α-Glucan, water dikinase (GWD): A plastidic enzyme with redox-regulated and coordinated catalytic activity and binding affinity

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The recently discovered potato tuber (*Solanum tuberosum*) α-glucan, water dikinase (GWD) (formerly known as R1) catalyzes the phosphorylation of starch by a dikinase-type reaction mechanism in which the β-phosphate of ATP is transferred to either the C-6 or the C-3 position of the glucosyl residue of starch. In the present study, we found that the GWD enzyme is inactive in the oxidized form, which is accompanied by the formation of a specific intramolecular disulfide bond as determined by disulfide-linked peptide mapping. The regulatory properties of this disulfide linkage were confirmed by site-directed mutagenesis studies. Both reduced thioredoxin (Trx) *f* and Trx *m* from spinach leaves reduced and activated oxidized GWD at very low concentrations, with Trx *f* being the more efficient, yielding an $S_{0.5}$ value of 0.4 μM. Interestingly, GWD displays a reversible and selective binding to starch granules depending on the illumination state of the plant. Here we show that starch granule-bound GWD isolated from dark-adapted plants exists in the inactive, oxidized form, which is capable of reactivation upon treatment with reduced Trx. Furthermore, the soluble form of GWD was found in its fully reduced state, providing evidence of a Trx-controlled regulation mechanism linking enzymatic activity and specific binding affinities of a protein to an intracellular surface. The regulatory site sequence, CFATC, of potato GWD is conserved in chloroplast-targeted GWDs from other species, suggesting an overall redox regulation of the GWD enzyme.

starch | redox regulation | thioredoxin

Starch is the primary energy reserve in higher plants, where it is found in both photosynthetic and nonphotosynthetic tissues. A typical 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. Starch found in the chloroplast of green leaves is termed "transitory starch," owing to the diurnal rise and fall of its levels in these tissues. Long-term storage of starch is deposited in the amyloplasts of storage organs such as seeds and tubers. A fundamental property of tuberous starch is the phosphate found in the amylopectin fraction, where it is monoesterified at either the C-3 or the C-6 of the glucosyl unit (1, 2). In potato storage starch, ≈0.5% of the glucose residues are phosphorylated, i.e., 1 of 200–300 units, whereas in transitory leaf starch, ≈0.1% of the glucose residues are phosphorylated (3).

Starch phosphorylation is an integral part of de novo starch synthesis (4, 5), and antisense repression of α-glucan, water dikinase (GWD) synthesis in potatoes results in a 90% reduction of starch-bound phosphate (6, 7). Mutations in a homologous gene in *Arabidopsis* termed *sex1* lead to a dramatic reduction or total suppression of the phosphate content in leaf starch (3). 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. This observation strongly suggests that starch phosphorylation is required for normal starch degradation (8), but the precise relation between starch phosphorylation and starch degradation remains to be resolved. GWD homologues have been reported in a variety of plants, e.g., in tubers of sweet potatoes and yams, seeds of maize and barley, and in banana fruits (9). This finding suggests that GWD is ubiquitous and exerts a general function throughout the plant kingdom.

GWD is an α-glucan, water dikinase (EC 2.7.9.4) catalyzing the transfer of the β-phosphate of ATP to either the C-3 or C-6 position of the glucosyl residue according to the following (10, 11):

$$ATP + \alpha\text{-glucan} + H_2O \leftrightarrow AMP + \alpha\text{-glucan-P} + P_i.$$

During catalysis, a His residue at position 992 of potato GWD is autophosphorylated, generating a stable phosphohistidine intermediate containing the β-phosphate. In addition, GWD displays a reversible binding to starch granules, partly existing in a soluble state in illuminated leaves and attached to the starch granule in darkened leaves (12).

The fact that GWD found in leaves is targeted to the chloroplast and appears to play a pivotal role in the turnover of transitory starch indicates that the regulation of the phosphorylation process could be exerted on a light–dark basis. Light regulation of photosynthetic enzymes is a process that links photosynthetic electron transport and the activity of specific chloroplast enzymes by means of the ferredoxin/thioredoxin (Trx) system (13, 14). In this system, the light signal is transferred by means of ferredoxin and ferredoxin:Trx reductase to Trxs. Trxs are ubiquitous, small (≈12 kDa) disulfide oxidoreductases that, in the chloroplasts, regulate the activity of selected target enzymes through reduction of their disulfide bridge (15). Chloroplasts of higher plants contain four types of Trxs (16–18). Two of them, the *f*-type (Trx *f*) and the *m*-type (Trx *m*), which are of different phylogenetic origin (19), have been shown to be involved in regulation of carbon metabolism and to exhibit a certain target enzyme specificity (15, 20, 21). In the present study, we describe the redox regulation of GWD. The enzymatic activity of GWD is regulated by the reduction of an internal, active, site-located disulfide bond by Trx. Furthermore, the redox state of GWD appears to influence binding affinity to starch granules, which is the first evidence of a Trx-mediated

change in the partitioning of a protein to structural elements of the cell.

