Amylose Is Synthesized in Vitro by Extension of and Cleavage from Amylopectin

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Marion van de Wall‡, Christophe D’Hulst§, jean-Paul vinchen‡, Alain Bule‘on†, Richard Visser†, and Steven Ball§

From the ‡Laboratoire de Chimie Biologique, Unité Mixte de Recherche du CNRS no. 111, Université des Sciences et Technologies de Lille, 59655 Villeneuve d’Ascq Cedex, France, §Institut National de la Recherche Agronomique, Centre de Recherches Agronomiques, Rue de la Géraudière, B.P. 71627, 44316 Nantes Cedex 03, France, and the †Department of Plant Breeding, Graduate School of Experimental Plant Sciences, Agricultural University of Wageningen, P. O. Box 386, 6700 AJ Wageningen, The Netherlands

Amylose synthesis was obtained in vitro from purified Chlamydomonas reinhardtii starch granules. Labeling experiments clearly indicate that initially the major granule-bound starch synthase extends glucans available on amylopectin. Amylose synthesis occurs thereafter at rates approaching or exceeding those of net polysaccharide synthesis. Although these results suggested that amylose originates from cleavage of a pre-existing external amylopectin chain, such transfer of chains from amylopectin to amylose was directly evidenced from pulse-chase experiments. The structure of the in vitro synthesized amylose could not be distinguished from in vivo synthesized amylose by a variety of methods. Moreover high molecular mass branched amylose synthesis preceded that of the low molecular mass, suggesting that chain termination occurs consequently to glucan cleavage. Short pulses of synthesis followed by incubation in buffer with or without ADP-Glc prove that transfer requires the presence of the glucosyl-nucleotide. Taken together, these observations make a compelling case for amylopectin acting as the in vivo primer for amylose synthesis. They further prove that extension is followed by cleavage. A model is presented that can explain the major features of amylose synthesis in plants. The consequences of intensive amylose synthesis on the crystal organization of amylopectin are reported through wide angle x-ray analysis of the in vitro synthesized polysaccharides.

Starch is usually defined as a mix of two distinct polymer fractions: amylopectin and amylose. Amylopectin the major compound is composed of intermediate size α(1→4)-linked glucans that are organized in clusters of parallel chains by a dense packing of α(1→6) linkages. Amylose, which accounts for 20–30% in weight of the starch granule, is often referred to as a smaller linear molecule with very few α(1→6) branches (for review, see Ref. 1). It has been apparent ever since the pioneering work of Leloir and Recondo (2, 3) that glucose is transferred from ADP-Glc to the nonreducing end of a growing α(1→4)-linked glucan, thus coupling an extra glucose residue to this chain with the simultaneous release of ADP.

The enzyme was identified by Fekete et al. (4) as associated with starch granules and was subsequently called granule-bound starch synthase. Due to the position of GBSSI inside the granule, diffusion of both donor and acceptor substrate might be a limiting factor for activity. GBSSI was first reported to use non-physiological concentrations of UDP-Glc (2), whereas ADP-Glc was shortly discovered thereafter as the preferred donor substrate (3). These observations opened an altogether new area of research for both glycogen and starch synthesis in bacteria and plants, respectively. It has been known, ever since the foundational work laid by Nelson and Rines (5), that GBSSI is responsible for the biosynthesis of the amylose fraction. Mutations leading to defects for GBSSI have been isolated in an ever increasing number of species including waxy (wx) maize (6), wx rice (7), wx barley (8), wx wheat (9), amylose-free (amf) potato (10), low amylose (lam) pea (11), wx amaranth (12), and sta2 Chlamydomonas reinhardtii (13). All mutants accumulate during storage normal amounts of starch granules containing amylopectin with wild-type crystalline organization (14). These important results establish that amylose is not required for the biogenesis of normal granules. A number of studies approaching the synthesis of amylose in vitro (2, 15-17), establish that GBSSI incorporates glucose both in amylopectin and amylose according to the conditions used. In vivo evidence supporting the involvement of GBSSI in amylopectin synthesis was produced by Maddelein et al. (18). In this study, genetic interaction experiments clearly showed that defects in GBSSI strongly reduced amylopectin synthesis in particular mutant backgrounds.

