Cellular Levels of Glutamyl-tRNA Reductase and Glutamate-1-Semialdehyde Aminotransferase Do Not Control Chlorophyll Synthesis in Chlamydomonas reinhardtii

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5-Aminolevulinic acid (ALA) is the first committed universal precursor in the tetrapyrrole biosynthesis pathway. In plants, algae, and most bacteria, ALA is generated from glutamate. First, glutamyl-tRNA synthetase activates glutamate by ligating it to tRNA^Glu. Activated glutamate is then converted to glutamate 1-semialdehyde (GSA) by glutamyl-tRNA reductase (GTR). Finally, GSA is rearranged to ALA by GSA aminotransferase (GSAT). In the unicellular green alga Chlamydomonas reinhardtii, GTR and GSAT were found in the chloroplasts and were not detected in the mitochondria by immunoblotting. The levels of both proteins (assayed by immunoblotting) and their mRNAs (assayed by RNA blotting) were approximately equally abundant in cells growing in continuous dark or continuous light (fluorescent tubes, 80 μmol photons s⁻¹ m⁻²), consistent with the ability of the cells to form chlorophyll under both conditions. In cells synchronized to a 12-h-light/12-h-dark cycle, chlorophyll accumulated only during the light phase. However, GTR and GSAT were present at all phases of the cycle. The GTR mRNA level increased in the light and peaked about 2-fold at 2 h into the light phase, and GTR protein levels also increased and peaked 2-fold at 4 to 6 h into the light phase. In contrast, although the GSAT mRNA level increased severalfold at 2 h into the light phase, the level of GSAT protein remained approximately constant in the light and dark phases. Under all growth conditions, the cells contained significantly more GSAT than GTR on a molar basis. Our results indicate that the rate of chlorophyll synthesis in C. reinhardtii is not directly controlled by the expression levels of the mRNAs for GTR or GSAT, or by the cellular abundance of these enzyme proteins.

5-Aminolevulinic acid (ALA) is the first committed precursor in the tetrapyrrole biosynthesis pathway. In animals and α-proteobacteria, ALA formation is catalyzed by ALA synthase through a one-step condensation reaction of succinyl-CoA and Gly (Kikuchi et al., 1958). In plants, algae, and most bacteria, ALA is generated from Glu in a three-step process (Beale, 1990). First, Glu is ligated to its cognate tRNA by the action of glutamyl-tRNA synthetase (GTS; Schön et al., 1986). Activated Glu is converted to Glu 1-semialdehyde (GSA) by glutamyl-tRNA reductase (GTR; Wang et al., 1984). Then, GSA is rearranged to ALA by GSA aminotransferase (GSAT; Hoober et al., 1988).

Light is required for chlorophyll synthesis in angiosperm plants, and light stimulates synthesis of ALA in these plants (Beale and Weinstein, 1990). Light also increases the activity of enzymes producing ALA that are extractable from plants germinated in the dark (Kannangara and Gough, 1979). Light- and developmentally regulated expression of genes for GTR has been reported in Arabidopsis (Arabidopsis thaliana), barley (Hordeum vulgare), and cucumber (Cucumis sativus; Ilag et al., 1994; Bougri and Grimm, 1996; Kumar et al., 1996; Tanaka et al., 1996, 1997; Vothknecht et al., 1996; Kruse et al., 1997; McCormac et al., 2001; Ujwal et al., 2002). Smaller variations in the expression of genes for GSAT have also been reported (Grimm, 1990; Ilag et al., 1994; Kruse et al., 1997).

The unicellular green alga Chlamydomonas reinhardtii differs from angiosperm plants in that it can synthesize chlorophyll both in the light and dark (Harris, 1989). However, C. reinhardtii can be entrained to a 12-h-light/12-h-dark cycle, and, under these conditions, the cells accumulate chlorophyll only during the light phase (Harris, 1989). We previously used this experimental system to show that in light/dark-synchronized cells, the genes for two early enzymes of chlorophyll synthesis, GSAT and porphobilinogen synthase, are transcribed primarily during the light phase (Matters and Beale, 1994, 1995).