Materials and Methods

Purification and Oxidation/Reduction of Trx. Recombinant Trxs *f* and *m* were purified as described in ref. 22 and kept at −80°C in small aliquots. The concentration was determined spectrophotometrically by using an extinction coefficient at 278 nm of 14,200 and 20,500 $M^{-1}\cdot cm^{-1}$ for Trx *f* (23) and *m* (22), respectively. Purified Trxs were reduced or oxidized with 5 mM reduced or oxidized DTT, respectively, in 100 mM Hepes-KOH (pH 7.0) at 37°C. After 5 min of incubation, DTT was removed on Bio-spin 6 columns (Bio-Rad) equilibrated in 100 mM Hepes-KOH (pH 7.0) and the protein was used immediately.

Construction of Site-Directed Mutant. The desired Cys-to-Ser mutation at residue 1008 of GWD was introduced by using the QuikChange site-directed mutagenesis kit from Stratagene with the pGWD plasmid as a template using two complementary oligonucleotides (new codons are indicated in bold): forward, 5′-GGGAAGGTTTGCTTTGCTACA**TCT**TTTGATCCC-AATATATTGGC-3′; reverse, 5′-GCCAATATATTGGGAT-CAAAA**AGA**TGTAGCAAAGCAAACCTTCCC-3′. DNA sequencing of the entire coding region confirmed the introduced mutation.

Oxidation and Redox Titrations of GWD. Oxidized GWD was produced by the method described in ref. 24, with a few modifications. GWD was incubated in 50 mM Tris·Cl, pH 7.5/50 μM $CuCl_2$ for 2 h at 25°C. GWD was then transferred to a Slide-A-Lyzer cassette (Pierce, 10-kDa molecular mass cutoff) and dialyzed against two changes of 1 liter of 50 mM Tris·Cl, pH 7.5/3 mM EDTA/0.5 mM PMSF/10% glycerol. Oxidation-reduction titrations of WT GWD and C1008S, measured by enzyme activity, were carried out to determine the oxidation state of the enzymes. Enzyme samples were incubated under aerobic conditions at 25°C in activation mixtures containing varying amounts of oxidized and reduced DTT to achieve equilibrium at defined redox potentials. Activation mixtures consisted of 50 μl of solution containing 1.2 μg of GWD, 100 mM Hepes-KOH (pH 7.9), catalytic amounts of Trx *f* (0.1 μM), and 80 mM total DTT to ensure a stable redox poise (25, 26). Samples were allowed to equilibrate at ambient potentials (E_h) defined by different ratios of reduced:oxidized DTT for 2 h. Subsequently, GWD activity was measured at 25°C and pH 7.9 over 30 min.

Expression and Purification of WT GWD and C1008S. The pGWD plasmid is an L-arabinose inducible pBAD/*Myc*-His C (Invitrogen)-derived expression vector that contains the potato *GWD* gene lacking the transit peptide and the *Myc*-His tag (11). Expression and purification of WT GWD and C1008S was performed as described in ref. 11.

Analytical Techniques. Protein concentration was measured by the method of Bradford (27), by using BSA as the standard. Starch content was measured as described in ref. 1.

Growth of Plants and Isolation of Chloroplasts from Potato Leaves. Potato plants (*Solanum tuberosum* cv. Dianella) were grown in a greenhouse with supplementary lighting in the winter. Plants were placed inside black plastic bags and kept in the dark overnight before the isolation of chloroplasts the next day.

Except where indicated, chloroplasts were isolated in the dark; a green safe-light was used when necessary. All healthy leaf blades from an ≈50-cm-tall plant (5–7 weeks old) were harvested, cut into ≈2 × 2-cm pieces and immediately transferred to an ice-cold domestic blender equipped with disposable razor blades (28). Leaves were covered with 700–800 ml of ice-cold

