The Two Plastidial Starch-Related Dikinases Sequentially Phosphorylate Glucosyl Residues at the Surface of Both the A- and B-Type Allomorphs of Crystallized Maltodextrins But the Mode of Action Differs\(^1\)

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In this study, two crystallized maltodextrins were generated that consist of the same oligoglucan pattern but differ strikingly in the physical order of double helices. As revealed by x-ray diffraction, they represent the highly ordered A- and B-type allomorphs. Both crystallized maltodextrins were similar in size distribution and birefringence. They were used as model substrates to study the consecutive action of the two starch-related dikinases, the glucan, water dikinase and the phosphoglucan, water dikinase. The glucan, water dikinase and the phosphoglucan, water dikinase selectively esterify glucosyl residues in the C6 and C3 positions, respectively. Recombinant glucan, water dikinase phosphorylated both allomorphs with similar rates and caused complete glucan solubilization. Soluble neutral maltodextrins inhibited the glucan, water dikinase-mediated phosphorylation of crystalline particles. Recombinant phosphoglucan, water dikinase phosphorylated both the A- and B-type allomorphs only following a prephosphorylation by the glucan, water dikinase, and the activity increased with the extent of prephosphorylation. The action of the phosphoglucan, water dikinase on the prephosphorylated A- and B-type allomorphs differed. When acting on the B-type allomorph, by far more phosphoglucans were solubilized as compared with the A type. However, with both allomorphs, the phosphoglucan, water dikinase formed significant amounts of monophosphorylated phosphoglucans. Thus, the enzyme is capable of acting on neutral maltodextrins. It is concluded that the actual carbohydrate substrate of the phosphoglucan, water dikinase is defined by physical rather than by chemical parameters. A model is proposed that explains, at the molecular level, the consecutive action of the two starch-related dikinases.

In terms of quantity, starch is one of the most prominent photosynthesis-derived products. The global starch production by land plants has been estimated to be approximately 2,850 million tons per year (Burrell, 2003). Starch is highly relevant for nutrition in animals and humans, but it is also used for many industrial applications, such as additives in paper or textiles and in pharmacy products as well. In addition, starch appears to be increasingly important as a photosynthesis-based renewable energy source that can be converted into technologically relevant products such as bioethanol and hydrogen (Hannah and James, 2008; Zhang et al., 2008).

Native starch is formed as a water-insoluble particle called a granule that is thought to comprise two types of polyglucans, amylopectin and amylose. The latter is an almost unbranched \(\alpha\)-1,4-glucan and usually is the minor constituent of the starch particle, accounting for 10% to 35% of the total starch dry weight (Ball, 2000). However, in some mutants, the relative amylose content is strongly diminished, resulting in an essentially amylose-free starch (such as in the \textit{waxy} mutant of maize \([\textit{Zea mays}]\), or, alternatively, it is increased, forming up to 70% of the starch mass (e.g. in the \textit{amylose extender} mutant from maize; Gérard et al., 2001). Nevertheless, in wild-type starches, amylopectin typically is the major constituent that also is essential for the molecular organization of the glucans within the entire starch granule (Ball and Morell, 2003). Like glycogen, amylopectin is a branched \(\alpha\)-glucan with 4% to 6% of the inter-Glc linkages being \(\alpha\)-1,6-bonds (Ball, 2000); however, as opposed to glycogen, the branching

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points occur as intramolecular clusters. Due to the length distribution of the side chains and the clustering of the branching points, neighboring glucan chains are capable of forming highly ordered double helices (Smith, 2001; Zeeman et al., 2002).

As revealed by x-ray diffraction analysis, two major native starch structures are known that differ in the arrangement of the double helices. The A-type allomorph, which is typical of wild-type cereal starches but also occurs in lower plants, is more compact, as compared with the B type, and consists of flat layers of double helices. By contrast, in the B-type allomorph, six double helices are thought to surround a central cavity that is filled with water molecules. The B-type allomorph is found in starch synthesized by dicotyledonal storage organs, such as potato (Solanum tuberosum) tubers, in some high-amylose starches from cereal mutants (Gallant et al., 1997; Gérard et al., 2001), and in assimilatory starches from potato and Arabidopsis (Arabidopsis thaliana) as well (Hejazi et al., 2008). Legume starches are believed to represent another allomorph that is designated the C type. However, this allomorph is actually a mixture of both the A- and B-type crystallites within a single native starch particle rather than a third distinct type of the double helical arrangement (Imbert et al., 1991; Bogracheva et al., 2001).

It should be noted that both the A- and B-type allomorphs of native starch granules often contain, as a minor constituent, an additional crystal structure designated the V type. Unlike the A- and B-type allomorphs, the V type is assumed to arise from single amylose helices, some of which are complexed with endogenous granular lipids. When estimated for the dry state, the V-type crystal structure accounts for only a small percentage of the total starch granule crystallinity (Lopez-Rubio et al., 2008).

The physical structure of the native starch particle is likely to have important biochemical implications, as it affects the performance of carbohydrate-active enzymes and, thereby, the transition of carbohydrates from the solid phase to the soluble phase. This conclusion has been reached by in vitro experiments demonstrating that the pancreas α-amylase hydrolyzes A-type starch faster than the B-type counterpart (Gérard et al., 2001).

Another metabolically important feature of amylopectin is the occurrence of covalent modification by phosphate esters that are found in a small proportion of the glucosyl residues. Most frequently phosphorylation occurs at the C6 position of the glucosyl residue, but C3 and, to a minor extent, C2 can also be esterified (Hizukuri et al., 1970). Recently, evidence has been presented that the esterification of the C6 and C3 positions of glucosyl residues differs in the structural effects on the neighboring inter-Glc bonds (Hansen et al., 2009). Phosphorylation at C6 is mediated by the recently identified α-glucan, water dikinase (GWD; EC 2.7.9.4), which utilizes ATP as dual phosphate donor and three distinct acceptors, two of which are sequentially used. The enzyme transfers the terminal phosphate group to water (thereby forming orthophosphate) and the β-phosphate group first to a conserved His residue within the catalytic domain of the monomeric GWD and, subsequently, to the C6 target of the glucosyl residue to be phosphorylated (Ritte et al., 2002, 2006). Phosphorylation at C3 is catalyzed by a second dikinase, designated phosphoglucaen water dikinase (PWD; EC 2.7.9.5; Ritte et al., 2006). The amino acid sequence of the catalytic (C-terminal) domain of PWD shares similarity with that of GWD, and in principle, the PWD-mediated catalysis follows the same mode of action as GWD, including the transient autophosphorylation at a conserved His residue (Baunsgaard et al., 2005; Köttig et al., 2005). However, PWD deviates from GWD in the amino acid sequence of the N-terminal domain, especially in the carbohydrate-binding region. PWD possesses a single carbohydrate-binding module that has been grouped into the family CBM20 (Machovič and Janaček, 2006a, 2006b). By contrast, the N-terminal domain of GWD contains two putative carbohydrate-binding motifs similar to those of an α-amylase that presumably is located in the chloroplasts (Yu et al., 2005). However, the structure of these motifs is still not known; therefore, a sequence-based prediction of the actual carbohydrate target is not yet possible.

GWD- and PWD-deficient Arabidopsis mutants possess to some extent similar but not equal phenotypes. Leaves of GWD-deficient lines (which contain essentially unchanged levels of functional PWD) have starch levels that are at least five times higher than those of the wild type and remain high even after prolonged darkness. Growth of the entire plant is strongly compromised. The phenotype of PWD-deficient mutants (which express functional GWD) is less severe, as growth is only slightly diminished and transitory starch levels are elevated but not as strongly as in the GWD-deficient lines. Mutants lacking functional PWD can degrade transitory starch, but net degradation occurs at a lower rate as compared with wild-type plants (Kötting et al., 2005). These data clearly indicate that, in vivo, PWD cannot substitute for GWD and that glucosyl 6-phosphate residues are involved in a more strict control of the starch turnover as compared with the C3 phosphate esters.