We have now extended these studies to include the mRNA and protein levels for both GSAT and GTR. Cell fractionation results indicate that GTR and GSAT are located only in the chloroplasts. Measurements of GTR and GSAT mRNA and protein levels in light/dark-synchronized cells and in cells growing in continuous...
light and continuous dark indicate that, in contrast to angiosperm plants, C. reinhardtii cells maintain both GTR and GSAT proteins at comparable levels in the light and dark, even in light/dark-synchronized cells that synthesize chlorophyll only in the light. We conclude that chlorophyll synthesis in C. reinhardtii is not directly controlled by the cellular levels of these two enzymes.

RESULTS

Expression of Recombinant GTR and GSAT and Generation of Specific Antibodies against Both Proteins

GTR and GSAT containing His tags were expressed in Escherichia coli. Both proteins were soluble and could be purified on nickel-nitrilotriacetic acid agarose (Ni-NTA) columns. Polyclonal antibodies generated against the recombinant forms of GTR and GSAT were specific, and immunoblots using these antibodies each produced only one band in crude cell extracts of C. reinhardtii (Fig. 1).

GSAT Activity

Purified GSAT and crude extracts of E. coli cells overexpressing GSAT were tested for GSAT activity at various temperatures and pH. The optimal GSAT activity in a 30-min incubation was obtained at 30°C and pH 7.9 (data not shown). GSAT activity was proportional to the amount of purified GSAT used in the assay up to a protein concentration of approximately 7.5 μg in the 600-μL assay mixture. At a concentration of 6 μg in the assay, purified GSAT had an activity of 7.14 ± 0.11 μmol mg⁻¹ protein in a 30-min assay. This value corresponds to a k_cat of 0.18 s⁻¹ per active site, on the basis of one active sites per 45,879-M, subunit of the native homodimeric GSAT enzyme.

Intracellular Localization of GTR and GSAT

Whole cells of C. reinhardtii were fractionated into chloroplast and mitochondrial fractions. Equal amounts of protein from whole cells, chloroplasts, and mitochondria were applied to gels, and the electrophoresed gels were either stained with Coomassie Blue or immunoblotted with anti-GTR, anti-GSAT, or anti-Rubisco antibodies. Anti-Rubisco antibody was used as a control to estimate the degree of contamination of mitochondrial fractions with chloroplast proteins. GTR and GSAT were both present in the chloroplast fraction, but neither could be detected in the mitochondrial fraction above background levels due to contamination from lysed chloroplasts (as assessed by traces of Rubisco in this fraction; Fig. 2). To verify the identification of the cell fractions, in a separate experiment, gels were stained with 3,3′,5,5′-tetramethylbenzidine (TMBZ)/H₂O₂ to detect protein-bound hemes of c-type cytochromes (cyt). The mitochondrial fraction exhibited bands of molecular masses corresponding to cyt c and cyt c₁, whereas the chloroplast fraction exhibited a major band of a molecular mass corresponding to cyt f and a minor band that is probably due to minor contamination by mitochondrial cyt c.

Levels of Chlorophyll, GTR, and GSAT in Cells Grown in Continuous Light and Dark

C. reinhardtii cells growing in continuous light or continuous dark for 7 to 8 d were harvested, and extracts were assayed for chlorophyll content and immunoblotted for determination of GTR and GSAT. The doubling times for cells growing in the light and dark were approximately 8 and 24 h, respectively. Light- and dark-grown cells contained 15.05 ± 0.65 and 8.94 ± 0.14 nmol chlorophyll in 10⁷ cells, respectively. The protein content of light- and dark-grown cells was 1.40 ± 0.06 and 1.20 ± 0.12 mg in 10⁷ cells, respectively. Therefore, there was 1.7-fold more chlorophyll in light-grown cells than in dark-grown cells on a per cell basis and 1.4-fold more on a cell protein basis. GTR protein was about 2-fold more abundant in light-grown cells than in dark-grown cells (Fig. 3). In contrast, GSAT protein was approximately equally abundant in cells growing in the light and dark. The molar ratio of GSAT/GTR was 1.9 in light-grown cells and 4.3 in dark-grown cells.

