Functional domain organization of the potato α -glucan, water dikinase (GWD): evidence for separate site catalysis as revealed by limited proteolysis and deletion mutants

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The potato tuber (*Solanum tuberosum*) GWD (α -glucan, water dikinase) catalyses the phosphorylation of starch by a dikinase-type reaction mechanism in which the β -phosphate of ATP is transferred to the glucosyl residue of amylopectin. GWD shows sequence similarity to bacterial pyruvate, water dikinase and PPKK (pyruvate, phosphate dikinase). In the present study, we examine the structure–function relationship of GWD. Analysis of proteolytic fragments of GWD, in conjunction with peptide microsequencing and the generation of deletion mutants, indicates that GWD is comprised of five discrete domains of 37, 24, 21, 36 and 38 kDa. The catalytic histidine, which mediates the phosphoryl group transfer from ATP to starch, is located on the 36 kDa fragment, whereas the 38 kDa C-terminal fragment contains the ATP-binding site. Binding of the glucan molecule

appears to be confined to regions containing the three N-terminal domains. Deletion mutants were generated to investigate the functional interdependency of the putative ATP- and glucan-binding domains. A truncated form of GWD expressing the 36 and 38 kDa C-terminal domains was found to catalyse the $E + ATP \rightarrow E-P + AMP + P_i$ (where P_i stands for orthophosphate) partial reaction, but not the $E-P + \text{glucan} \rightarrow E + \text{glucan-P}$ partial reaction. CD experiments provided evidence for large structural changes on autophosphorylation of GWD, indicating that GWD employs a swivelling-domain mechanism for enzymic phosphotransfer similar to that seen for PPKK.

Key words: dikinase, domain structure, limited proteolysis, phosphorylation, starch.

INTRODUCTION

The starch granule contains two distinct polysaccharides: amylose and amylopectin. Whereas 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. 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 C_6 (~70%) position of the glucosyl unit [1,2]. In potato storage starch, approx. 0.5% of the glucose residues are phosphorylated, i.e. one of 200–300 units, whereas cereal endosperm starches contain much less covalently linked phosphate (<0.01%) [3,4]. In transitory leaf starch, approx. 0.1% of the glucose residues are phosphorylated [5].

Starch phosphorylation is an integral part of *de novo* starch synthesis as documented by ³²P-radiolabelling studies [6], and by *in vitro* studies using isolated intact potato amyloplasts [7]. Suppression of GWD (α -glucan, water dikinase) synthesis in potato using antisense technology resulted in a 90% reduction of starch-bound phosphate [8,9]. A mutation in a homologous gene in *Arabidopsis*, termed *sex1*, led to a lowering of the phosphate content of leaf starch [5]. Interestingly, the transgenic potato plants as well as the *sex1* mutant show a starch excess (sex) phenotype proposed to be a result of impaired starch degradation [9]. This was corroborated by the fact that anti-sense GWD potato tubers stored at low temperatures show a decreased cold sweetening [9]. This strongly suggests that starch phosphorylation is required for normal starch degradation [10], but the precise relation between starch phosphorylation and starch degradation remains to be resolved.

GWD homologues recognized by potato GWD antibodies have been reported in a variety of plants, e.g. in tubers of sweet potato

and yam, seeds of maize and barley, and in banana fruits [11]. 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 low or undetectable phosphate content.

The enzymic mechanism responsible for starch phosphorylation has recently been elucidated, and it has been shown that a 155 kDa (mature protein) potato starch-granule-bound protein, GWD, phosphorylates starch according to reaction 1 [12,13]:

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 C_3 position of the glucosyl residue. In this reaction γ -phosphate of ATP is concomitantly transferred to water to produce stoichiometric amounts of orthophosphate (P_i) and AMP in the overall reaction. During catalysis a histidine residue at position 992 of potato GWD is autophosphorylated generating a stable phosphohistidine intermediate containing the β -phosphate, which is subsequently transferred to either the C_6 or C_3 position of the glucosyl residue according to the reactions below [13]:

From gel-filtration chromatography experiments it has been demonstrated that the active form of GWD exists as a homodimer [13]. GWD activity was determined to be optimal at pH 7.0 and 35°C. Analysis of the GWD substrate requirements show that amylopectin is a much better substrate than amylose, which

Abbreviations used: DTT, dithiothreitol; GWD, α -glucan, water dikinase; PPKK, pyruvate, phosphate dikinase; WT, wild-type.

