Light Induces Phosphorylation of Glucan Water Dikinase, Which Precedes Starch Degradation in Turions of the Duckweed *Spirodela polyrhiza*[1,2]

Rezarta Reimann, Michael Hippler, Bernd Machelett, and Klaus-J. Appenroth*

Institute of General Botany and Plant Physiology (R.R., M.H., K.-J.A.) and Institute of Nutritional Sciences (B.M.), University of Jena, 07743 Jena, Germany

Degradation of storage starch in turions, survival organs of *Spirodela polyrhiza*, is induced by light. Starch granules isolated from irradiated (24 h red light) or dark-stored turions were used as an in vitro test system to study initial events of starch degradation. The starch-associated pool of glucan water dikinase (GWD) was investigated by two-dimensional gel electrophoresis and by western blotting using antibodies raised against GWD. Application of this technique allowed us to detect spots of GWD, which are light induced and absent on immunoblots prepared from dark-adapted plants. These spots, showing increased signal intensity following incubation of the starch granules with ATP, became labeled by randomized [$\beta\gamma$-^{33}P]ATP but not by [γ-^{33}P]ATP and were removed by acid phosphatase treatment. This strongly suggests that they represent a phosphorylated form(s) of GWD. The same light signal that induces starch degradation was thus demonstrated for the first time to induce autophosphorylation of starch-associated GWD. The in vitro assay system has been used to study further effects of the light signal that induces autophosphorylation of GWD and starch degradation. In comparison with starch granules from dark-adapted plants, those from irradiated plants showed increase in (1) binding capacity of GWD by ATP treatment decreased after phosphatase treatment; (2) incorporation of the β-phosphate group of ATP into starch granules; and (3) rate of degradation of isolated granules by starch-associated proteins, further enhanced by phosphorylation of starch. The presented results provide evidence that autophosphorylation of GWD precedes the initiation of starch degradation under physiological conditions.

Degradation of starch in cereal endosperm has been thoroughly investigated over a long period (see Ritchie et al., 2000). However, the mechanisms discovered in these systems are not applicable to the metabolic mechanisms of starch in other plant cells, e.g. transitory starch in chloroplasts of leaves or storage starch in amyloplasts of tubers, roots, or turions (see Smith et al., 2003). The main reason for this difference is the a-cellular character of endosperm at the time of starch degradation. The network of regulatory processes is certainly different in living cells. In the last few years, a hitherto unexpected aspect of starch degradation has been revealed (see Blennow et al., 2002). A protein was discovered associated with starch granule surfaces (Lorberth et al., 1998; Ritte et al., 2000b), which showed reversible binding depending on the metabolic status of the cell (Ritte et al., 2000b; Reimann et al., 2002). By analysis of antisense plants and mutants, it became clear that this protein is necessary for phosphorylation of starch and that starch degradation is impaired in its absence (Lorberth et al., 1998; Yu et al., 2001). Later, the biochemical function of this protein, now named α-glucan water dikinase (GWD; EC 2.7.9.4), was revealed (Ritte et al., 2002; Mikkelsen et al., 2004). This protein becomes autophosphorylated by the β-phosphate group of ATP and transfers this phosphate group further to starch. An in vitro assay of GWD has recently been described (Ritte et al., 2003). Site-directed mutagenesis of a conserved His residue resulted in an inactive enzyme and loss of autophosphorylation (Mikkelsen et al., 2004). The Glc moieties were phosphorylated at C6 and C3 positions; possible structural consequences have been described (Blennow et al., 2003; Engelsen et al., 2003).

In turions, survival organs of the duckweed *Spirodela polyrhiza*, the main storage compound is starch (Henssen, 1954). Germination and degradation of storage starch are induced by light absorbed by phytochrome (Appenroth and Augsten, 1990; Doelger et al., 1997; Appenroth and Gabrys, 2001). In darkness, no starch degradation is detected. This creates an opportunity to start degradation of storage starch at any time using a suitable light treatment of turions. In contrast to transitory starch, whose level oscillates according to the daily light/dark cycle and circadian rhythm, the level of storage starch depends on seasonal changes. In turions, the main function of starch is to support growth of newly formed fronds following germination in spring (Ley et al., 1997). Formation of

[1] This work was supported by the Deutsche Forschungsgemeinschaft, Bonn (grant no. Ap54/6 to K.-J.A.), and the Freistaat Thüringen (Nachwuchsgruppe Pflanzenphysiologie; grant to M.H.).

[2] Dedicated to Professor Dr. Aino Henssen, Marburg, Germany. Her pioneer work about starch metabolism in turions of *Spirodela polyrhiza* was published 50 years ago.

* Corresponding author; e-mail klaus.appenroth@uni-jena.de; fax 49 3641 949232.

Article, publication date, and citation information can be found at www.plantphysiol.org/cgi/doi/10.1104/pp.103.036236.

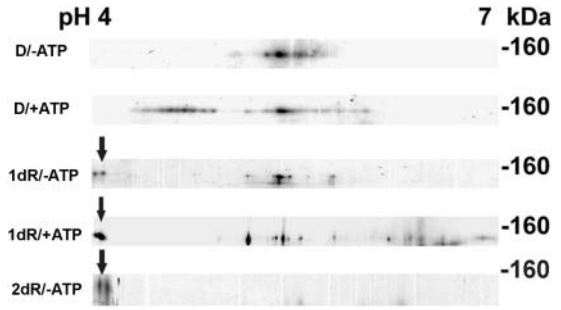

Figure 1. Western analysis of the influence of R irradiation of *S. polyrhiza* and ATP treatment on starch-associated GWD. Turions were irradiated with R for 1 d (1 d R), for 2 d (2 d R), or were dark-adapted (D). Starch granules were isolated, incubated with (+ATP) or without ATP (−ATP), and starch-associated proteins were extracted and finally investigated by 2D-GE/western analysis using antibodies raised against GWD. Two-dimensional gels were focused between pH 3 and pH 10, and the region of 160 kD after immunostaining is shown. Arrows indicate the position of spots that appeared after irradiation of turions. D/±ATP, dark control (no irradiation of turions) and incubation of isolated starch granules with or without ATP; 1dR/±ATP, irradiation with R for 1 d and incubation with or without ATP; 2dR/−ATP, irradiation with R for 2 d, starch granules not incubated with ATP.

turions and of storage starch proceeds in late summer or autumn (Henssen, 1954). Thus, in these storage organs, synthesis and degradation of starch are well separated, which makes them additionally advantageous as an experimental system.