We have shown that growth-arrested (nitrogen-starved) C. reinhardtii cells accumulate a polysaccharide that bears strong structural resemblance to maize endosperm storage starch (13, 18, 19). Moreover, we have demonstrated that similar enzymes synthesize it and that it responds in an identical fashion to mutations affecting these activities (12, 20). We have reported that the starch accumulated during log-phase growth differs markedly from storage starch (21). The polysaccharide, which is similar to vascular plant leaf (transient) starch, harbors little or no amylose whereas the amylopectin displays an altered chain-length distribution. Van den Koonhuysen et al. (22) showed that, in C. reinhardtii, mutants defective either for

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† To whom correspondence should be addressed. Tel.: 33-3-20-43-65-43; Fax: 33-3-20-43-65-55; E-mail: steven.ball@univ-lille1.fr.

§ The abbreviations used are: GBSSI, granule-bound starch synthase I; BE, branching enzyme; Λmax, maximal absorbance wavelength of the iodine-polysaccharide complex.
phosphoglucomutase or for the large subunit of ADP-Glc pyrophosphorylase, isoamylase was purchased from Hayashibara Biochemical Laboratories (Okayama, Japan).

Chlamydomonas Strains, Growth Conditions, and Media—The reference strain of C. reinhardtii used in this study is 137C (mt nit nii2). It, defective for the large subunit of ADP-Glc pyrophosphorylase (sta1-1), was generated by X-ray mutagenesis from 137C and has been described previously (20). The GBSSI-defective strain BAFR1 (mt nit1 nii2 sta2-29 ARG7) contains a disruption of the STA2 gene that was generated through random integration of the pARG7 plasmid in the nuclear DNA of C. reinhardtii (17). Standard media are fully detailed in Ref. 23, while growth conditions and nitrogen-starved media are described in Refs. 13, 20, 21, and 24.

Amylopectin/Amylose Content, Starch Purification, and Spectral Properties of the Iodine-Starch Complex—A full account of amyloglucosidase assays, starch purification on Percoll gradients, and λmax determinations can be found in Ref. 13.

In Vitro Synthesis of Amylose, Protease Protection Experiments—500 μg of starch was incubated with 3.2 mM ADP-Glc in the presence of 50 mM glucose (pH 9.0), 100 mM (NH4)2SO4, 0.4% β-mercaptoethanol, 5 mM MgCl2, 0.05% bovine serum albumin, and 2.2 μM ADP[U-14C]Glc at 10.5 Gbq/mmol in a total volume of 2 ml at 30°C for different periods of time. The reaction was terminated by adding three volumes of 96% ethanol. After centrifugation at 3000 × g for 10 min, the supernatant was discarded and the starch was suspended in 100% Me2SO and boiled for 20 min. The polysaccharide was precipitated overnight at –20°C by adding three volumes of 96% ethanol. After centrifugation at 15,000 × g for 20 min at 4°C, the pellet was dried in air for at least 10 min, dissolved in 500 μl of 10 mM NaOH, and subjected to gel permeation chromatography. To investigate the sensitivity of GBSSI to proteases, concentrations of 0.1 and 0.2 mg/ml were used, respectively, for protease K and Pronase using the standard GBSSI assays at 30 min and 1 h after a 30-min preincubation.

Separation of Starch Polysaccharides by Gel Permeation Chromatography—0.5–1.0 mg of starch dissolved in 500 μl of 10 mM NaOH was applied to a column (0.5 cm inner diameter) × 65 cm) of Sepharose CL2B or CL4B, which was equilibrated and eluted with 10 mM NaOH. Fractions of 300–320 μl were collected at a rate of one fraction per 1.5 min. Radioactivity was determined by liquid scintillation counting. Glucans in the fractions were detected by their reaction with iodine, and the levels of amylopectin and amylose were quantified by determining the amount of glucose after amyloglucosidase treatment.

Debranching Analysis—Isoamylase-mediated debranching of gel permeation chromatography-purified fractions of amylopectin and amylose was achieved as described previously (21). After completion of the debranching reaction, samples were kept at 80°C in 10% Me2SO to avoid retrogradation of the long glucans into insoluble material. The debranched polysaccharides were subjected to TSK HW-50(F) chromatography as detailed in Ref. 21. Debranching of amylopectin for CL4B gel permeation chromatography was performed in 50 mM NaAc (pH 4.0) containing 59 units of isoamylase.