grinding buffer [50 mM Hepes-KOH, pH 7.5/330 mM sorbitol/1 mM $MgCl_2$/1 mM $MnCl_2$/2 mM EDTA/0.1% (wt/vol) BSA fraction V/10 μg·ml^{-1} leupeptin/0.5 mM PMSF] and homogenized in four 1-s pulses. The homogenate was filtered through two layers of nylon mesh moistened with grinding buffer and was centrifuged at 3000 × g for 4 min at 4°C in four 250-ml Sorvall GSA bottles. Two pellets ("intact") were gently resuspended in a total of 2 ml of grinding buffer; cotton swabs were used to avoid breaking the intact chloroplasts. The other two chloroplast pellets ("lysate") were resuspended by pipetting in a total of 2 ml of lysis buffer [25 mM Hepes-KOH (pH 7.0) containing 1× Roche Complete Protease Inhibitor Mixture]. The respective suspensions were overlaid on two Percoll gradients (prepared by centrifuging two SS34 tubes, each containing 35 ml of 50% Percoll in grinding buffer, at 47,800 × g for 40 min at 4°C in a Sorvall SS34 rotor). The gradients were then centrifuged at 7,700 × g for 15 min at 4°C in a Sorvall HB-4 swinging bucket rotor. Intact chloroplasts were recovered as a green band floating near the bottom of the intact gradient. Lysed and aggregated chloroplasts were recovered from upper and middle sections of the lysate gradient; starch was recovered as the pellet. Intact chloroplasts were washed in ice-cold HS [50 mM Hepes (pH 8.0) and 330 mM sorbitol containing 1× Roche Complete Protease Inhibitor Mixture) and centrifuged at 1,500 × g for 5 min at 4°C in an HB-4 rotor. Lysed chloroplasts were washed in lysis buffer and centrifuged at 8,000 × g for 10 min at 4°C. The chlorophyll content of all green samples was determined according to the method of Arnon (29). The starch pellet was washed once with lysis buffer.

Where the activity of light-activated GWD was analyzed, dark-adapted plants were placed in normal laboratory light for 2.5 h, and chloroplasts were isolated under these light conditions.

Preparation of Stromal Extract. Intact chloroplasts were resuspended at a chlorophyll concentration of 2 mg·ml^{-1} in ice-cold lysis buffer and were incubated for 5 min on ice in the dark. The chloroplast lysate was centrifuged in a microcentrifuge at 20,000 × g for 5 min at 4°C, and the supernatant was carefully collected. The thylakoid pellet was washed once in a small volume (300–500 μl) of lysis buffer and recentrifuged, and the supernatant was collected in a second tube. For the analysis of light-activated GWD, stromal extract was prepared under normal laboratory light.

Assay of GWD Activity. GWD activity was measured as described in ref. 11 by using radiolabeled [β-^{33}P]ATP, with minor modifications. As glucan substrates, we used soluble potato starch (Sigma) and the more efficient chain-elongated amylopectin AP150, prepared as described in ref. 11. In standard assays, we used 20 μM ATP, and, when measuring starch granule-bound GWD activity, we used 40 μM ATP.

Mass Spectrometric Analysis of GWD Cysteine-Containing Peptides.
Peptide preparation. 10 μg of oxidized GWD was digested with 0.3 μg of trypsin (sequence grade from Promega) in 50 mM NH_4HCO_3 (pH 7.8) at 37°C overnight. The digest was then micropurified by using Poros R2 reversed-phase chromatographic media from Applied Biosystems. The Poros R2 was washed with methanol and then equilibrated with 5% formic acid. The peptides were eluted with either 70% acetonitrile in 0.1% trifluoroacetic acid (TFA) or 50% methanol in 0.1% TFA. From this, 0.5 μl of the peptide solution and 0.5 μl of either 2,5-dihydroxybenzoic acid or a α-cyano-4-hydroxycinnamic acid matrix was applied to the MALDI target. Cocrystallization was achieved at room temperature.
MALDI-TOF MS. Mass spectra were acquired with an Ultraflex TOF/TOF (Bruker, Billerica, MA). The instrument is equipped with delayed extraction, and spectra were acquired in the positive
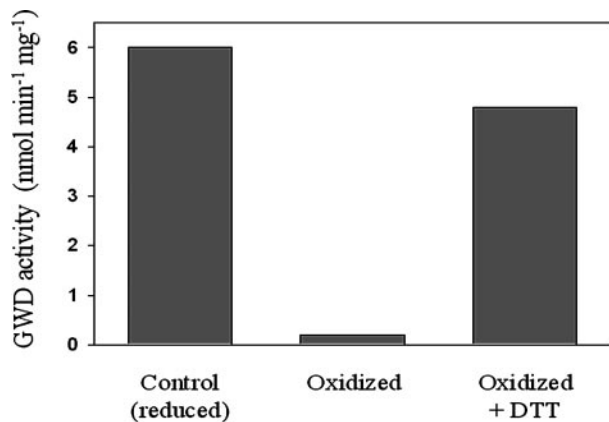
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Fig. 1. Reversibility of the reductive activation of recombinant potato GWD. In the control, freshly purified GWD was incubated in 100 mM Hepes-KOH (pH 7.0). $CuCl_2$ was added to 50 μM to initiate the oxidation process, and, after 2 h at 25°C, $CuCl_2$ was removed. Reactivation of the oxidized GWD was performed by incubation with 5 mM DTT at 30°C for 30 min. Aliquots of enzyme were withdrawn at different times to measure the activity.

reflector mode. An acceleration voltage of 25 kV and a laser frequency of 50 Hz were used. Pulsed ion extraction at 10 ns was used. Calibration of the instrument was performed by using a lactoglobulin tryptic digest. An average of 200 spectra were generated for each analysis.