In this paper, the newly discovered enzyme GWD has been investigated in the turion system. An in vitro assay has been developed, which uses isolated starch granules and investigates their starch-associated proteins, phosphorylation of starch, and degradation of starch. The granules were isolated from irradiated (starch degradation-induced) or dark-adapted (no starch degradation in vivo) turions. This assay allows us to characterize the effect of the inducing light signal on different steps of starch degradation. We tested a working hypothesis that a light signal, which induces starch degradation, also induces autophosphorylation of GWD, resulting in the phosphorylation of starch. By this mechanism, early events of starch degradation are initiated.

RESULTS

Influence of Light on GWD

Turions of *S. polyrhiza* were irradiated with red light (R) for 24 h or 48 h, or were dark-adapted as a control. Starch granules were isolated, and starch-associated proteins were extracted and investigated by two-dimensional SDS gel electrophoresis (2D-GE), followed by western analysis using antiserum raised against GWD (Fig. 1). The analysis of the dark control (Fig. 1; D/−ATP) showed a cluster of immunosignals at a molecular mass of 154 kD and a pI of approximately 5.5. To investigate a possible influence of autophosphorylation on starch-associated GWD, starch granules were incubated with ATP and further investigated by 2D-GE/western analysis. In the dark-control sample incubated with ATP (Fig. 1; D/+ATP), the antibodies detected a range of new spots slightly shifted toward the acidic region. When samples from irradiated turions (1 d R) were analyzed, two new signals appeared in the acidic region with a pI of approximately 4.0 (see arrow in Fig. 1; 1dR/−ATP), and one weaker signal at approximately 5.0. These new signals correspond to the same molecular weight as that of the polypeptides detected in the dark control. After 2 d of irradiation (Fig. 1; 2dR/−ATP), the original cluster of signals observed in the dark control disappeared completely so that only the two spots in the acidic region were detected. When irradiated samples were treated with ATP (1 d R), no new spots were found. Instead, the signal intensities of the R-produced spots were significantly enhanced (Fig. 1; 1dR/+ATP). The comparison of ATP-treated starch granules isolated from dark-adapted and R-irradiated turions suggests that the ATP treatment enhances the effect of R irradiation in vivo on GWD but cannot replace it.

To confirm that the light-induced spots in the acidic region represent phosphorylated forms of GWD, starch granules were incubated with radioactive ATP. The granules were isolated from irradiated turions (1 d R), and either common γ-ATP or randomized $\beta\gamma$-ATP was used for incubation. Subsequently, the starch-associated proteins were extracted, separated

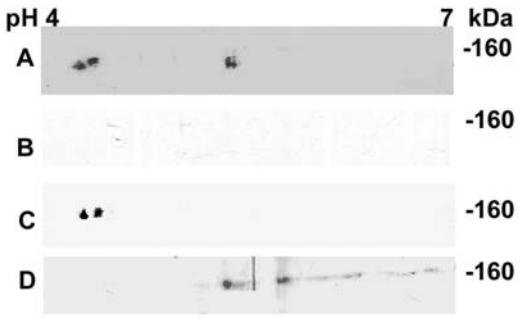

Figure 2. Comparison of western blots analyzed by immunostaining with GWD antibodies and by phosphor imaging. A, Starch granules were isolated from irradiated turions (1 d R) and incubated with [γ-^{33}P]ATP. Starch-associated proteins were extracted thereafter and analyzed after 2D-GE and western transfer by immunodetection using antibodies raised against GWD. Almost identical results were obtained after incubation with [$\beta\gamma$-^{33}P]ATP instead of [γ-^{33}P]ATP (data not shown). B, The same membranes as in A but were analyzed by phosphor imaging to detect radioactive labeled proteins. Starch granules were isolated from irradiated turions (1 d R) and incubated with [γ-^{33}P]ATP. C, The experiments were carried out as described in A, but randomized [$\beta\gamma$-^{33}P]ATP was used for incubation followed by phosphor imaging as described in B. D, Starch granules were isolated from irradiated turions (1 d R) and treated with acid phosphatase before being investigated by 2D-GE/western analysis using antibodies raised against GWD.

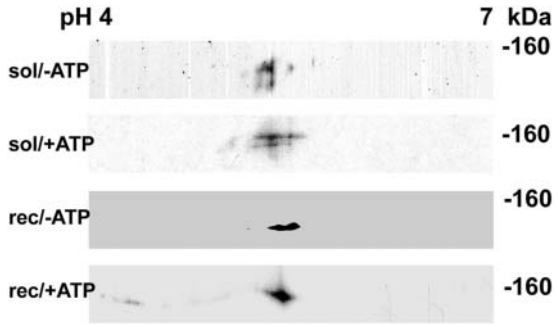

Figure 3. Effects of ATP treatment of soluble GWD on 2D-GE and western blots. Soluble GWD (sol) was extracted from turions irradiated for 24 h in R. Alternatively, recombinant purified GWD (rec) was used. The protein solutions were incubated with (+ATP) or without ATP (−ATP) and also analyzed by 2D-GE/western analysis. For further explanations, see Figure 1.

by 2D-GE, and transferred to a polyvinylidene difluoride membrane. To ensure comparison of the signals, the same membranes were used for immunodetection of GWD using antibodies and for phosphor imaging (Fig. 2). The intensity of the immunosignals in the acidic range (Fig. 2A) clearly showed successful phosphorylation of GWD by the ATP treatment (compare with Fig. 1). No radioactive signal of any polypeptide in the investigated range of molecular weights was detected when γ-ATP was used (Fig. 2B). However, using randomized $\beta\gamma$-ATP, the spots detected by immunostaining in the acidic region were also labeled by radioactivity (Fig. 2C). This shows that phosphate in the β-position of ATP specifically autophosphorylated the protein recognized by GWD antibodies. Additionally, starch granules isolated from irradiated turions (1 d R) were treated with acid phosphatase. As a result of this enzymatic treatment, no polypeptides in the acidic region were recognized by the GWD antibodies, demonstrating independently that these GWD spots represent phosphorylated forms of the protein (Fig. 2D).