In a parallel experiment, cells were treated with 200 μg/mL cycloheximide to arrest protein synthesis. The GTR content declined to 50% of the initial value in 5.5 h in light-grown cells and 8 h in dark-grown cells. The GSAT content declined more slowly, and remained at 50% to 70% of the initial value even at 24 h.

Levels of GTR and GSAT mRNA in Cells Grown in Continuous Light and Dark

The levels of mRNA for GTR and GSAT in cells growing in continuous light and dark were determined by RNA blots. Each mRNA was approximately equally abundant in light- and dark-grown cells, differing by not more than ±13% in each case (data not shown).
GSAT Activity in Extracts of Light- and Dark-Grown Cells

GSAT activity was measured in extracts of \textit{C. reinhardtii} as described in “Materials and Methods.” GSAT was approximately equally active in cells grown in the light and the dark. The values were $125 \pm 3$ and $109 \pm 6$ nmol ALA formed in the 30-min assay per mg cell protein in light- and dark-grown cells, respectively.

Levels of GTR and GSAT and Their mRNAs in Light/Dark-Synchronized Cells

Synchronized cells of \textit{C. reinhardtii} grow in size during the light phase and double in number in the dark (Harris, 1989). A good indication that cells are synchronized is the phase-dependent rate of chlorophyll accumulation. Total chlorophyll was extracted from cells at the indicated time points in the light and dark phases. The chlorophyll level increased approximately 4-fold from 0.22 nmol per mL of cell culture at the beginning of a light phase (hour 0) to 1.0 nmol per mL of culture at the end of the light phase (hour 12; Fig. 4). The chlorophyll level remained the same throughout the following dark phase (hours 12 to 24). During the first 9 h of the second light phase (hours 24 to 33), the chlorophyll levels increased approximately 2.5-fold from 1.0 to 2.65 nmol per mL of culture. These results indicate that under our growth conditions, the \textit{C. reinhardtii} cells used in these experiments were successfully synchronized.

The levels of mRNA for GTR and GSAT in synchronized cells were determined at various points in the cycle. The mRNA levels for both GTR and GSAT peaked at 2 h into the light phase (Fig. 5). Then, the levels of GTR mRNA leveled off and remained approximately constant during the remainder of the light phase. The GSAT mRNA level increased approximately 5-fold at L2 over the level at L0 (hours during the light phase designated L0–L12). This increase was greater than the approximately 1.7-fold increase for GTR mRNA but not as great as was previously reported for GSAT mRNA (Matters and Beale, 1994). The difference between the earlier and the present results in the degree of increase of GSAT mRNA could possibly be due to the peak occurring between the sampling times in the present experiments.

GTR and GSAT protein levels in light/dark-synchronized cells were quantitated by immunoblotting. The GTR protein level peaked at 4 to 6 h into the light phase, and the degree of increase was approximately 2-fold, following the earlier 2-fold increase in GTR mRNA at 2 h into the light phase (Fig. 6). In contrast, the GSAT protein level remained approximately constant throughout the light/dark cycle and did not follow the changes in GSAT mRNA levels.

In a parallel experiment, cells were treated with 200 \( \mu \text{g/mL} \) cycloheximide at the beginning of the

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![Figure 2. Localization of GTR and GSAT within \textit{C. reinhardtii} cells. Extracts of whole cells (1), the chloroplast fraction (2), and the mitochondrial fraction (3) were stained using Coomassie Blue (A) or immunoblotted with anti-GTR (B), anti-GSAT (C), and anti-Rubisco (D). In a separate experiment, the gel was stained with TMBZ/H\(_2\)O\(_2\) to detect hemoproteins, which were identified on the basis of their apparent molecular masses (E). Positions of molecular mass marker proteins are indicated on the right sides of the sections.](image-url)