Determination of the β-Amylolytic Limit—Amylose and amylopectin were dissolved in 25 μl of 0.8 M NaOH, diluted with 25 μl of distilled water and incubated for 24 h at 30°C in 200 μl of 50 mM NaAc (pH 3.5). The latter contained no β-amylase, 22 units of β-amylase, or both 22 units of β-amylase and 59 units of isoamylase. After adding 150 μl of 3,5-dinitrosalicylic acid (10 mg/ml) to 50 μl of sample, the mixture was boiled for 10 min and the number of reducing ends was determined spectrophotometrically at 540 nm. Maltose was used as a standard. As a control, the β-amylolysis limit was determined for maize amylopectin, potato amylose, and potato starch.

X-ray Diffraction Measurements—Samples (10 mg) were sealed between two aluminum foils, to prevent any significant change in water content during the measurement. Diffraction diagrams were recorded using Inel (Orléans, France) X-ray equipment operating at 40 kV and 30 mA. CuKα (λ = 0.15405 nm) was selected using a quartz monochromator. A curved position-sensitive detector (Inel CPS120) was used to monitor the diffracted intensities using 2-h exposure periods. Relative crystallinity was determined, after bringing all recorded diagrams at the same scale using normalization of the total scattering between 3 and 30° (29), following a method derived from Wakelam et al. (26). Dry extruded starch and spherolithic crystals of amylose were used as amorphous and crystalline standards, respectively.

RESULTS

Synthesis of Amylose Occurs in Vitro from Transient Starches in the Absence of Added Maltooligosaccharides—Starch was purified both from nitrogen-starved cultures of mutants defective for the large subunit of ADP-Glc pyrophosphorylase and from nitrogen supplied wild-type algae. The granules extracted from these strains displayed GBSSI activities ranging between 15 and 20 nmol of ADP-Glc incorporated into insoluble polysaccharide per min and per mg of starch. This activity displayed an apparent Km of 3.5 mM for ADP-Glc and was entirely protected from Pronase (0.2 mg/ml) and (or) proteinase K (0.1 mg/ml) action. When using 3.2 mM ADP-Glc, incorporation was linear with time for periods ranging from 10 min to 2 h at 30°C. Although all experiments reported in this paper were performed from freshly purified material, no loss of GBSSI activity could be evidenced after 1 week of storage at 4°C. Starch purified from strain BAFR1 (containing a gene-disrupted GBSSI structural gene) displayed less than 0.5% of the wild-type activity, which fell below background when fractionated on Sepharose CL2B columns. This proves that only GBSSI was monitored under our experimental conditions. Fig. 1 (A and B) shows the separation of amylopectin and amylose by CL2B chromatography before and after in vitro synthesis, respectively. After 24 h, we were able to raise the amylose content from less than 2% to over 24% of the total starch. The polysaccharide synthesized under these conditions could not be distinguished from standard C. reinhardtii amylose. Fig. 1C displays an experiment where 0.05 mg of starch subjected to in vitro synthesis for 24 h in the presence of labeled ADP-Glc was mixed and chromatographed with 1 mg of storage starch (20% amylose) extracted from wild-type cultures. We can conclude from these experiments that the molecular mass distribution of the in vitro synthesized material is identical to that of native amylose. Moreover, the fine structure of the in vitro synthesized product was investigated using debranching analysis followed by gel permeation (Fig. 2A). By all these criteria, the in vitro synthesized polysaccharide proved to be identical to native C. reinhardtii amylose. As with algal native amylose, we detected up to 1% branches within the in vitro synthesized product. We confirmed the presence of a similar branching pattern by measuring the β-amylolysis limit. β-Amylase is known to be an exo-type of enzyme digesting external chains up to 2–3 residues from a branch point. The β-amylolysis limit (the percentage of digested material) for both the in vitro and in vitro synthesized amyloses ranged between 70 and 75%. Together with the identical chain-length distributions revealed by our debranching analyses (see above), these results prove that both polysaccharides contain an identical distribution of α(1–6) linkages with a strong bias toward the reducing end of the molecules (for review, see Ref. 25). From all these
experiments, we conclude that authentic amylose biosynthesis has been achieved. It must be stressed that this synthesis occurred at the expense of major changes in the structure of amylopectin. The $\lambda_{\text{max}}$ of the iodine-polysaccharide complex of amylopectin increased from 570 to 600 nm (Fig. 1), while debranching analysis (Fig. 2B) clearly shows that the label is incorporated in the fraction excluded from TSK-HW50 chromatography. The OD of the intermediate and small chain-length amylopectin fractions (26–39) before and after in vitro synthesis were identical, whereas the OD of the long glucan fractions increased dramatically after in vitro synthesis (data not shown). We believe these chains to be external as the $\beta$-amylolysis limit of amylopectin increased before and after synthesis from 50–55% to 58–63%, respectively. That these chains are covalently bound to amylopectin is confirmed by the fact that all attempts to dissociate them from amylopectin were unsuccessful. These attempts consisted of dispersing starch with aqueous Me$_2$SO at 100 °C precipitating the chains with ethanol, resuspending the starch in 10 mM NaOH and running columns at various dilutions. We have also submitted the amylopectin to several rounds of Sepharose CL-2B chromatography. Indeed, the long glucan fraction of amylopectin could only be recovered by enzymatic debranching as was reported both by Baba et al. (16) and by Denyer et al. (17).

