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δ-Aminolevulinic Acid Synthase of *Euglena gracilis*: Regulation of Activity[1]

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ABSTRACT

δ-Aminolevulinic acid (ALA), a key precursor of the tetrapyrroles heme and chlorophyll, is capable of being synthesized by two different routes in cells of the unicellular green alga *Euglena gracilis*: from the intact carbon skeleton of glutamate, and via the condensation of glycine and succinyl CoA, mediated by the enzyme ALA synthase. The regulatory properties of ALA synthase were examined in order to establish its role in *Euglena*.

Partially purified *Euglena* ALA synthase, unlike the case with the bacterial or animal-derived enzyme, does not exhibit allosteric inhibition by the tetrapyrrole pathway products heme, protoporphyrin IX, and porphobilinogen, at concentrations up to 100 micromolar.

In aplastidic mutant cells, extractable ALA synthase activity is constant during exponential growth, and decreases to low levels as the cells reach the stationary state. Rapid exponential decline of ALA synthase ($t_{1/2}$ = 55 min) occurs after administration of 43 micromolar cycloheximide, but not 6.2 millimolar chloramphenicol. These results suggest that, as in other eukaryotic cells, ALA synthase is synthesized on cytoplasmic ribosomes and is subject to rapid turnover *in vivo*.

Extractable ALA synthase activity increases 2.5-fold within 6 hours after administration of 100 millimolar ethanol, a stimulator of mitochondrial development, and 4.5-fold within 12 hours after administration of 1 millimolar 4,6-dioxoheptanoic acid, which blocks ALA utilization, suggesting that activity is controlled *in vivo* by a feedback induction-repression mechanism, coupled with rapid enzyme turnover.

In heterotrophically grown wild-type cells, low levels of ALA synthase rapidly increase 4.5-fold within 12 hours after cells are transferred from the light to the dark, and decrease exponentially ($t_{1/2}$ = 75 min) when cells are transferred from the dark to light. The dark levels are equal to those in light- or dark-grown aplastidic mutant cells. The low level occurring in light-grown wild-type cells is not altered by the presence of 10 micromolar 3-(3,4-dichlorophenyl)-1,1-dimethylurea, which blocks photosynthetic O_2 production. The decrease that occurs on dark-to-light transfer can be diminished by 12- or 24-hour prior incubation with 6.2 millimolar chloramphenicol, which also retards chlorophyll synthesis after the transfer to light.

The positive relationship of ALA synthase activity to degree of mitochondrial expression, and the inverse relationship to plastid development and chlorophyll synthesis, suggests that ALA synthase functions to provide precursors to nonplastid tetrapyrroles in *Euglena*. In light-grown, wild-type cells, the diminished levels of ALA synthase may be due to the ability of developing plastids to export heme or a heme precursor to other cellular regions, which thereby supplants the necessity for ALA formation via the ALA synthase route.

Chls and hemes share a common biosynthetic pathway starting with the formation of ALA,[3] the first identified compound that is a committed precursor to the tetrapyrroles, and ending with protoporphyrin IX, the branch-point intermediate before insertion of Fe or Mg, leading to hemes or Chls, respectively. In animals, fungi, and bacteria, ALA is formed from succinyl-CoA and glycine, mediated by the enzyme ALA synthase [succinyl-CoA: glycine *C*-succinyl transferase (decarboxylating), EC 2.3.1.37]. Plants and algae, however, appear to synthesize ALA via another route that utilizes the intact carbon skeleton of glutamate or a closely related compound, rather than succinyl-CoA and glycine (for a review, see 1). Although the plant pathway has not been completely characterized, it has been found to exist in all O_2-evolving photosynthetic organisms examined, including bluegreen, red, and green algae, *Euglena*, and greening tissues of higher plants. Until recently, ALA synthase had not been detected in extracts of any of these organisms.

The occurrence of ALA synthase was recently reported in extracts of *Euglena gracilis* (4). Relatively high levels of activity were extracted from dark-grown, wild-type cells and nongreening aplastidic mutant cells grown in the light or dark. Light-grown green wild-type cells yielded measurable, but considerably lower, levels of ALA synthase. It was proposed that ALA synthase in *E. gracilis* functions to provide precursors for nonplastid tetrapyrrole synthesis.

A study of the physical and kinetic properties of the ALA synthase of *Euglena gracilis* revealed that it is similar to ALA synthase isolated from other sources with respect to molecular weight, pH optimum, and substrate affinities (8). The regulatory behavior of ALA synthase in *Euglena* reported here provides information concerning its role in this organism. Portions of this work have been reported previously in abstract form (2).

MATERIALS AND METHODS

***Euglena* Cells.** Cultures of *Euglena gracilis* Klebs strain Z Pringsheim and an aplastidic mutant derived from this strain, W_{14}ZNalL (25) were kindly provided by H. Lyman (State University of New York, Stony Brook, NY). The cells were cultured at 23°C in light or dark, in a glucose-based heterotrophic medium as previously described (4). 4,6-Dioxoheptanoic acid and cycloheximide were added to some cultures in the form of concentrated aqueous solutions. Ethanol was added in undiluted form, chlor-

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[3] Abbreviations: ALA, δ-aminolevulinic acid; PBG, porphobilinogen.

amphenicol was added as a dried powder, and DCMU was added as a concentrated solution in dimethylsulfoxide.

Cell Growth Measurement. Cell population densities were determined with a Coulter Counter (model ZBI, Coulter Electronics).

Chl. Cells were extracted in methanol and Chl was determined spectrophotometrically with a Cary model 219 instrument. The absorption coefficients of Mackinney (18) were utilized.

Protein Content. Protein concentration in broken cell supernatants was determined by the method of Bradford (5), using BSA as a standard.

