Molecular and Biochemical Analysis of Periplastidial Starch Metabolism in the Cryptophyte *Guillardia theta*

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Starch in synchronously grown *Guillardia theta* cells accumulates throughout the light phase, followed by a linear degradation during the night. In contrast to the case for other unicellular algae such as *Chlamydomonas reinhardtii*, no starch turnover occurred in this organism under continuous light. The gene encoding granule-bound starch synthase (GBSSI), the enzyme responsible for amylose synthesis, displays a diurnal expression cycle. The pattern consisted of a maximal transcript abundance around the middle of the light phase and a very low level during the night. This diurnal regulation of GBSSI transcript abundance was demonstrated to be independent of the circadian clock but tightly light regulated. A similar yet opposite type of regulation pattern was found for two α-amylase isoforms and for one of the two plastidic triose phosphate transporter genes investigated. In these cases, however, the transcript abundance peaked in the night phase. The second plastidic triose phosphate transporter gene had the GBSSI mRNA abundance pattern. Quantification of the GBSSI activity revealed that not only gene expression but also total enzyme activity exhibited a maximum in the middle of the light phase. To gain a first insight into the transport processes involved in starch biosynthesis in cryptophytes, we demonstrated the presence of both plastidic triose phosphate transporter and plastidic ATP/ADP transporter activities in proteoliposomes harboring either total membranes or plastid envelope membranes from *G. theta*. These molecular and biochemical data are discussed with respect to the environmental conditions experienced by *G. theta* and with respect to the unique subcellular location of starch in cryptophytes.

One of the most fascinating and important biological processes on Earth is the light-driven fixation of carbon dioxide called photosynthesis. Apart from cyanobacteria, photosynthesis is performed by red algae (Rhodophyta), green plants (Virididaeplanta), and blue-green Glaucophyta, and all of these groups arose by a single ancient symbiosis (primary endosymbiosis) between a protozoan host and a cyanobacterium. During this process the cyanobacterium converted into a primary chloroplast surrounded by an envelope consisting of an inner membrane and an outer membrane (1).

Interestingly, many eukaryotic algae acquired photosynthesis through a process called “secondary endosymbiosis,” which gave rise to even more complex chimeric eukaryotes (chlorarachneans and chromalveolates). The nonphotosynthetic host obtained its plastid by engulfing a phototrophic eukaryote with a primary chloroplast. It is an open question whether these eukaryotes are derived from the plastid envelope of the red algal symbiont, the second outermost membrane encloses the former cytosol of the symbiont (periplastid compartment), and in chromobiotes and cryptomonads the outer membrane is connected with the endoplasmic reticulum of the host cell (34).

Photosynthetic fixation of carbon dioxide leads to the generation of storage products such as soluble sugars (e.g., sucrose) or insoluble starch. While sucrose acts as a major transport form of carbohydrates, chloroplastic starch functions as temporary storage for carbon and energy required during the night phases (38). In higher plants and green algae, the plastidic (stromal) localized starch biosynthesis starts with the provision of ADP-glucose, which serves as a carbon donor for elongation of an existing starch core (15, 33, 37). This elongation is mediated by two isoforms of starch synthases, a soluble starch synthase and a granule-bound starch synthase (GBSS) (27).

However, the organization of starch metabolism in the red lineage of algae is totally different from that in the green lineage of photoautotrophs. For example, red algae store starch outside the plastid in the cytosol (23), and the starch synthases accept UDP-glucose as a substrate (3, 21, 37).

Remarkably, although algae harboring complex plastids contribute significantly to annual CO₂ fixation on Earth (29), our knowledge about starch synthesis in these species is much less than that about starch synthesis in higher plants. In recent years *Guillardia theta* has been developed as a model cryptomonad, because the genome of the vestigial nucleus (nucleomorph), residing in the periplastid compartment, has been
sequenced (5) and isolated plastids are able to import preproteins exhibiting cryptomonad-specific import/target signatures (35). Moreover, *G. theta* is also interesting since starch resides neither in the stroma (as in green algae) but in the periplastid compartment (9). This subcellular location of starch concurs with the unique bipartite structure of the GBSSI transit sequence identified by us (10).