Spectral analysis. The program GPMAW (Lighthouse Data, Odense, Denmark) was used to calculate the expected masses for the GWD tryptic peptides.

Results

Reversible Activation of Oxidized GWD by DTT. The activity of several chloroplast enzymes is regulated by the redox level, which is mediated by reversible thiol/disulfide interchange (30, 31). Many of these enzymes are involved in carbon metabolism, so we decided to investigate whether GWD could be a potential thiol-regulated enzyme. The effect of two thiol-oxidizing agents, 5,5′-dithiobis(2nitro-benzoic acid) (DTNB) and $CuCl_2$, on GWD enzymatic activity was examined. Treatment of GWD with 100 μM DTNB, which covalently modifies cysteine residues, followed by desalting completely inactivated the enzyme (data not shown). $CuCl_2$ does not covalently modify cysteine residues; instead, it generates an oxidizing environment that mimics that observed inside the chloroplast during the dark period (24). Incubation of GWD with a specific activity of 6.0 nmol·min^{-1}·mg^{-1} (using soluble starch as the glucan substrate) in 50 μM $CuCl_2$ followed by dialysis to remove $CuCl_2$ led to almost complete inactivation of the enzyme to 0.2 nmol·min^{-1}·mg^{-1} (Fig. 1). The $CuCl_2$-oxidized form of GWD remained soluble, and incubation of the oxidized GWD with 10 mM DTT reactivated the enzyme to 4.8 nmol·min^{-1}·mg^{-1}. Incubation of the DTNB-treated enzyme with DTT yielded similar results (data not shown). These results may suggest that GWD is a redox-regulated enzyme and that reduction of a disulfide bridge or bridges is involved in the activation process.

Identification of an Intramolecular Disulfide Bridge. To determine whether the oxidation–reduction properties of GWD involved the formation and reduction of a disulfide bridge or bridges, reduced and oxidized GWD were subjected to analysis by MS. Nine cysteine residues are present in the amino acid sequence of GWD, and peptide mass fingerprints of tryptic digests of GWD in the reduced and fully active state demonstrated that all nine Cys residues were found in a reduced state (data not shown). This finding agrees well with the general observation that
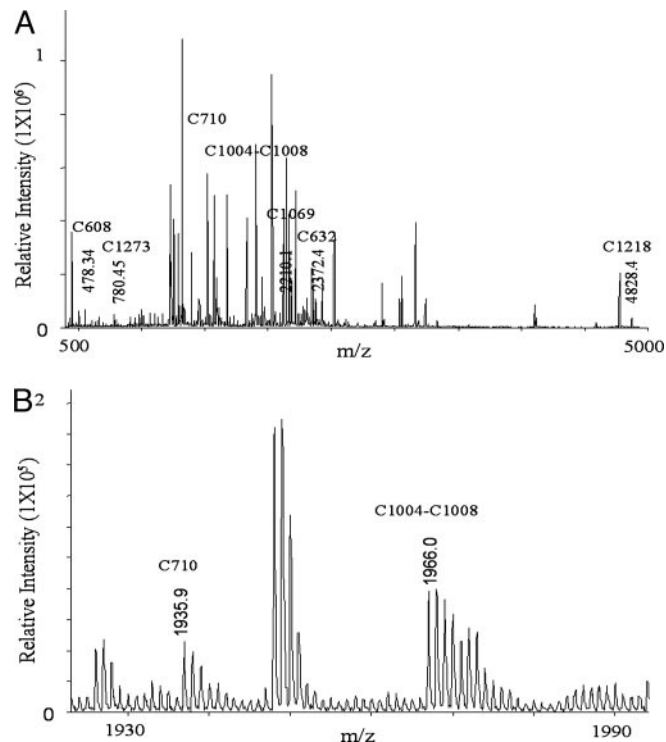

Fig. 2. Positive ion MS spectrum of oxidized GWD tryptic digest by using a 2,5-dihydroxybenzoic acid matrix. (*A*) Entire spectrum, with only the peaks corresponding to C-containing peptides shown. (*B*) Zoomed for peptides with C710, C1004, and C1008.