![Figure 3. Immunoblots of GTR and GSAT of \textit{C. reinhardtii} cells grown in continuous light or continuous dark. Equal amounts of total cell protein were loaded in each lane. Molar amounts of GTR (black bars) and GSAT (gray bars) per gram total cell protein were calculated on the basis of immunoblotting using recombinant GTR and GSAT as protein standards. The error bars indicate the sos for triplicate samples.](image-url)
light phase to arrest protein synthesis. The level of GTR declined to 50% of the initial value at 8 h and was 25% of the initial value at 24 h. The GSAT content declined more slowly and was at 55% of the initial value even at 24 h.

**GTR/GSAT Stoichiometry in the Cells**

Purified recombinant GTR and GSAT proteins were used as standards for quantitation of GTR and GSAT protein levels in synchronized cells by immunoblotting. Under synchronized growth conditions, there was always significantly more GSAT than GTR, on a molar basis (Fig. 6). The GSAT/GTR molar ratio varied from 1.5 at hour L5 to 4.8 at hour L11.

**DISCUSSION**

Expression of recombinant His-tagged *C. reinhardtii* GTR and GSAT proteins in *E. coli* allowed for purification of the proteins and generation of specific polyclonal antibodies against them. The antibodies were used to examine GTR and GSAT levels in *C. reinhardtii* cells under several growth conditions and to compare the protein levels with the levels of their encoding mRNAs. The antibodies, in conjunction with cell fractionation, were also used to determine that both GTR and GSAT reside primarily or exclusively in the chloroplasts of *C. reinhardtii* cells.

Because ALA synthesis is the first universal committed step of tetrapyrrole formation, it would be expected to be a key control point that regulates the entry of precursors into this biosynthetic pathway. In photosynthetic cells and tissues, where chlorophyll is the major tetrapyrrole end product, several lines of evidence indicate that ALA formation is a physiologically important rate-limiting step of chlorophyll synthesis. For example, administration of exogenous ALA to etiolated leaves or cotyledons causes the accumulation of high concentrations of later chlorophyll intermediates (Nadler and Granick, 1970; Gough, 1972). Antisense repression of GTR expression in Arabidopsis inhibits synthesis of heme and chlorophyll (Kumar and Söll, 2000). Although overexpression of GTR in *E. coli* leads to the accumulation of ALA and porphyrins (Chen et al., 1996; Srivastava and Beale, 2005; Srivastava et al., 2005), overexpression of GTR in plants has apparently not been reported.

Angiosperm plants contain at least two genes that encode GTR (Ilag et al., 1994; Bougri and Grimm, 1996; Kumar et al., 1996; Tanaka et al., 1996, 1997; Vothknecht et al., 1996; Kruse et al., 1997; McCormac et al., 2001; Ujwal et al., 2002). These genes are differentially expressed in different tissues and under different developmental conditions (Gough et al., 2003). It appears that there is a constitutively expressed gene that is expressed in many tissues and one or more genes that...
are specifically expressed in rapidly greening cells. Induction in the light of genes for GTR enzymes that supply precursors for the rapid synthesis of chlorophyll can explain the increase in extractable ALA-forming activity that has been observed in tissues upon exposure to light (Weinstein and Castelfranco, 1978; Kannangara and Gough, 1979; Harel and Ne’eman, 1983; Weinstein and Beale, 1985).

Plants may contain either one or two genes for GSAT (Hess et al., 1992; Frustaci et al., 1995; Hansson et al., 1998; Polking et al., 1999; Tsang et al., 2003). The expression of the genes for GSAT, like those for GTR, is influenced by the light and developmental status (Ilag et al., 1994; Kruse et al., 1997; Polking et al., 1999). However, the effects of light are difficult to interpret. In etiolated barley seedlings, GSAT mRNA levels decrease upon transfer to light (Grimm, 1990), and, in cucumber leaves, GSAT mRNA levels do not change in the light (Masuda et al., 1996). The GSAT protein level does not appear to follow the mRNA level (Kannangara and Gough, 1978; Masuda et al., 1996; Kruse et al., 1997; Kumar et al., 1999; Polking et al., 1999).

