Pathway of Cytosolic Starch Synthesis in the Model Glaucophyte Cyanophora paradoxa

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Received 9 October 2007; Accepted 23 November 2007

The nature of the cytoplasmic pathway of starch biosynthesis was investigated in the model glaucophyte Cyanophora paradoxa. The storage polysaccharide granules are shown to be composed of both amylose and amylopectin fractions, with a chain length distribution and crystalline organization similar to those of green algae and land plant starch. A preliminary characterization of the starch pathway demonstrates that Cyanophora paradoxa contains several UDP-glucose-utilizing soluble starch synthase activities related to those of the Rhodophyceae. In addition, Cyanophora paradoxa synthesizes amylose with a granule-bound starch synthase displaying a preference for UDP-glucose. A debranching enzyme of isoamylase specificity and multiple starch phosphorylases also are evidenced in the model glaucophyte. The picture emerging from our biochemical and molecular characterizations consists of the presence of a UDP-glucose-based pathway similar to that recently proposed for the red algae, the cryptophytes, and the alveolates. The correlative presence of isoamylase and starch among photosynthetic eukaryotes is discussed.

Glaucophyta are single-cell freshwater algae carrying peptidoglycan-containing plastids, called cyanelles, that strongly resemble their cyanobacterial ancestors. Together with the Rhodophyceae (red algae) and the Chloroplastida (green algae and land plants), they have been demonstrated to originate from a single endosymbiosis that gave birth to the Archaeplastida (including the plant kingdom) (1). This endosymbiosis involved a cyanobiont related to present-day cyanobacteria and a unicellular biflagellated heterotrophic eukaryotic host (43). In addition to acquiring photosynthesis, all three lines gained the ability to synthesize starch, a novel form of storage polysaccharide found in living cells.

Starch is composed of chains organized with the same chemical linkages as those of glycogen but defines a far more elaborate organization (for a review of starch structure, see reference 7). It consists of several distinct polysaccharide fractions. Amylose is composed of mostly linear α1,4-linked glucan chains. It is synthesized through the granule-bound starch synthase (GBSS), an elongation enzyme bound to and active within the starch granule (reviewed in reference 4). The latter is mostly composed of amylopectin, a moderately (5 to 6%) branched polymer that has an asymmetrical distribution of branches that allows the formation of chain clusters hooked together through a number of longer spacer chains. This organization enables the polymers to partially crystallize in the form of huge insoluble semicrystalline granules that come in various highly specific shapes and sizes within the plant kingdom. Unlike GBSS, the enzymes of amylopectin synthesis are active mostly within the soluble phase (reviewed in reference 5). The pathways of storage polysaccharide synthesis have been intensively studied in animals, fungi, and bacteria (for reviews, see references 20, 37, and 42). The bacterial and eukaryotic pathways of glycogen synthesis can be distinguished by a number of very important features. These include, but are not limited to, the nature of the glycosyl nucleotide used for polymerization, UDP-glucose or ADP-glucose in heterotrophic eukaryotes and bacteria, respectively.

Starch metabolism has been intensively studied in green plants and algae (reviewed in references 5, 23, 28, 46, and 54). While 6 to 12 genes generally are found in heterotrophic eukaryotes or bacteria for glycogen metabolism, green algae and land plants synthesize starch from ADP-glucose only (27, 53), with the help of 30 to 40 genes that are phylogenetically related to both cyanobacterial and heterotrophic eukaryote sequences (10, 36).
Red algae and glycoproteins can be distinguished from the green algae and land plants by the fact that they synthesize starch in the cytoplasm and not in their plastids.

In red algae and their secondary endosymbiotic starch-stor- ing derivatives, such as cryptophytes and alveolates, both biochemical and bioinformatic data suggest the presence of a UDP-glucose-based pathway (34, 50). However, this pathway remains to be validated by genetic approaches.

In this paper we report the first detailed characterization of glycoptephy starch structure and metabolism. Supported by a combination of biochemical and molecular approaches, we propose that the model glycoptephy Cyanophora paradoxa synthesizes starch through a UDP-glucose pathway similar to those that recently were proposed for rhodophyceae, cryptophytes, and alveolates (dinoflagellates and apicomplexan parasites).

The presence of a functional isoamylase complex in glycoptephy further strengthens the correlation established between this activity and the synthesis of starch in photosynthetic eukaryotes.

**Materials and Methods**

Materials. ADP-[U-14C]glucose, UDP-[U-14C]glucose, and [α-32P]ATP were purchased from Amersham (Buckinghamshire, United Kingdom). ADP-glucose and UDP-glucose were obtained from Sigma. Sepharose CL-2B columns and Percoll were obtained from Amersham Pharmacia Biotech. The starch amylo- glucosidase assay kit was obtained from Roche (Germany).