chloroplast-targeted enzymes are active when fully reduced, except for glucose 6-P dehydrogenase, which is inactivated by reduction (32). Upon examination of the inactive, oxidized form of GWD, we identified eight of the expected nine cysteine-containing peptides. Analysis of the tryptic peptides showed that C1004 and C1008 are linked. The other six peptides with C608, C632, C710, C1069, C1218, and C1273 were detected as reduced. The peptide containing C834 was not detectable under the conditions used. However, this cysteine is not expected to be linked, because the rest of the other cysteines were shown to be reduced. Fig. 2*A* shows the mass spectrum of the tryptic digest, and the identified cysteine-containing peptides are labeled. Fig. 2*B* shows the zoomed peaks for the C710-, C1004-, and C1008-containing peptides. The expected mass for the peptide containing linked C1004 and C1008 is 1965.94, and the observed mass was 1966.0. The same pattern was observed when α-cyano-4-hydroxycinnamic acid was used as a matrix (spectra not shown). These observations indicate that oxidation and, hence, inactivation of GWD is closely linked to the formation of an intramolecular disulfide bridge between C1004 and C1008.

C1004-C1008 Is the Regulatory Disulfide of GWD. The above results suggest that the oxidation-reduction regulated enzymatic activity of GWD is mediated by the formation of a reversible disulfide linkage between C1004 and C1008. A mutant GWD enzyme, C1008S, that was incapable of forming the particular disulfide linkage was generated by site-directed mutagenesis and purified to homogeneity (data not shown). Analysis of the C1008S mutant showed a specific GWD activity of 5.7 nmol·min^{-1}·mg^{-1} (using soluble starch as the glucan substrate), which corresponds to 95% of WT GWD activity. Fig. 3 shows the result of a representative oxidation–reduction titration of WT GWD at pH 7.9, mimicking the stromal pH in the light (33). Three independent titrations at this pH gave an average value for $E_{m,7.9}$ of
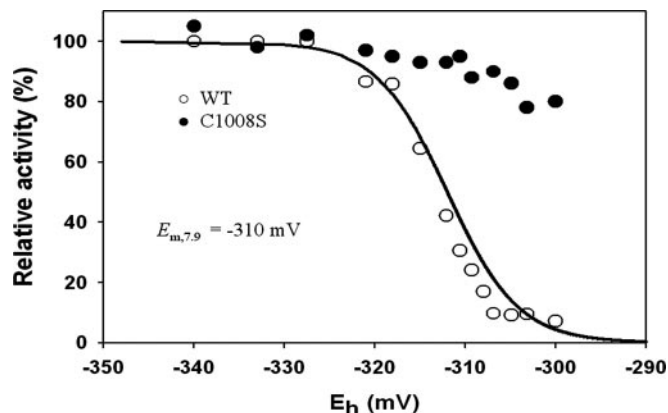
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Fig. 3. Oxidation–reduction titration of recombinant WT GWD (○) and C1008S (●) at pH 7.9. Oxidation–reduction equilibration was carried out for 2 h at 25°C and at ambient potentials (E_h) defined by different ratios of reduced: oxidized DTT. Aliquots were withdrawn and assayed for GWD activity as described in *Materials and Methods*. The solid curve was calculated by fitting the data to the Nernst equation.

-310 ± 20 mV. Fig. 3 also shows a titration of the C1008S mutant enzyme, which is permanently active over the titrated redox range, and thus demonstrates that the bridging of C1004 and C1008 is responsible for the loss of GWD activity observed in the oxidized form.

GWD Is a Target of Plant Trxs. The ability of GWD to interact with the physiological reducing agent, Trx, was tested by using purified Trx *f* and Trx *m* from spinach leaves. Analyses showed that both reduced Trx *f* and Trx *m* were capable of activating the oxidized form of GWD. To test whether Trxs transfer the reducing power more efficiently than the nonphysiological reducing agent DTT, the activation was performed at different concentrations. It was observed that both Trxs were effective at concentrations 3 orders of magnitude lower than DTT (Fig. 4 and Table 1). Chloroplast Trxs show selectivity in their interaction with target enzymes (15, 20, 21), and Trx *f* proved more effective than Trx *m*, displaying almost 3-fold higher efficiency
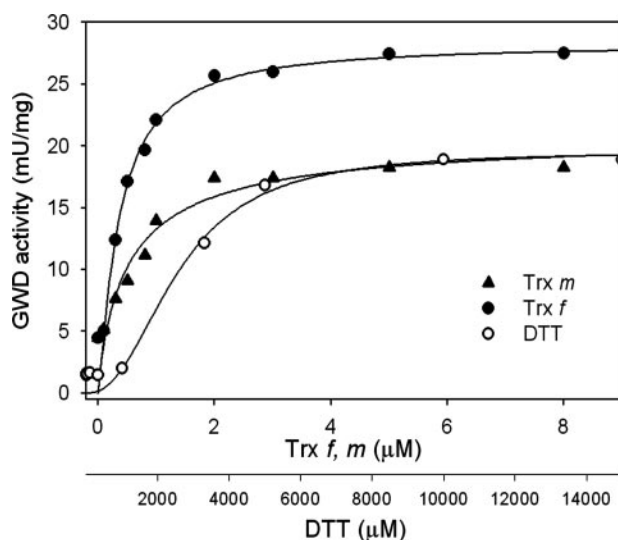