The soluble pool of GWD, not associated with the starch granule surface, was also investigated (Fig. 3). This pool represents more than 90% of the total amount of GWD (compare with Reimann et al., 2002). Regardless of the light conditions applied (1 d R or darkness), the immunoblots were almost identical to each other and comparable to the dark control of starch-associated GWD (Fig. 1; D/−ATP). No effect of ATP treatment (100 μM) was detected (Fig. 3; sol/−ATP, sol/+ATP). When soluble recombinant GWD from potato (*Solanum tuberosum*) was used for ATP treatment, two signals were detected in the acidic region that are very weak in comparison with the signals detected at the pI of approximately 5.5 (compare with Fig. 3; rec/−ATP, rec/+ATP). The position of these signals was the same as those of starch-associated GWD from irradiated samples treated with ATP (Fig. 1; 1dR/+ATP).

Binding of GWD to Starch

The phosphate content of starch granules isolated from dark-adapted and irradiated turions was investigated by colorimetric measurements following acid digestion of starch samples. Irradiation of turions (1 d R) increased the phosphate content from 3.1 ± 0.2 μmol g^{-1} starch in dark-control samples to 3.8 ± 0.2 g^{-1} that is significant at the 5% level according to Student's *t* test. Approximately every 2,000th Glc moiety of starch is therefore phosphorylated in turions. This very low phosphate content in starch of turions is the reason why no phosphate was detectable by enzymatic determination of Glc-6-P as described by Nielsen et al. (1994) or by anion-exchange chromatography with pulsed amperometric detection of phosphorylated Glc as described by Ritte et al. (2000a; data not shown).

To confirm that phosphorylation of starch influences, the binding capacity of GWD in vitro and starch granules isolated from irradiated turions (1 d R) or from dark-adapted ones were incubated with ATP. These starch granules were deproteinized and then incubated with soluble proteins from irradiated turions (1 d R). Proteins bound to the granules during this incubation were extracted by SDS, and the amount of GWD was determined by one-dimensional SDS gel electrophoresis (1D-GE)/western analysis. In the starch granules isolated from irradiated turions, the amount of starch-associated GWD was enhanced by ATP treatment (Fig. 4). By contrast, the granules from dark-adapted turions did not show any effect of ATP treatment. It can be concluded that GWD has

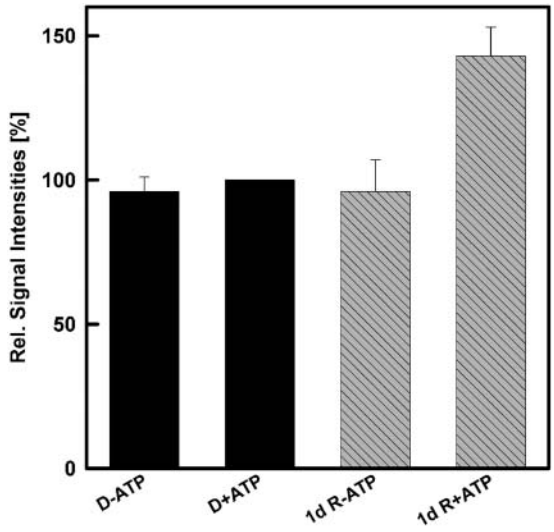

Figure 4. Effect of ATP treatment on the level of starch-associated GWD. Starch granules from irradiated (1 d R) or dark-adapted (D) turions were incubated with ATP (+ATP) or in buffer without ATP as control (−ATP). Following deproteinization, these granules were incubated with soluble proteins from irradiated turions (1 d R). Thereafter, proteins bound to the granules were extracted, and the amount of GWD was determined by 1D-GE/western analysis.

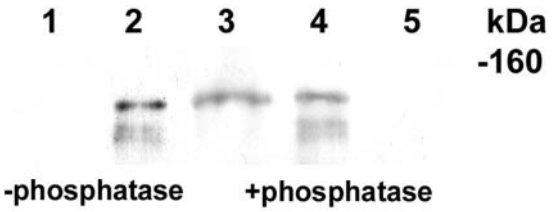

Figure 5. Effect of acid phosphatase on the level of starch-associated GWD. Starch granules from irradiated turions (1 d R) were incubated with (+phosphatase) or without (–phosphatase) acid phosphatase. After separation of supernatant and starch pellet by centrifugation, the two pools were investigated by 1D-GE/western analysis. The amount of protein loaded in each lane represents the entire fraction released from or associated with 10 mg of starch. The signal intensities were quantified from six independent samples using the ImageMaster VDS system. Data (relative western signal intensities) are given as average ± SE in brackets below. Lane 1, Soluble GWD without acid phosphatase treatment (−phosphatase; no signal detectable). Lane 2, Starch-associated GWD without acid phosphatase treatment (−phosphatase; signal intensity 1.0). Lane 3, GWD released during the acid phosphatase treatment (+phosphatase; signal intensity 0.18 ± 0.02). Lane 4, Starch-associated GWD after acid phosphatase treatment (+phosphatase; signal intensity 0.84 ± 0.05). Lane 5, Phosphatase in the same amount as used for lanes 3 and 4 (control; no signal detectable).

higher affinity to phosphorylated starch in an ATP-dependent manner in starch granules from irradiated turions.

As shown before (Fig. 1), starch-associated GWD is partly phosphorylated on the granules from irradiated turions, and the level of phosphorylation can be decreased by treatment with acid phosphatase (Fig. 2D). To investigate whether the phosphorylation status of GWD itself has an influence on the capacity of starch binding, the granules were isolated from irradiated turions (1 d R) and incubated in the presence of acid phosphatase (20 min, 25°C). Following centrifugation (5 min, 15,000*g*), the relative amount of GWD was determined in the supernatant (released from starch surface) and in the pellet (starch associated) by 1D-GE/western analysis (Fig. 5). In controls without phosphatase treatment, GWD was detected exclusively in the starch-associated form but not in the supernatant. During the dephosphorylation of GWD by phosphatase, a significant amount of GWD was detected in the supernatant, released from the starch surface. This result indicates that dephosphorylation of GWD in vitro decreased its capacity to bind starch.