Cyanophora strains, growth, and media. Cyanophora paradoxa strain 29380 was purchased at the EPSAG (University of Gottingen, Germany). The algae was grown in D1Y medium (R. A. Anderson and M. D. Keller, unpublished data) at 25°C under a 12-h day/12-h night cycle (30 µmol photons m−2 s−1) and vigorous shaking.

Determination of starch levels, starch purification, and amylpectin structures. A full account of amyloglucosidase assays, starch purification, and β-mann (maximum wavelength of the iodine-polyacrylamide complex) measurements can be found in Delreux et al. (16). The separation of starch polysaccharides by gel permeation chromatography (GPC) on a Sepharose CL-2B column was performed as described in Wotteble et al. (51). The percentages of amylopectin and amylepectin were determined by pooling the large-mass amylpectin excluded from the column and the lower-mass amylepectin separately. The pooled amylepectin and amylepectin fractions were assayed with the help of the amyloglucoside assay. The chain length distributions of glucopaty and chloroplastida amylopectins were obtained by debranching them with isoamylase and separating the chains through high-performance anion exchange with pulsed amperometric detection, as described in Delrue et al. (16).

Crude extract preparation and compartmentalization studies. Cells (3 liters at 2 × 106 cells ml−1) were harvested by centrifugation (4,000 × g for 15 min). The pellet was resuspended in 10 ml of 0.25 M Tris-HCl, pH 8.0, and disrupted by sonication. The lysate was centrifuged for 10 min at 10,000 × g. This supernatant was taken as the crude extract. For compartmentalization studies, a similar cell pellet was lysed by an osmotic shock procedure. This procedure consisted of resuspending the pellet in 2 ml of 1 M Tris-HCl, pH 8.0, for 4 min and subse- quently adding 8 ml of 0.05 M Tris-HCl, pH 8.0, to this suspension for another 6 min. After centrifugation at 750 × g for 5 min, the supernatant was spun at 10,000 × g for 10 min. This defined our cytosolic extract. The chain length distributions of glucopaty and chloroplastida amylopectins were obtained by debranching them with isoamylase and separating the chains through high-performance anion exchange with pulsed amperometric detection, as described in Delrue et al. (16).

Enzyme purification and assays. GBSS was assayed as described previously (16). In vitro synthesis of amylase was performed by using a previously detailed method (49).

Starch synthase and phosphorylase purifications. The crude cytoxic extract for starch synthase and phosphorylase purifications was prepared as described above. The supernatant (17 ml) was loaded on an anion-exchange chromatog- raphy (AEC) column (MonoQ HR 5.5; 1 ml column volume) preequilibrated in buffer A (25 mM Tris-acetate, pH 7.5, 10 mM dithiothreitol [DTT]). The pro- teins were eluted at 1 ml · min−1 with a 50-ml linear gradient of 0 to 0.5 M NaCl in buffer A containing 1 M NaCl. Starch synthase and phosphorylase activities were monitored by zymogram analyses. Briefly, proteins from each fraction (0.5 ml) were separated by nondenaturating polyacrylamide gel electrophoresis (PAGE) containing 0.15% of rabbit glycogen (Sigma). After electrophoresis, gels were incubated overnight at room temperature either in starch synthase buffer [25 mM HEPES, pH 7.5, 30 mM (NH4)2SO4, 20 mM MgCl2, 5 mM β-mercap- toethanol, 1.2 mM UDP-glucose] or phosphorylase buffer (10 mM glucose-1-phosphate [G-1-P], 25 mM Tris-acetate, 10 mM DTT). The activities then were visualized as dark bands after the gels were stained with iodine solution. The fraction containing the starch synthase and phosphorylase activities were pooled and concentrated at 0.1 ml by using centrifugal filter devices (Amicon-ultra; Millipore). Starch synthase and phosphorylase activities were further purified by gel filtration chromatography (Superose 6 prepacked column; 1 cm by 30 cm; Amersham Biosciences). The samples (0.1 ml) were loaded onto a Superose 6 column preequilibrated in buffer A with a flow rate of 0.3 ml · min−1 using a fast-protein liquid chromatography system. Starch synthase and phosphorylase activities were monitored by zymogamy analyses. The fractions (free of hydrolytic activities) were pooled and used for enzymatic characterization.