Extraction of ALA Synthase. All operations were carried out at 0 to 5°C. Cells in exponential growth were harvested at a culture density of approximately 3×10^6 cells · ml^{-1}. Preliminary sedimentation and washing was accomplished by 10-s centrifugation periods at 13,000*g* in a microcentrifuge (Eppendorf model 5412). Cells were washed once with cold water and once with cell extraction medium (75 mM Hepes; 50 mM glycine; 25 mM succinic acid; 20 mM $MgCl_2$; 5 mM EDTA; 0.1 mM pyridoxal phosphate; pH adjusted to 7.80 with NaOH), and finally resuspended in extraction medium. Sonication was performed directly in the 1.5-ml conical centrifuge tubes by applying four 5-s sonic bursts, using a microprobe-equipped Sonifier (model W 185, Heat Systems—Ultrasonics). The broken cell suspensions were centrifuged in the microcentrifuge for 1 min at 13,000*g* to yield supernatant for the ALA synthase assay. This extraction procedure consistently yielded ALA synthase with greater activity per number of extracted cells than the previously used procedure (4, 8).

ALA Synthase Assay. Cell extracts were incubated for 20 min at 37°C in 1 ml of extraction medium plus 1 mM GTP, 0.1 mM CoA, and sufficient succinic thiokinase to catalyze the formation of 0.2 μmol of succinyl-CoA per min at 30°C. In some cases, the succinyl-CoA generating system was replaced by 2.5×10^{-4} M succinyl-CoA, prepared by the method of Simon and Shemin (27). In some assays, PBG was added as a concentrated solution in incubation medium. Protoporphyrin IX and hemin were first dissolved in 100 mM NaOH, then diluted with incubation medium and neutralized to the incubation pH before addition. Incubations were terminated by addition of 50 μl of 100% (w/v) TCA solution in water. Precipitate was removed by centrifugation for 1 min at 13,000*g* in the microcentrifuge. One-half milliliter of supernatant was adjusted to pH 6.8 with approximately 170 μl of 0.5 M Na_3PO_4, then 25 μl of ethylacetoacetate was added and the solution was heated to 95°C for 15 min to for 2-methyl-3-carbethoxy-4-(3-propionic acid) pyrrole (19). After cooling to room temperature, an equal volume of Ehrlich-Hg reagent (28) was added, and the light absorption at 552 nm was determined after 5 min, using the Cary model 219 spectrophotometer. Standards containing different known amounts of ALA were prepared for each assay, to quantitate the ALA content in the experimental samples and to assure linearity of the color yield. The calculated molar absorption coefficient for the Ehrlich-Hg reagent-pyrrole complex derived from authentic ALA was approximately 8.0×10^4. Assays of incubations containing added PBG were corrected for the Ehrlich color product contribution due to the PBG.

Chemicals. 4,6-Dioxoheptanoic acid was purchased from Calbiochem-Behring. All other reagents, except solvents and growth medium ingredients, were obtained from Sigma. The others were from Fisher. Succinic thiokinase was purchased from Sigma or Boehringer Mannheim.

RESULTS

Changes in Activity during Cell Growth. Aplastidic cells were planted at an initial population density of 4×10^3 cells · ml^{-1}. After they had grown to approximately 1×10^5 cells · ml^{-1}, samples were taken periodically during the next 110 h, and analyzed for cell density, soluble protein content, and ALA synthase activity. When these quantities are expressed per unit of culture volume and plotted on a logarithmic scale *versus* time (Fig. 1), it can be seen that all increased approximately exponentially until the cells neared the end of their growth. Upon approach to the stationary state, total ALA synthase activity leveled off, then declined rapidly as the cells attained their maximum population density of 1.1×10^7 cells · ml^{-1}. Total soluble protein content also decreased after the cells ceased dividing. All other results reported here were obtained with cells while they were within the exponential phase of growth.

Changes in Activity during Greening. As reported previously (4), light-grown, wild-type *Euglena* cells have only approximately one-third as much extractable ALA synthase activity as wild-type cells grown in complete darkness. The time course of changes in extractable ALA synthase activity in exponentially growing, wild-type cells was followed after transfer from the dark to the light. ALA synthase activity began to decrease 15 min after the cells were first exposed to the light (see Fig. 2). The decline in activity continued for the next 4 h, following first-order kinetics, with a half-life of approximately 75 min. Chl synthesis began almost immediately after the transfer from the dark to the light (Fig. 2). Chl was formed at an exponentially increasing rate with a doubling time of 75 min.

Changes in Activity when Greening is Halted. ALA synthase activity was extracted from exponentially growing, wild-type cells