In Vitro Phosphorylation of Starch

The in vitro assay was used to investigate whether preirradiation of turions has any influence on the phosphorylation of isolated starch granules. This type of experiment was carried out because it has been shown before that autophosphorylation of GWD precedes the phosphate transfer to glucan (Ritte et al., 2002). Starch granules from preirradiated turions (1 d R) and dark-adapted ones were incubated with randomized $\beta\gamma$-ATP. After starch-associated proteins were removed by washing with SDS-containing buffer, the incorporation of radioactive phosphate into starch was measured by scintillation counting. Figure 6 shows that incorporation of phosphate into starch was higher in preirradiated than in dark-adapted samples. γ-ATP, however, had no significant influence on the phosphate content of starch. Because preirradiation of turions (1 d R) did not increase the amount of GWD bound to the granule in vivo (Reimann et al., 2002), the higher phosphorylation of starch in preirradiated samples could not be a consequence of the amount of GWD attached to starch.

In Vitro Degradation of Starch Granules

As stated in preliminary experiments, incubation of isolated starch granules in buffer at room temperature led to their degradation, and low molecular carbohydrates like Glc, maltose, and carbohydrates with polymerization degrees of 3 and 4 were abundant products (data not shown). Using the in vitro assay, we characterized degradation of native starch granules. The soluble carbohydrates formed by starch hydrolysis were further degraded by acid hydrolysis, and the resulting amount of Glc was measured. Starch degradation was higher in starch granules isolated from irradiated turions (1 d R), and it was strongly enhanced by the ATP treatment (Fig. 7). ATP did not affect the weaker degradation of the granules isolated from dark-adapted turions. In control experiments with deproteinized starch granules, no release of Glc was detected (data not shown).

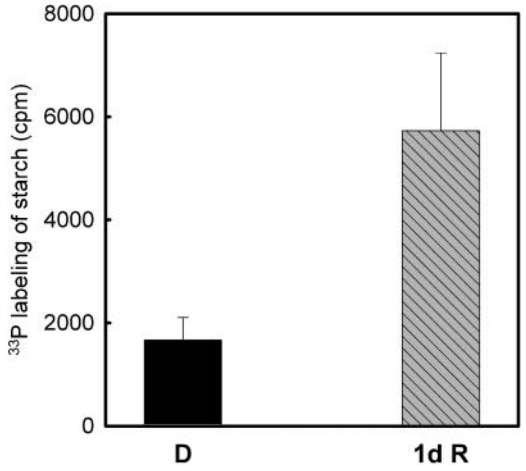

Figure 6. Labeling of starch granules with [$\beta\gamma$-^{33}P]ATP. Starch granules were isolated from irradiated (1 d R) or dark-adapted (D) turions and incubated with enzymatic randomized [$\beta\gamma$-^{33}P]ATP. Starch-associated proteins were removed, and the granules were thoroughly washed, suspended in water, and measured in a scintillation counter. As control, starch granules from irradiated turions were incubated with [γ-^{33}P]ATP, which resulted in 400 cpm.

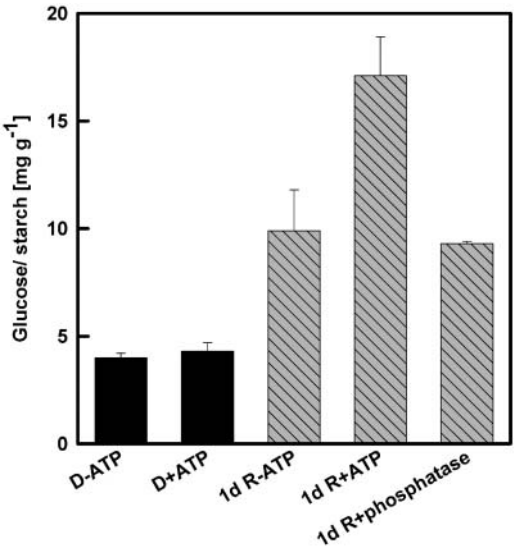

Figure 7. Effect of incubation of isolated starch granules with ATP or acid phosphatase on starch degradation rates in vitro. Starch granules were isolated from dark-adapted turions (D) or irradiated turions (1 d R), and starch degradation in vitro was investigated in the presence (+ATP) or absence (−ATP) of ATP or with acid phosphatase (+phosphatase).

Finally, starch granules were incubated with acid phosphatase to decrease the phosphorylation level of starch-associated GWD, and the influence of this treatment on starch degradation in vitro was tested. Starch granules were isolated from irradiated turions (1 d R) and incubated with acid phosphatase for 15 min at 25°C. Thereafter, starch granules were washed twice to remove acid phosphatase, and the rate of starch degradation was determined as described before. The phosphatase treatment had no influence on the rate of starch degradation (Fig. 7). Therefore, the phosphorylation status of GWD itself did not influence starch degradation in vitro.

DISCUSSION

In this study we discovered that R induces autophosphorylation of the recently described enzyme GWD (Lorberth et al., 1998; Yu et al., 2001; Ritte et al., 2002; Mikkelsen et al., 2004), a key enzyme in starch degradation. We conducted our experiments with turions of *S. polyrhiza*, which represent an excellent model system to study starch degradation since they are starch storage organs in these plants. Moreover, in contrast to degradation of transitory starch in chloroplasts of leafs (Lorberth et al., 1998), degradation of storage starch in amyloplasts of turions is induced by light (Doelger et al., 1997; Appenroth and Gabrys, 2001, 2003). Within the 24 h-period of irradiation, degradation of starch was induced by light, but significant breakdown of starch could be detected only in the following (light or dark) period because this biochemical process needs more time (Appenroth and Gabrys, 2001). Most interestingly, irradiation of turions induced autophosphorylation of GWD. This is evident by (1) two new forms of immunodetectable GWD shifted toward the acidic region of 2D maps. These two protein spots are induced by light. (2) The increase in staining intensity of the two acidic protein spots resulted from incubation of starch granules with ATP. The same two proteins, which were recognized by GWD antibodies, were labeled by incubation of isolated starch granules with [^{33}P]ATP. This reaction uses specifically β-ATP, whereas γ-ATP has no effect. (3) Phosphorylation of starch proceeds during incubation of isolated starch granules with β-ATP, whereas γ-ATP has no effect. All these results are in agreement with the properties of GWD as revealed by biochemical investigations (Ritte et al., 2002, 2003; Mikkelsen et al., 2004). It can be concluded that the light signal starts a chain of events, which produces autophosphorylation of GWD and, finally, results in starch degradation. To the best of our knowledge, this is shown here for the first time.