Kinetics of Amylose Deposition—Denyer et al. (17) have reported that, in the absence of maltooligosaccharides, after 1 h of incubation, amylopectin was the predominant if not only labeled species. Although these results could be confirmed by us, it was furthermore evident that this situation changed dramatically when longer incubations were analyzed. Our results reported above show that after 24 h of incubation more than 40% of the label is incorporated in the amylose fraction. To study amylose deposition, we followed the kinetics of in vitro synthesis in the presence of labeled ADP-Glc. For this purpose, we used amylose-less starch granules purified from a nitrogen starved mutant defective for the large subunit of ADP-Glc pyrophosphorylase (22). For each time point, a complete analysis involving Sepharose CL2B chromatography of dissolved starch granules was performed. The OD at $\lambda_{\text{max}}$ and amount of labeled material were thus recorded in each fraction and are displayed in Fig. 3A (from 10 min to 2 h) and Fig. 3B (from 2 to 24 h). We were thus able to monitor total incorporation of label, as well as the amount selectively synthesized within amylose and amylopectin (Fig. 4). It is clear that Chlamydomonas
Amylose.

In vitro synthesis of amylopectin was progressively replaced by that of amylose (\(\text{ADP-Glc}\), the amylopectin and amylose were separated by CL2B-Sepharose chromatography. In vitro synthesis of amylose is shown for starch (white column), amylopectin (black column), and amylose (gray column). After 2 h of incubation, amylopectin synthesis was progressively replaced by that of amylose.

Starch behaves very much like that of peas in the initial steps. Indeed, very little if any amylose synthesis occurred during the first hour (Fig. 3A), while active amylopectin elongation is witnessed. That external amylopectin chains are getting longer is proven by the increase in \(\lambda_{\text{max}}\) of the iodine-polysaccharide complex from 570 to 600 nm that is completed within the very first 30 min (Table I). To ascertain that single chains are indeed getting longer, we debranched amylopectin and compared the length of these chains to those characterizing mature debranched C. reinhardtii amylopectin. Results displayed in Fig. 5 together with our \(\beta\)-amylolysis (see above) studies confirm that external amylopectin chains are getting elongated by GBSSI. Moreover, a bimodal distribution of the long glucans of amylopectin is detected. After 2 h of incubation, amylopectin synthesis becomes progressively substituted by that of amylose (Figs. 3B and 4). After 12 h, amylose synthesis rates exceeded those of incorporation by GBSSI. In addition and at the same time, the \(\lambda_{\text{max}}\) of the amylopectin-iodine complex is decreasing from 615 to 605 nm (Table I). This result indicates that incorporation in amylose is accomplished at the expense of amylopectin. It is striking to note that high molecular mass amylose biosynthesis occurs before that of the low molecular mass species. thus mimicking the bimodal distribution of the amylopectin external long glucan fractions. At this point of our analysis, we already suspected that external amylopectin chains were used to generate mature amylose by a single endo-type of cleavage event. We thus undertook experiments specifically designed to test this hypothesis.