When considering the metabolic function(s) of starch phosphorylation, it should be noted that phosphorylation occurs during both net starch synthesis and degradation, although the rates of phosphorylation are likely to be different (Nielsen et al., 1994; Ritte et al., 2004). It is reasonable, therefore, to assume that starch phosphorylation exerts an important role in the entire transitory starch metabolism, rather than being functional only during the degrading process (and, consequently, the starch-related dikinases cannot, in a strict sense, be considered as “starch-degrading enzymes”).

Depending on the botanical source, the degree of starch phosphorylation varies strongly. In potato tuber
starch, approximately 0.1% to 0.5% of the glucosyl residues are phosphorylated (Ritte et al., 2002), and this value is considered to be indicative of a high level of phosphorylation. By contrast, cereal starches contain a far lower relative phosphate content that often is close to the limit of detection (approximately 0.002%; Glaring et al., 2006). In principle, these differences could be due to different rates of phosphorylation, as catalyzed by the two starch-related dikinases, and this assumption seems to be supported by the observation that, in general, starches of the B-type allomorph appear to have a higher degree of phosphorylation as compared with those of the A-type allomorph. If so, the dikinases may preferentially act on the B-type allomorph. Alternatively, the phosphorylation catalyzed by the two dikinases could be balanced by counteracting phosphatases, such as SEX4. This plastidial enzyme has been shown to act as a (phospho) glucan phosphatase that is involved in leaf starch metabolism (Kötting et al., 2009). If antagonistic enzyme activities are taken into consideration, the actual level of starch phosphorylation is determined by the rate of both phosphorylation and the subsequent hydrolysis of phosphate esters and, consequently, does not necessarily reflect the action of the starch-related dikinases.

Recently, crystallized maltodextrins (MDcryst) have been prepared that, by using x-ray diffraction, were identified as being the B-type allomorph and to possess a highly ordered structure (which exceeds that of native starch granules). MDcryst have been applied as a substrate for a recombinant GWD from potato. Using a carefully optimized assay, the rate of phosphorylation was by far higher than that observed with any other carbohydrate substrate, such as native starch granules or starch-derived polysaccharides. By contrast, solubilization by heat treatment of the MDcryst almost completely abolished the activity of GWD. Phosphorylation resulted in the formation of singly, doubly, and triply phosphorylated glucans and favored the solubilization of both neutral glucans and phosphoryl-glucans (Hejazi et al., 2008). Recombinant PWD also phosphorylated MDcryst provided the MDcryst had been prephosphorylated by GWD and were not solubilized by heat treatment (Hejazi et al., 2008).

Because of the high phosphorylation rates and the phosphorylation pattern obtained, MDcryst are a suitable model carbohydrate that mimics phosphorylation-relevant features of highly ordered regions within the native starch granule. It allows study of the action of the two starch-related dikinases and the transition of carbohydrates from the solid to the soluble state without any other starch-related enzyme being required.

Until now, only the B-type allomorph of the MDcryst has been applied as substrate of the two dikinases. Using native starch granules as a target, the rates of phosphorylation as obtained with recombinant GWD varied largely within the B-type allomorph (Hejazi et al., 2008); therefore, it is reasonable to assume that additional but largely unknown features of the native starch granule also strongly affect the action of GWD. This implies that any preference or specificity of the starch-related dikinases for a given allomorph can be analyzed most convincingly if MDcryst preparations representing both the B- and A-type allomorphs are available.

In this study, we used two MDcryst preparations that are indistinguishable in their oligoglucan patterns but differ in the physical arrangement of the double helices and represent the highly ordered A- and B-type allomorphs. Using these two MDcryst preparations, we analyzed the action of the two starch-related dikinases. The size distribution of the MDcryst particles has been determined using the Coulter counter, and surface properties of both allomorphs were monitored by scanning electron microscopy. Thermal stability of the two allomorphs was analyzed by measuring the temperature dependence of light scattering. Finally, the phosphorylation-dependent solubilization of both allomorphs and the transition of (phospho)glucans into the soluble state have been studied.

RESULTS

Physical and (Physico)chemical Properties of the A- and B-Type Allomorphs of MDcryst

In a previous study, we used commercially available maltodextrins to prepare MDcryst representing the B-type allomorph (Hejazi et al., 2008). However, we were unable to prepare any A-type MDcryst when using the same starting material and following the procedure described by Gidley and Bulpin (1987). Therefore, no experiments could be designed that allowed us to elucidate any possible allomorph preference of the two starch-related dikinases.

As we assumed that a more homogeneous starting material could facilitate the crystallization of the two allomorphs, we established a procedure enabling us to select a relatively narrow size range of linear α-glucans. Many commercially available MDs are dominated by small-sized constituents having a degree of polymerization (DP) ranging from 1 to 4 that do not (co)crystallize. In addition, they usually contain medium-sized dextrans (whose DP ranges from 6 to approximately 40) and, as very minor constituents, larger glucans with a DP exceeding 40. In principle, the complexity of a given MD preparation can be diminished by conventional fractionating techniques, such as precipitation within a narrow range of ethanol-water mixtures. However, when applied in a preparative scale, the efficiency of these techniques is often limited, as less abundant larger dextrans tend to coprecipitate with highly abundant smaller ones.

To overcome these limitations, a two-step fractionation procedure was established that precedes the actual crystallization of the A- and B-type allomorphs. It includes, first, the crystallization of a commercially
available MD preparation that results in the B-type allomorph and, more importantly, quantitatively excludes maltodextrins with a DP of less than 5. Second, following solubilization of the crystallized dextrins by heat treatment, they were subjected to a fractionated precipitation between 60% and 70% (v/v) ethanol. Under the conditions established, precipitation with 60% (v/v) ethanol reliably removes higher M<sub>m</sub> glucans, but medium-sized maltodextrins are retained in solution even when performed in a preparative scale. Finally, the ethanol concentration is raised to 70% (v/v) and the MDs that are insoluble between 60% and 70% (v/v) ethanol are used as starting material for either of the two crystallization procedures described by Gidley and Bulpin (1987). In the following, the crystallization starting with a 30% (w/v) MD solution at 4°C is designated procedure I and the other one (50% [w/v]; 37°C) is designated procedure II.

The MD<sub>cryst</sub> obtained by either procedure were characterized by various analytical techniques. The results are shown in Figure 1 and Table I. First, aliquots of the two MD<sub>cryst</sub> suspensions were solubilized by heat treatment, and the oligoglucan patterns were determined by high-performance anion-exchange chromatography pulsed-amperometric detection (HPAEC-PAD). For both preparations, the MD patterns were indistinguishable. They consist of DP ranging from 5 to approximately 30 (Fig. 1A). It should be noted that the sensitivity of the PAD tends to decrease with increasing size of the carbohydrates; therefore, the signal intensities do not strictly reflect the relative abundance of the various maltodextrins. Nevertheless, the conclusion is safe that the two crystallization procedures do not cause any noticeable chemical diversification of the MD<sub>cryst</sub> preparations. Second, the size distribution of the two MD<sub>cryst</sub> particles was determined using a Coulter counter (Fig. 1B). The particles obtained by procedure I possessed a slightly smaller size and a relatively wider size distribution than those prepared by procedure II. For the former, the most frequently observed particle diameter was 7 to 8 μm, whereas the corresponding value for the latter was 12 to 13 μm. For both MD<sub>cryst</sub> preparations, the maximum particle size was indistinguishable. Importantly, both MD<sub>cryst</sub> preparations are sufficiently stable when suspended in buffer and agitated using exactly the conditions applied for the dikinase assays (see below). Stability was ensured by monitoring the size distribution following the resuspension of both MD<sub>cryst</sub> preparations in the reaction buffer (lacking any recombinant dikinase) and continuous agitation (Fig. 1B).