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correlates well with the fact that only amylopectin is phosphorylated in plants.

Sequence analyses of GWD have revealed that the C-terminus of GWD shows homology with the nucleotide-binding domain and phosphohistidine domain of bacterial pyruvate, water dikinase (EC 2.7.9.2) and PPK (pyruvate, phosphate dikinase; EC 2.7.9.1). For PPK it has been shown that the catalytic histidine commutes between the nucleotide site and the pyruvate site, where it delivers the β -phosphoryl group to pyruvate [14,15]. This reaction involves a domain swivel where the phosphohistidine domain changes its position such that it alternately interacts with the nucleotide and pyruvate [14]. In the N-terminus of GWD two regions show homology (amino acid residues 51–128 and 334–511 with 31 and 25 % identity respectively) with the N-terminal region (amino acid residues 196–370) of a putative plastidic α -amylase (accession no. AAG52558) from *Arabidopsis thaliana* that might represent starch-binding domains of GWD. This motif, however, is not present in other amylases from bacteria, animals, fungi or plants.

In the present study, we employed limited proteolysis as a tool to investigate the overall functional domain structure of GWD, and results support a five-domain structure. Furthermore, deletion mutants of GWD and CD experiments indicate that the nucleotide and starch-binding sites are non-overlapping, kinetically independent sites and that the catalytic histidine could move between the two sites during turnover.

MATERIALS AND METHODS

Expression and purification of WT (wild-type) GWD

The pGWD plasmid is an L-arabinose-inducible pBAD/Myc-His C (Invitrogen) derived expression vector, which contains the potato GWD gene devoid of the transit peptide and the Myc-His tag [13]. Expression and purification of WT GWD was performed as described previously [13].

Limited proteolysis of GWD with proteinase K

Purified GWD (0.4 mg/ml) was digested at 25°C with proteinase K (Roche, Indianapolis, IN, U.S.A.) in 50 mM Tris/HCl (pH 7.5), 1 mM CaCl₂ and 0.1 mM DTT (dithiothreitol). At the indicated time points, a 25 μ l aliquot was removed and the digestion terminated by the addition of 2.5 μ l of 20 mM PMSF in propan-2-ol.

Protein quantification, SDS/PAGE and Western blotting

Protein concentration was measured by the method of Bradford [16], and with the BCA protein assay reagent (Pierce, Rockford, IL, U.S.A.) using BSA as the standard [17]. SDS/PAGE was performed using 8 % homogeneous or 8–25 % linear-gradient polyacrylamide gels according to Laemmli [18] and protein visualized with Coomassie Brilliant Blue. Western blotting was performed as described by Burnette [19]. GWD was immunologically detected by rabbit anti-GWD IgG (1:1000 dilution) and horseradish peroxidase-conjugated swine anti-rabbit IgG (1:5000 dilution; DAKO P0217) using a horseradish peroxidase substrate (Bio-Rad Laboratories, Hercules, CA, U.S.A.). Chemiluminescence was detected with the UVP AutoChemi System (UVP, Upland, CA, U.S.A.).

Protein sequencing

N-terminal protein sequencing was performed at the Macromolecular Structure, Sequencing and Synthesis Facility (Michigan State University, U.S.A.).

Assay of GWD activity

GWD activity was measured as described previously using radiolabelled [β -³³P]ATP [13]. One unit of GWD activity is defined as 1 μ mol of phosphate incorporated into α -glucan per min at 30°C.