The starch synthase activities were measured in the synthesis direction. Standard assays were performed for 15 min at 35°C with 20 µl of enzyme preparation and 80 µl of a mixture containing HEPES-KOH (50 mM; pH 7.5), (NH4)2SO4 (0.1 M), β-mercaptoethanol (5 mM), MgCl2 (5 mM), bovine serum albumin (BSA) (0.5 mg · ml−1), glycogen or amylepectin (10 mg · ml−1), UDP-Glc (2 mM), and UDP-[U-14C]Glc (2 µM). At the end of the incubation, glycogen or amylepectin was precipitated with 1 ml methanol-KCl (45), incubated at −20°C for 10 min, and collected by centrifugation (3,000 × g, 5 min, 4°C). The pellet was resuspended in 0.2 ml distilled water; the precipitation, centrifugation, and resuspension procedure was repeated twice more before the pellets were resus- pended in 0.2 ml distilled water and mixed with 2.25 ml scintillation liquid. The activity determined by measuring 32P incorporation into glycogen was determined by adding 50 µl of 10% P32 glucose (32P-Glc) (2000 Ci mmol−1; ICN) into 50 µl of enzyme preparation and at time zero (0 min) 50 µl of 1% (wt/vol) potato amylopectin dispersed in buffer A. The reaction was stopped by being boiled for 5 min at 95°C. The production of G-1-P was assayed by adding 300 µl of 50 mM Tris-HCl, 100 mM MgCl2, 0.05 mM glucose-1,6-diphosphate, and 0.5 µM NADP buffer. The production of NADP(H)+ was monitored spectrophotometrically at 365 nm after the addition of 4 U of phosphoglucomutase (Sigma Chemical Co., St. Louis, MO) and 2 U of glucose-6-phosphate dehydrogenase (Sigma). The phosphor- ylase activity is expressed as nanomoles of G-1-P produced per minute per milligram of protein. Apparent Km values were determined by using GraphPad Prism 5 software.

The starch hydrolyase activities were purified by loading 42 ml of supernatant on an AEC column (HiTrap FF [5 ml]) preequilibrated in buffer A (25 mM Tris-acetate, pH 7.5, 10 mM DTT). The proteins were eluted at 2 ml · min−1 with a 15-ml linear gradient of 0 to 0.5 M NaCl in buffer A containing 1 M NaCl. Starch metabolism enzymes were monitored by zymogamy analyses. Proteins from each fraction (2.5 ml) were separated by nondenaturing PAGE. After electrophoresis, proteins were transferred overnight onto polyacrylamide gel (20 cm × 20 cm × 0.15 cm) containing 3% (wt/vol) polyacrylamide (SIGMA). Starch synthase activities were monitored after being electrotransferred (2 h, 30 V, 300 mA) to glycogen-containing gels. The gels were incubated overnight at room tempera- ture in the incubation buffer described above containing either 1.2 mM ADP-Glc or 1.2 mM UDP-Glc. The electrophrodes were stained with iodine solution. Because blue-staining isomylase-like activities migrated very slowly in this zymogamy system, in a fashion similar to that of isomylase in green algae and plants, we chose to purify this enzyme by GPC. Isoamylase was purified by gel filtration chromatography (Superose 6 prepacked column; 1 cm by 30 cm; Amersham Biosciences). The isomylase-containing fractions (2 ml by 2.5 ml) that were free of all other starch hydrolyases were pooled and concentrated to 0.5 ml by using centrifugal filter devices (Amicon-ultra; Millipore). The sample (0.1 ml) was loaded onto a Superose 6 column preequilibrated in buffer A with a flow rate of 0.3 ml · min−1 using a fast-protein liquid chromatography system. The isomylase activity was monitored according to the procedure of Miller (30) based on the chemical interaction between the 3.5 diminoisocyclic acid (DIN) and sugar reducing ends. For each fraction (0.6 ml), 50 µl of isomylase activity was mixed with 50 µl of 1% (wt/vol) potato amylpectin dispersed in buffer A. The reaction was stopped by adding 100 µl of DNS. The mixtures were heated for 10 min at 100°C. The optical density was measured at 570 nm. The glucose was used as a standard to obtain the reducing end value. The isomylase activity was expressed as the equivalent of 1 µg of glucose reducing end produced min−1 mg−1 of protein. The isomylase-containing fractions were checked for the absence of other starch hydrolytic enzymes by zymogamy analysis. The fractions containing the purified isomylase were pooled and used to perform further characterizations. De-
branching enzyme activity toward amylpectin, glycogen, beta-limit dextrin (BLD), and pullulan was monitored by measuring the production of reducing ends over a time course. Fifty microliters of isoamylase activity (20 μg of reducing ends produced/min) was incubated at 37°C in 950 μl of 1% (wt/vol) polysaccharide dispersed in buffer A. The production of reducing ends was monitored over a time course of 0 to 3 h. Aliquots of 100 μl were removed after each half hour and mixed with 100 μl of DNS as described above. The total quantity of reducing ends released in the same experimental conditions for each polysaccharide was determined using commercial isomylase (Pseudomonas amyloderma amylase). The percentage of debranched polysaccharide was calculated by the formula (microgram of glucose equivalent of reducing ends measured/microgram of glucose equivalent of reducing ends released after complete debranching of the polysaccharide) × 100, thereby reducing all errors due to the different reactions of glucose and MOS with respect to DNS. The relative debranching activity toward AP and BLD was determined with respect to Gly, which was chosen arbitrarily as the reference value (set as 1) after taking into consideration the differences in the percentage of branching points for each polysaccharide for amylpectin (4.17%), BLD (9.25%), and Gly (7.12%) obtained from Manners (29).