Both the MD<sub>cryst</sub> prepared by either procedure I or II were analyzed by x-ray diffraction using a NanoStar instrument that allows carbohydrate particles to be analyzed in a fully hydrated state (for details, see Hejazi et al., 2008). For comparison, native starch granules from potato tubers (B type) and from wild-type maize kernels (A type) were also analyzed using the same conditions. The x-ray diffraction patterns clearly show that the MD<sub>cryst</sub> prepared by procedure I resemble native starch granules from potato tubers and represent the B-type allomorph. By contrast, the MD<sub>cryst</sub> obtained by procedure II is clearly the A-type allomorph, which is similar to the native starch from wild-type maize kernels (Fig. 1C). We also isolated native starch from leaves of greenhouse-grown wild-type maize plants and analyzed the transitory starch under exactly the same conditions. These maize starch particles exhibited the B-type allomorph, as does potato leaf starch (data not shown). When applying the method of Nara and Komiy (1983), for both MD<sub>cryst</sub> preparations a degree of crystallinity indistinguishable from unity was observed. By contrast, the two native starch preparations possess a lower degree of order (Fig. 1C; Table I). A similar observation had been made for a previously described MD<sub>cryst</sub> preparation representing the B-type allomorph (Hejazi et al., 2008). However, the latter preparation contained a wider DP range covering values from 6 to approximately 40 (Hejazi et al., 2008).

Based on the x-ray diffraction patterns, the crystallite sizes of the two MD<sub>cryst</sub> preparations and both native starch granules as well were determined using the Scherrer equation (Table I). Both MD<sub>cryst</sub> preparations possess a crystallite size larger than that of the two native starch granules.

Light microscopic analyses using polarized light are consistent with the data shown in Figure 1, B and C. Both the A-type (Fig. 1D) and B-type (Fig. 1E) allomorphs of the MD<sub>cryst</sub> possess birefringence and exhibit a characteristic polarization cross. Size distribution of the A-type particles is more homogeneous than that of the B type.

The thermal stability of the two MD<sub>cryst</sub> preparations was studied in more detail by monitoring the temperature-dependent light scattering. Under continuous stirring, a diluted suspension of the two MD<sub>cryst</sub> preparations was subjected to a controlled heating (5°C min<sup>−1</sup>), and scattering at 532 nm was continuously monitored. During solubilization of the MD<sub>cryst</sub> particles, the light scattering decreases by several orders of magnitude (Fig. 1F); therefore, a temperature can be defined that reflects 50% loss of scattering (T<sub>50</sub>). For the A- and B-type allomorphs, the T<sub>50</sub> value is 79°C and 61°C, respectively. It should be noted that similar differences have been observed for native starch granules: B-type granules tend to be disrupted at lower temperatures as compared with A-type granules (Brogachova et al., 1997).

When analyzing several independently prepared A-type MD<sub>cryst</sub> we consistently observed an increase in scattering that takes place in a relatively narrow range of elevated temperatures at or immediately before the onset of the disintegration of the particles and that is maximal at approximately 75°C (Fig. 1F). As revealed by light microscopic evaluation, this phenomenon (that never has been observed with the B-type MD<sub>cryst</sub> allomorph) is due to a significant degree of aggregation of the A-type MD<sub>cryst</sub> particles at higher temperatures (data not shown).
Figure 1. Physical and (physico)chemical properties of the A- and B-type allomorphs of MD\textsubscript{cryst}. A, MD patterns of MD\textsubscript{cryst} of the B-type (procedure I) and the A-type (procedure II) allomorphs as revealed by HPAEC-PAD. Aliquots of both particle suspensions were solubilized by heat treatment, and equal amounts (30 µg of glucosyl residues each) were applied to a PA100 column. In the eluate, carbohydrates were monitored by PAD. Signal intensities (relative units but equal sensitivity) are plotted against the elution time. DP6 and DP15, MD having DP of 6 and 15, respectively. B, Size distribution of the A- and B-type allomorphs of MD\textsubscript{cryst} as revealed by Coulter counter. For both particle preparations, size distribution was monitored before incubation (solid line) and after incubation for 10 min (30°C) in the absence of GWD but in the presence of the reaction buffer under continuous agitation (broken line). C, X-ray diffraction patterns of MD\textsubscript{cryst} obtained by procedure I (B type) and procedure II (A type) as determined using a NanoStar instrument. For comparison, native starch granules from kernels of wild-type maize or from tubers of potato were analyzed under identical conditions. D and E, Polarization light microscopic evaluation of A-type (D) and B-type (E) MD\textsubscript{cryst}. Magnification, ×600. F, Temperature-dependent light scattering of A- or B-type MD\textsubscript{cryst}. Light scattering at 532 nm (relative units) is plotted against the temperature. a.u., Arbitrary units.
Structural features of A- and B-type MD\textsubscript{cryst} and of native starch granules as revealed by x-ray diffraction analysis

<table>
<thead>
<tr>
<th>Sample</th>
<th>Degree of Crystallinity</th>
<th>Crystallite Size (nm)</th>
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<tbody>
<tr>
<td>Potato tuber</td>
<td>0.78</td>
<td>9.8</td>
</tr>
<tr>
<td>Maize kernel</td>
<td>0.52</td>
<td>8.8</td>
</tr>
<tr>
<td>A-type MD\textsubscript{cryst}</td>
<td>1</td>
<td>15.2</td>
</tr>
<tr>
<td>B-type MD\textsubscript{cryst}</td>
<td>1</td>
<td>18.5</td>
</tr>
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</table>

Presumably, another observation is more relevant for the enzymological studies described below: the graphs shown in Figure 1F clearly rule out that the preparation of the A-type allomorph is, to any noticeable extent, cross-contaminated with that of the B-allomorph and vice versa. The same conclusion is derived from the x-ray patterns (Fig. 1C). However, due to the largely different T\textsubscript{50} values, the temperature-dependent light scattering permits a more sensitive test for the homogeneity of each of the two MD\textsubscript{cryst} preparations. Several independent preparations of each allomorph were obtained using the two crystallization procedures. The chemical and physical features of the various MD\textsubscript{cryst} preparations were very similar to those shown in Figure 1 and Table I.

In summary, the data shown in Figure 1 and Table I clearly demonstrate that the two MD\textsubscript{cryst} preparations possess indistinguishable chemical features but differ in structural properties (i.e. the physical arrangement of double helices). Therefore, in biochemical studies, they can be used as model carbohydrate particles that mimic structural features of native starch granules representing either the A-type or the B-type allomorph.

Recombinant stGWD Phosphorylates Both the A- and B-Type MD\textsubscript{cryst}

The two MD\textsubscript{cryst} allomorphs were used to determine the initial rates of the C6 phosphorylation as catalyzed by recombinant stGWD. The GWD standard assay was applied, but varying amounts of the A- and B-type allomorphs (up to 12 mg each) were added. Reaction mixtures were incubated for 10 min under continuous agitation. Subsequently, the MD\textsubscript{cryst} preparations were solubilized by heat treatment, and the total 33P content of the (phospho)maltodextrins was monitored by phosphorimaging following thin-layer chromatography. The assay conditions (such as the continuous agitation in the reaction buffer) did not alter the size distribution of the MD\textsubscript{cryst} (compare with Fig. 1B). Recombinant stGWD did phosphorylate both the A- and B-type allomorphs, and the phosphorylation rates obtained were similar. For both the A- and B-type allomorphs, the rates increased with increasing amounts of MD\textsubscript{cryst} but saturating levels of the maltodextrin particles were not achieved (Fig. 2). When the stGWD-catalyzed reaction was terminated after 5 min using otherwise identical assay conditions, essentially the same specific activities were obtained (data not shown).