Construction of truncated GWDs

The pGWD plasmid was used as a PCR template to generate truncated GWDs with the following primer combinations: N1 forward, 5'-GAGGAATAATAAATGAGTTCTTTTGCCGTTG-AA-3'; N1 reverse, 5'-AGTTTCAACTTCTACCAAGTTAC-3'; N2 forward, 5'-GAGGAATAATAAATGGTTGGAGTCCAGAT-AAATCC-3'; N2 reverse, 5'-CATCTGTGGTCTTGTCTGAA-CGAC-3'; N3 forward, 5'-GAGGAATAATAAATGGTTGGAG-TCCAGATAAATCC-3'; N3 reverse, 5'-AGTTTCAACTTCTA-CCAAGTTAC-3'.

For the PCR we used the proofreading, single 3'-thymidine overhang producing DNA polymerase Easy A (Stratagene), which enabled the subsequent TA-cloning into the pBAD-TOPO vector (Invitrogen). The constructs were confirmed by restriction enzyme analysis and DNA sequencing of the entire coding region.

Expression and purification of truncated GWDs

Expression of N1, N2 and N3 was performed as described for WT, except that 0.02 % (w/v) L-arabinose was used for the induction of protein expression. Cells were resuspended and sonicated on ice in 50 mM Tris/HCl (pH 7.5), 3 mM EDTA, 2.5 mM DTT, 10 % (v/v) glycerol, 0.5 mM PMSF, 1 mM benzamidinium and 5 μ g/ml leupeptin.

Autocatalytic phosphorylation of GWD

Proteins were incubated in 25 mM Hepes/KOH (pH 7.0), 5 μ Ci of [β -³³P]ATP, 10 mM MgCl₂ and 0.5 mM DTT for 25 min at 30°C. Following denaturation by addition of 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.

CD spectroscopy

CD spectra were recorded on a JASCO J-810 CD spectrophotometer. The measurements were made at 20°C using a quartz cell with a 0.1 cm path length, and the wavelength was scanned from 200 to 250 nm at 20 nm/min. The buffer spectrum was subtracted from the sample spectrum, and each spectrum reported is an average of ten scans.

The CD measurements of GWD were recorded in a 20 mM potassium phosphate buffer (pH 7.0) containing 10 mM MgCl₂. Autophosphorylation of GWD was initiated by adding ATP to 20 μ M from a 100 $\times$ stock to decrease the dilution of the sample. The CD spectrum of the autophosphorylated GWD was recorded after 20 min of incubation. To dephosphorylate the autophosphorylated form of GWD it was treated with 0.2 % (w/w) alkaline phosphatase (Sigma). The amount of alkaline phosphatase used in this experiment was below the detection limit of the CD instrument. Potential structural changes induced by incubating GWD with the very slowly hydrolysable ATP analogue, ATP[S] (Roche), was tested using ATP[S] at 100 μ M final concentration.

The effect of binding of starch was analysed by adding soluble potato starch (Sigma) to 1 mg/ml with GWD in a 20 mM potassium phosphate buffer (pH 7.0) containing 10 mM MgCl₂.

Figure 1 Coomassie Brilliant Blue-stained SDS/PAGE gel of the GWD generated proteolytic fragments

GWD was digested with proteinase K at an enzyme to protease molar ratio of 200:1. At the indicated time points, an aliquot was taken and the digestion terminated by the addition of PMSF and 8 μ g of total protein was loaded per lane. Std, molecular-mass standards (sizes given in kDa).

The secondary structure composition was predicted using the program 'K2d' (<http://www.embl-heidelberg.de/~andrade/k2d>) [20].

RESULTS

Proteinase K treatment and domain mapping of GWD

Proteinase K is a mostly non-specific serine protease, and generally it is believed that peptide regions located between protein structural domains are solvent exposed and less ordered and therefore, are more susceptible to proteolysis [21,22]. For the purpose of identifying stable folding domains within the GWD three-dimensional structure, native GWD was subjected to limited proteolysis by proteinase K. The action by proteinase K on purified GWD (155 kDa) was monitored over time by SDS/PAGE, and at each time point the proteolysis reaction was terminated by the addition of PMSF. In initial experiments we used a 1:1000 molar ratio of proteinase K to GWD, which resulted in a slow digestion of GWD with approximately half of the native GWD left undigested after 60 min (results not shown). Experiments with a 1:200 molar ratio resulted in a more rapid digestion of GWD producing two major fragments of approx. 120 and 95 kDa after 5 min of digestion with about half of the native GWD left apparently undigested (Figure 1). No native GWD remained after 60 min of digestion, and instead fragments below 40 kDa were formed, representing putative separate domains of GWD. These results indicate that cleavage of native GWD to either the 120 or 95 kDa fragment represents the major proteolytic pathway. The digestion pattern did not significantly change between 60 and 90 min of digestion (results not shown), indicating that stable domain structures were formed.