Starch granules isolated from dark-adapted turions (which do not undergo starch degradation in vivo) show in vitro different properties than those from irradiated turions. The starch-associated GWD is not phosphorylated, as the two GWD isoforms present after $\beta\gamma$-ATP treatment are not detectable. Moreover, treatment of the isolated granules with ATP results in a different pattern of immunosignals in the 2D-GE/western blot. Evidently, R irradiation of the turion cells makes the starch-associated fraction of GWD accessible to [β-P]ATP-dependent autophosphorylation. This indicates another undiscovered effect of light, which represents a prerequisite for the initiation of starch degradation in the turion storage tissue. The

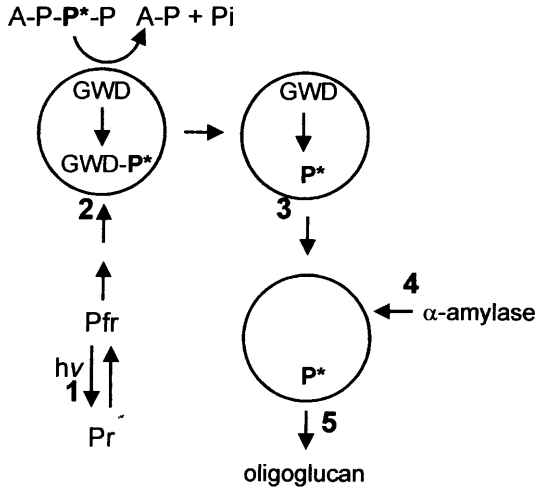

Figure 8. Schema representing possible sequential steps of light-induced degradation of storage starch in turions. 1, Phototransformation of phytochrome from its Pr in its Pfr form, which starts a signal transduction chain. 2, Activation of the starch-associated dikinase GWD and autophosphorylation of GWD using the β-phosphate group of ATP. 3, Phosphorylation of starch. 4, Enhanced association of enzymes like α-amylase. 5, Initiation of starch degradation and release of oligoglucans from starch granules.

nature of this light effect is unknown and might involve modifications of GWD or of starch granule surfaces. We describe this process operational as activation of GWD because it creates a prerequisite for its [β-P]ATP-dependent autophosphorylation.

The shift into acidic region of the 2D-GE described above (approximately 1.5 units) that is caused by phosphorylation of GWD cannot be solely explained by phosphorylation itself. In the case of GWD sequences from potato and Arabidopsis (Lorberth et al., 1998; Yu et al., 2001), the calculated shift caused by one phosphorylation site is approximately 0.04 units. Even 10 phosphorylation events would shift the pI by not more than 0.33 units. It is therefore tempting to speculate that phosphorylation induces a conformational change of the GWD protein, which could be responsible for the large shift of the pI. Surprisingly, two different spots were detected in the acidic region after phosphorylation. Moreover, several spots were detected in dark samples (control) or in dark samples treated with ATP. The reason of this phenomenon is not known.

The results presented here show that effective autophosphorylation of GWD requires association of this protein with starch granule surfaces. This starch-associated fraction of GWD (Reimann et al., 2002) is the target of the light signal, whereas only a very small part of soluble GWD becomes phosphorylated by ATP treatment. Whether this requirement is true also for other organs, e.g. potato tubers, is not yet known.

GWD binds reversibly to the starch surface in dependence on the physiological conditions (Ritte et al., 2000b; Reimann et al., 2002). This represents an additional step of regulation of starch degradation as suggested before (Reimann et al., 2002). From the results presented, it can be concluded that binding of GWD to starch granules in vitro depends on phosphorylation of GWD as well as on phosphorylation of starch. This holds true not just for GWD, whose function consists in the phosphorylation of starch (Ritte et al., 2002; Mikkelsen et al., 2004) preceding its degradation (Lorberth et al., 1998; Yu et al., 2001). For example, α-amylase, which plays an important role in starch degradation itself (see Smith et al., 2003), was also shown to be associated with starch in a light-dependent way (Reimann et al., 2002). It can be postulated that phosphorylation of starch modifies its binding capacity also to various other proteins (compare with Reimann et al., 2002) that may participate in the degradation process of starch granules. Thus, phosphorylation of starch is crucial for the binding of starch-degrading enzymes, representing one of the key events in the initiation of starch degradation in turions of *S. polyrhiza*.

From starch excess mutants of potato (Lorberth et al., 1998) and Arabidopsis (Yu et al., 2001), it is known that the phosphate content of starch correlates with the level of GWD. Autophosphorylation of GWD and transfer of phosphate to glucan were described as ''ping-pong type reaction mechanism'' (Ritte et al., 2002). Our data show that starch granules from irradiated turions (1 d R) become more phosphorylated and show higher rates of degradation than those from dark-adapted turions. These results do not prove a causal link in the system investigated here. However, the level of phosphorylation of starch correlates to the level of starch degradation activity. As shown by Reimann et al. (2002), the binding of α-amylase is correlated to the phosphorylation of starch, which would link the action of GWD to degradation of starch by enzymes like α-amylase. Increased amount of starch-associated α-amylase and enhanced degradability of starch account for the rate of light-induced starch degradation in plant cells of *S. polyrhiza*.

Taken together, the sequence of events leading to starch degradation may include the following steps (Fig. 8). (1) Absorption of light by phytochrome, which leads through some undiscovered intermediary steps to the activation (responsiveness toward phosphorylation) of starch-associated GWD. (2) Starch-associated GWD becomes autophosphorylated. (3) Starch becomes phosphorylated, which (4) enhances the binding capacity of enzymes as already known from α-amylase (Reimann et al., 2002) as well as the accessibility of starch. Finally, (5) hydrolysis of starch is initiated. Additional but at the moment unknown hypothetical reactions (e.g. influence of phytochrome-induced signal transduction chains on other proteins) may further complicate the complex regulation of starch degradation.