Fig. 4. Activation of recombinant potato GWD by Trxs and DTT. Enzyme samples were incubated at 30°C in 50 μl of a solution containing 50 mM Hepes-KOH (pH 7.0) and varying concentrations of reductants. After 30 min, aliquots were withdrawn and assayed for GWD activity.

Table 1. Activation of recombinant GWD by Trxs and DTT

	V_{max}, mU·mg^{-1}	$S_{0.5}$, μM	$V_{max}/S_{0.5}$, mU·mg^{-1}·μM^{-1}
Trx *f*	28.3 ± 1.2	0.4 ± 0.1	70.8
Trx *m*	20.9 ± 3.6	0.8 ± 0.2	26.1
DTT	19.6 ± 0.9	2550 ± 300	0.01

One unit of GWD activity is defined as 1 μmol of phosphate incorporated into α-glucan per min at 30°C.

($V_{max}/S_{0.5}$) and a higher V_{max} (using the efficient AP150 as the glucan substrate). This observation agrees well with previous observations that most Trx-regulated enzymes are more efficiently activated by Trx *f*. The determined $S_{0.5}$ for Trx *f* (0.4 μM) is even lower than that observed for the well characterized fructose-1,6-bisphosphatase, which displays an $S_{0.5}$ for Trx *f* of 0.9 μM (34). Furthermore, it was investigated whether reduced glutathione could activate the oxidized form of GWD. However, incubation of oxidized GWD with up to 20 mM reduced glutathione did not result in activation of the enzyme, indicating that the activation is specific to Trx. The above results suggest that GWD is a Trx-regulated enzyme, with Trx *f* being the most efficient activator. The *in vivo* oxidation mechanism of GWD is not yet well understood. By using oxidized Trx *f* or Trx *m*, it was not possible to inactivate recombinant GWD (data not shown), indicating that some other oxidant or even other form of chloroplast Trx is responsible for the oxidation *in vivo*.

Starch Granule-Bound GWD Isolated from Dark-Adapted Plants Exists in the Inactive, Oxidized Form. Previously, it was shown that in potato leaves, GWD displays a reversible and selective binding to starch granules depending on the illumination state of the plant (9, 12). Based on Western blots, Ritte *et al.* (12) determined that a major fraction of GWD was attached to the surface of the starch granule when isolated from dark-adapted plants, whereas GWD was dissociated from the granule and predominantly found in a soluble form upon isolation from illuminated plants. To investigate whether this observation could be correlated to the specific redox state of GWD, we isolated intact chloroplasts from dark-adapted potato plants (16 h of darkness) or illuminated plants (16 h of darkness and 2.5 h of light). The isolation procedure was performed quickly at 4°C omitting redox agents, and the following measurements of GWD activity were performed immediately. Measurement of granule-bound GWD activity by using starch granules isolated from dark-adapted plants showed a low activity of 3.2 pmol (mg starch)$^{-1}$h^{-1} (Fig. 5); however, this measurement increased 13-fold after treatment with reduced Trx *f*. Treatment with CuCl$_2$ resulted in a decrease in activity to 0.9 pmol (mg starch)$^{-1}$h^{-1}. Analysis of starch granules isolated from illuminated plants showed very little granule-bound GWD activity [1.2 pmol (mg starch)$^{-1}$h^{-1}], which could only be increased 2-fold after treatment with Trx *f*. These results demonstrate that in the dark period, GWD predominantly binds to the starch granule in the inactive, oxidized form, which can be reactivated by Trx. Furthermore, very little GWD was found attached to the granule during the light period, which agrees well with previous reports (12).