In this study we were interested in revealing mechanisms causing changes of starch levels in *Guillardia theta* during consecutive day/night cycles. Therefore, we focused on the regulation of genes involved in starch turnover, and we wanted to elucidate the nature of carrier proteins involved in photosynthetic export and energy provision in complex plastids. The regulatory processes and their implications for starch levels in *G. theta* are discussed in comparison to regulation of starch synthesis in green algae and higher plants and with respect to the environmental conditions experienced by free-living *G. theta* cells.

**MATERIALS AND METHODS**

**Growth of Guillardia theta.** A culture of *Guillardia theta*, strain CCMP 327 (Provasoli-Guillard National Center for Culture of Marine Phytoplankton), was maintained and grown in h/2 marine medium (11) (lacking silica) under alternating light/dark conditions (12 h light/12 h dark). For studies concerning enzyme activity, starch quantification, Northern blot analysis, membrane preparations, and plastid isolation, synchronized cultures were grown for a minimum of 2 days in 1,000-ml aerated glass tubes under the light regimen stated above.

Cultures were inoculated at the early log phase and sampled after 2 to 3 days. For analysis of diurnal starch turnover and regulation of transcription, actively growing cells were sampled every 2 to 3 hours and fresh medium was added immediately to compensate medium volume reduction and to obtain a constant cell density. Cells were harvested by centrifugation (2,000 × g, 10 min).

**Isolation of plastids and preparation of total membranes.** Actively growing synchronized cells were harvested at the middle of the light phase, and plastids were purified by density gradient centrifugation (35). The purity and integrity of the plastid fraction were proven microscopically, by latency analysis of stromally located NADP-GAPDH (intactness of >90%), and by quantification of the mitochondrial marker enzyme fumarase and the peroxisomal marker catalase (36). The purity and integrity of the isolated plastids were assessed by quantification of total polysaccharide, protein, and DNA by the anthrone reaction (43), by RNA isolation and gel estimation (37), and by spectrophotometric determination of total chlorophyll concentration (38).

**Extraction and preparation of total RNA and RNA gel blot hybridization.** Total RNA was extracted from 400 ml of actively growing cell suspension by using the Purescript extraction kit (Gentra Systems, North Minneapolis, MN). RNA gel blot hybridization analysis was carried out using standard methods (26) and visualized using a phosphorimager (Packard, Frankfurt, Germany).

**RESULTS**

Mechanisms controlling diurnal turnover of starch. Within three consecutive day/night cycles, starch accumulates in the light phases and declines in the corresponding night phases (Fig. 1A). The maximal starch level in the first light phase after inoculation of the *Guillardia theta* culture was only 60% of the value present in the consecutive light phases (phases two and three) (Fig. 1A). Taking light phases two (24 to 36 h) and three (48 to 60 h) after the start of inoculation as representative, we observed a net rate of starch synthesis of 4.13 mg/mg chlorophyll/12 h (Fig. 1A). This value corresponds to a carbon flux equivalent in 1.91 μmol C₅/mg chlorophyll/h.

To investigate the putative persistence of the starch turnover in continuous light, we also monitored the levels of this polysaccharide over 72 h of constant illumination (Fig. 1A). During continuous light phase which followed the three consecutive day/night cycles, no starch breakdown in *Guillardia* occurred.
This observation is a first indication that the diurnal starch accumulation in *Guillardia* is governed by light and not by the circadian clock.