The N-terminal sequences of all major proteolytic fragments are presented in Table 1. Analyses of the sites of proteolytic cleavage within the GWD amino acid sequence revealed four major cleavage sites. The regions spanned by these proteolytic fragments as well as their exact cleavage sites are indicated in Figure 2. In the putative ATP-binding region located in the C-ter-

Table 1 Amino acid sequences of the N-terminals of the fragments generated from GWD by limited proteolysis with proteinase K

The fragments were separated by SDS/PAGE and blotted on to a PVDF membrane and sequenced by Edman degradation.

Fragment size (kDa)	N-terminal sequence	Location in amino acid sequence
155	MVLTTDT	1–7
119	KVLEEPALS	318–326
95	LIWNKNY	538–544
74	MVGQIN	721–727
45	KVLEEPA	318–324
38	QSSSNLVEVE	1045–1054
37	SATRLRVKK	1055–1063
37	MVLTTDTSSQ	1–10
36	MVGQINPV	721–729
24	KVLEEPALSKI	318–328
21	LIWNKNYNV	538–546

Figure 2 Domain organization of GWD as judged by limited proteolysis

Major proteolytic fragments of GWD resulting from limited proteolysis with proteinase K are shown according to their individual locations. Major proteinase K cutting sites representing putative interdomain junctions of GWD are indicated as denoted by amino acid number in the sequence of GWD. The size of the putative individual domains is indicated. H* denotes the catalytic histidine.

minus of GWD, proteinase K performs a staggered cut cleaving before either amino acid residue 1045 or 1055 producing fragments of 38 and 37 kDa respectively. This could be due to an extended loop in this region, which produces two highly exposed sites for proteolytic attack. For simplistic reasons, this most C-terminal putative ATP-binding fragment will be denoted as the 38 kDa domain. As shown in Figure 2, the proteolysis experiments indicate a five-domain structure of GWD composed of 37, 24, 21, 36 and 38 kDa domains spanning the N- to C-terminal.

Catalytic activity of proteolysed GWD

The proteolysis of GWD was monitored by SDS/PAGE (Figure 1) with no apparent native GWD left after 60 min of digestion with proteinase K. Analysis showed background levels of starch phosphorylating activity (<0.005 m-unit/mg) after 60 min of digestion, demonstrating that no component of the degradation mixture is able to carry out the full starch phosphorylating reaction. The components of the degradation mixtures at each time point were tested for the ability to catalyse the first nucleotide partial reaction, autophosphorylation ($E^{-\text{His}} + \text{ATP} \rightarrow E^{-\text{His-P}} + \text{AMP} + P_i$). Autophosphorylation was checked by incubation with [β - ^{33}P]ATP, and after SDS/PAGE the radiolabelled fragments were detected by autoradiography as described in the Materials and methods section. The fragments of 120 and 95 kDa, which

Figure 3 Autocatalytic phosphorylation of proteinase K generated fragments

GWD was digested with proteinase K and as described under Figure 1. After termination of the proteolytic digestion, aliquots (8 μ g) were incubated with [β - 33 P]ATP (5 μ Ci). After SDS/PAGE, radioactivity was visualized using autoradiography (see the Materials and methods section). The position of the 120, 95, 36 + 38 and 36 kDa fragments are indicated. Std, molecular-mass standards (sizes given in kDa).