In the next series of experiments, the 33P contents of the insoluble and soluble (phospho)glucans were determined separately. At 10-min intervals, aliquots of the reaction mixtures were withdrawn and centrifuged. The supernatant was immediately analyzed by thin-layer chromatography and phosphorimaging. The pellet was washed with water, solubilized by heat treatment, and then processed as described before. The total reaction time was extended to 60 min using the conditions defined for GWD standard assay. For both the A- and B-type allomorphs, the ratio between the insoluble and the soluble 33P labels changed during incubation: during the first 30 min, the majority of the radioactivity was recovered in the insoluble state, but during the second half of the incubation period, the 33P content of the soluble fraction increased more strongly than that of the pellet (Fig. 3). These data clearly show that the GWD-mediated phosphorylation of both the A- and B-type allomorphs of MD\textsubscript{cryst} initiates solubilization of the (phospho)glucans.

For a more detailed analysis of the GWD action on both allomorph types, the phosphorylation patterns in the soluble and insoluble (phospho)glucans were determined. In addition, the total amount of the solubilized glucosyl residues was determined enzymatically following acid hydrolysis. After 30 or 60 min of incubation, aliquots of the soluble (phospho)glucans were subjected to HPAEC-PAD. Following the separation into singly, doubly, and triply phosphorylated glucans, the label of the (phospho)glucans was quantified by liquid scintillation counting. Likewise, insol...
uble (phospho)glucans were solubilized by heating. Subsequently, they were chromatographed and quantified as described above. The degree of phosphorylation was confirmed by matrix-assisted laser-desorption ionization-mass spectrometry (for details, see Hejazi et al., 2008). The data obtained for the 60-min reaction period are shown in Table II. (Phospho)glucans released from both the A- and B-type MD<sub>cryst</sub> consisted mostly of monophosphorylated glucans, but doubly phosphorylated (phospho)glucans and, although as a very minor constituent, triply phosphorylated (phospho)glucans were clearly detectable. For the A-type-derived soluble (phospho)glucans, the abundance of the monophosphorylated maltodextrins was slightly higher than that for the B-type-derived compounds. In the insoluble fractions of both the A- and B-type allomorphs, singly phosphorylated dextrins were dominant, whereas incorporation into the doubly phosphorylated compounds accounted for lesser than 1% of the total label (Table II). For both allomorphs, triply phosphorylated glucans were below the limit of detection. The phosphorylation patterns obtained after a phosphorylation period of 30 min are similar to those shown in Table II, but as expected, the incorporation of phosphate is lower (data not shown).

During the GWD action on both the A- and B-type allomorphs, neutral maltodextrins were released in addition to the phosphoglucans, but quantitatively the two allomorphs differed. From the B type, 4-fold more neutral maltodextrins were converted into the soluble state as compared with the A-type allomorph (Table II). Similarly, more neutral maltodextrins were detected during separation by HPAEC-PAD (data not shown).

The experiments described above were restricted to the initiation of the GWD-dependent solubilization process; therefore, it remained uncertain whether or not the GWD action results in an almost complete solubilization of both allomorphs. For the following

### Figure 3.

stGWD-dependent <sup>32</sup>P incorporation into soluble or insoluble MD derived from the A-type (A) or the B-type (B) allomorph of MD<sub>cryst</sub>. The GWD standard assay was used except that the incubation time was extended to 60 min. At intervals, the soluble and insoluble maltodextrins were separated by centrifugation for 10 min at RT. The pellets were washed twice with water and dissolved in water. Following solubilization (5 min at 95°C), aliquots of both fractions were analyzed by thin-layer chromatography and phosphorimaging. Black symbols, <sup>32</sup>P content of the pelleted (phospho)glucans; white symbols, <sup>32</sup>P content of the soluble (phospho)glucans. \( n = 3 \).

### Table II. GWD-mediated phosphorylation and solubilization of A- and B-type MD<sub>cryst</sub>

Patterns of singly, doubly, and triply phosphorylated α-glucans in the soluble and insoluble state. Four milligrams of A- or B-type maltodextrin was incubated with GWD for 1 h at 30°C under continuous agitation. The samples were separated into soluble and insoluble fractions by centrifugation for 10 min at RT. The pellets were resuspended in water using an equal volume to that of the respective supernatant. Following solubilization for 10 min at 95°C, samples were passed through a 10-kD membrane filter, and the resulting filtrate was loaded onto the HPAEC column. Eluting compounds were detected by PAD. The 0.5-mL fractions were collected and mixed with 3 mL of scintillation cocktail, and radioactivity was monitored using a liquid scintillation counter. Radioactivity of the various phosphoglucans is given as percentage of the total <sup>32</sup>P content. Total glucosyl residues solubilized were subjected to acid hydrolysis and were then quantified using the photometric hexokinase/Glc-6-P dehydrogenase assay \(( n = 3 )\). n.d., Not detected.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Fraction</th>
<th>Phosphorylated MD</th>
<th>Glucosyl Residues Released</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Singly</td>
<td>Doubly</td>
</tr>
<tr>
<td>A-type MD&lt;sub&gt;cryst&lt;/sub&gt;</td>
<td>Supernatant</td>
<td>65.5</td>
<td>11.4</td>
</tr>
<tr>
<td></td>
<td>Pellet</td>
<td>21.3</td>
<td>0.33</td>
</tr>
<tr>
<td>B-type MD&lt;sub&gt;cryst&lt;/sub&gt;</td>
<td>Supernatant</td>
<td>53.6</td>
<td>12.7</td>
</tr>
<tr>
<td></td>
<td>Pellet</td>
<td>29.2</td>
<td>0.9</td>
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</tbody>
</table>
reasons, this is an important point to be clarified. Physical properties, as revealed by x-ray diffraction and temperature-dependent light scattering, reflect structural features of the entire MDcryst particles; therefore, they do not exclude the possibility that the surface of the particles may possess structural features deviating from those of the interior parts of MDcryst. If so, these surface-related features are difficult to detect, as they contribute little to the signal intensities derived from the entire particles. However, they could be essential for biochemical reactions, such as the action of GWD. Therefore, we tested for a massive solubilization of MDcryst using unlabeled ATP and monitored the release of glucans into the soluble phase. Over an extended period of time, the reaction mixtures were separated into an insoluble and a soluble fraction. The latter was subjected to an exhaustive acid hydrolysis, and the Glc formed was quantified by a coupled enzymatic assay. Preliminary experiments clearly indicated that during prolonged incubation, the activity of recombinant stGWD is not retained (see below). Therefore, at each time point, the reaction mixtures were centrifuged and, following the removal of the supernatant, the pelleted MDcryst were resuspended in a freshly prepared incubation buffer having the same composition as the previous one but containing a new sample of recombinant stGWD. The volume of the reaction mixtures remained unchanged over the entire incubation period. As a control, samples were treated identically but GWD was omitted.

During 12 h of GWD action, the majority of both the A- and B-type allomorph MDcryst was converted into the soluble state. This conversion was strictly dependent on the presence of GWD (data not shown). Because of the massive phosphorylation-dependent solubilization, it can be excluded that the action of GWD is restricted to the initial surface area of either of the MDcryst preparations (Table III).