The soluble form of GWD was located in the stromal fraction, which was generated by lysis of intact chloroplasts after isolation. Further analyses of the redox-dependant GWD activity showed that neither in the dark-isolated nor in the light-isolated stromal fractions could the activity of GWD be enhanced by a pretreatment with reduced Trx. In addition, treatment with CuCl$_2$ resulted in near background levels of GWD activity, suggesting that the soluble form of GWD exists as a fully reduced and active form.
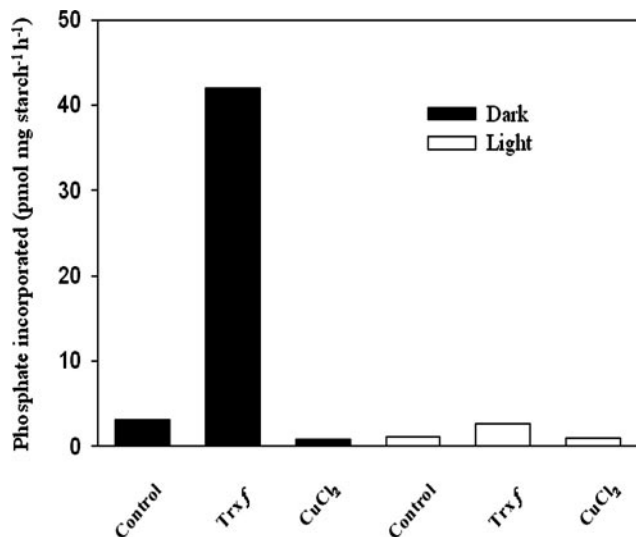
Fig. 5. Activation of starch granule-bound potato GWD. Starch granules were prepared after isolation of intact chloroplasts from leaves of dark-adapted potato plants (black bars, 16 h of darkness) and light-adapted plants (white bars, 16 h of darkness and 2.5 h of light). In the control, granules were incubated in 100 mM Hepes-KOH (pH 7.0). Reduced Trx f was added to 10 μM, or CuCl$_2$ was added to 50 μM. After 30 min at 30°C, aliquots were withdrawn to measure GWD activity.

The Regulatory Site Sequence of Potato GWD Is Conserved in Other Chloroplast-Targeted GWDs. For most of the Trx-regulated enzymes, cytosolic isoforms exist that are not regulated by thiols (35, 36). Comparisons of the amino acid sequences of the Trx-regulated enzymes reveal no cysteine-containing consensus motif (15, 20). However, in some cases, the responsible element or part of it is a CXXXXC sequence that bears close resemblance to the CFATC regulatory site sequence located in this study. This observation prompted a search for similar motifs in GWD sequences from other species. Fig. 6 shows part of an alignment of various GWD sequences and, furthermore, illustrates that the CFATC motif is located very close to the catalytic histidine of GWD. Because of the fairly recent discovery of the GWD enzyme, very little is known about the role and subcellular localization of different homologs within the same species, e.g., *Arabidopsis thaliana*. As shown in Fig. 6, the CFATC motif is present in other GWDs known or believed to be chloroplast-targeted, e.g., potato GWD, *Arabidopsis* GWD1, and citrus GWD, which all are potentially Trx-regulated. The *Arabidopsis* GWD3 homolog has been demonstrated to be plastid-targeted (41), but, interestingly, it lacks the CFATC motif. No further

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potato GWD          DMPDVLSHVSVRARNGKVCFATCFDPN
Arabidopsis GWD1    DMPDVLSHVSVRARNGKICFATCFDSG
Citrus GWD          DMPDVLSHVSVRARNCKVCFATCFDPN
Arabidopsis GWD2    SMIDVLSHVSIRARNSKICFATCFDQN
Ostreococcus GWD    DMPDILSHCAVRARNEKVLFATAFDVS
Arabidopsis GWD3    ELPH-LSHLGVRARQEKIVFVTCDDDD
Chlamydomonas GWDb  SVPDVLSHSAVRARNMGVLLAGCHCAQ
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Fig. 6. Alignment of the conserved active-site region of various GWDs. The region surrounding the active-site histidine (marked with asterisk) and the regulatory CFATC sequence of potato GWD are shown. The putative regulatory disulfides are shown in boldface. GenBank protein accession nos. are as follows: potato GWD, AAK11735; *Arabidopsis* GWD1, AAG47821; citrus GWD, AAM18228; *Arabidopsis* GWD2, AAO42141; *Ostreococus* GWD, AAS88899; *Arabidopsis* GWD3, AAU93516; *Chlamydomonas* GWDb, BF866967/AW661031.

information is available on the localization of *Ostreococcus* GWD and *Chlamydomonas reinhardtii* GWDb, but these proteins could be localized in the cytosol, non-redox-regulated, or other cysteines could be involved in the regulation.

Discussion

GWD appears conserved throughout the plant kingdom, where it is responsible for the phosphorylation of starch, and seems to exert a general function as a key enzyme in the regulation of starch degradation. In higher plants, a GWD deficiency results in a phenotype unable to mobilize transitory leaf starch, but the precise relation between starch phosphorylation and degradation remains to be resolved. These observations suggest that the molecular regulation of GWD activity and its reversible partitioning to the starch granule play pivotal roles in the mobilization of starch.