MATERIALS AND METHODS

Cultivation of Plants, Light Sources, and Irradiation

All experiments were carried out with etiolated nondormant turions of the duckweed (Araceae) *Spirodela polyrhiza* L. Schleiden strain SJ (Appenroth et al., 1996). Cultivation of plants, formation of turions, light sources, and irradiation were described before (Appenroth et al., 1996; Appenroth and Gabrys, 2001; Reimann et al., 2002). The fluence rate of R (658 nm, half-band width 25 nm) was 12 μmol m^{-2} s^{-1} (Reimann et al., 2002).

One- and Two-Dimensional Gel Electrophoresis, Electrophoretic Protein Transfer, and Immunostaining

The isolation of starch granules and the extraction of soluble and starch-associated proteins have been described previously (Reimann et al., 2002). Starch-associated proteins were extracted in 0.125 M Tris-HCl (pH 6.8) buffer containing 2% (w/v) SDS. A total of 0.02 mL of buffer per milligram of starch was used. Soluble proteins were extracted in 50 mM HEPES-KOH (pH 7.5) buffer (Reimann et al., 2002) without SDS and quantified according to Bradford (Appenroth et al., 1982).

One-dimensional SDS gel electrophoresis, electrophoretic transfer, and immunostaining were carried out as described before (Reimann et al., 2002). Sample preparation and 2D-GE were described by Hippler et al. (2001). The loaded starch-associated proteins corresponded to the same amount of starch dry weight. Proteins were dissolved in 380 μL of solubilization buffer (2 M thiourea, 8 M urea, 4% [w/v] CHAPS, 20 mM Tris base, 30 mM dithioerythritol, 0.5% [v/v] immobilized pH gradient [IPG] buffer, pH 3–10 [Amersham, Buckinghamshire, UK], 0.05% [w/v] β-dodecyl-maltoside, and 0.5% [w/v] bromphenol blue) and incubated for 1 h at room temperature, then centrifuged at 9,000*g* for 5 min. A total of 350 μL of the supernatant was carefully removed, filled into a fresh tube, and 0.5% (v/v) IPG buffer

(Amersham) was added again. This solution was loaded into the groove of a ceramic strip tray (Amersham). The IPG strip, with a linear pH gradient from 3.0 to 10.0, was overlaid upside down, covered with paraffin, and incubated for 12 h at 20°C. After rehydration of the IPG strips in the presence of the sample, electrophoresis was performed using an IPGphor apparatus (Amersham) at 15°C until 50 kVh were reached.

After electrophoresis in the first dimension was finished, the IPG strips were removed from the tray and equilibrated for SDS-PAGE using two different equilibration solutions. The IPG strips were first incubated with solution 1 (50 mM Tris-HCl, pH 6.8; 6 M urea; 30% [v/v] glycerol; 2% [w/v] SDS; and 2% [w/v] dithioerythritol) for 12 min and afterward with solution 2 (Tris-HCl, pH 6.8; 6 M urea; 30% [v/v] glycerol; 2% [w/v] SDS; 2.5% [w/v] iodoacetamide; and 0.5% [w/v] bromphenol blue) for 5 min. The strips were then briefly washed with water, loaded on top of a prepared SDS-PAGE (8% acrylamide and 0.42% piperazine diacrylamide), and covered with 0.5% agarose. The SDS-PAGE was run at 8°C and 30 mA per gel using a Bio-Rad multi-cell apparatus (Hercules, CA).

For the immunostaining, antibodies raised against GWD (EC 2.7.9.4) from potato (*Solanum tuberosum*; Lorberth et al., 1998) were used. The antiserum was diluted 1:5,000 when used following 1D-GE and 1:3,000 after 2D-GE. The intensities of detected protein bands were quantified using the ImageMaster VDS system (Amersham-Pharmacia, Freiburg, Germany) and the software supplied by the producer.

ATP Treatment and Analysis of GWD

Starch granules (30 mg dry weight) were incubated in 0.5 mL of the following buffer: 50 mM HEPES-KOH (pH 7.5), 1 mM EDTA, 6 mM $MgCl_2$, 5 μL of protease inhibitor (cocktail for plant cell and tissue extract; Sigma-Aldrich, Taufkirchen, Germany), and 0.1 mM ATP (Ritte et al., 2002, 2003). ATP was omitted in control probes. The suspension was rotated at approximately 15 rpm for 20 min at 25°C using the mixing rotor Variospeed (Renner, Darmstadt, Germany). The suspension was centrifuged for 5 min at 16,000*g*, and the starch-associated proteins were extracted from the pellet and investigated by 2D-GE/western analysis (see above).

For the incubation of starch granules with radioactive ATP, two different labeled ATP preparations were used: [γ-^{33}P]ATP (Hartmann Analytic, Braunschweig, Germany) and a mixture of [β-^{33}P]ATP and [γ-^{33}P]ATP (designated as γ-ATP and $\gamma\beta$-ATP) obtained by enzymatic randomization with myokinase and pyruvate kinase (Ritte et al., 2002). Starch granules (30 mg dry weight) were incubated at 25°C for 30 min in 400 μL of the following solution: 50 mM HEPES-KOH, pH 7.5, 1 mM EDTA, 10% (v/v) glycerol, 5 mM $MgCl_2$, 5 mM KCl, and 100 μM ATP containing 4 MBq $\gamma\beta$-ATP or γ-ATP. Following incubation, starch-associated proteins were separated by 2D-GE and electrophoretic transferred on polyvinylidene difluoride membrane (Hybond-P; Amersham-Pharmacia). These membranes were used in a PhosphorImager (Storm 820; Molecular Dynamics, Krefeld, Germany) to detect radioactive labeled proteins and for immunodetection using GWD antiserum as described above.

Soluble proteins were extracted from turions (1g fresh weight) that had been irradiated with R for 24 h. The concentration of the total soluble protein was 0.1 mg in 0.5 mL. The plant extract was incubated with (100 μM) or without ATP and thereafter used for 2D-GE/western analysis. The same experiment was carried out with recombinant, partially purified GWD from potato (0.6 μg mL^{-1}) as described before (Ritte et al., 2002).