The Morphology of the A- and B-Type MDcryst Particles during the Action of the Two Starch-Related Dikinases

This conclusion is supported by scanning electron microscopic analyses of the A- and B-type allomorphs of the MDcryst. Analyses were performed before and during the action of GWD. Consistent with the size distribution (as revealed by the Coulter counter) and the light microscopic evaluation (Fig. 1), both allomorphs occurred as a relatively homogeneous population of particles, but the morphology of both types differed. A-type particles possess a more complex particle structure and a more uneven surface (Fig. 4, A and B). By contrast, the B-type particles are more evenly formed and have a smooth surface (Fig. 4, C and D). During the action of stGWD, the entire structure of both types of particles is altered, but, depending on the allomorph, the alterations differ. In the entire population of the A-type allomorph particles, the surface is gradually smoothed (Fig. 4, E and F). This clearly indicates that the stGWD-mediated solubilization is not restricted to distinct areas of the particle surface but rather takes place on the entire surface of the particles and proceeds toward the interior part of the particles. By contrast, the GWD action on B-type allomorph particles results in structural diversification that takes place at the particle surface. The typical heterogeneity of the surface of the MD particles consists of structures that are vertically oriented toward the particle surface. These structures, which are never observed with the A-type allomorph, are shown in Figure 4, G and H. It should be noted that these alterations require both a catalytically active stGWD and the presence of ATP. When either ATP was omitted or the recombinant GWD had been inactivated by heat treatment, the structures of both types of particles remained unchanged during the entire incubation period (data not shown). Based on kinetic studies, it appears that the structural alterations as observed with the B-type allomorph reflect transitory states within the solubilization process (data not shown). Nevertheless, the phosphorylation-dependent transition of MDcryst to the soluble state occurs on the entire surface of both the A- and B-type allomorphs.

The Activity of Recombinant stGWD Is Inhibited by Soluble Maltodextrins

The decrease of the stGWD activity during the massive solubilization of MDcryst is due, at least in part, to an inhibitory effect that is exerted by soluble maltodextrins on the recombinant enzyme. This conclusion was reached in experiments in which a fixed amount of MDcryst (B-type allomorph) was incubated with a constant amount of stGWD and varying amounts of soluble maltodextrins. Two soluble maltodextrins were applied. First, soluble maltodextrins

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Table III. Prolonged GWD action results in a massive solubilization of the MDcryst

<table>
<thead>
<tr>
<th>Incubation Time</th>
<th>Glucosyl Residues Released</th>
</tr>
</thead>
<tbody>
<tr>
<td>h</td>
<td>A Type</td>
</tr>
<tr>
<td>1</td>
<td>6.2 ± 3.1</td>
</tr>
<tr>
<td>2</td>
<td>7.9 ± 1.1</td>
</tr>
<tr>
<td>3</td>
<td>11.4 ± 0.9</td>
</tr>
<tr>
<td>4</td>
<td>13.8 ± 3.3</td>
</tr>
<tr>
<td>5</td>
<td>15.3 ± 2.4</td>
</tr>
<tr>
<td>6</td>
<td>16 ± 0.8</td>
</tr>
<tr>
<td>7</td>
<td>21.9 ± 0.5</td>
</tr>
<tr>
<td>8</td>
<td>28.6 ± 1.4</td>
</tr>
<tr>
<td>9</td>
<td>31 ± 1.1</td>
</tr>
<tr>
<td>10</td>
<td>38.9 ± 1.1</td>
</tr>
<tr>
<td>11</td>
<td>43.3 ± 0.1</td>
</tr>
<tr>
<td>12</td>
<td>45 ± 1.1</td>
</tr>
</tbody>
</table>
were obtained by heat treatment of MD$_{cryst}$ (B type). These soluble maltodextrins possess exactly the same size distribution as the insoluble MD$_{cryst}$ acting as GWD substrate (Fig. 1A). The other soluble MD applied was a commercial sample (ICN Biomedicals; see “Materials and Methods”). The size distribution of this MD sample, as revealed by HPAEC-PAD, was dominated by DP 1 to 11; therefore, it clearly deviated from

**Figure 4.** Scanning electron micrographs of both the A- and B-type allomorphs of MD$_{cryst}$ without phosphorylation and following stGWD-mediated phosphorylation. A and B, A-type allomorph. C and D, B-type allomorph. E and F, A-type allomorph following 3 h of phosphorylation by stGWD. G and H, B-type allomorph following 3 h of phosphorylation by stGWD. Bars = 20 μm (A, C, E, and G) and 10 μm (B, D, F, and H).
that of the MD<sub>cryst</sub>-derived MD. The two MD preparations were similarly efficient at inhibiting stGWD despite the difference in size distribution. As a control, varying amounts of a commercially available sample of soluble dextrans from <i>Leuconostoc mesenteroides</i> were added to the reaction mixture. The dextran sample consists of α-1,6-linked oligoglucans, the majority of which have a DP ranging from 1 to 17 (data not shown). The soluble dextrans exerted a far less inhibitory effect on GWD (Table IV).

**The Action of Recombinant atPWD on Both the A- and B-Type Allomorphs of MD<sub>cryst</sub>**

The phosphorylation at the C3 position was studied using recombinant atPWD (EC 2.7.9.5). Both the A- and B-type allomorphs of the MD<sub>cryst</sub> served as carbohydrate substrates. Preliminary in vitro experiments clearly indicated that any PWD activity requires a prephosphorylation of both allomorphs by GWD. In order to selectively monitor the esterification catalyzed by PWD, prephosphorylation was performed using unlabeled ATP and [β-<sup>33</sup>P]ATP was exclusively used for the subsequent phosphorylation by atPWD. In order to vary the degree of prephosphorylation, both the A- and B-type MD<sub>cryst</sub> were prephosphorylated for varying periods of time. At intervals, stGWD was inactivated by adding SDS. Subsequently, the prephosphorylated MD<sub>cryst</sub> were incubated for 10 min with recombinant atPWD (250 ng each) and [β-<sup>33</sup>P]ATP. Following the inactivation of atPWD by adding EDTA, the reaction mixtures were separated into soluble and insoluble fractions by centrifugation. Both fractions were heated, and aliquots of the two solutions were analyzed by phosphorimaging following thin-layer chromatography.

**Table IV. The inhibition of recombinant stGWD by two soluble maltodextrin preparations**

<table>
<thead>
<tr>
<th>MD</th>
<th>GWD Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MD&lt;sub&gt;rel&lt;/sub&gt;</td>
</tr>
<tr>
<td></td>
<td>%</td>
</tr>
<tr>
<td>0</td>
<td>100 ± 3.0</td>
</tr>
<tr>
<td>0.5</td>
<td>67.4 ± 0.6</td>
</tr>
<tr>
<td>1.0</td>
<td>41.9 ± 1.5</td>
</tr>
<tr>
<td>1.5</td>
<td>33.7 ± 1.0</td>
</tr>
<tr>
<td>2.0</td>
<td>23.3 ± 0.8</td>
</tr>
</tbody>
</table>

**In both the A- and B-type allomorphs of the MD<sub>cryst</sub>**

the activity of PWD increased with increasing prephosphorylation catalyzed by stGWD (i.e. with the length of the prephosphorylation period). However, in the A-type allomorph, approximately 80% of the PWD-catalyzed <sup>33</sup>P incorporation remained in the insoluble state. By contrast, phosphorylation by atPWD of the prephosphorylated B-type allomorph resulted in solubilization of approximately two-thirds of the <sup>33</sup>P label in the soluble state (Fig. 5).