In contrast to plants, C. reinhardtii contains only one gene for GTR and one for GSAT (Matters and Beale, 1994; Srivastava et al., 2005). Also, in contrast to angiosperm plants, C. reinhardtii can synthesize and accumulate chlorophyll in both the light and dark. However, C. reinhardtii cells can be entrained to a light/dark cycle, and under these conditions they synthesize chlorophyll only during the light phase. This flexibility provides an opportunity to assess correlations between expression of GTR and GSAT mRNAs, GTR and GSAT protein levels, and chlorophyll levels under a wide range of conditions.

Our results indicate that the mRNA levels for both GTR and GSAT respond to the light environment, and higher levels of both mRNAs are present in cells growing in the light than in cells growing in the dark. Although the GTR protein level correlates well with the GTR mRNA level, the GSAT protein level is approximately constant under all growth conditions and does not correlate with the GSAT mRNA level. In addition, under all growth conditions, the cellular abundance of GSAT is significantly greater than that of GTR, on a molar basis. When combined with in vitro evidence showing that the specific activity of GSAT is much greater than that of GTR, on a molar basis (see below), the results lead to the conclusion that GSAT is never rate limiting for tetrapyrrole biosynthesis. Because the cells contain significant quantities of both GTR and GSAT even under conditions when they are not accumulating chlorophyll, it can be concluded that the primary mode of regulation of chlorophyll synthesis is not exerted via regulation of the synthesis or cellular abundance of GTR or GSAT.

We are aware that our results showing that GSAT protein and enzyme activity levels are approximately equal in cells growing in continuous light and dark are somewhat contrary to the earlier preliminary observations of Mau et al. (1992), who reported that when cells were switched from growing in the dark to continuous light, the extractable GSAT activity increased 16-fold. At present, we cannot account for this discrepancy. It is possible that the cells used by Mau et al. are a different strain and/or had been growing in the dark for a much longer time than our cells, which were cultured in the dark for 7 to 8 d. Under prolonged culture in the dark, cells of some strains eventually become “dark adapted” and lose a large portion of their chlorophyll and chlorophyll-binding proteins (R. van Lis, A. Atteia, L.A. Nogaj, and S.I. Beale, unpublished data), and it is possible that the enzyme levels also decline under these conditions.

Previously, we reported a specific activity for purified C. reinhardtii GTR of 21 nmol mg$^{-1}$ protein in a 30-min assay (Srivastava et al., 2005). This value corresponds to a $k_{cat}$ of $6.3 \times 10^{-4}$ s$^{-1}$ per active site, on the basis of one active site per 52,502-M, subunit of the native homodimeric GTR enzyme (Srivastava et al., 2005). In this report, purified GSAT had a $k_{cat}$ of 0.18 s$^{-1}$ per active site. Therefore, under these in vitro conditions, GSAT was approximately 300 times more active than GTR on a molar basis. Extrapolation from these in vitro values to the in vivo activities of these enzymes can yield only an approximation. Nevertheless, on the basis of our finding that there is always more GSAT than GTR in the cells, on a molar basis, it is very likely that it is always GTR, rather than GSAT,
that limits ALA formation in the cells. This conclusion is consistent with other observations showing that the levels of neither GTS (Mau et al., 1992) nor tRNA 

Chlamydomonas reinhardtii strain CC124 was obtained from the Chlamydomonas culture collection at Duke University, Durham, North Carolina. Cells were grown in Tris-acetate phosphate medium (Harris, 1989) at 26°C in continuous light (fluorescent tubes, 80