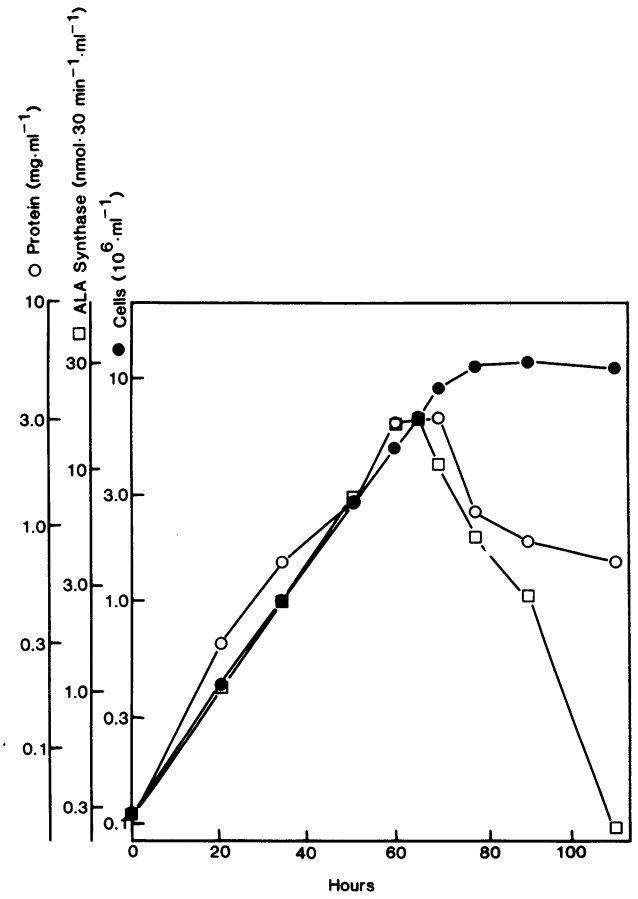

FIG. 1. Protein synthesis and ALA synthase activity during cell growth. Aplastidic cells were grown in light at an initial density of 4×10^3 cells · ml^{-1}. Starting at 1×10^5 cells · ml^{-1}, samples were taken periodically. Cell number (●), soluble protein content (○), and extractable ALA synthase activity (□) were measured. These are expressed per milliliter of culture medium and shown on a logarithmic scale *versus* time after initial sample was taken.

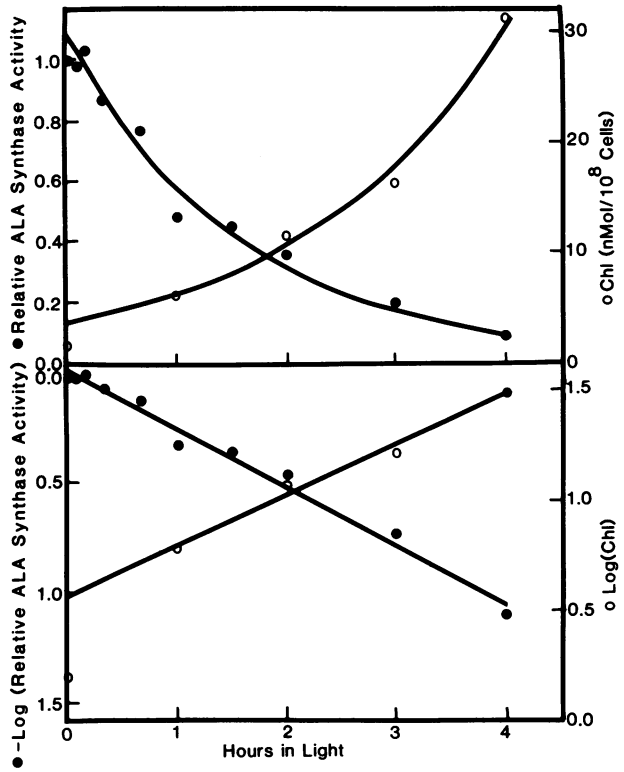

FIG. 2. Effects of dark-to-light transfer of wild-type cells. Relative ALA synthase activity (●) and cellular content of chlorophylls (nmol · 10^8 $cells^{-1}$) (○) are plotted *versus* hours after transfer of wild-type cells from dark to light (top). Data were replotted as negative logarithms of relative ALA synthase activity (●) and Chl content (○) *versus* hours in light to illustrate exponential nature of changes (bottom). Initial ALA synthase activity was 21 nmol ALA · 30 min^{-1} · 10^7 $cells^{-1}$.

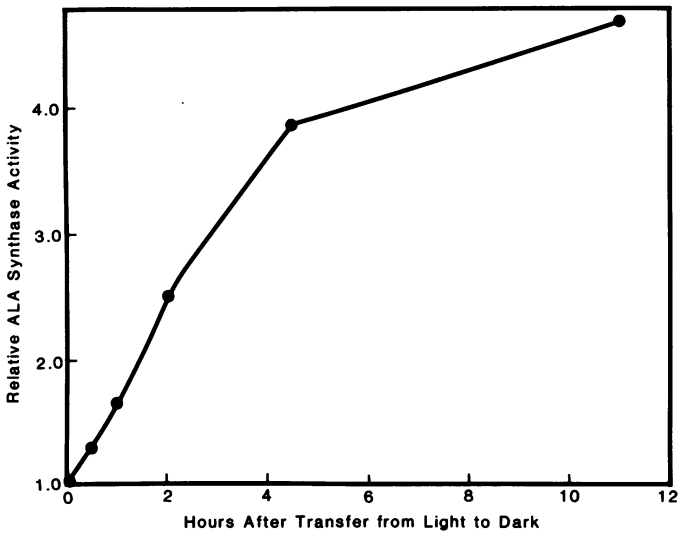

FIG. 3. Effects of light-to-dark transfer of wild-type cells. ALA synthase activity in cell extracts is plotted *versus* hours after transfer of light-grown, wild-type cells to darkness. Initial ALA synthase activity was 4.6 nmol ALA · 30 min^{-1} · 10^7 $cells^{-1}$.

at various times after transfer from the light to the dark. Activity began to increase almost immediately after the transfer, and continued to increase linearly for the next 4 h before reaching a constant level which was 4.5-fold higher than the initial level (Fig. 3).

Activity of ALA Synthase in Mixed Extracts from Wild-Type and Aplastidic Cells. In order to test whether the difference in ALA synthase activity in extracts of light-grown, wild-type and aplastidic cells was due to the presence of an inhibitor or activator, the ALA synthase levels in extracts of green wild-type and white aplastidic cells were assayed separately and combined. The activity in the mixed extract was nearly equal to the mean of the activities of the separate extracts (Table I).

Table I. *ALA Synthase Activity in Mixed Extracts from Wild-Type and Aplastidic Cells*

ALA synthase activity was measured in extracts from light-grown, wild-type cells, aplastidic cells, and an equal mixture of the two cell extracts. The mean value of activities of the two unmixed extracts is tabulated for comparison purposes.