Pulse-Chase Experiments—The best way to probe the function of amylopectin as a primer for amylose biosynthesis would be to pulse-label the former and check if we can subsequently chase the label into the latter. We chose to pulse-label amylopectin for 30 min at \(t_0\) from starch extracted from the same strain as that was used in the time-course experiments described above. As predicted from our previous experiments, amylopectin was found as the sole labeled species immediately after the radioactive pulse. As synthesis proceeded with unlabeled substrate, the label was slowly but clearly chased into amylose (Fig. 6A). Again, the label appeared first in the high molecular mass amylose fraction (Fig. 6B). We double-checked that pulse-labeled amylopectin external chains could also be chased into amylose in the case of both transient and storage starches from wild-type algae. An example of such an experiment can be found in Fig. 6C, which displays a pulse-chase experiment with storage starch from wild-type algae. Similar pulses gave similar results, yielding a net chase from amylopectin into amylose. In this case, a low but substantial amount of radioactivity in the amylose fraction was immediately detected following the pulse. This result can be simply explained by assuming that a percentage of amylopectin outer chains are already physically ready to be transferred into amylose at \(t_0\). It is worth noting that there again, high molecular mass amylose appears first. Pulse-chase experiments performed on transient starches from wild-type strains behaved in a fashion virtually identical to that which we reported for the low ADP-Glc synthesizing mutants. In addition, during time-course experiments extension of amylopectin chains also preceded the appearance of amylose. Moreover, we double-checked that time-course and pulse-label results obtained from transient starch using 0.5 mM ADP-Glc and pH 7.8 were identical after 4 days of incubation to those obtained after 24 h at 3.2 mM ADP-Glc and pH 8.2. At this stage, the only reasonable interpretation of our results would be to assume that GBSSI extends amylopectin chains. When these chains become “long enough,” they are

![Fig. 3. Kinetics of in vitro synthesis. Starch from the mutant defective for the large subunit of ADP-Glc pyrophosphorylase (I7) was subjected to in vitro synthesis in the presence of \(^{14}\text{C}\)-labeled ADP-Glc. After in vitro synthesis, the amylopectin and amylose were separated by CL2B-Sepharose chromatography. A shows the incorporation of \(^{14}\text{C}\) from ADP-[U-\(^{14}\text{C}\)] Glc after 10 min (---), 30 min (----), and 2 h (---) of in vitro synthesis. B shows the incorporation of \(^{14}\text{C}\) after 2 h (-----), 6 h (-----), 12 h (-----), and 24 h (-----). After 2 h, the amount of label incorporated in the amylose fractions increased, whereas there was no further increase in the amount of label incorporated in the amylopectin fractions.](image-url)

![Fig. 4. Incorporation of \(^{14}\text{C}\) from ADP-[U-\(^{14}\text{C}\)] Glc in amylopectin and amylose. After in vitro synthesis in the presence of \(^{14}\text{C}\)-labeled ADP-Glc, the amylopectin and amylose were separated by CL2B-Sepharose chromatography. The total incorporation of label after different times of in vitro synthesis is shown for starch (white column), amylopectin (black column), and amylose (gray column). After 2 h of incubation, amylopectin synthesis was progressively replaced by that of amylose.](image-url)

| Table I | \(\lambda_{\text{max}}\) of the amylopectin-iodine complex after different times of in vitro synthesis |
|---------|-------------------------------------------------|
| Time of in vitro synthesis (h) | \(\lambda_{\text{max}}\) |
| 0 | 570 |
| 1/6 | 580 |
| 1/2 | 600 |
| 2 | 615 |
| 6 | 615 |
| 12 | 610 |
| 24 | 605 |

Wavelength of the maximal absorbance of the iodine polysaccharide complex is expressed in nm.
CL-4B-Sepharose chromatography. One milligram of wild-type amylopectin separated by CL-2B-Sepharose chromatography was mixed with 0.05 mg of amylopectin from the I7 mutant subjected to 1 h of in vitro synthesis. After debranching with isoamylase, the amylopectin was subjected to CL-4B-Sepharose chromatography. The optical density (\(\lambda_{\text{max}}\)) of the iodine-polysaccharide complex was measured at \(\lambda_{\text{max}}\) (---). The incorporation of \(^{14}\text{C}\) from ADP[U-\(^{14}\text{C}\)]Glc (---) was determined by liquid scintillation counting. Because of the relative abundance of cold wild-type reference polysaccharides, the OD reflects exclusively the wild-type amylopectin chain-length distribution, whereas the radioactive material represents the in vitro synthesized glucans. The chains elongated by GBSSI are considerably longer than the average chains of wild-type amylopectin. A bimodal distribution reflecting the existence of two types of external amylopectin chains elongated by GBSSI was already evidenced after 1 h of in vitro synthesis.