For a more detailed analysis of the distribution of the <sup>33</sup>P label within the soluble and pelletable maltodextrins, in each of the four (phospho)maltodextrin samples, singly, doubly, and triply phosphorylated α-glucans were separated and the <sup>33</sup>P contents were quantified; in addition, the total amount of glucosyl residues of the soluble maltodextrins was determined enzymatically. For monitoring the <sup>33</sup>P distribution, neutral maltodextrins were separated from (phospho)glucans by HPAC-PAD. The (phospho)glucans were separated into singly, doubly, and triply phosphorylated maltodextrins as described previously (Hejazi et al., 2008). The data are shown in Table V.

Four observations can be made. First, due to the PWD action on prephosphorylated B-type MD<sub>cryst</sub>, approximately 3-fold more glucosyl residues are converted into the soluble form as compared with the A-type allomorph. Second, the ratio of <sup>33</sup>P labeling of the soluble and insoluble MD differs depending on the A- or B-type allomorph. The soluble maltodextrins derived from the A-type MD<sub>cryst</sub> contain less than 10% of the <sup>33</sup>P-labeled glucosyl-3-phosphate residues, whereas approximately 90% of the <sup>33</sup>P label is recovered in the pellet fraction. By contrast, following the PWD action on the B-type allomorph, most of the <sup>33</sup>P label is observed in soluble MD. Third, the phosphorylation patterns of the soluble phosphoglucans differ depending upon the A- or B-type allomorph. In the A-type-derived soluble MD, monophosphorylated glucans are predominant. By contrast, the soluble (phospho)glucans derived from the B-type allomorph were dominated by triply phosphorylated glucans, accounting for 52% of the total label of soluble MD. Fourth, in the A-type-derived insoluble MD, more than 70% of the phosphoglucans exist as singly and triply phosphorylated dextrans, whereas in the B-type-derived pelletable (phospho)glucans, monophosphorylated glucans are more prominent (Table V).

It is important to note that in all samples analyzed, a significant proportion of the <sup>33</sup>P label was recovered as monophosphorylated chains. This implies that atPWD, although its catalytic activity depends on a prephosphorylation of MD<sub>cryst</sub> by GWD, is capable of utilizing neutral α-glucan chains as a substrate and that, at the level of the individual glucan chains, activity of atPWD is not restricted to (phospho)glucans. Thus, when considering the interaction of stGWD and atPWD, these data strongly suggest that the preceding GWD-mediated phosphorylation is essential for PWD as it alters physical properties of...
MD$_{\text{cryst}}$, such as the arrangement of (double) helices. For the PWD-mediated phosphorylation, the alterations of physical properties of MD$_{\text{cryst}}$ appear to be far more important than any chemical modification of single $\alpha$-glucan chains.

Furthermore, it should be noted that both the doubly and triply phosphorylated glucan chains, as separated by HPAEC-PAD, may consist of a mixture of chemically heterogeneous compounds. In principle, doubly phosphorylated $\alpha$-glucan chains can include phosphoglucans that carry a phosphate ester in both the C6 and C3 positions or, alternatively, two C3 esters. If phosphate esters occur in both the C6 and C3 positions, the latter is labeled with $^{33}$P. If, however, the phosphoglucan chain contains two C3 phosphate esters, at least one of them needs to carry $^{33}$P. Similarly, the triply phosphorylated glucan chains, in principle, consist of a mixture of up to three phosphoglucan chains that contain at least one $^{33}$P-labeled C3 ester but up to two C6-bound phosphates. Thus, the triply phosphorylated glucans may contain three C3 phosphate esters or two C3 plus one C6 phosphate esters or one C3 plus two C6 phosphate esters per molecule.

**DISCUSSION**

In this study, we used an in vitro assay in order to analyze the action of two starch-related dikinases. The assay comprises (at least) three distinct phases. One phase is soluble, and the products of the catalysis of GWD (or of PWD) finally enter this phase. The second phase is restricted to the surface of the MD$_{\text{cryst}}$, and in this phase, the dikinase-mediated $\alpha$-glucan phosphorylation takes place. As shown in Figures 3 and 4, the second phase is dynamic and gradually moves toward the center of the crystalline particles as the phosphorylation-dependent solubilization proceeds. The third phase (the size of which gradually decreases) occurs in the interior space of the crystalline particles. The $\alpha$-glucans in this phase are not immediately accessible to the dikinase; therefore, they do not participate in the phosphorylation (and the subsequent solubilization) unless the surface of the particles has progressively moved.

As both the phosphorylation and the solubilization of the MD$_{\text{cryst}}$ require the action of only one enzyme (i.e. GWD), it is likely that, in a spontaneous process, the

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**Table V.** PWD-mediated phosphorylation of A- and B-type MD$_{\text{cryst}}$

<table>
<thead>
<tr>
<th>Sample Fraction</th>
<th>Phosphorylated MD Glucosyl Residues Released</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>A-type MD$_{\text{cryst}}$</td>
<td>Supernatant</td>
</tr>
<tr>
<td></td>
<td>Pellet</td>
</tr>
<tr>
<td>B-type MD$_{\text{cryst}}$</td>
<td>Supernatant</td>
</tr>
<tr>
<td></td>
<td>Pellet</td>
</tr>
</tbody>
</table>
Phosphorylation of A- and B-Type Crystallized Maltodextrins

Phosphate esters formed locally increase the hydration of the (phospho)glucan chains and thereby favor solubilization at room temperature. Heat treatment results in a complete solubilization of both allomorphs without any enzymatic action being required.

In this study, both the A- and B-type allomorphs of MD<sub>cryst</sub> were included. Both allomorphs were derived from the same maltodextrin preparation, and following crystallization, their maltodextrin patterns are indistinguishable. This implies that (at least when using a relatively narrow MD size distribution) the two crystallization procedures chosen do not exert any size-dependent selectivity. Consequently, the two maltodextrins allowed us to experimentally test for any allomorph specificity or preference of GWD or PWD.

In in vitro experiments, stGWD phosphorylates both the A- and B-type allomorphs of MD<sub>cryst</sub> and the rates obtained with both allomorphs exceed those obtained with native starch granules (Hejazi et al., 2008). In fact, stGWD appears to phosphorylate the A type with a higher rate than the B type (Fig. 2). For a detailed evaluation of the two rates obtained, the physical features of the two MD<sub>cryst</sub> preparations should be compared (Fig. 1). Both particle preparations possess a similar but not identical size distribution. Therefore, equal amounts of both crystalline particles added to a reaction mixture are expected to result in a similar but not necessarily equal total surface area. As a further complication, the surface of the A-type particles is less even than that of the B type (Fig. 4). With both the A- and B-type allomorphs, no substrate saturation has been achieved (Fig. 2). Therefore, the initial phosphorylation rates are measured under similar but not necessarily identical conditions. The recombinant stGWD applied in the in vitro assays is derived from potato, which synthesizes the B-type allomorph both as a transitory and a reserve starch (Hejazi et al., 2008); therefore, under in vivo conditions, the enzyme is unlikely to ever act on an A-type allomorph starch. The high activity of stGWD observed with both allomorphs of MD<sub>cryst</sub> clearly rules out any obvious allomorph specificity or preference exerted by GWD phosphorylation. By contrast, the total amount of maltodextrins that are solubilized due to the action of GWD differs significantly. Solubilization is 4-fold higher with the B-type allomorph as compared with the A type. Thus, it appears that the phosphorylation-dependent transition into the soluble phase is more efficient with the B-type allomorph than with the A type (Tables II and III). Presumably, this difference is reflected by the lower thermal stability of the B-type allomorph (compare with Fig. 1).