Cell Strain	Synthase Activity	
	Specific	Relative
	nmol ALA · 30 min^{-1} · 10^7 $cells^{-1}$	*%*
Aplastidic	8.3	100
Wild-type	1.9	23
Mixed	5.4	66
Mean of unmixed extracts	5.1	62

Table II. *Effects of DCMU on Cell Growth, Chl Content, and Extractable ALA Synthase Activity*

Cell density, cellular chlorophyll content, and extractable ALA synthase activity were measured in light-grown, wild-type cells at 0, 6, and 12 h after addition of 10 μM DCMU. Values for untreated control cells are tabulated for comparison purposes.

Time	Cell Treatment	Cell Density	Chl	ALA Synthase
h		*cells · ml^{-1}*	*nmol · 10^7 $cells^{-1}$*	*nmol ALA · 30 min^{-1} · 10^7 $cell^{-1}$*
0	Control	1.41×10^6	151	5.6
	DCMU	1.37×10^6	152	5.5
6	Control	2.09×10^6	152	5.8
	DCMU	2.04×10^6	153	5.6
12	Control	2.80×10^6	151	5.4
	DCMU	3.09×10^6	150	5.7

Effects of DCMU. The contribution of photosynthetic O_2 production and CO_2 fixation to the regulation of ALA synthase activity was assessed by adding DCMU at 10 μM to wild-type cells growing heterotrophically in the light. At this concentration, DCMU completely abolishes photosynthetic O_2 production in *Euglena* (24; S. I. Beale, unpublished data). DCMU was added as a concentrated solution in dimethyl sulfoxide. The final concentration of dimethyl sulfoxide was 10 μl/60 ml culture and an equivalent amount had no effect when added to a control culture. The presence of DCMU for 6 or 12 h appeared to have no effect on growth, Chl content, or the level of extractable ALA synthase in wild-type cells (Table II).

Induction of ALA Synthase Activity by Ethanol. Because ethanol has been shown to induce respiration and the levels of mitochondrial marker enzymes in *Euglena* (10, 15), the possibility of an effect on ALA synthase activity was examined. Ethanol was administered at 100 mM to aplastidic cells growing exponentially in the light, and extractable ALA synthase activity was measured as a function of time after ethanol addition. Activity of the enzyme began to increase after 1 h, and continued to increase through 6 h of incubation with ethanol (Fig. 4).

Induction of ALA Synthase Activity by 4,6-Dioxoheptanoic Acid. 4,6-Dioxoheptanoic acid, an inhibitor of ALA dehydrase *in vitro* (9) and *in vivo* (20), was administered at 1 mM to aplastidic cells growing exponentially in the light. One hour after the addi-

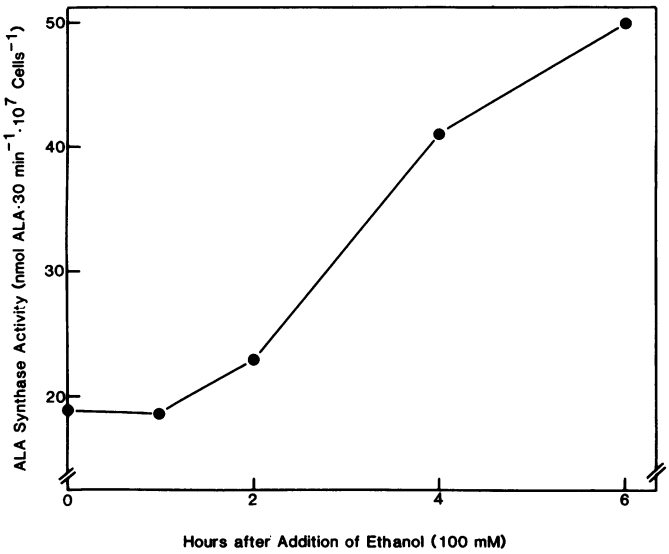

FIG. 4. Induction of ALA synthase activity by ethanol. ALA synthase activity in extracts of exponentially growing aplastidic cells is plotted against hours after addition of 100 mM ethanol. Cells were extracted as described in (Ref. 4) and initial ALA synthase activity was 19 nmol ALA · 30 min^{-1} · 10^7 cells^{-1}.

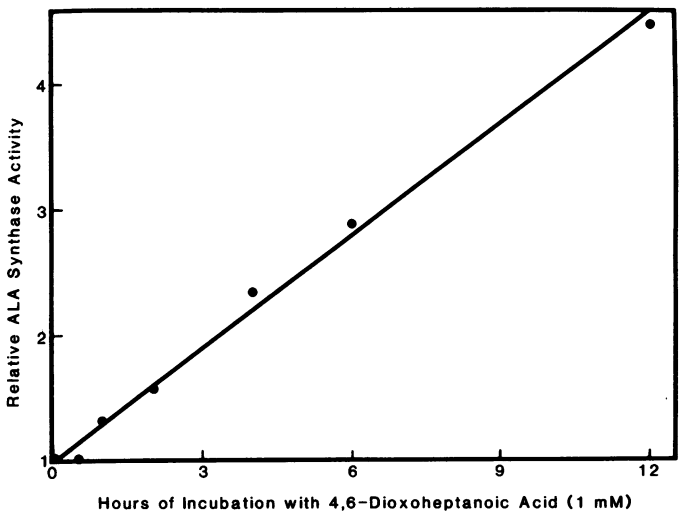

FIG. 5. Induction of ALA synthase activity by 4,6-dioxoheptanoic acid. ALA synthase activity in extracts of exponentially growing aplastidic cells is plotted against hours after addition of 1 mM 4,6-dioxoheptanoic acid. Initial ALA synthase activity was 22 nmol of ALA · 30 min^{-1} · 10^7 cells^{-1}.

tion, an increase in the level of extractable ALA synthase activity occurred (Fig. 5). The increase in ALA synthase activity proceeded linearly for the next 11 h, with a doubling of the initial activity after 3 h of incubation.