NMR analysis. Fifty microliters of the purified isoamylase activity (20 μg of reducing end produced min⁻¹) was mixed with a 950-μl solution containing 5 mg of rabbit liver glycogen dispersed in 25 mM Tris-acetate, pH 7, 10 mM DTT overnight at 37°C. The reaction was boiled for 5 min at 90°C. Proteins were removed by loading them onto a Dowex 50x2 (1 cm by 6 cm) column immediately coupled to a Dowex 1x2 (1 cm by 6 cm) column equilibrated with water as described previously. The lyophilized samples then were subjected to proton nuclear magnetic resonance (NMR) as described previously (8, 31). The spectra were calibrated on δ-CH₃ of dimethyl sulfoxide (2.6 parts/million).

Apparent Km determination of GBSSI. The protocols used for the apparent Km determination of granule-bound starch synthase I (GBSSI) were described by Ral et al. (39).

cDNA library screening and cloning for starch synthase, starch phosphorylase, and GBSS of Cyanophora paradoxa. The Cyanophora paradoxa ZAP cDNA library was provided by W. Löffelhardt. The cDNA probes were designed using expressed sequence tag (EST) sequences obtained from the TbestDB project web site (http://tbestdb.bcm.umontreal.ca/searches/login.php). The protocol used for cDNA library screening is described in Jakowitsch et al. (21) with the following modifications. DNA probe labeling and detection were performed with the DIG luminescent detection kit (Roche Molecular Biochemicals) using the protocol provided by the manufacturer.

Nucleotide sequence accession numbers. Sequences determined in the course of this work have been deposited in GenBank under accession numbers EU165054 to EU165056.

RESULTS

Cyanophora paradoxa accumulates semicrystalline high-amylose storage polysaccharide granules that are similar to plant starch. Starch was purified from late-log-phase Cyanophora paradoxa cells by sedimentation and density gradient purification. The dispersed polysaccharide was subjected to the separation of polysaccharide fractions through GPC. Results displayed in Fig. 1A demonstrate that this material contained both a high- and low-mass polysaccharide fraction similar to amylpectin and amylose, respectively, from the unicellular green alga Chlamydomonas reinhardtii (Fig. 1B). The amylose/amylopectin ratio was calculated by harvesting their respective fractions after GPC and measuring the two pooled fractions with the amylglucosidase assay. The amylose content of the glaucophyte was systematically high to very high, varying from 30% of the total polysaccharide in nitrogen-supplied medium in alternating day and night cultures to 60% in continuous light. At variance with the results obtained with green algae, nitrogen starvation had little impact on the relative amylose content. Chlamydomonas reinhardtii wild-type strain 137C accumulates from 2% to at most 10% amylose when supplied with nitrogen and from 10 to 30% under nitrogen starvation.
Amylopectin from *Cyanophora paradoxa* was collected after GPC and subjected to debranching through the action of an isoamylase. The chain length distribution of the debranched chains then was examined by high-performance anion exchange with pulsed amperometric detection. Results displayed in Fig. 1C show that *Cyanophora paradoxa* contains a starch-type bimodal distribution of glucans that is selectively enriched in very short chains compared to those of *Chlamydomonas reinhardtii* (Fig. 1D). Starch granules diffract and give two types of X-ray diffraction patterns corresponding to two different assembly geometries that have been named A type and B type, respectively (7). The starch granule typically is considered semicrystalline. Indeed, only sections of the amylpectin molecule assemble into crystals. Amylose usually is thought to be mostly amorphous. Nevertheless, a high amylose content has a tendency to yield a B type of diffraction pattern for the remaining amylpectin. The *Cyanophora paradoxa* starch granules thus were further analyzed using X-ray diffraction. The samples used for the analysis produced clear B-type diffractograms with a crystallinity percentage around 30% (Fig. 2A). The morphology of the starch granules was further investigated by scanning electron microscopy (Fig. 2B). Classical starch granules were evidenced with occasional cavities or morphology alterations that could be due either to in vivo polysaccharide degradation or to the growth of granules in close contact with other cellular structures. However, when present, the cavities did not match the size and frequency recently observed in the cryptophyte floridean starch granules (18). In *Cyanophora paradoxa* granules varied between 0.5 and 2 to 3 µm in diameter, figures comparable to those of the starch found in green algae and plant leaves.