**Diurnal regulation of genes and enzymes involved in starch turnover.** To get a better understanding of the underlying molecular mechanisms governing starch turnover in *Guillardia theta*, we monitored the patterns of expression of genes involved in starch metabolism. *GBSS1* mRNA accumulated rapidly to a high level at 1 hour after start of the light phase (Fig. 1B). This level of *GBSS1* mRNA stayed constant for about 6 hours within the light phase and declined towards the end of the photosynthetic period (Fig. 1B). At the end of the light phase (the last 4 hours), the level of *GBSS1* mRNA was already very low, and it remained close to the detection limit in the dark phase (Fig. 1B). Interestingly, these substantial diurnal changes of *GBSS1* mRNA levels disappeared within the first 36 h of permanent illumination (Fig. 1B). Subsequently, *GBSS1* mRNA levels remained constantly low (Fig. 1B).

As shown above, the level of *G. theta GBSS1* mRNA displayed large variations within a single light/dark cycle (Fig. 1B). As starch synthesis seems to be strictly coregulated (Fig. 1A), it was interesting to analyze whether *GBSS1* activity levels correlated to *GBSS1* mRNA. *GBSS1* activity during the night phase remained constant at about 175 nmol/mg protein/h (Fig. 2). However, upon onset of light, this activity increased by about 43% to a maximum of 250 nmol/mg protein/h within the first 8 hours of light and stayed nearly constant at that level until the end of the light phase. After the onset of darkness, *GBSS1* activity declined to about 200 nmol/mg protein/h at 12 p.m. (Fig. 2).

Two partial mRNA sequences coding for the starch-degrading enzymes α-amylase 1 (*Amy1*) and α-amylase 2 (*Amy2*) were discovered in the data from the ongoing *Guillardia* EST project. Interestingly, both mRNA species accumulated similarly after the onset of darkness and reached a maximal level at 7 hours past the end of the light phase (Fig. 3). After the start...
of illumination, Amy1 and Amy2 mRNAs declined rapidly, and they remained at a constant low level throughout the entire light phase (Fig. 3). The mRNAs of both amylase genes show an expression pattern opposite to that for the GBSS1 mRNA (Fig. 3).

Within the data from the ongoing EST project we also discovered two mRNAs coding for proteins annotated as a putative plastidic triose phosphate/phosphate transporters (TPT1 and TPT2). Since this carrier activity is crucially involved in carbohydrate transport across the envelopes of plastids from higher plants (7), it was interesting to analyze the expression patterns of the corresponding genes in G. theta.

The level of TPT1 mRNA followed the Amy1 and Amy2 mRNA oscillation pattern (Fig. 3). TPT1 mRNA accumulated within the first 5 hours of the night phase and remained at a high level until the end of the night (Fig. 3). Immediately after the onset of illumination, the TPT1 mRNA level dropped, and it reached the lower detection limit after about 5 hours (Fig. 3). During the complete night phase, TPT2 mRNA remained at a low level, and then it accumulated substantially between 6 and 8 a.m. (Fig. 3). The TPT2 mRNA level further increased until 10 a.m., remained high until 4 p.m., and slightly declined thereafter towards the end of the light phase (Fig. 3). This slight decrease towards the end of the light phase resembles the expression pattern of GBSS1 (Fig. 1B), and both examples indicate that gene expression in G. theta is not triggered exclusively by the light stimulus.

**Effect of a sudden light/dark switch on the expression of genes involved in starch biosynthesis.** Constant illumination of Guillardia theta following alternating light/dark phases yielded a stable high expression level of GBSS1 mRNA (Fig. 1B). Data from the experiment presented in Fig. 3 argue for the presence of light-controlled up-regulation of GBSS1 and TPT2. To analyze the effect of illumination in more detail, we first grew G. theta for 2 days in the standard light/dark cycle and then delayed the start of the third light phase for 2 hours (Fig. 4A).

As displayed in Fig. 4A, GBSS1 and TPT2 mRNAs were either absent or at low levels, respectively, without the light signal occurring normally at 7 a.m. However, after onset of the delayed illumination, both mRNA species accumulated in Guillardia (Fig. 4A). As demonstrated above, GBSS1 mRNA declined towards the end of the light phase in standard light/dark cycles (Fig. 1B). Therefore, it was interesting to analyze how sudden darkness might also affect the accumulation of GBSS1 mRNA. For this experiment we first grew G. theta for 2 days in standard light/dark conditions. In the third cycle we turned the lights on for only 2 hours and then switched them off (Fig. 4B). As displayed in Fig. 4B, the level of GBSS1 mRNA declined rapidly after onset of darkness and remained at a constant low level.