Acid Phosphatase Treatment and Analysis of Starch-Associated GWD

Starch granules (10 mg dry weight) were incubated in 1 mL of buffer containing 100 mM PIPES-HCl (pH 6.0) and 1 mM dithiothreitol in the presence of 10 units of acid phosphatase from potato (Roche Diagnostics, Mannheim, Germany). The suspension was incubated for 20 min at 26°C using a mixing rotor (15 rpm) and thereafter centrifuged for 5 min at 16,000*g*. The supernatant (which contained the starch-associated proteins released from starch grains during the phosphatase treatment) was precipitated with phenol-ether (Sauve et al., 1995), redissolved in 0.1 mL of sample buffer (Laemmli, 1970), and further used for 1D-GE/western analysis. This fraction was designated as released proteins. The pellet (which contained the starch granules) was used for the extraction of starch-associated proteins (Reimann et al., 2002). This starch-associated fraction was investigated by 1D-GE and 2D-GE, followed by western analysis of GWD. Controls were treated as the samples, but phosphatase was omitted during suspension.

Calculation of pIs of GWD following Potential Phosphorylations

The pIs of GWD subjected to multiple potential phosphorylations were calculated using the algorithm from ExPASy's Compute pI/Mw program provided under http://scansite.mit.edu (see also Bjellqvist et al., 1993). Two GWD proteins were used for the calculations: R1 from Solanum tuberosum (CAD 88974) and sex1 from Arabidopsis (NP 563877).

In Vitro Binding of Soluble GWD to Starch Granules

Isolated starch granules (10 mg dry weight) were incubated with nonradioactive ATP as described above. The suspension was then centrifuged (5 min, 16,000*g*), and starch was deproteinized using toluene (Morrison and Karkalas, 1990). The starch granules were vigorously mixed for 30 s with 1.2 mL water:toluene (3:1) and separated by centrifugation (5 min, 16,000*g*). This procedure was repeated four times, and thereafter the starch granules were washed three times with 1 mL of water. The starch granules were then incubated under rotation for 1 h at 4°C with soluble proteins (0.6 mg in 500 μL), extracted from turions that had been irradiated for 24 h in R. The resulting suspension was loaded on top of a 5-mL cushion consisting of 95% (v/v) Percoll (Amersham-Pharmacia) and 5% (v/v) 0.5 M HEPES-KOH (pH 7.0). After centrifugation (15 min, 2,000*g*; Ritte et al., 2000b), the starch granules were washed twice with 1 mL of 0.1 M HEPES-KOH (pH 8.0), and the starch-associated proteins were extracted using 0.1 mL of 2% (w/v) SDS. Starch-associated GWD was investigated by 2D-GE/western analysis.

In Vitro Degradation of Starch Granules

Starch granules (10 mg dry weight) were incubated in 0.5 mL of buffer containing 50 mM HEPES-KOH (pH 7.5) and 1 mM EDTA at 25°C under rotation. After the incubation, the granules were separated from the supernatant by centrifugation (5 min, 16,000*g*), and the sugar released during the incubation was measured (Waffenschmidt and Jaenicke, 1987). An aliquot of 0.4 mL from the supernatant was hydrolyzed together with 0.4 mL of 1 M HCl for 100 min at 100°C. After centrifugation (5 min, 16,000*g*), 0.5 mL from supernatant was neutralized by adding 0.5 mL of 0.5 M NaOH. For the colorimetric determination (A_{560}) of the released Glc, 0.2 mL of the neutralized solution was mixed with 0.3 mL of water and 0.5 mL of reagent as described by Waffenschmidt and Jaenicke (1987). The amount of Glc released is given in micrograms of Glc per gram starch granules (dry weight).

To investigate the influence of ATP on starch degradation, 0.1 mM ATP and 6 mM $MgCl_2$ were added to the reaction mixture. The treatment with acid phosphatase was carried out in the same way as described above (see "Acid Phosphatase Treatment and Analysis of Starch-Associated GWD"). In all experiments, six independent samples were investigated. Data are given as average ± SE.

[^{33}P]ATP Treatment and Analysis of Starch Granules

$\beta\gamma$-ATP was prepared as described above. Starch (10 mg dry weight) was incubated in 0.5 mL of the following buffer: 50 mM HEPES-KOH, pH 7.5, 1 mM EDTA, 6 mM $MgCl_2$, and 25 μM ATP with 0.4 MBq $\beta\gamma$-ATP. After 20 and 120 min of incubation under rotation (15 rpm) at 25°C, the reaction was stopped by adding 120 μL of 0.1 M HCl. The starch granules were thoroughly washed (once with 2% [w/v] SDS and six times with phosphate-buffered ATP [2 mM]) as described recently (Ritte et al., 2002, 2003). Following centrifugation (5 min, 16,000*g*), the starch granules were suspended in 0.8 mL of water and mixed with 1.2 mL of scintillation solution (Rothiszint mini; Roth, Karlsruhe, Germany). Radioactivity was measured in a scintillation counter (LS6500; Beckmann, Munich). Data were given as differences between the results measured after 20 min and after 120 min of incubation to eliminate unspecific associated ATP. As control, γ-ATP was used instead of randomized $\beta\gamma$-ATP for incubation. Six independent samples were investigated, and results were given as average ± SE.

For the determination of the phosphate content of starch, 15 mg of isolated starch granules were deproteinized by toluene treatment (see "In Vitro

Binding of Soluble GWD to Starch Granules''), lyophilized, and digested by pressurized microwave decomposition (equipment PMD2; Kürner, Rosenheim, Germany) with 1 mL of concentrated HNO_3 (suprapur; Merck, Darmstadt, Germany) for 8 min. The resulting solutions were diluted with water to 5.0 mL, adjusted to pH 3.0 to 4.0 by 5 M NaOH, and the phosphate content was measured by the molybdate method (Worsfold et al., 1987). Phosphomolybdenum blue was formed and measured at 880 nm in a spectrophotometer. Six independent samples were investigated for each treatment. Results were given as average ± SE.

ACKNOWLEDGMENTS

Without the support by Prof. Dr. M. Steup and Dr. G. Ritte, University of Potsdam, Germany, this work would not have been possible. We also thank Ms. Gabriele Lenk for skillful technical assistance and Prof. Halina Gabrys, Jagiellonian University, Krakow, Poland, for critical proofreading of the manuscript.