C. reinhardtii is subject to complex regulation that is not completely understood. GTR activity in vitro is sensitive to allosteric inhibition by the end-product heme, but this inhibition is dependent on the presence of as yet unidentified soluble cellular proteins (Srivastava et al., 2005). C. reinhardtii GTR is also inhibited by certain membrane-associated proteins designated FLP (Falcioni et al., 2005). Although the FLP proteins appear to be important for the regulation of GTR activity in response to light chlorophyll precursors, the mechanism of action of these apparently constitutively expressed proteins is not clear. Finally, GTR is able to form functional complexes with both GTS and GSAT. Formation of a GTS-GTR complex requires the presence of glutamyl-tRNA, and this complex has been proposed to be important for diverting glutamyl-tRNA away from protein synthesis and toward ALA synthesis (Jahn, 1992a). A stable 1:1 GTR-GSAT complex is formed even in the absence of substrates and intermediates, and this complex has been shown to facilitate channelling of the unstable intermediate GSA from GTR to GSAT (Nogaj and Beale, 2005). Moreover, formation of the complex with GSAT appears to increase the in vitro activity of GTR severalfold (Nogaj and Beale, 2005). It is clear that additional work will be required to fully understand the regulation of GTR in C. reinhardtii and its key role in controlling the supply of tetrapyrrole precursors.

In summary, our results support the conclusion that ALA synthesis in C. reinhardtii is regulated principally by modulation of the activity of the enzyme that catalyzes the initial, committed step, GTR. The rate of chlorophyll formation is not directly controlled by modulation of the activity that limits ALA formation in the cells. This conclusion is consistent with other observations showing that the levels of neither GTS (Mau et al., 1992) nor tRNA

In the dark, or 12-h-light/12-h-dark synchronized conditions. Cells were grown to a density of 2 to 5 × 10^6 cells mL^-1 under synchronized conditions and in continuous light or dark.

**Overexpression and Purification of C. reinhardtii GTR and GSAT**

Recombinant, enzymatically active His-tagged C. reinhardtii proteins corresponding to the mature forms of GTR and GSAT were expressed and purified by Ni-NTA column chromatography as described (Nogaj and Beale, 2005). Specific polyclonal rabbit antibodies directed against each of these proteins were obtained as described (Nogaj and Beale, 2005).

**Heme Detection in Polyacrylamide Gels**

SDS-PAGE gels were stained for heme-associated peroxidase activity by the method of Thomas et al. (1976). A 6.3 mM solution of TMBZ was freshly prepared in methanol. Immediately before use, three parts of the TMBZ solution was mixed with seven parts of 0.25% sodium acetate, pH 5.0. The SDS-PAGE gel was immersed in this mixture and kept at room temperature in the dark with gentle shaking. After 1 h, H_2O_2 was added to a final concentration of 30 mM. The staining was visible within 3 min.

**RNA Isolation and RNA-Blot Analysis**

Total RNA was isolated using the RNeasy Mini kit (Qiagen) according to the manufacturer’s protocol. For better RNA yield, whole cell pellets were resuspended in RLT lysis buffer (from the RNeasy Mini kit) and incubated for 3 to 5 min at 56°C. The rest of the protocol was unchanged. Total RNA was eluted from the RNeasy column with 60 µL of RNase-free water.

For RNA blots, 10 µg of total RNA was separated on a 1% FA gel (as described in the RNeasy Mini Handbook) and blotted onto a Nytran membrane (Schleicher and Schuell). Blots were UV-crosslinked, prewashed at 68°C in a solution of 10 mM Tris, pH 8.0, and 0.1% (w/v) SDS, and then prewashed and hybridized at 38°C in UltraHyb solution (Ambion). The final two washes were done at 68°C in a solution containing 5% SET, 0.1% (w/v) sodium pyrophosphate, and 0.01% (w/v) SDS. DNA probes were made using the Random Primers DNA labeling system (Invitrogen) and purified with Quick Spin Sephadex G-50 columns (Boehringer Mannheim). Blots were exposed on a phosphoimaging plate for 20 h, and densitometric data were obtained using Image Gauge (FujiFilm USA).