Effects of Cycloheximide. Cycloheximide is an inhibitor of protein synthesis on cytoplasmic ribosomes in many eukaryotic cells, including *Euglena* (22, 26). This drug was administered at 43 μM to aplastidic cells growing exponentially in the light. A decrease in extractable ALA synthase activity was observed almost immediately (Fig. 6). The decline in activity followed an exponential course for the next 90 min, with a half-life of approximately 55 min. In order for the decrease in ALA synthase activity to occur, it was necessary for the cells to be maintained at the growth temperature of 23°C. If the cells were cooled on ice, the level of extractable ALA synthase activity remained constant in the presence or absence of cycloheximide (data not shown).

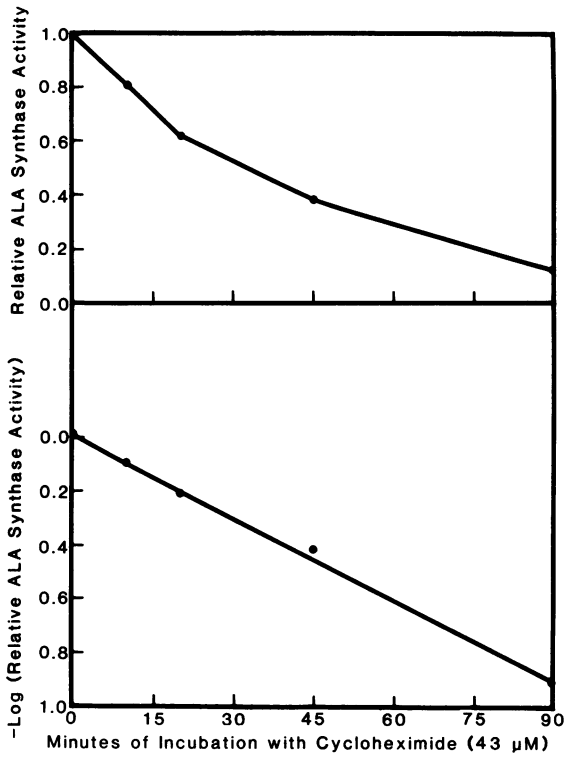

FIG. 6. Decay of ALA synthase activity after cycloheximide treatment. ALA synthase activity in extracts of growing aplastidic cells is plotted *versus* minutes after addition of 43 μM cycloheximide (top). Data are replotted as the negative logarithm of relative ALA synthase activity *versus* minutes after cycloheximide addition to illustrate exponential nature of the decay of activity (bottom). Cells were extracted as described in (Ref. 4) and initial activity was 12.2 nmol ALA · 30 min^{-1} · 10^7 cells^{-1}.

Table III. *ALA Synthase Activity in Mixed Extracts from Cycloheximide-Treated and Control Cells*

ALA synthase activity was measured in extracts from light-grown, aplastidic cells, untreated, or incubated for 4 h with 43 μM cycloheximide before extraction, and an equal mixture of the two cell extracts. The mean of activities of the two unmixed extracts is tabulated for comparison purposes.

Cell Treatment	ALA Synthase	
	Specific	Relative
	nmol ALA · 30 min^{-1} · 10^7 cells^{-1}	%
Control	28.4	100
Cycloheximide	2.0	7
Mixed extracts	14.9	52
Mean of unmixed extracts	15.2	54

Aplastidic cells were grown in the presence of 43 μM cycloheximide for 4 h and then extracted. This extract was mixed with extract from untreated cells. The ALA synthase activity in the mixed extract assay was close to the mean of the activities of the extracts from treated and untreated cells (Table III).

Aplastidic cells were treated with cycloheximide at 43 μM, 4.6-dioxoheptanoic acid at 1 mM, or a combination of the two, and the levels of extractable ALA synthase activity were observed for 4 h after treatment during continued growth in the light. Cycloheximide completely blocked the induction of ALA synthase activity by 4,6-dioxoheptanoic acid (Fig. 7). The combination of drugs caused a decline in ALA synthase activity which was somewhat less rapid than in the culture containing only cyclohex-

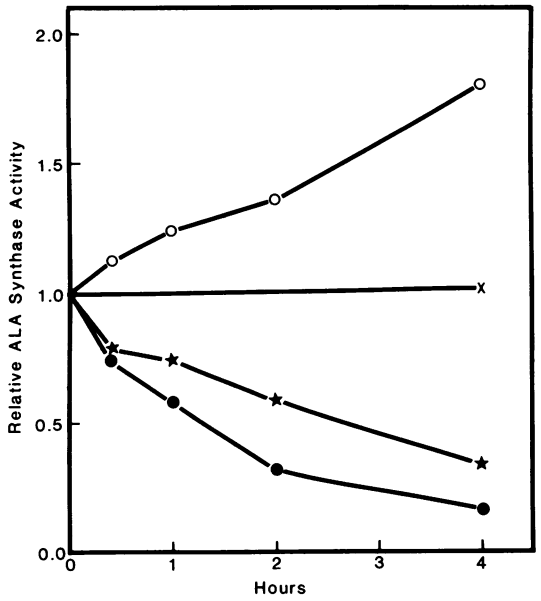

FIG. 7. Effects of cycloheximide on induction by 4,6-dioxoheptanoic acid. ALA synthase activity in extracts of growing aplastidic cells is plotted *versus* hours after addition of 43 μM cycloheximide (●), 1 mM 4,6-dioxoheptanoic acid (○), neither (X), and both compounds (★). Initial activity was 45 nmol ALA · 30 min^{-1} · 10^7 $cells^{-1}$.

imide.

