twice before the divergence of algae and higher plants. The database search revealed additional EST sequences from potato and soybean which appeared to group with AtGWD3. These, as well as ESTs representing putative GWDs from e.g. rape and grape, were not included in the final tree because the available sequence information only partially covered the aligned protein region.

In the phylogenetic tree, the GWD nucleotide-binding domain exhibits a closer phylogenetic relationship to prokaryotic PPSs than to the PPDKs. This sequence-based close relationship between these two types of dikinases is supported by the dikinase activity of the GWD enzymes being mechanistically more similar to that of the PPSs, as water and not phosphate is used as one of the two phosphorylation targets in the dikinase reaction mechanism.

The ability of GWD to carry out α-glucan phosphorylation is highly dependent on the type of polyglucan substrate used. Amylose proved to be an extremely poor substrate, whereas amylose molecules branched by the activity of SBE I provided a much better glucan substrate. This demonstrates that the presence of α-1,6 branch points within the substrate is important for substrate recognition by GWD. These results provide the biochemical basis to explain why amylopectin, but not amylose, is phosphorylated in plants. An increase in the average length of the amylopectin side chain from DP 24.9 to DP 29.5 resulted in a dramatic 20-fold increase in the specific activity of GWD.

From analysis of the chains phosphorylated by GWD *in vitro*, we found that GWD phosphorylates longer glucan chains of

DP 30–100 independently of the specific glucan substrate used. These results indicate that the chain preference is due strictly to size. This observation explains structural analyses that document a high degree of phosphorylation of similar-length amylopectin chains in native tuberous starch [20]. This may also explain why starches from cereal seeds, which have shorter amylopectin unit chains, are phosphorylated only slightly, in spite of the presence of comparatively high levels of GWD [10]. Branching enzymes play a dominant role in determining amylopectin structure, and antisense inhibition of branching enzyme (SBE I + SBE II) in potato results in increased amylopectin unit chain lengths and a dramatic 3-fold increase in the level of starch-bound phosphate [23]. Most probably, structures formed by folding of the long amylopectin unit chains provide very good substrates for phosphorylation by GWD. Apparently, further elongation does not enhance the probability of forming structures suitable for phosphorylation. It is not fully understood why GWD exhibits a sharp increase in activity towards glucans of DP 29.

Gel filtration experiments showed that GWD exists as a dimeric enzyme in its native and active state. Furthermore, autophosphorylation of GWD did not seem to alter the ability of the enzyme to dimerize. It is tempting to speculate that dimerization of GWD may offer a means by which the regulation of GWD could be understood *in vivo*.

Currently, no molecular structure is available for GWD. Solution of the crystal structure will be important in order to identify those structural features that determine the catalytic activity of GWD, and may also resolve how the dikinase domain found in PPS and PPDK is structurally adapted to process the phosphorylation of starch instead of pyruvate. No GWD-type sequences are found in heterotrophic organisms such as *E. coli*, despite the fact that glycogen is slightly phosphorylated [24,25]. This could be interpreted to suggest that heterotrophic organisms may use kinase-dependent-type glucan phosphorylation instead of the dikinase mechanism demonstrated for the GWD enzyme.

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