In order to uncouple GBSSI from that of the postulated cleavage reaction, we performed by Denyer \(\text{et al.}\) (17) using 50 mM maltotriose and starch. A number of reports in the literature suggest that amylose biosynthesis is stimulated by addition of maltodextrins such as maltotriose. Therefore, we repeated the experiments performed by Denyer \(\text{et al.}\) (17) using 50 mM maltotriose and 3.2 mM ADP-Glc concentrations. Synthesis of amylose-like material was indeed massive within 1 h of incubation (Fig. 7), whereas relatively little glucose was incorporated in amylopectin. GBSSI incorporated 2-3 times more glucose in the starch granules under these conditions. Two important conclusions can be drawn from this experiment. (i) The approximately 3-fold reduction of label in amylopectin in the presence of maltotriose suggests that the nonreducing ends of maltotriose compete with amylopectin molecules as acceptor substrates. Apparently, a large number of maltotriose molecules have been elongated to such an extent that they became too large too escape from the granule. (ii) The increase in GBSSI activity upon maltotriose supply indicates that the amount of available acceptor substrate in the granule is limiting. This suggests that the immobilized GBSSI cannot use the full potential of nonreducing termini within the starch granule. However, in the presence of a diffusible acceptor substrate such as maltotriose, a larger number of GBSSI molecules can participate in the synthesis reaction. To illustrate the number of nonreducing ends, the following estimation was made. Assuming an amylopectin content of 30%, a volume for one glucose residue of 0.125 nm\(^3\), and an average chain-length of 20 glucose units, we have calculated a nonreducing end concentration of 450 mM within the granule.

The above observations prompted us to investigate \(^{14}\text{C}\) incorporation in starch granules with varying ADP-Glc and maltotriose concentrations (Table II). In the absence of maltotriose, \(^{14}\text{C}\) incorporation increased proportionally with the ADP-Glc concentration until a concentration of 1 mM is reached. At this stage, the donor substrate concentration was limiting. After this point, the \(^{14}\text{C}\) incorporation versus ADP-Glc concentration curve started to level off. At an ADP-Glc concentration of 3.2 mM, the incorporation of label increased with the maltotriose...
same A-type patterns. However, high amylose mutants from SSI-defective mutants from algae and cereals display the very
found in potato tuber and high amylose mutant starches. GB-S
switch is due a modification in amylopectin structure or to the
radioactivity incorporated doubled, and amylose was the predominantly
species. In the presence of 50 mM maltotriose (- - -), the total amount of
reached. At 200 mM maltotriose, a reduced 14C incorporation
was observed. Because of the abundance of maltotriose, most of
the label was transferred to this acceptor molecule instead of to
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\begin{array}{cccccccc}
\text{ADP-Glc} & \text{Maltooligosaccharides} & 0 & 2 & 5 & 10 & 50 & 200 \\
\text{concentration (mM)} & \text{concentration (mM)} & & & & & & \\
0.01 & 0.12 & 0.10 & 0.09 & 0.08 & 0.07 & 0.06 \\
0.04 & 0.44 & 0.49 & 0.48 & 0.46 & 0.39 & 0.29 \\
0.2 & 2.0 & 2.6 & 2.7 & 2.7 & 2.5 & 2.1 \\
1.0 & 8.8 & 10 & 12 & 14 & 14 & 12 \\
3.2 & 18 & 26 & 33 & 42 & 47 & 43 \\
\end{array}
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A-type diffraction patterns are found in cereal
and B-types. A-type starches fall into two distinct types, namely the so-called A-
in vitro
pectin biosynthesis. Crystalline diffraction patterns of plant
crease peaks at 2θ values of 9.9, 11.2, 15, 17, 18.1, and 23.3° characterize the A-type starches, whereas diff-
crease peaks at 2θ values of 5.6, 15, 17, 22, and 24° typify B-type
It is clear that the t48 (B) starch sample has switched from the
A-type (A) to the B-type.

The x-ray diffraction diagrams are displayed in Fig. 8. The crystallinities measured for the t48 and t48 samples amounted to
27 and 16%, respectively. Transient starch displays an A-type
diffraction diagram with a crystallinity of about 27%, very close
to those described previously for Chlamydomonas storage
starch (15). After prolonged synthesis, the diagram clearly
switched to the B-type with a lower crystallinity (16%). Never-
theless, the degree of crystallinity of B-type starches is well
known to depend strongly on the water content (27). Therefore,
the calculated value is only relative, as it was not possible to
manage the hydration level on a so small amount of substrate.
Moreover, some A-type can be still present in t48 starch. In-
deed, it is impossible in B-type starch diffraction diagrams to
detect less than 15% A-type (34), because of the high similarity
of spectra and the low crystallinity of native starches.