RNA blots were stripped and reprobed with another radiolabeled probe. The membranes were incubated twice with 15 min with a boiling solution of 0.1% SSC and 0.1% (w/v) SDS, and then prewashed and hybridized as described above. Comparison of mRNA levels of both GTR and GSAT under different growth conditions was done relative to the mRNA level of a reference gene, glyp, which has been shown to be expressed at a constant level in C. reinhardtii cells (Schloss et al., 1984; Schloss, 1990). The glyp clone was obtained from K.L. Kindle, Cornell University, Ithaca, New York.

**Protein Preparation and Immunoprecipitation**

Light/dark-synchronized cells (10 mL) were harvested 1 h before the beginning of the light phase (D11) and at every hour during the light phase (L0–L12). Cell pellets were resuspended in 200 µL of 50 mM Na_2CO_3 and disrupted with a Sonifier Cell Disruptor (Heat Systems-Ultrasonics) on ice for five 30-s periods with 30-s intervening cooling periods. Total protein concentration was determined with a bicinchoninic acid kit for protein determination (Sigma) and bovine serum albumin as the standard. Proteins were separated by SDS-PAGE and blotted onto a nitrocellulose membrane (Osmonics). The membrane was blocked with 5% (w/v) dry nonfat milk in TBST buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% [w/v] Tween 20). The blots were probed with monoclonal antibodies directed against the 58-kDa subunit of the GTS-GTR complex or the 36-kDa subunit of the GSAT complex. The blots were washed with TBST buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% [w/v] Tween 20). The blots were washed with TBST buffer and incubated with horseradish peroxidase-conjugated secondary antibodies. The heme staining was visible within 3 min.
Twee 20, CTR and GSAT protein levels were detected using anti-CTR and anti-GSAT antibodies. As a positive control for isolated organelles, anti-Rubisco antibody was used (obtained from J.V. Morony, Louisiana State University, Baton Rouge, LA). Alkaline phosphatase-conjugated secondary antibodies (Sigma) were used to detect primary antibodies. Immunocomplexes were visualized by incubation of the membrane with NBT/BCIP Liquid substrate system (Sigma). Band intensities were calculated using the public domain NIH Image program (developed at the United States National Institutes of Health and available on the Internet at http://rnb.info.nih.gov/ nih-image).

Chlorophyll Determination

Light/dark-synchronized cultures were sampled at various times extending over 33 h. Culture samples containing approximately 1.7 × 10^6 cells were centrifuged for 2 min at 2,000g. The cells were resuspended by vortex mixing in 100% (v/v) methanol and extracted twice. The combined extracts were adjusted to 5 mL with methanol, and the A_{663} and A_{645} were determined. The chlorophyll concentrations were calculated according to the following equations derived from the absorption coefficients (Mackinney, 1941): [chlorophyll a] = 18.46 × 10^{-3} × A_{663} - 9.28 × 10^{-3} × A_{645} and [chlorophyll b] = 37.21 × 10^{-3} × A_{600} - 13.78 × 10^{-3} × A_{645}.

GSAT Activity Assays

GSAT was incubated with 5 mM levulinic acid and 2 mM GSA (synthesized according to Gough et al., 1989) in 0.5 mL of assay buffer (50 mM Tricine, pH 7.9, 1 mM glycerol, 15 mM MgCl₂, 1 mM DTT-dithiothreitol, 20 μM pyridoxal phosphate) for 30 min at 30°C. The reaction was stopped by adding, with mixing, 25 μL of 100% (w/v) TCA, and the mixture was incubated for 10 min on ice. The chilled mixture was centrifuged for 10 min at 12,000g, and the supernatant was decanted into 150 μL of 100% (w/v) TCA, and the mixture was incubated for 10 min and then cooled on ice. An equal volume of freshly made Ehrlich-Hg reagent (German and Granick, 1936) was added to a cool reaction, with mixing. ALA concentration was determined by measuring the A_{650} and using an absorption coefficient of 7.2 × 10^{3} M⁻¹ cm⁻¹ (Mauzerall and Granick, 1956). For calculating net enzyme activity, background A_{650} of blank assays not containing enzyme were subtracted from all assay values to correct for nonenzymatic conversion of GSA to ALA and other colored products.

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LITERATURE CITED


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