Received November 20, 2003; returned for revision January 27, 2004; accepted January 28, 2004.

LITERATURE CITED

Appenroth K-J, Augsten H (1990) Photophysiology of turion germination in *Spirodela polyrhiza* (L.) SCHLEIDEN. V. Demonstration of a calcium-requiring phase during phytochrome-mediated germination. Photochem Photobiol **52:** 61–66

Appenroth K-J, Augsten H, Liebermann B, Feist H (1982) Effects of light quality on amino acid composition of proteins in *Wolffia arrhiza* (L.) WIMM. using a specially modified BRADFORD method. Biochem Physiol Pflanzen **177:** 251–258

Appenroth K-J, Gabrys H (2001) Light-induced starch degradation in non-dormant turions of *Spirodela polyrhiza*. Photochem Photobiol **73:** 77–82

Appenroth K-J, Gabrys H (2003) Ion antagonism between calcium and magnesium in phytochrome-mediated degradation of storage starch in *Spirodela polyrhiza*. Plant Sci **165:** 1261–1265

Appenroth K-J, Teller S, Horn M (1996) Photophysiology of turion formation and germination in *Spirodela polyrhiza*. Biol Plant **38:** 95–106

Bjellqvist B, Hughes GJ, Pasquali Ch, Paquet N, Ravier F, Sanchez J-C, Frutiger S, Hochstrasser DF (1993) The focusing positions of polypetides in immobilized pH gradients can be predicted from their amino-acid-sequences. Electrophoresis **14:** 1023–1031

Blennow A, Engelsen SB, Nielsen TH, Baunsgaard L, Mikkelsen R (2002) Starch phosphorylation: a new front line in starch research. Trends Plant Sci **7:** 445–450

Blennow A, Hansen M, Schulz A, Jørgensen K, Donald AM, Sanderson J (2003) The molecular deposition of transgenically modified starch in the starch granule as imaged by functional microscopy. J Struct Biol **143:** 229–241

Doelger K, Tirlapur UK, Appenroth K-J (1997) Phytochrome-regulated starch degradation in germinating turions of *Spirodela polyrhiza*. Photochem Photobiol **66:** 124–127

Engelsen SB, Madsen AO, Blennow A, Mottawia SM, Moller BL, Larsen S (2003) The phosphorylation site in double helical amylopectin as investigated by a combined approach using chemical synthesis, crystallography and molecular modelling. FEBS Lett **541:** 137–144

Henssen A (1954) Die Dauerorgane von *Spirodela polyrrhiza* (L.) Schleid. in physiologischer Betrachtung. Flora **141:** 523–566

Hippler M, Klein J, Fink A, Allinger T, Hoerth P (2001) Towards functional proteomics of membrane protein complexes: analysis of thylakoid membranes from *Chlamydomonas reinhardtii*. Plant J **28:** 595–606

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:** 680–685

Ley S, Doelger K, Appenroth K-J (1997) Carbohydrate metabolism as a possible modulator of dormancy in turions of *Spirodela polyrhiza* (L.) Schleiden. Plant Sci **129:** 1–7

Lorberth R, Ritte G, Willmitzer L, Kossmann J (1998) Inhibition of a starch-granule-bound protein leads to modified starch and repression of cold sweetening. Nat Biotechnol **16:** 473–477

Mikkelsen R, Baunsgaard L, Blennow A (2004) Functional characterization of α-glucan, water dikinase, the starch phosphorylating enzyme. Biochem J **377:** 525–532

Morrison WR, Karkalas J (1990) Starch. *In* PJ Lea, ed, Methods in Plant Biochemistry, Vol 2. Academic Press, London, pp 323–352

Nielsen TH, Wischmann B, Enevoldsen K, Moller BL (1994) Starch phosphorylation in potato-tubers proceeds concurrently with de-novo biosynthesis of starch. Plant Physiol **105:** 111–117

Reimann R, Ritte G, Steup M, Appenroth K-J (2002) Association of α-amylase and the R1 protein with starch granules precedes the initiation of net starch degradation in turions of *Spirodela polyrhiza*. Physiol Plant **114:** 2–12

Ritchie S, Swanson SJ, Gilroy S (2000) Physiology of the aleurone layer and starchy endosperm during grain development and early seedling growth: new insights from cell and molecular biology. Seed Sci Res **10:** 193–212

Ritte G, Eckermann N, Haebel S, Lorberth R, Steup M (2000a) Compartmentation of the starch-related R1 protein in higher plants. Starch Staerke **52:** 179–185

Ritte G, Lloyd JR, Eckermann N, Rottmann A, Kossmann J, Steup M (2002) The starch-related R1 protein is an α-glucan, water dikinase. Proc Natl Acad Sci USA **99:** 7166–7171

Ritte G, Lorberth R, Steup M (2000b) Reversible binding of the starch-related R1 protein to the surface of transitory starch granules. Plant J **21:** 387–391

Ritte G, Steup M, Kossmann J, Lloyd JR (2003) Determination of the starch-phosphorylating enzyme activity in plant extracts. Planta **216:** 798–801

Sauve DM, Ho DT, Roberge M (1995) Concentration of dilute protein for gel electrophoresis. Anal Biochem **226:** 382–383

Smith AM, Zeeman SC, Thorneycroft D, Smith AM (2003) Starch mobilization in leaves. J Exp Bot **54:** 577–583

Waffenschmidt S, Jaenicke L (1987) Assay of reducing sugars in the nanomole range with 2,2′-bicinchoninate. Anal Biochem **165:** 337–340

Worsfold PJ, Clinch JR, Casey H (1987) Spectrophotometric field-monitor for water-quality parameters: the determination of phosphate. Anal Chim Acta **197:** 43–50

Yu T-S, Kofler H, Hausler RE, Hille D, Flugge UI, Zeeman SC, Smith AM, Kossmann J, Lloyd J, Ritte G, et al. (2001) The *Arabidopsis sex1* mutant is defective in the R1 protein, a general regulator of starch degradation in plants, and not in the chloroplast hexose transporter. Plant Cell **13:** 1907–1918