**Activity of transport proteins involved in starch metabolism in Guillardia theta.** In Guillardia theta starch resides outside the stroma in the periplastid compartment. This spatial separation predicts comparable large metabolic fluxes between the plastid and the surrounding periplastid compartment. In higher plants the chloroplastic triose phosphate/phosphate antiporter is the major carrier protein catalyzing the efflux of photosynthate into the cytosol (7). In addition both heterotrophic plastids and certain types of chloroplasts have been found to contain hexose phosphate/phosphate transporters mediating transmembrane movement of either glucose 6-phosphate (Glc6P) or, possibly, glucose 1-phosphate (Glc1P) (7, 18). However, up to now the plastidic phosphate transporter complement of cryptophytes has never been analyzed. Therefore, we conducted uptake experiments with reconstituted total membranes either from
independent biological replicates. Analysis represent a typical expression pattern as observed in three boxes represent phases of unexpected darkening. The Northern blot and white bars indicate the corresponding light phase. The gray shaded reveals equal RNA loading. Dark gray bars indicate the dark phase, nized with a

ern blot analysis. Total RNA was extracted from 400 ml of synchro-
der sudden light/dark changes. Alterations of GBSS1 gene expression under sudden light/dark changes were detected by Northern blot analysis. Total RNA was extracted from 400 ml of synchronized algae harvested at the indicated time points. Blots were hybridized with a TPT2- or GBSS1-specific probe. Ethidium bromide staining reveals equal RNA loading. Dark gray bars indicate the dark phase, and white bars indicate the corresponding light phase. The gray shaded boxes represent phases of unexpected darkening. The Northern blot analysis represent a typical expression pattern as observed in three independent biological replicates.

whole G. theta cells or from purified intact plastids harboring the two innermost membranes (35).

As shown in Fig. 5A, total G. theta membranes contain a phosphate/phosphate (P\textsubscript{i}/P\textsubscript{i}) antiport activity as is present in plastids from higher plants. In addition to P\textsubscript{i}/P\textsubscript{i} exchange, the corresponding carrier is also able to catalyze a dihydroxyacetone phosphate (DHAP)- or phosphoenolpyruvate-driven counterexchange of P\textsubscript{i} (Fig. 5B and D), whereas glycerate 3-phosphate, Glc6P, or Glc1P does not stimulate uptake of \textsuperscript{32}P\textsubscript{i} into proteoliposomes (Fig. 5C, E, and F).

After reconstitution of membranes from enriched G. theta plastids, we were able to determine the apparent affinities (K\textsubscript{m}) and the V\textsubscript{max} of the plastidic phosphate transport system within a time-linear phase of 2 to 5 min. The plastidic phosphate transporter imports P\textsubscript{i} (in counterexchange with DHAP) at a V\textsubscript{max} of 33 \mu mol/mg chlorophyll/h and exhibits an apparent K\textsubscript{m} of 0.62 mM (Fig. 6A).

In addition to the transport of phosphorylated sugars, we also monitored the transport of nucleotides via the plastidic nucleotide transporter NTT. Proteoliposomes containing proteins from enriched G. theta plastid membranes exhibited a time-linear uptake of ATP (data not shown). Analysis of the concentration dependency of ATP uptake into these proteoliposomes revealed an apparent K\textsubscript{m} of 0.19 mM and a V\textsubscript{max} of 0.9 \mu mol/mg chlorophyll/h (Fig. 6B). Proteoliposomes harboring total membranes exhibited a maximal bongkrekic acid-insensitive ATP uptake of 20 \mu mol/mg chlorophyll/h (SE, \pm 0.5 \mu mol/mg chlorophyll/h), indicating that most of the plastidic ATP/ADP transport activity resides in the two outermost and not in the two innermost membranes from G. theta plastids.