Based on the results of all of these studies, we conclude that *Cyanophora paradoxa* accumulates a typical semicrystalline high-amylose starch.

Glaucophyte granule-bound starch synthase activity can use both ADP-glucose and UDP-glucose. Amylose generally is associated with the presence of granule-bound starch synthase, the enzyme that, in plants, is responsible for the processive synthesis of such long glucans (for a review, see reference 4). We thus measured the granule-associated starch synthase activity from freshly purified starch granules. The *Cyanophora paradoxa* GBSS was able to use both ADP-glucose and UDP-glucose (apparent $K_m$ values of 8 mM ADP-Glc and 15 mM UDP-Glc) but displayed a sixfold higher $V_{max}$ with UDP-glucose ($0.23 \pm 0.04$ nmol ADP-Glc incorporated/min/mg of starch and $1.48 \pm 0.5$ nmol UDP-Glc incorporated/min/mg of starch). We further characterized the structure of the polysaccharide produced with both ADP-glucose and UDP-glucose. Results displayed in Fig. 3 demonstrate that the behavior of the starch-bound activity of the two glycosyl nucleotides was identical and was analogous to that evidenced for cryptophyte (18), green alga (49), and plant (17) GBSSI. Indeed, GBSSI is known in the absence of added malto-oligosaccharides to polymerize glucose residues to the outer chains of amylpectin. These chains subsequently are cleaved off amylpectin to generate mature amylose (Fig. 3A and B). On the other hand, in the presence of malto-oligosaccharides that act as competitive primers with amylpectin, the enzyme is observed to directly synthesize amylose-like molecules, and very little incorporation occurs in the amyllopectin (Fig. 3C and D). In addition to this typical GBSS type of activity, we found a 64-kDa major granule-associated protein (Fig. 4) that displayed significant homology to plant GBSSI sequences. The protein was hydrolyzed with trypsin, and peptides were sequenced. This enabled us to correlate the protein with the full GBSSI-like sequence that we have cloned from our *Cyanophora paradoxa* cDNA bank (GenBank accession no. EU165054).

*Cyanophora paradoxa* contains a major GT5 family UDP-glucose-utilizing soluble starch synthase. Because the kinetic characterization of GBSSI did not support a very dramatic preference for UDP-glucose over ADP-glucose, we investigated the properties of the soluble starch synthases. Our glycogen-primed zymogram gels demonstrated the presence of several UDP-glucose-utilizing soluble starch synthase activity bands with close mobility. These activities were localized in the cytoplasmic compartment (Fig. 5A). No ADP-glucose-utilizing elongation enzymes were evidenced by these procedures (results not shown). We chose to partly purify the enzyme activities and investigate their kinetic properties. Results displayed in Fig. 5B demonstrate that two types of soluble starch synthase activities could be purified free of contaminating starch hydrolases by AEC followed by GPC. Peaks 1 and 2 amounted to 87 and 13%, respectively, of the total assayable starch synthase activities and investigate their kinetic properties. Results displayed in Fig. 5B demonstrate that two types of soluble starch synthase activities could be purified free of contaminating starch hydrolases by AEC followed by GPC. Peaks 1 and 2 amounted to 87 and 13%, respectively, of the total assayable starch synthase activity, with an optimal temperature of 35 and 25°C, respectively, and an optimal pH of 7.5 and 8, respectively (data not shown). These activities display a marked preference for UDP-glucose and cannot use ADP-glucose at significant rates (<2% of the activity measured in the presence of UDP-glucose) (data not shown). Interestingly, the second activity (peak 2) displayed clear sigmoidal kinetics in the presence of increasing concentrations of amylpectin (Fig. 5D), while peak 1 showed a classical Michaelis-Menten type of kinetics in the
presence of glycogen or amylopectin, as did peak 2 in the presence of glycogen (Fig. 5C and D). The behavior of peak 2 recalls that of the starch synthase that recently was purified from *Gracilaria tenuistipitata* (34). For peak 1, increasing concentrations of UDP-glucose in the presence of either glycogen or amylopectin enabled us to measure apparent $K_m$ values of $2.2 \pm 0.2$ mM in the presence of 10 mg ml$^{-1}$ glycogen and $5.2 \pm 0.9$ mM in the presence of 10 mg ml$^{-1}$ amylopectin (Fig. 5E). Peak 2 displayed $K_m$ values of $4 \pm 0.3$ mM in the presence of 10 mg ml$^{-1}$ glycogen and $4.6 \pm 2.6$ mM in the presence of 10 mg ml$^{-1}$ amylopectin (Fig. 5F). We then searched for the presence of ESTs encoding soluble starch synthase and found a small sequence, which was used to select out longer cDNAs. We obtained an incomplete 1,669-bp cDNA containing most of the core sequence corresponding to soluble starch synthases of the GT5 family according to the CAZY classification, but it still lacked the variable N-terminal extension evidenced both in rhodophyceae and chloroplastida (GenBank accession no. EU165055). Phylogenetic trees built with this core sequence clearly support the grouping of this cDNA sequence with those evidenced in rhodophyceae and alveolates (Fig. 6).