Figure 4 Construction of GWD deletion mutants

The putative domain structure and the sizes of the individual domains are shown. Three deletion mutants N1, N2 and N3 of GWD were constructed as described in the Materials and methods section, and the domains spanned by each deletion mutant is indicated. H* denotes the catalytic histidine.

lack the 37 and 37 + 24 kDa N-terminal domains respectively, are both capable of being autophosphorylated (Figure 3) suggesting that the ATP-binding is located in the C-terminus of GWD. Furthermore, autophosphorylation is also seen for the fragment consisting of only the C-terminal 36 + 38 kDa domains. Interestingly, after 60 min, we saw major autophosphorylation of the 36 kDa domain, containing the catalytic histidine. This indicates that either the 36 kDa domain is capable of both binding ATP and catalysing the histidine phosphorylation or the ATP molecule is bound by another domain, which can still transfer the β -phosphate to the histidine domain even though it resides on a different polypeptide.

Construction of GWD deletion mutants

In an attempt to study further the GWD domain structure–function and enable us to investigate a possible separate site catalysis using isolated GWD deletion mutants, we constructed three GWD deletion mutants as illustrated in Figure 4. N1 lacks the C-terminal putative ATP-binding domain. N2 encodes the two C-terminal 36 and 38 kDa catalytic histidine and putative ATP-binding domains respectively. N3 was constructed to encode only the 36 kDa catalytic histidine domain.

Catalytic activity of GWD deletion mutants

WT GWD and N1–N3 were expressed in *Escherichia coli*, and the catalytic activities of WT GWD and N1–N3 were examined

Figure 5 Autocatalytic phosphorylation of GWD deletion mutants

Constructs encoding WT GWD, N1, N2 and N3 were expressed in *E. coli*. Crude extract (100 μ g) was incubated with [β - 33 P]ATP (5 μ Ci). After SDS/PAGE, radioactivity was visualized using autoradiography (see the Materials and methods section). Std, molecular-mass standards (sizes given in kDa).

using crude protein extracts. Starch phosphorylating activity of WT GWD was measured to 0.8 m-unit/mg, whereas the GWD deletion mutants, N1–N3, showed background levels of starch phosphorylating activity (<0.005 m-unit/mg). Next, we examined the ability of the proteins to catalyse the nucleotide partial reaction, autophosphorylation ($E^{-His} + ATP \rightarrow E^{-His-P} + AMP + P_i$). Accordingly, WT GWD and N1–N3 were reacted with [β - 33 P]ATP, and the 33 P-labelled fragments are shown in Figure 5. These results show that WT GWD and N2 (Figure 5) can catalyse the nucleotide partial reaction, whereas N1 and N3 cannot. The fact that N1 is incapable of autophosphorylation (Figure 5, lane 2) compared with N2, which readily autophosphorylates (Figure 5, lane 3), confirms that the C-terminal 38 kDa domain is responsible for nucleotide binding. We could not detect any autonomous phosphorylation of the 36 kDa histidine domain, N3 (Figure 5, lane 4). Thus, the catalytic histidine, the nucleotide and the starch-binding sites appear to be located on separate structural domains. The above results further suggest that the nucleotide partial reaction ($E^{-His} + ATP \rightarrow E^{-His-P} + AMP + P_i$) and the starch partial reaction ($E^{-His-P} + \alpha$ -glucan $\rightarrow E^{-His} + \alpha$ -glucan-P) take place at separate active sites. The N2 deletion mutant was able to autophosphorylate (Figure 5, lane 3), but was unable to carry out the full starch phosphorylating reaction (<0.005 m-unit/mg) indicating that the three N-terminal domains are involved in the binding of the glucan substrate. Attempts were made to construct a deletion mutant lacking the two N-terminal 37 and 24 kDa putative starch binding domains. However, despite considerable efforts this deletion mutant failed to express.