Effects of Chloramphenicol. Cultures of wild-type cells were grown in complete darkness until they had reached 1 × 10^6 cells · ml^{-1}, at which time 6.2 mM chloramphenicol was added. Twelve and 24 h later, cultures with and without chloramphenicol were transferred to the light. Duplicate control flasks were kept in the dark. Growth and extractable ALA synthase levels were monitored in all cultures over the next 12 h. Also, Chl content was monitored in the cultures that were transferred to the light. Growth rates of all cultures remained about equal (data not shown, but see Ref. 4). After transfer to the light, the rate of Chl synthesis was lower in the chloramphenicol-pretreated cultures (Fig. 8). Cellular level of extractable ALA synthase activity remained constant in the dark control cultures, whereas the levels increased somewhat in the dark chloramphenicol-pretreated cultures. ALA synthase levels decreased sharply in all cultures that were transferred to the light, but the decrease was less pronounced in the chloramphenicol-pretreated cultures (Fig. 8). After 12 h in the light, untreated cells had 20% of the extractable ALA synthase activity of the dark control cells, whereas 12-h chloramphenicol-pretreated cells that had been transferred to the light still had 43% of the activity of the 12-h chloramphenicol-pretreated cells that had remained in the dark. Twenty-four-hour chloramphenicol-pretreated cells that had been transferred to the light had 68% of the activity of the 24-h chloramphenicol-pretreated cells that had remained in the dark.

Light-grown aplastidic cells were pretreated with chloramphenicol at 6.2 mM for 16 h prior to the administration of cycloheximide at 43 μM. Separate chloramphenicol-pretreated and cycloheximide-treated cultures were prepared for comparison. Pretreatment of the cells with chloramphenicol 16 h before cycloheximide addition did not significantly affect the kinetics of the decrease in ALA synthase activity after treatment with cycloheximide (Table IV). Chloramphenicol alone appeared to cause a small increase in ALA synthase level.

Effects of Possible Feedback Inhibitors *In Vitro*. ALA synthase isolated from animal and bacteria sources has been reported to be inhibited by micromolar concentrations of hemes and porphyrins (6, 21, 29, 31). Because ALA synthase from *Euglena* has physical and kinetic properties similar to those enzymes (8), the possibility of allosteric inhibition by hemin, protoporphyrin IX, and PBG was examined at concentrations up to 100 μM in extracts of aplastidic cells that had been partially purified by passage through a column of Sephadex G-25 in extraction medium to remove low molecular weight components. None of these potential allosteric feedback inhibitors had a pronounced effect on ALA synthase activity within the range of concentrations examined (Table V).

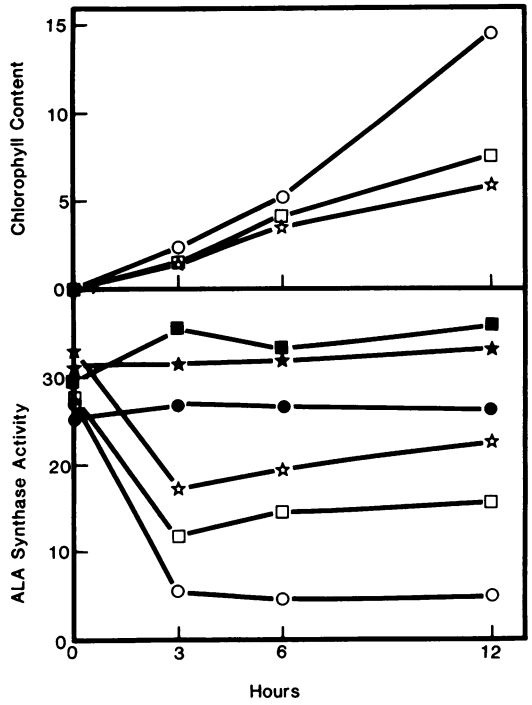

FIG. 8. Effects of chloramphenicol on Chl and ALA synthase. Dark-grown, wild-type cells were transferred to light after earlier addition of 6.2 mM chloramphenicol. Chl content (nmol · 10^7 $cells^{-1}$) and *in vitro* ALA synthase activity (nmol · 30 min^{-1} · 10^7 $cells^{-1}$) were measured at 0, 3, 6, and 12 h after transfer to light. ALA synthase activity was also measured in control cultures left in dark. (●, ■, ★) cultures left in dark; (● □, ☆) cultures transferred to light at 0 h; (○, ●) no chloramphenicol added; (□, ■) chloramphenicol added 12 h previously; (☆, ★) chloramphenicol added 24 h previously.

Table IV. *Effects of Chloramphenicol Pretreatment on ALA Synthase Activity*

ALA synthase activity was measured in extracts of aplastidic cells pretreated with 6.2 mM chloramphenicol 16 h prior to the addition of 43 μM cycloheximide, in untreated cells, and in cells given one or the other drug only.

	ALA synthase activity after addition of cycloheximide		
	Initial	1 h	2 h
	nmol ALA · 30 min^{-1} · 10^7 $cells^{-1}$		
Control	30.2	38.6	39.9
Chloramphenicol-pretreated only	39.5	45.1	51.1
Cycloheximide	33.4	16.9	7.0
Cycloheximide plus chloramphenicol pretreatment	46.2	19.3	8.2

DISCUSSION

Euglena gracilis is the only green oxygenic organism that has thus far yielded ALA synthase activity *in vitro*, and it is therefore

Table V. *Effects of Hemin, Protoporphyrin IX, and Porphobilinogen on ALA Synthase Activity in Vitro*

Extract from aplastidic cells was passed through Sephadex G-25 in extraction medium to remove endogenous low molecular weight compounds. ALA synthase activity was then measured in the presence of the indicated compound.