**FIG. 3.** Fractionation of starch granules isolated from *Cyanophora* and subjected to in vitro synthesis of amylose. After incubation with radiolabeled ADP-glucose (A) or UDP-glucose (B), starch granules were subjected to CL-2B size-exclusion chromatography in order to detect the fractions in which radiolabeled glucose residues were incorporated. The isotopic dilution for ADP-glucose was three times lower than that for UDP-glucose. The same experiment with ADP-glucose (C) or UDP-glucose (D) was done with the addition of 50 mM maltotriose. The $x$ axis indicates the elution volume, the left axis shows the iodine-polysaccharide complex absorbance of each fraction eluted from the column (○), and the right axis shows the radioactivity of each fraction (○). Without the addition of maltotriose (DP3), radioactive material was incorporated in both the excluded amylopectin fraction and the amylose fraction. The addition of DP3 restricts incorporation in the amylose fraction only. This behavior is a specific property of GBSS-like starch synthases. OD, optical density.

**FIG. 4.** Visualization of the proteins associated with *Cyanophora paradoxa* starch granules. Granule-bound proteins were extracted by boiling them in sodium dodecyl sulfate (SDS) and were separated by SDS-PAGE (18). A molecular mass marker also is displayed. The major band at around 64 kDa was hydrolyzed with trypsin, and the resulting peptides were eluted from the gel and identified by matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) and mass spectrometry–mass spectrometry (MS-MS) sequencing. The peptides YDQYFADWTSVR, VTFLLHNLYQGR, DLPVNALATR, VFYETKGKDR, and SVPTPLAFVGR correspond to a GBSS and were found in the sequence isolated by cDNA screening. The band at around 92 kDa was hydrolyzed with trypsin, and the resulting peptides were eluted from the gel and identified by MALDI-TOF and MS-MS sequencing. The peptide MQAVQORY corresponds to a starch phosphorylase and was found in recent *Cyanophora paradoxa* EST resources (GenBank accession no. EC661027) (35).

**FIG. 5.** Visualizations of the proteins associated with *Cyanophora paradoxa* starch granules. Granule-bound proteins were extracted by boiling them in sodium dodecyl sulfate (SDS) and were separated by SDS-PAGE (18). A molecular mass marker also is displayed. The major band at around 64 kDa was hydrolyzed with trypsin, and the resulting peptides were eluted from the gel and identified by matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) and mass spectrometry–mass spectrometry (MS-MS) sequencing. The peptides YDQYFADWTSVR, VTFLLHNLYQGR, DLPVNALATR, VFYETKGKDR, and SVPTPLAFVGR correspond to a GBSS and were found in the sequence isolated by cDNA screening. The band at around 92 kDa was hydrolyzed with trypsin, and the resulting peptides were eluted from the gel and identified by MALDI-TOF and MS-MS sequencing. The peptide MQAVQORY corresponds to a starch phosphorylase and was found in recent *Cyanophora paradoxa* EST resources (GenBank accession no. EC661027) (35).