Both the A- and B-type allomorphs of native starch granules contain highly ordered and less ordered regions as well. Probably, the MD<sub>cryst</sub> that were used in this study are suitable model substances only if those metabolic processes to be studied take place at the ordered regions of the granule. By contrast, the biochemistry of the less ordered regions appears to be different and may even not include the action of GWD. However, even the highly ordered areas of the native starch granules differ in an important feature from the MD<sub>cryst</sub>. Phosphorylation and hydration of the chains within the highly ordered regions of the starch granules do not result in a transition of the (phospho) glucans into the soluble phase, as they remain covalently bound via α-1,6-inter-Glc bonds; therefore, further enzyme activities are required for solubilization.

The data presented in this in vitro study clearly demonstrate that an efficient glucan phosphorylation catalyzed by GWD (and by PWD as well) does not require the occurrence or vicinity of any α-1,6-inter-Glc bonds. Obviously, these results do not necessarily exclude the possibility that, during native starch granule biosynthesis, the occurrence of branching points might be affected by phosphorylated sites.

The carbohydrate substrate of GWD appears to be defined by physical properties rather than by chemical features. As GWD phosphorylates both the A- and B-type allomorphs with similar rates, it is tempting to speculate that the vicinity of two α-glucan double helices may be an essential structural feature of the carbohydrate substrate of GWD.

The data shown in Figure 2 are consistent with in vitro experiments in which phosphorylation of native starch granules from *Chlamydomonas reinhardtii* was achieved by stGWD (data not shown). Under both normal photoautotrophic growth conditions and mineral deficiency (nitrogen or phosphorus deficiency), the starch formed by *Chlamydomonas* is the A-type allomorph (data not shown). Furthermore, the genome of *Chlamydomonas* contains at least four genes that putatively encode starch-related dikinases (Deschamps et al., 2008). It is reasonable, therefore, to assume that initial steps in the mobilization of the highly ordered regions of the native starch granules are essentially the same, irrespective of the A- or B-type allomorph, but the processes downstream of phosphorylation at the C6 position differ between the two allomorphs. Furthermore, the low stationary level of phosphate esters that is frequently observed in A-type starch granules (0.002%) is likely to be caused by the regulation of the starch-related dikinases and/or that of counteracting enzymes, such as the SEX4 laforin-type phosphatase (Kötting et al., 2009).

The data presented in Table IV clearly indicate that the recombinant stGWD is inhibited by soluble maltodextrins. This effect, however, is difficult to analyze in more detail, as the two soluble maltodextrin preparations used consist of a complex mixture of maltodextrins. Therefore, it is not inconceivable that, depending on the size of the maltodextrins, the inhibitory effect varies and some of the constituents of the soluble maltodextrins may even be ineffective.

Recombinant atPWD did phosphorylate both the A- and B-type allomorphs of MD<sub>cryst</sub> and provided a GWD-mediated prephosphorylation at the C6 position of some glucosyl residues (Table V); therefore, PWD acts downstream of GWD. A similar conclusion has been reached by in vitro assays using native starch
granules (Baunsgaard et al., 2005; Köttig et al., 2005). However, the data presented in Table V provide a mechanistic explanation of the strict dependence of PWD on a preceding phosphorylation. Due to the atPWD action, monophosphorylated α-glucans are formed in addition to doubly and triply phosphorylated MD. This implies that atPWD acts on neutral glucans and strongly suggests that the preceding phosphorylation at the C6 position generates the carbohydrate substrate of atPWD by altering physical rather than chemical features of MDcryst. Therefore, we propose that the actual carbohydrate substrate of PWD is a transition state from a highly ordered to a less ordered structure that is defined by the physical order of glucan chains and/or glucan double helices (Fettke et al., 2009). This conclusion is supported by the high rate of atPWD-mediated phosphorylation that has been observed with native starch granules from some mutant plants even without any preceding GWD-mediated phosphorylation (Fettke et al., 2009). Presumably, in these cases, starch-related mutations result in structural alterations at the starch granule surface that are similar to those achieved in wild-type starches as a consequence of the GWD-mediated phosphorylation. Unfortunately, the transition state that represents the carbohydrate substrate of PWD is difficult to analyze. In the case of the B-type allomorph of MDcryst, it is tempting to speculate that the structural changes that are visible during the stGWD-mediated solubilization are related to this transition state (Fig. 4, G and H), but this speculation is difficult to prove or disprove experimentally.

MATERIALS AND METHODS

Plant Materials

Wild-type maize (Zea mays) plants were grown in a local greenhouse.

Chemicals and Enzymes

\((\beta-3')\)ATP (3,000 Ci mmol\(^{-1}\)) was purchased from Hartmann Analytic. Maltodextrins were obtained either from Sigma-Aldrich (product no. EC 232-940-4) or from ICN Biomedicals (product no. 960048). Dextrins from Leuco-nostoc mesenteroides were purchased from Fluka (product no. 2326775). Native starch granules from maize kernels (product no. EC 232-679-6) were obtained from Sigma-Aldrich, and native potato (Solanum tuberosum) tuber starch (product no. 102954) was purchased from ICN Biomedicals. Polyglucans were quantified using the starch kit from Roche Diagnostics. PEI-Cellulose F thin-layer plates were purchased from Merck. Recombinant GWD from potato and PWD from Arabidopsis (Arabidopsis thaliana; At5g26570) were heterologously expressed and purified as described previously (Ritte et al., 2002; Köttig et al., 2005).

Fractionation and Crystallization of Maltodextrins

**Fractionation**

For crystallization procedures I and II described below, the same maltodextrin fractionation method was applied. Commercial maltodextrins (Sigma-Aldrich) were freed from low dextrin fractionation method was applied. Commercial maltodextrins (Sigma-Aldrich) were freed from low dextrin fractionation method was applied. Commercial maltodextrins (Sigma-Aldrich) were freed from low dextrin fractionation method was applied. Commercial maltodextrins (Sigma-Aldrich) were freed from low dextrin fractionation method was applied. Commercial maltodextrins (Sigma-Aldrich) were freed from low dextrin fractionation method was applied. Commercial maltodextrins (Sigma-Aldrich) were freed from low dextrin fractionation method was applied. Commercial maltodextrins (Sigma-Aldrich) were freed from low dextrin fractionation method was applied. Commercial maltodextrins (Sigma-Aldrich) were freed from low dextrin fractionation method was applied. Commercial maltodextrins (Sigma-Aldrich) were freed from low dextrin fractionation method was applied. Commercial maltodextrins (Sigma-Aldrich) were freed from low dextrin fractionation method was applied. Commercial maltodextrins (Sigma-Aldrich) were freed from low dextrin fractionation method was applied. Commercial maltodextrins (Sigma-Aldrich) were freed from low dextrin fractionation method was applied.

The precipitated maltodextrins were removed by centrifugation (10 min at 8,000g; RT) and then kept at 4°C overnight. Crystalline material was collected by centrifugation (10 min at 800g; RT). The precipitated maltodextrins were washed five times with water (centrifugation as above). Finally, sodium acid was added (final concentration of 0.05% [w/v]) and the suspension was stored at 4°C until use. For procedure II, a solution containing 30% (w/v) MD was prepared by heating (15 min at 95°C) and then kept at 4°C overnight.

**Crystallization**

For procedure I, a solution containing 30% (w/v) MD was prepared by heating (15 min at 120°C) and then kept at 4°C overnight. Crystalline material was collected by centrifugation (10 min at 800g; RT). The precipitated maltodextrins were washed five times with water (centrifugation as above). Finally, sodium acid was added (final concentration of 0.05% [w/v]) and the suspension was stored at 4°C until use. For procedure II, a solution containing 30% (w/v) MD was prepared by heating (as above) and then kept overnight at 30°C. MDcryst were collected, washed by centrifugation, and stored as in procedure I. Lyophilization of the MDcryst tended to diminish the stability of the particles. B-type allomorph MDcryst particles were more labile than the A-type allomorph.