**DISCUSSION**

In the present study, we have used both mutant and wild-
type Chlamydomonas starch granules to elucidate the process
of amylose biosynthesis. Comparison of amylose synthesis in
these backgrounds with that of starch purified from a strain
containing a gene-disrupted GBSSI structural gene showed that
only GBSSI enzyme activity was measured under our
experimental conditions. We have proven, by pulse-chase ex-
periments among other techniques, that extension of amylopect-
A-type diffraction patterns are found in cereal
and C. reinhardtii starches, whereas the B-type is
found in potato tuber and high amylose mutant starches. GB-
SSI-defective mutants from algae and cereals display the very
same A-type patterns. However, high amylose mutants from
maize and C. reinhardtii switch to the B-type of crystalline
organization. Because these high amylose mutants are affected
in the amylopectin biosynthesis pathway, it is not known if the
switch is due a modification in amylopectin structure or to the
increase in amylose, which by itself could influence the amylo-
pectin crystalline organization. We therefore compared the
wide angle x-ray diffraction analysis of a sample of t48 transient
starch with less than 1% in weight amylose to that of the very
same starch that was subjected to intensive in vitro synthesis
for over 48 h with 3.2 mM ADP-Glc leading to 45% final amylose
content.

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in the amylopectin biosynthesis pathway, it is not known if the
switch is due a modification in amylopectin structure or to the
Active GBSSI can be localized bound at the surface, bound within the granule, or both. The physical location of the active enzyme is of paramount importance because it is only at the surface that the enzyme can eventually be considered as moving with the growing amylose molecules. Within the polysaccharide matrix itself, there is very little room if any for enzyme movement. We have chosen to discuss only the latter possibility. Indeed, evidence for the presence of actively moving and readily dissociating surface enzyme is presently lacking. Three possible models accounting for amylose synthesis within the starch granule are displayed in Fig. 9. The three models share a number of major assumptions, which will be discussed first.

We assume that each GBSSI enzyme is tightly bound to the amylopectin matrix and is in fact an immobilized enzyme. Although this assumption remains to be formally proven the resistance of GBSSI activity to proteases, the pronounced decrease of the apparent $K_m$ following solubilization of the enzyme (28) and the immunolocalization of this enzyme within the granule (29) are all in agreement with it. Moreover, we must take into account the inability of large proteins to diffuse into the polysaccharide matrix, the likelihood of amylose deposition within the amorphous cavities of the granule, and the availability of GBSSI for multiple rounds of amylose chain synthesis. Assuming an amylose content of 30%, an average molecular weight of 500,000 (25), and an amount of approximately 2.5 μg of 76-kDa GBSSI protein/mg of starch (estimated by SDS gel electrophoresis), we have calculated a 1:18 ratio of GBSSI to amylose molecules. The major difference between model A on the one hand and models B and C on the other is that the former uses maltooligosaccharide-primed amylose synthesis, whereas B and C show the amylopectin-primed amylose biosynthesis. In B, we show a BE-mediated cleavage through intra-molecular transglycosylation. Cleavage by a hydrolytic enzyme is also possible but not shown in this figure. In contrast to B, C shows that hydrolysis (h) can occur very near the site of polymerization (p) within GBSSI, ensuring that a non-reducing end is close to the site of synthesis for reinitiating the next round of amylose biosynthesis.
the glucan situated the furthest away from the nonreducing end of the glucan is more likely to encounter the less abundant branching enzyme (BE) trapped in the granule (Fig. 9). Amylose branching is thus a stochastic event requiring a close encounter with BE within the path of the growing glucan.

We have been able to reproduce the effect of maltoligosaccharides on in vitro amylose synthesis previously reported by Denyer et al. (17) in our system (Fig. 7). The reduction of label in amylopectin in the presence of maltotriose indicated that high concentrations of maltoligosaccharides compete with amylopectin as acceptor substrate for GBSSI. Incorporation of 14C in the insoluble polysaccharide at different concentrations of ADP-Glc and maltotriose (Table II) suggests that at high ADP-Glc concentrations the length of the glucans produced is long enough to be retained within the granule whereas at lower concentrations the relatively short oligosaccharides will escape from the granule. Such small labeled oligosaccharides were indeed reported by Leloir and colleagues (2) in experiments involving UDP-Glc concentrations well below the apparent $K_m$ of the enzyme. Our present evidence points to normal amylose synthesis occurring in the absence of maltoligosaccharides. After prolonged incubation in the absence of maltoligosaccharides, massive amylose synthesis was achieved whereas our incubation media contained less than 1 $\mu$mol maltoligosaccharides (data not shown). Whether or not maltoligosaccharide-primed amylose biosynthesis occurs in vivo is a matter of available acceptor substrate concentrations. The cluster-like structure of amylopectin provides a formidable potential of nonreducing ends in the starch granule (see “Results”). The increase of 14C incorporation upon addition of diffusible maltoligosaccharides (Table II) strongly indicates that GBSSI cannot use this full potential. However, the large number of possible priming sites within the starch granule, together with the presence of amounts of maltoligosaccharides below 1 $\mu$mol in wild-type Chlamydomonas undergoing amylose biosynthesis, make it very unlikely that maltoligosaccharide priming is important in vivo.