*Cyanophora paradoxa* contains an isoamylase type of debranching enzyme and multiple starch phosphorylases. Starch phosphorylase zymograms display activity bands that barely
entered the polysaccharide-containing gels. We tracked this phosphorylase activity during the semipurification and assayed the enzyme(s) in semipure fractions lacking starch synthase or hydrolase activities. The kinetics displayed in Fig. 7A demonstrate the presence of classical starch phosphorylase activities with high affinity toward both glycogen (apparent $K_m$, 0.5 ± 0.08 mg · ml$^{-1}$) and amyllopectin (apparent $K_m$, 0.65 ± 0.05 mg · ml$^{-1}$). However, the enzyme clearly was inhibited by high amounts of amyllopectin substrate. Figure 7B displays a similar experiment performed with increasing concentrations of orthophosphate in the presence of either 10 mg · ml$^{-1}$ glycogen (apparent $K_m$, 2 ± 0.8 mM P$_i$) or 10 mg · ml$^{-1}$ amyllopectin (apparent $K_m$, 3 ± 0.7 mM P$_i$). Interestingly, as displayed in Fig. 7C, this activity was inhibited by both UDP-glucose ($K_i$, 0.35 ± 0.1 mM) and ADP-glucose ($K_i$, 0.1 ± 0.05 mM) in a fashion similar to that evidenced in green algae (2, 14, 32). As was described for other phosphorylases, the inhibition was competitive with respect to orthophosphate. The finding of a short EST that possibly could encode starch phosphorylase gave us a probe to select out the full-length cDNA and therefore enabled us to get the full enzyme sequence (GenBank accession no. EU165056). Another EST recently was selected from the growing genomic resources (GenBank accession no. EC661027) (35). This EST displayed a clearly distinct sequence, suggesting the presence of several different phosphorylase subunits or enzymes. The mass of several peptides generated by the analysis of a 92-kDa starch granule-associated protein (Fig. 4) matched that predicted by the EST encoding this second phosphorylase.

We then probed for the existence of starch hydrolases and...
found the usual set of zymogram bands on starch-containing gels from crude extracts (Fig. 8A) and after semipurification (Fig. 8B). In addition, we looked for the presence of these enzymes within cyanelles and found no traces of such activities. Finally, among the starch hydrolases, we probed for the existence of isoamylase, a starch debranching enzyme, through standard zymogram procedures and enzyme purification. Mutant work performed with green algae and vascular plants strongly suggested that isoamylase is involved in trimming the misplaced chains in a hydrophilic precursor of amylopectin to generate an insoluble semicrystalline form of the polysaccharide (3). This suggestion now is supported by a wealth of molecular and biochemical data obtained solely from green plants and algae (12, 13, 15, 22, 31, 33, 38, 52).

We searched for the presence of isoamylase by tracking a slow-migrating set of blue-stained bands evidenced on amylopectin- or starch-containing zymogram gels after iodine staining of the incubated gels. A blue-stained band is indicative of a debranching-type hydrolase digesting the polysaccharide trapped within the gels. The blue stain is due to the remaining slowly diffusing longer debranched oligosaccharides. Because this is only an indication of the presence of a debranching enzyme, we subjected crude extracts (Fig. 8A) to purification by AEC (Fig. 8B) followed by GPC until the fraction suspected to contain debranching enzyme was devoid of all other types of starch hydrolases (amylases, branching enzyme, and D-enzyme) (Fig. 8C).

The substrate specificity of the suspected debranching enzyme was measured with enzyme assays involving the production of reducing ends (Fig. 8D and E). Definitive proof that the purified fraction contained only debranching enzyme activity finally was obtained by proton NMR analysis (Fig. 8F). The signals at 5.3 to 5.1 ppm and 4.85 ppm correspond to the proton resonances from the carbon atoms engaged in an \(-1,4\) linkage and an \(-1,6\) linkage, respectively. The NMR analysis performed at 70°C instead of 90°C yields a two- to threefold underestimation of the number of branching points. After incubation with isoamylase activity, the proton resonances from the carbon atoms of the reducing end appear as two signals, at 5.05 and 4.45 ppm, corresponding to \(1H_{\text{A}}\) and \(1H_{\text{B}}\) anomers, respectively. This is correlated with a dramatic decrease of the signal at 4.85 ppm, corresponding to the \(1H\) from the carbon atoms engaged in an \(-1,6\) linkage. The results clearly indicated the presence in *Cyanophora paradoxa* of a high-mass (390 kDa) isoamylase complex (Fig. 8C), as measured by GPC on a Superose 6 column. The *Cyanophora* enzyme was able to debranch glycogen, amylopectin, and amylopectin \(-\) limit dextrin with comparable efficiencies (Fig. 8D and E). It was, however, unable to debranch pullulan at significant rates.