In the present study, we have investigated a possible redox regulation of GWD mediated by Trx. Initial findings showed that oxidation of GWD resulted in a nearly complete loss of enzymatic activity (Fig. 1), which could be reversed by reduction with DTT. Redox titration of GWD yielded an $E_{m,7.9}$ value of -310 mV (Fig. 3), which corresponds to ≈ -255 mV at pH 7.0, assuming a slope of E_m vs. a pH of ≈ 59 mV/pH unit. This value is the most positive value among the known redox-regulated enzymes (21, 26) and might explain why not all GWD is oxidized in the dark. Mass spectrometric analysis revealed that oxidation of GWD is accompanied by the formation of a putative regulatory intramolecular disulfide linkage close to the active site between C1004 and C1008 (Fig. 2). Replacement of Cys residue 1008 with a Ser yielded a permanently active, redox-insensitive enzyme, demonstrating that the bridging of C1004 and C1008 is responsible for the inactivation of GWD upon oxidation. Further evidence that the observed redox regulation of GWD could play an important role *in vivo* came when testing GWD as a possible target protein for the physiological reducing agent, Trx. GWD proved very sensitive to reduction by Trx f and Trx m from spinach (Fig. 4). Trx could activate GWD in concentrations 3 orders of magnitude lower than DTT (Table 1), and selectivity was observed for Trx, with Trx f being 3-fold more efficient than Trx m. The higher efficiency seen for Trx f resulted in a higher V_{max} and an $S_{0.5}$ value of 0.4 μM, which is comparable to that observed for the well characterized fructose-1,6-bisphosphatase (0.9 μM) (34).

In chloroplasts, the physiological interpretation of a Trx regulation is straightforward; starch biosynthesis and phosphorylation occur concurrently during the light period (4). Moreover, the enzyme responsible for the first committed step in starch biosynthesis, ADP-glucose pyrophosphorylase (EC 2.7.7.27), has recently been shown to be redox-regulated (37–39). When searching the databases, we found the regulatory site sequence of potato GWD conserved in other GWD homologs (Fig. 6). Interestingly, the CFATC sequence is conserved in *Arabidopsis* GWD1, which is a chloroplast enzyme and is known to be the homolog that plays the dominant role in determining the phosphate content of *Arabidopsis* leaf starch. The CFATC sequence was not conserved in all GWD homologs, indicating that these are cytosolic, not regulated by Trx, or other cysteines could be involved in the regulation.

Previously, it was shown that a major fraction of GWD was attached to the surface of the starch granule when isolated from dark-adapted plants, whereas GWD was predominantly found in a soluble form upon isolation from illuminated plants (12). From isolation of chloroplasts from dark- and light-adapted potato plants, we were able to show that GWD attached to the starch granule in the dark-adapted plants almost exclusively exists in the inactive, oxidized form, which can be reactivated by treatment with reduced Trx (Fig. 5). Furthermore, the soluble fraction of GWD was shown to exist as the fully active, reduced

form. These results strongly suggest that the redox state of GWD plays a role in partitioning GWD between the granule-bound form and the soluble stromal form. To our knowledge, this is the first time it has been shown that the Trx-mediated redox status of a protein is capable of influencing the binding to structural elements of the cell by modulation of its binding affinity. It is tempting to speculate that binding of the oxidized form of GWD to the starch granule at the onset of darkness plays a role in initiating the degradation of transitory leaf starch.

An expressed sequence tag sequence from the potato (PDB accession no. CK251207) shows homology (57% identity, 73% similarity; amino acid residues 710-1057) with GWD3 from *Arabidopsis* and possibly represents a redox-insensitive potato GWD homolog because of the missing regulatory cysteines. This recently discovered potato enzyme could potentially play a role in determining the recently observed increase in the phosphate content of the starch granule surface occurring at the early stages of starch degradation in potato leaves (40).

The relevance of Trx regulation for GWD located in the amyloplasts is not clear. As in autotrophic tissue, starch phosphorylation must be balanced with available assimilated carbon transported to the tuber. Comparison of transitory and storage starch in potatoes shows a 5-fold higher level of phosphorylation for starch located in the potato tuber, indicating an overall higher GWD activity in the tuber, in line with the general reducing conditions in the potato tuber (38). It is currently not known whether Trx exists in the amyloplasts and the links to heterotrophic carbon metabolism, but, because Trxs have been detected in many plant tissues and different subcellular locations, it is likely that Trx is present in the amyloplast. In the amyloplast, as in other heterotrophic plastids, the oxidative pentose phosphate pathway could provide the reducing power either for the NADP/Trx reductase system, or, by means of ferredoxin:NADP reductase and ferredoxin, to the ferredoxin/Trx system.

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