**DISCUSSION**

We demonstrated that starch levels in synchronized Guillardia theta cells increase throughout the light phase, reach a maximal level in the last part of the light period, and decrease within the subsequent night phase (Fig. 1A). This pattern of starch accumulation resembles diurnal changes of the starch levels in leaves from higher plants but not those found in other unicellular algae such as, e.g., Chlamydomonas reinhardtii. Higher plants such as Arabidopsis thaliana accumulate transitory starch throughout the entire light phase and start degradation of this polysaccharide right after the onset of the dark phase (8). In contrast C. reinhardtii exhibits the lowest starch levels in the middle of the day, demonstrating rapid degradation of starch during the early phase of photosynthesis (32; S. Ball et al., unpublished data).

It appears remarkable that all genes tested that are involved either in starch turnover (GBSS1, \alpha-Amy-1, and \alpha-Amy-2) or in transport of photosynthetic intermediates (TPT1 and TPT2) exhibit a diurnal regulation of the expression level (Fig. 1A and 3). Moreover, we showed that changes of GBSS1 mRNA accumulation correlate to some extent with changes in the extractable enzyme activity (Fig. 1B and 2). However, we have to consider that altered GBSS1 activity (Fig. 2) might also be caused by a so-far-unknown type of posttranslational modification.

In general, circadian clock-controlled processes have to persist under constant conditions after a previous phase characterized by a diurnal pattern (16). In case of both diurnally changing starch levels and diurnally changing GBSS1 mRNA levels, we showed that constant illumination, after alternating light/dark cycles, yielded an uncontrolled stable behavior (Fig. 1A and B). This observation clearly reveals that light and not a circadian clock triggers the diurnal rhythm of both factors. In species such as C. reinhardtii or the dinoflagellate Gonyaulax polyedra, the diurnal rhythms are governed by light-independent endogenous circadian clocks (25; Ball et al., unpublished data). As the diurnal rhythm of several genes in Guillardia is controlled by light (Fig. 3 and 4), we have to conclude that different unicellular algae exploit individual molecular mechanisms to control their diurnal rhythms.

So far it is unknown why G. theta, in contrast to other unicellular algae, exhibits a light-governed regulation of the diurnal rhythm. However, we might speculate that this is due to specific environmental conditions experienced by Guillardia. So far this species has been found in coastal regions of the United States (24) and in the Wadden Sea in Denmark (12). These zones are characterized by wind-driven upwelling (13, 24) and tide changes (29), which are known to provoke substantially changing light intensities. Therefore, the rapid light-controlled regulation of enzymes and processes ultimately connected to the presence of light is crucial. However, light-
FIG. 5. Time dependency of $^{32}$P uptake into proteoliposomes. Proteoliposomes harboring 50 μg of total membrane proteins from *G. theta* were incubated in uptake medium containing 0.1 mM α-$^{32}$P, for the indicated time periods. Proteoliposomes were preloaded with 10 mM of various putative counterexchange substrates (■) or did not contain the putative counterexchange substrate (unloaded controls, ○). (A) Pi-stimulated uptake; (B) DHAP-stimulated uptake; (C) glycerate 3-phosphate-stimulated uptake; (D) phosphoenolpyruvate-stimulated uptake; (E) Glc6P-stimulated uptake; (F) Glc1P-stimulated uptake. Data represent the means from three independent experiments (±SE).
what is the putative energy source allowing starch biosynthesis in the periplastid compartment?

We demonstrated that plastids of *G. theta* possess a triose phosphate/phosphate transporter using mainly dihydroxyacetone phosphate as a counterexchange substrate against inorganic phosphate (Fig. 5). Interestingly, this carrier is not able to transport 3-phosphoglycerate (Fig. 5C), which is known to be a highly efficient substrate for the TPT from higher plants (6, 7). However, this feature of the *G. theta* TPT carrier resembles biochemical characteristics of the TPT from the red alga *Galdieria sulfuraria* (36). Considering that cryptomonads acquired photosynthesis through secondary endosymbiosis of a red alga (14), we have to assume that the biochemical properties of TPT have not been modified dramatically after entry of the symbiont into the host cell.