CD analyses

To investigate further the nature of the various domains of GWD on binding of the two substrates and subsequent catalysis, we decided to employ CD, which is a potent technique for detecting conformational changes. CD spectra were recorded as described in the Materials and methods section. In the far-UV region (below 250 nm), the spectral characteristics are determined primarily by the polypeptide backbone conformation of the protein and hence its secondary structure [23,24]. Analysis of native GWD generated a CD spectrum (Figure 6A), which is characteristic for a protein where the secondary structural elements are dominated by α -helices. The α -helix makes a dominant contribution with its negative CD bands at 208 and 222 nm. The spectrum of the autophosphorylated form of GWD

Figure 6 CD analyses of GWD

CD spectra were recorded and samples were prepared as described in the Materials and methods section. Each spectrum reported is an average of ten scans. (A) CD spectrum of native GWD is shown by —. After incubation with ATP, the spectrum of the autophosphorylated GWD, GWD-P, was recorded (---). The autophosphorylated form of GWD, GWD-P, was then treated with phosphatase and the CD spectrum was taken (.....). (B) CD spectrum of native GWD (—) and that following incubation with ATP[S] (γ S-ATP) (---). (C) CD spectrum of native GWD (—) and that of GWD incubated with soluble potato starch (---).

showed a similar overall shape (Figure 6A) as for the native GWD. However, a clear shift towards more positive CD values is observed, which is a strong indication of less α -helical content in the autophosphorylated form. This was further confirmed by estimation of the α -helix and β -sheet contents in the polypeptide calculated by the neural network program 'K2d' [20]. Using this a 5% decrease in the α -helical content, from 41 to 36% of the native GWD and autophosphorylated GWD, was observed. From these computations the amount of β -sheet remained constant at 16%. Treatment of the autophosphorylated form of GWD with small amounts of phosphatase resulted in a CD spectrum

identical with that seen for native GWD (Figure 6A). These results suggest that autophosphorylation of the catalytic histidine of GWD induces a major conformational change, which is reversible on dephosphorylation. Binding of ATP could potentially induce structural changes of GWD. To test this, we incubated GWD with ATP[S], which is a very slow hydrolysable analogue of ATP that in several other studies has been shown to bind to ATP-utilizing enzymes [25,26]. Assay of GWD activity using a 10-fold excess (100 μ M) of ATP[S] resulted in 64% inhibition of GWD activity, indicating that the ATP[S] molecule binds at the ATP-binding site of GWD. Incubation of GWD with 100 μ M ATP[S] did not result in significant changes in the CD spectrum (Figure 6B).

The effect of binding of starch was tested as shown in Figure 6(C), and incubation of GWD with starch results in a slightly more negative CD spectrum at 208 nm, suggesting a minor increase in α -helical secondary structural elements generated on binding of starch to GWD. However, quantification of the CD spectra did not indicate a significant change in the secondary-structure composition as a result of starch binding.

DISCUSSION

In the present study, the technique of limited proteolysis has been employed for the purpose of identifying stable folding domains within the GWD structure. Physical data are presented that support a five-domain structure of GWD composed of 37, 24, 21, 36 and 38 kDa domains spanning the N- to C-terminal (Figure 2). From analyses of the proteolytic fragments it appeared that the 36 kDa domain containing the catalytic histidine was able to phosphorylate autonomously (Figure 3). This further implies that the 36 kDa domain would also contain the ATP-binding site. However, identity with the ATP-binding site of pyruvate, water dikinase and PPDK was confined to the 38 kDa most C-terminal domain. To investigate this further, three deletion mutants of GWD were generated (Figure 4). Neither N1, which lacks the most C-terminal 38 kDa domain, nor N3, which encodes the 36 kDa domain comprising the catalytic histidine, was able to carry out autophosphorylation (Figure 5). N2 encoding the fusion of the 36 + 38 kDa C-terminal domains proved capable of autophosphorylation (Figure 5) independent of the three missing N-terminal domains. These results are somewhat contradictory to that observed when investigating the proteolytic degradation mixture (Figure 3). A plausible explanation could be that in the proteolytic degradation mixture the ATP molecule still binds to the nucleotide-binding domain, and subsequently transfers the β -phosphate to the catalytic histidine regardless of the fact that these two domains are not covalently linked. It is probable that the structure of these two domains will allow for a close docking, bringing the nucleotide in close proximity with the catalytic histidine to allow for catalysis to proceed. Overall, our results confirm that the nucleotide binding is confined to the 38 kDa C-terminal domain, as indicated by bioinformatics predictions [5]. Furthermore, the ability of N2 to autophosphorylate independently demonstrates that GWD is comprised of two independently active sites. If GWD consisted of only one active site, deletion mutants like N2 would quite probably disrupt the overall active-site structure. An enzymic mechanism with two independent active sites will require a movement of the catalytic histidine between the two sites. CD experiments provided evidence for large structural changes on autophosphorylation of the catalytic histidine of GWD resulting in an estimated 5% decrease in the α -helical content (Figure 6A), which could be reversed by treatment with phosphatase. Additionally, no significant structural changes of GWD were observed after incubation with ATP[S]