Compound	Concentration	ALA Synthase	
		Specific	Relative
	μM	*nmol ALA · 30 min*$^{-1}$	%
Hemin	0	22.3	100
	1	22.3	100
	5	21.3	93
	10	21.6	94
	50	21.8	95
	100	21.3	93
Protoporphyrin IX	0	23.1	100
	1	23.5	102
	5	22.9	99
	10	23.0	100
	50	20.3	88
	100	17.9	78
Porphobilinogen	0	23.1	100
	1	24.0	104
	5	23.2	100
	10	21.5	93
	50	19.9	86
	100	19.3	84

the only such organism that is unambiguously capable of forming ALA from succinyl-CoA and glycine. Because *Euglena* cells are also capable of ALA formation from glutamate or α-ketoglutarate via the 'plant' pathway (23, S. I. Beale, unpublished data), it becomes important to establish which pathway is responsible for each major class of end products: Chl, plastid hemes, and mitochondrial hemes. By studying the conditions leading to induction or repression of extractable ALA synthase activity in wild-type and aplastidic mutant cells, we have obtained results that are consistent with the hypothesis that ALA synthase in *Euglena* is responsible for the synthesis of precursors to nonplastid tetrapyrroles.

In various organisms, ALA synthase activity has been reported to be regulated by a number of different mechanisms. These include control of the activity of the enzyme by various inhibitors and activators (6, 16, 21, 29, 31), control of the formation of the enzyme at the translational and perhaps at the transcriptional levels (11–14, 24), and control of the translocation of the enzyme from the cytoplasm into the mitochondria (30). Allosteric feedback inhibition by micromolar concentrations of heme occurs with enzymes derived from photosynthetic (6, 29, 31) and nonphotosynthetic bacteria (7), and animal cells (21). ALA synthase from the photosynthetic bacterium *Rhodopseudomonas spheroides* is also inhibited by low concentrations of protoporphyrin IX (31). ALA synthase from *Euglena* cells did not respond *in vitro* to physiologically relevant concentrations of heme or protoporphyrin IX. PBG, which is the immediate successor to ALA in the heme biosynthetic pathway, also had little, if any, effect on ALA synthase activity *in vitro*.

Cycloheximide, an inhibitor of cytoplasmic protein synthesis (26), caused an exponential decrease in extractable ALA synthase activity during the course of incubation with intact growing cells. The calculated half-life of of the enzyme *in vivo* was 55 min, after the addition of cycloheximide. The exponential decay of enzyme activity after treatment of the cells with this inhibitor of cytoplasmic protein synthesis suggests that ALA is synthesized in the cytoplasm (as in other eukaryotic cells) and the enzyme is labile *in vivo*, having a turnover half-life of less than 1 h. The similarity of calculated ALA synthase half-life time after cycloheximide treatment to the half-life after transfer of wild-type cells from darkness to light, suggests that the mechanism of suppression of enzyme activity, after the transfer to the light, involves blockage of formation of an enzyme that is rapidly turned over. Rapid turnover of ALA synthase was previously reported in rat liver cells, where the half-life value ranges from 35 (14) to 70 min (17).

The fact that mixed extracts of control and cycloheximide-treated cells had an ALA synthase activity equal to the mean value of the separate unmixed extracts indicates that cycloheximide does not act by causing the accumulation of a soluble inhibitor of ALA synthase or the removal of a soluble activator of the enzyme.

It was found that in order for the observed decrease in extractable ALA synthase activity to occur after administration of cycloheximide, the cells must be maintained at their normal growth temperature of 23°C. The stabilization of the activity at 0°C in the presence or absence of cycloheximide suggests that the turnover of the enzyme is mediated by an enzymic mechanism, rather than being a result of an intrinsically unstable protein.

If the rate of ALA synthase formation were under feedback regulation by one or more end products of the tetrapyrrole pathway, then blocking the formation of these products would be predicted to lead to the induction of ALA synthase. 4,6-Dioxoheptanoic acid has been reported to be a powerful and specific inhibitor of ALA dehydrase (9), the enzyme that catalyzes the condensation of two ALA molecules to form PBG. Dioxoheptanoic acid, when administered to growing cells, causes a rapid increase in the level of extractable ALA synthase. The dioxoheptanoic acid-induced increase in extractable ALA synthase activity is largely blocked by cycloheximide, supporting the hypothesis that the increase is mediated by enzyme synthesis on cytoplasmic ribosomes.

The pronounced decrease in extractable ALA synthase activity, which is observed as *Euglena* cultures approach the end of the exponential growth phase and enter the stationary phase, further supports the hypothesis that activity *in vivo* is regulated by differential rates of formation and breakdown of the enzyme. If ALA synthase were the rate-controlling enzyme of the pathway to heme, its activity *in vivo* would be expected to reflect closely the rate of heme formation. Heme formation would be required in growing cells that are actively synthesizing respiratory Cyts, and the demand for heme would be expected to slacken as cells cease growing. The correlation of expected demand for heme with observed ALA synthase activity *in vitro* is consistent with regulation by demand for heme via modulation of the amount of enzyme present.