Models B and C account for the amylopectin-primed amylose synthesis that we have observed (Fig. 6). In these models, the external amylopectin chain is secured in the active site of GBSSI and we assume that enzyme and substrate do not easily dissociate, due to the immobilized character and processivity of the enzyme and the organized structure of amylopectin. Amylose is formed when the side chain is detached from the amylopectin molecule. Model B suggests that cleavage occurs far from GBSSI, either by BE through an intramolecular transglycosylation (Fig. 9B) or by a hydrolytic enzyme trapped within the granule, e.g. a-amylase (not indicated in the figure). Takata et al. (30, 31) have demonstrated that BE from Bacillus stearothermophilus can catalyze inter- and intramolecular branching of both amylose and amylopectin, and they suggest that these reactions are common to BEs from various sources. As a result of the intramolecular transfer by BE, the newly formed amylose will not have a reducing end and is cyclic at the point of cleavage. However, because the number of amylose molecules more or less agrees with the number of reducing ends documented for amylose, we believe that downstream cleavage through hydrolyses is presently a more likely hypothesis. Cleavage far from GBSSI implies that the nonreducing end of the amylopectin chain will not be easily available for a next round of synthesis. In this case, multiple rounds of amylose synthesis will depend on the accessibility of new amylopectin nonreducing ends to GBSSI and the possibility of disso-
ciation of the GBSSI-substrate complex. In model C, the assumption is made of a hydrolytic event occurring very near the site of synthesis within GBSSI. We postulate that steric hindrance of the glucan’s progress will trigger hydrolysis by the GBSSI enzyme itself, assuming that GBSSI has a dual activity, i.e. synthase (or polymerase) and hydrolase. Such a dual activity has been observed before in the Klenow fragment of DNA polymerase of Escherichia coli. The N terminus contains a 3'-5' exonuclease (hydrolase) activity, whereas the C-terminal part contains a polymerase function (32). After release of the amylose chain, the old external amylopectin chain, which has never left the acceptor binding sites, can reinitiate the next round of amylose biosynthesis. In this model, growth of the glucan will be finally stopped by the lack of space within the amylopectin matrix. This will be a late event at the beginning of amylose synthesis and will happen progressively sooner as the starch granule fills with amylose. This can explain why long chain amylose precedes that of the low molecular mass material. Also, the position of GBSSI within the granule can be of influence on the synthesis of low and high molecular mass amylose. Jane and Shen (33) have shown that both concentration and size of amylose is dependent on its position within the starch granule. A detailed biochemical characterization of GBSSI is required to determine whether amylose biosynthesis proceeds via mechanism B or C.

Our results also prove that filling the starch granule with amylose in vitro in the absence of concomitant crystalline amyo-
lopectin synthesis is sufficient to change the crystalline organ-
ization within the granule. There are presently two possi-
bilities to explain these results. First, because of the large amount of amylose present after synthesis, it is possible that the newly synthesized material crystallizes in the B-type under in vitro conditions. Another intriguing and perhaps more likely hypoth-
thesis would be that the massive amylose synthesis within the amylopectin matrix would push the preexisting A-type into B-type crystals. Indeed, the structure proposed for the B-type crystals displays a central cavity that could be easily filled by one or two amorphous amylose chains (35), whereas the denser A-type packing does not allow for amylose infiltration within the crystal. Our present estimates of crystallinity levels before and after in vitro synthesis of amylose do not allow us to discriminate between these two possibilities. However, our re-
results do establish in vitro synthesis of amylose from transient Chlamydomonas starches as an extremely powerful system to investigate the selective impact of amylose on the structure of amylopectin within the granule.

We believe that the model we propose is useful in that it makes a number of experimentally testable predictions. One of them is that a radioactive pulse given at the time of amylose synthesis should lead to a net chase of radioactive material from amylopectin to amylose. We are thus proceeding to con-
firm our in vitro approach by similar experiments performed in vivo in the presence of normal amylopectin synthesis.

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In Vitro Synthesis of Amylose