**Assay of the Activity of Recombinant GWD and PWD**

**GWD (Standard Assay)**

This assay was used to measure the initial rates of the stGWD-catalyzed glucan phosphorylation. Except where stated otherwise, reaction times were restricted to 5 or 10 min. Within 10 min, the rate of phosphorylation was constant. In a final volume of 50 μL, the standard reaction mixture contained 4 mg of A- or B-type MD (as stated), reaction buffer (consisting of 50 mM HEPES-KOH, pH 7.5, 6 mM MgCl₂, 1 mM EDTA, and 2 mM diethiothreitol), 20 μg of bovine serum albumin, and 25 μM ATP including 2 μCi of \([\beta-3']\)ATP. As a control, GWD was inactivated by heat treatment prior to addition to the assay mixture. Phosphorylation was started by the addition of recombinant stGWD (250 ng except where stated). The reaction mixture was incubated for 10 min (except where stated at 30°C under continuous agitation. Incubation was terminated by heating for 5 min at 95°C under agitation and centrifugation (10 min at 10,000g). Subsequently, 2 μL of the supernatant was spotted onto PEI-Cellulose plates and subjected to thin-layer chromatography (see above). In some experiments, the standard conditions were modified as stated.

**GWD (Modified Assays)**

In some experiments, the standard assay was modified. For scanning electron microscopy, both the A- and B-type MD were incubated with recombinant stGWD. In a final volume of 50 μL, the reaction mixture contained A- or B-type MDcryst (100 μg each), reaction buffer (as in the standard assay), 50 μM unlabeled ATP, 400 μg mL\(^{-1}\) bovine serum albumin, and 100 μg of recombinant stGWD. As controls, either ATP was omitted (but functional stGWD was added) or heat-inactivated stGWD was added (but ATP was included). The total incubation time of the reaction mixtures was 3 h (30°C under continuous agitation). However, at 60-min intervals, the entire reaction mixtures were centrifuged (5 min at 10,000g; RT) and the pellet of MDcryst were resuspended in a freshly prepared reaction mixture including freshly diluted stGWD. Finally, the reaction was terminated by adding SDS (final concentration of 2% [w/v]) and centrifugation (as above). For massive GWD-mediated solubilization of MDcryst, the same assay was applied, except that the amount of MDcryst and stGWD was 40 μg. At intervals, the soluble and insoluble maltodextrins were separated by centrifugation for 5 min at RT. The pelleted maltodextrins were treated as above, and the soluble maltodextrins were hydrolyzed in 0.7 N HCl for 2 h at 95°C. Following neutralization with 0.7 N NaOH, Glc was quantified enzymatically using the starch kit.

**PWD (Standard Assay)**

atPWD activity was measured using A- or B-type MDcryst following a prephosphorylation using unlabeled ATP and stGWD. Prephosphorylation was performed using the assay mixture described above except that...
Phosphorylation of A- and B-Type Crystallized Maltodextrins

[$\beta^{32}$P]ATP was omitted and the time of the phosphorylation varied as stated. Prephosphorylation was started by the addition of 250 ng of recombiant sGWD. Following incubation (as indicated), prephosphorylation was terminated by adding SDS to give a final concentration of 2% (w/v) and the resulting mixture was centrifuged for 10 min at 10,000g. The insoluble prephosphorylated MD$_{cryst}$ was freed from SDS by washing with water (five times, 10 min each). Subsequently, aliquots (4 mg each) of the prephosphorylated (but unlabeled) MD$_{cryst}$ were resuspended in the reaction mixture described above (including 2 $\mu$L of [$\beta^{32}$P]ATP; final volume of 50 $\mu$L) and used as a substrate for PWD. Labeling was started by adding recombiant atPWD (250 ng). A reaction mixture containing heat-inactivated atPWD served as a control. Reaction mixtures were incubated for 5 or 10 min at 30°C under continuous agitation and were then inactivated by adding a final concentration of 20 mM EDTA (w/v). Samples were separated into soluble and insoluble fractions by centrifugation (10 min at 10,000g), and the insoluble fraction was resuspended in 50 $\mu$L of water. Both fractions were heated for 5 min at 95°C, and 2 $\mu$L of the solution was spotted onto PEI-Cellulose plates and subjected to thin-layer chromatography (see above).

Analytical Techniques

**Light Microscopy**

MD$_{cryst}$ were analyzed by polarization light microscopy using an AX 70 microscope from Olympus.

**X-Ray Diffraction**

X-ray diffraction analyses were performed with MD$_{cryst}$ and native starch granules in a fully hydrated state using a NanoStar (Hejazi et al., 2008). Crystalline size was quantified according to the Scherrer equation.

**Thin-Layer Chromatography**

Neutral and phosphorylated glucans were separated by thin-layer chromatography using PEI-Cellulose plates according to Hejazi et al. (2008). Following chromatography, radioactivity was quantified using a phosphor-imager (Hejazi et al., 2008).

**Protein Quantification**

Buffer-soluble proteins were quantified using the microversion of the Bio-Rad protein assay kit according to Bradford (1976).

**HPAEC-PAD Analysis**

Neutral and phosphorylated maltodextrins were analyzed using a HPAEC-PAD device (DX-600 system; Dionex) and a CarboPac PA 100 or PA 1 column. Prior to sample application, the column was equilibrated for 10 min with a mixture of 5 mM sodium acetate and 100 mM NaOH. Following sample application, maltodextrins were eluted using a linear sodium acetate gradient (1 mL, min $^{-1}$, 5–500 mM) dissolved in 100 mM NaOH. Eluate fractions (0.5 mL each) were collected, and each fraction was mixed with 3 mL of scintillation cocktail. Radioactivity was monitored using a liquid scintillation counter (for details, see Hejazi et al., 2008).

**Coulter Counter**

The size distribution of the MD$_{cryst}$ was determined using a Coulter Counter Multisizer 3 using a 100-$\mu$m aperture tube (range, 2–60 $\mu$m; Beckman). For measurements, 150 $\mu$L of MD$_{cryst}$ was resuspended in 75 mL of Coulter Isoton II Diluent (Beckman).

**Mass Spectrometry**

Neutral and phosphorylated maltodextrins were separated by chromatography on a graphitized carbon black column, and mass spectrometry was performed using a Bruker Reflex matrix-assisted laser-desorption ionization time of flight apparatus (Bruker Daltonik) in the positive-ion mode (Hejazi et al., 2008).

**Light-Scattering Experiments**

The relative changes in the light-scattering intensities of suspensions of both the A- and B-type allomorphs were monitored at an angle of 90° using the fluorescence spectrometer Fluorolog-3 (Horiba Jobin Yvon). The wavelengths of both the excitation and emission monochromators were set at 532 nm. Measurements were performed using a 1-cm fluorescence cell (Hellma) placed into a temperature-controlled sample holder equipped with peltier elements. To ensure homogeneity, the suspension was continuously stirred. The temperature of the suspension increased at a rate of 5°C min$^{-1}$. To avoid multiple scattering, the MD$_{cryst}$ particle suspensions were diluted with water to give an extinction at 532 nm not exceeding 0.7.

**Scanning Electron Microscopy**

Following three steps of washing and centrifugation (as above), the pelletred MD$_{cryst}$ were resuspended in water and lyophilized. Specimens were coated with gold and analyzed by scanning electron microscopy using a Quanta apparatus (Philips).

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**LITERATURE CITED**


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