Remarkably, although *G. theta* is only a unicellular organism, this species contains at least two genes coding for plastidic phosphate transporters. We classified two of these genes as putative triose phosphate/phosphate transporters (TPT1 and TPT2) because proteoliposomes harboring total *G. theta* membranes import P_i in counterexchange with DHAP (Fig. 5A) but not in counterexchange with hexose phosphates such as Glc6P or Glc1P (Fig. 5E and F). Interestingly, the genes encoding TPT1 and TPT2 are regulated in opposite directions. TPT1 mRNA accumulates during the night phase, whereas TPT2 accumulates during the light phase (Fig. 3). Assuming that this expression pattern reflects the metabolic involvement of corresponding carrier proteins, we might assume that TPT2 resides in the innermost envelope membrane from *G. theta* plastids and catalyzes the export of triose phosphates generated during photosynthesis. In contrast, TPT1 might reside in the third and/or fourth membrane separating the periplastid space from the host cell cytoplasm. In the latter case, one function of TPT1 would be the export of starch degradation products during the night phase.

Assuming that the *G. theta* pyrenoid contains, similarly to green algae, ribulose-1,5-bisphosphate carboxylase/oxygenase and other Calvin cycle enzymes (31) and assuming that the pyrenoid in *G. theta* is involved in starch biosynthesis, we have to predict that growth of the starch grain occurs on the inner surface of the ball-shaped cavity. For this process, not only triose phosphates have to enter the periplastid compartment but also energy in form of nucleotides. We cannot exclude the possibility that the highly specific ATP/ADP transporter of the NTT type located in the two innermost envelope membranes (Fig. 6B) might contribute to the nucleotide provision into the periplastid compartment. However, it seems more likely that most of the nucleotides required for starch biosynthesis in the periplastid compartment derive from the host cytosol. This assumption is in line with our finding that the two outermost membranes of *Guillardia* chloroplasts harbor more than 20-fold-higher NTT activity than the two innermost envelopes (Fig. 6B) (see Results).

As was reported for red algae (21, 36), starch synthases in *G. theta* depend upon the presence of UDP-glucose and not on ADP-glucose (4a). Therefore, the ball-shaped cavity must contain a nucleoside diphosphate kinase equilibrating the adenylate and the uridinylate pools. At least for higher plants, the presence of this enzyme in various cellular compartments (mitochondria, plastids, and the cytosol) has been documented.

controlled diurnal rhythms might also turn out to be a general feature of cryptomonads.

We showed in our accompanying paper that isolated starch grains of *G. theta* typically exhibit a ball-shaped cavity (4a). Such cavities are also known to occur in starch grains extracted from other unicellular algae and are caused by the large pyrenoid (31). However, the cellular situation in *G. theta* is much more complex, since the pyrenoid remains in the stroma (4a) whereas starch resides outside in the periplastid compartment (9). In this context two questions arise. First, what kind of metabolites cross the two innermost envelope membranes separating the stroma from the periplastid compartment? Second,
(4), and we have verified that this enzyme is also highly active in G. theta (data not shown).

Our assumption that exported triose phosphates are converted within the periplastid compartment into UDP-glucose is supported by the fact that this compartment represents the cytosol of the formerly free-living red algae. Starch synthesis in red algae takes place in the cytosol (23) and requires the presence of all enzymes involved in this compartment. Moreover, although we have considerable experience in analyzing transport processes, we were unable to observe UDP-glucose transport activities on proteoliposomes harboring total G. theta membranes (data not shown). Of course, further work is required to analyze the subcellular distribution of critical enzymes required for starch biosynthesis in G. theta, and this work is in progress.

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