Figure 7 Hypothetical model illustrating the GWD catalytic cycle

For simplistic reasons, the proteolytic fragments are represented as spatially separate structural domains connected by loops. As shown previously, GWD can bind ATP and autophosphorylate without the presence of the glucan substrate. Hence, in this model we imagine that binding of the nucleotide substrate occurs before binding of the glucan substrate. The reverse situation or simultaneous binding could possibly occur *in vivo*. For binding of the nucleotide molecule, GWD is considered to be in a conformation state (conformer 1) where the catalytic histidine domain is spatially close to the ATP-binding site. Catalysis and autophosphorylation induce a major conformational change (conformer 2), here illustrated as a rearrangement of the catalytic histidine domain, which allows for the phosphohistidine to interact with the glucan-binding site. On binding of the glucan molecule, the second round of catalysis is initiated and the glucan substrate is phosphorylated. The enzyme then reverts to the initial conformational state (conformer 1) ready for yet another nucleotide molecule to bind. The ATP-binding site is shown to be located on the 38 kDa domain (white sphere), the catalytic histidine on the 36 kDa domain (black sphere), and the glucan-binding sites on the 37 and 24 kDa domains (grey spheres).

(Figure 6B). These observations could be explained by a domain rearrangement within GWD as a direct result of autophosphorylation. This hypothesis is supported by recent investigations of GWD from duckweed *Spirodele polyrhiza*. Here it was found that autophosphorylation of GWD resulted in a major shift in its apparent isoelectric point of 1.5 units, which is far more than what can be accounted for solely by the introduction of the phosphohistidine [27]. X-ray crystal structures of *Clostridium symbiosum* PPK have shown that two separate active sites are linked by a mobile domain containing the phosphoryl group carrying catalytic histidine. In PPK the two active sites are located ≈ 45 Å (1 Å = 0.1 nm) apart [14], and the catalytic histidine domain is responsible for communication between these remote active sites, which requires continual cycling between two conformational states. In one conformational state the catalytic histidine is in close proximity to the ATP/ P_i active site to allow for catalysis generating the phosphohistidine. This enables a conformational change where the histidine domain will pivot around two α -helical linkers to associate with the pyruvate/PEP active site to undergo the second partial reaction where the phosphoryl group is transferred to pyruvate [28]. We envision that a similar mechanism could be employed by GWD. On the basis of the results presented in this report, we propose a mechanism for GWD as illustrated in Figure 7. We propose that two conformers of GWD could exist where the phosphohistidine domain changes its position such that it alternately interacts with the nucleotide and glucan substrates. We imagine one conformer of GWD, conformer 1, where the catalytic histidine domain will interact with the nucleotide. The first round of catalysis could then generate the phosphohistidine that induces a conformational change to the second conformer, conformer 2. In this state, the phospho-

histidine domain can interact with the starch/glucan binding domain(s) to allow for the second round of catalysis generating the phosphorylated glucan polymer. After catalysis GWD can then revert to the first conformational state where another nucleotide molecule can bind. CD analysis indicated small structural changes on incubation of native GWD with starch (Figure 6C). This indicates that it could be possible for the glucan substrate to bind to conformer 1 before autophosphorylation. As shown previously, GWD can autophosphorylate without the presence of a glucan substrate, demonstrating that autophosphorylation can occur before binding of the glucan molecule. The exact order of substrate binding and autophosphorylation, which occurs during starch phosphorylation remains to be investigated.

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