**DISCUSSION**

Our characterization of *Cyanophora paradoxa* soluble starch synthases clearly shows that these activities are able to elongate...
Kinetic characterization of both soluble and granule-bound starch synthases was evidenced by our purification studies. It is presently unknown if the activities reflect alternative regulated states of the same enzyme or if they are encoded by distinct proteins.

Another important finding reported in this work consists in the characterization of a glucohyde GBSSI responsible for amylose synthesis. This enzyme could use both ADP-glucose and UDP-glucose, but the specific activity measured with UDP-glucose was sixfold higher. The ambiguous selectivity of GBSSI toward ADP-glucose and UDP-glucose is a common trait for the GBSSI genes currently reported. Indeed, the maize enzyme initially was detected through its activity toward amylose synthesis. This enzyme could use both ADP-glucose and UDP-glucose, but the specific activity measured with ADP-glucose (25), while ADP-glucose was recognized only recently as the preferred substrate (40). Similarly, we have recently characterized a GBSSI from the cryptophyte *Guilardia theta* that showed only a slight preference for UDP-glucose (18). The reason for the low selectivity of the bound enzyme compared to that of the soluble starch synthases is discussed elsewhere (P. Deschamps et al., submitted).

Kinetic characterization of both soluble and granule-bound enzymes showed that both activities display comparable affinities toward their UDP-glucose substrate. This distinguishes glucohydes from their chloroplastida relatives. In green algae.
and land plants, the affinity of the soluble enzyme for ADP-glucose is at least 4-fold to more than 10-fold higher than that of the bound activity (4). When the supply of ADP-glucose is limited, amylopectin synthesis is relatively favored over that of amylose. This explains why mutants partly defective for ADP-glucose synthesis display a significant relative enrichment of amylopectin over that of amylose (48). In *Chlamydomonas*, cultures supplied with normal levels of nutrients contain little amylose (<5%), while nutrient-starved cultures accumulate between 10 to 25% of this starch fraction (26). In *Cyanophora paradoxa*, the relative amount of amylose always remains at a very high level (between 30 to 60%), which is only rarely reached in some plant mutants. Moreover, this high level is not significantly affected by the supply of nutrients. These levels are three- to sixfold higher than those reported for green algae and plants. We believe this to be due solely to the comparable affinities of the granule-bound and soluble starch synthase toward UDP-glucose. Indeed, the amount of protein within the granule and the enzyme-specific activity are significantly inferior to those we measure for the *Chlamydomonas* enzyme and therefore cannot explain the relatively high amylose content.

The finding we report for GBSSI in glaucophytes echoes the description that was recently made for rhodophyceae (44). GBSSI thus is found in all three lineages that presently define the archaeplastida and very likely was present in the ancestor of all plants. This has important implications with respect to our understanding of the evolution of storage polysaccharide metabolism. It has been demonstrated previously that GBSSI
requires the presence of a preformed semicrystalline starch granule to be normally active (11). This in turn suggests that the ancestor of all plants synthesized such a structure, probably in its cytoplasm. An ancient cytoplasmic location for the synthesis of starch is suggested by the finding of a similar compartmentalization of storage polysaccharide in rhodophyceae and glaucophytes. Although some nuclear gene phylogenies have not been able to resolve the divergence order of the three archaeaplastida lineages, both more recent nuclear gene phylogenies as well as plastid phylogenies indicate that glaucophytes diverged first from the common ancestor (41, 43). In addition, a recent study concerning the emergence of the light-harvesting complex (LHC) proteins can be explained only with a similar divergence order (24). The hypothesis of a cytoplasmic location of starch synthesis in the common ancestor of all plants thus is more parsimonious in this respect.

It must be stated that the phylogeny of all sequences obtained either in this work or by bioinformatics analysis of the growing Cyanophora paradoxa (Deshamps et al., submitted) genomic resources are in complete agreement with the monophyletic origin of plants. Of special relevance are the phosphorylase enzyme sequences, which, as in red and green algae, display a common eukaryotic (host) origin.

All positive findings reported in this work support the existence of a UDP-glucose-based pathway of starch synthesis in glaucophytes that is very similar to that that has been proposed for rhodophyceae. We cannot exclude, however, the existence of minor forms of soluble starch synthases able to use ADP-glucose that would be phylogenetically related to the chloroplastic or cyanobacterial enzymes, especially when faced with glucose that would be phylogenetically related to the chloroplastic or cyanobacterial enzymes, especially when faced with glucose (185:1–26).

We thank Curtis Hannah for providing us with the antibody directed against the small subunit of ADP-glucose pyrophosphorylase. We thank Emmanuel Maes for performing the NMR analysis.

REFERENCES


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