Ethanol has been reported to stimulate the rate of respiration and synthesis of mitochondrial marker enzymes in *Euglena* while inhibiting chloroplast development, generally promoting 'animal-like' characteristics and suppressing 'plant-like' characteristics of the cells (10, 15). Addition of 100 mM ethanol to aplastidic cells growing in the light results in a rapid increase in extractable ALA synthase activity. This result further supports a role for ALA synthase in the development of respiratory Cyt hemes. Heme is a likely candidate for a repressor of ALA synthase in *Euglena*, because it is the end product of the nonplastid tetrapyrrole pathway. Other workers (11–14) have shown that heme functions as an allosteric inhibitor and repressor of ALA synthase extracted from other cell types, and acts as the principal regulator of bacteriochlorophyll synthesis in *Rhodopseudomonas spheroides* (6). An induction in extractable ALA synthase activity occurs when either wild-type or aplastidic *Euglena* cells are incubated with *N*-methyl mesoporphyrin, a powerful inhibitor of ferrochelatase [protoheme ferrolyase, EC 4.99.1.1] (3). This compound caused a

decrease in protoheme level without affecting Chl synthesis in wild-type cells, supporting the hypothesis that, in *Euglena*, ALA synthase provides precursors to nonplastid tetrapyrroles and that heme acts as a repressor of ALA synthase activity.

It was previously noted that green, light-grown, wild-type cells contain only approximately one-third as much ALA synthase activity as dark-grown, wild-type cells or aplastidic mutant cells (4). In mixed extracts of light-grown, wild-type and aplastidic mutant cells, the ALA synthase activity is close to the mean value of the activities of the separate extracts. This result suggests that the difference in activity is not due to a soluble inhibitor or activator acting during the ALA synthase assay, but does not rule out the existence of a tightly bound modulator of activity.

Extractable ALA synthase activity decreases rapidly after transfer of growing wild-type cells from darkness to light. The exponential decrease in activity during the first 4 h after the transfer to light indicates a half-life of 75 min for the enzyme activity under these conditions. While the decrease in ALA synthase activity is occurring, Chl synthesis increases at an exponential rate. When growing wild-type cells are transferred from the light to the dark, Chl synthesis ceases and the extractable ALA synthase activity increases 4-fold during the first 5 h after the transfer, and then continues to increase more slowly until levels approaching those found in dark-grown cells are attained at approximately 12 h. The inverse correspondence between rates of Chl formation and ALA synthase activity suggests strongly that ALA synthase is not involved in providing Chl precursors in *Euglena*.

The level of extractable ALA synthase in light-grown, wild-type cells is equally low whether the cells are grown in the presence or absence of DCMU at 10^{-5} M, a concentration that completely abolishes photosynthetic O_2 evolution. This observation indicates that the lower ALA synthase level is not a response to the presence of a photosynthetic product, but may instead by a consequence of some other aspect of chloroplast development.

The rapidity of the responses to shifts from light to dark, and from dark to light, suggests that the level of ALA synthase is closely controlled by the developmental status of the chloroplast. The similarity of the time course for these changes to those that occur after the administration of cycloheximide, can be most simply explained by the operation of an induction-repression mechanism coupled with rapid degradation of enzyme.

The effects of chloramphenicol on ALA synthase levels are of interest. This drug is an inhibitor of protein synthesis on 70S-type ribosomes, and has been used to inhibit *in vivo* plastid protein synthesis, specifically in *Euglena* (22). Growth in the presence of chloramphenicol does not decrease the level of extractable ALA synthase in aplastidic cells, and prior application of chloramphenicol does not prevent the rapid decrease in activity induced by a subsequent application of cycloheximide.

In wild-type cells, chloramphenicol preincubation lessens the magnitude of decrease in extractable ALA synthase activity after transfer from the dark to the light. The degree of diminution of the effect of the dark-to-light transfer is proportional to the degree of inhibition of greening caused by progressively longer durations of preincubation of the cells with chloramphenicol in the dark before transfer to the light. These effects of chloramphenicol provide further evidence that the ALA synthase level responds to the rate or state of chloroplast development in wild-type cells, with higher degrees of plastid development leading to increasing suppression of ALA synthase activity. In aplastidic and dark-grown wild-type cells, chloramphenicol by itself appears to elevate the level of extractable ALA synthase activity slightly. This phenomenon has not been studied further.

In summary, the results reported here suggest that the role of ALA synthase in *Euglena* is restricted to the formation of precursors to nonplastid (*e.g.* mitochondrial) tetrapyrroles, that the enzyme is synthesized on cytoplasmic ribosomes, and that the principal mode of regulation is via differential rates of synthesis and degradation of the enzyme, rather than by direct allosteric end product feedback regulation of activity.

Finally, there is the question of the significance of the lower levels of ALA synthase activity found in green or greening cells compared with aplastidic or dark-grown, wild-type cells. All of these cell types grow at similar rates and have similar respiratory rates (S. I. Beale, unpublished data), suggesting that their rates of synthesis of mitochondrial cytochromes are similar. The decrease of ALA synthase that occurs in wild-type cells after transfer from the dark to the light cannot be ascribed to repression in response to a product of photosynthesis, because photosynthesis was reported not to begin to occur at measurable levels until many hours after the transfer to the light (24), and also because the effect was not blocked by DCMU, which completely inhibits photosynthetic O_2 evolution at the concentration used (24).

We propose that in green or greening *Euglena* cells, the plastids, while synthesizing ALA at high rates via the plant pathway, are able to supply most of the mitochondrial requirement for heme precursors, perhaps by exporting ALA or another intermediate from the plastid to the cytoplasm or mitochondria. In the presence of the plastid source of heme precursors, the mitochondrial ALA-forming system via ALA synthase then becomes repressed. Other algae and higher plants, which have no detectable ALA synthase activity, may have lost their ability to form ALA outside of the plastids. One important reason why viable aplastidic strains have not been found among other algae and plants may be that these species depend entirely on the plastids for all heme precursor requirements, and thus aplastidic strains would have no means of carrying out either respiration or photosynthesis. This hypothesis can be tested by using *in vivo* techniques to determine the biosynthetic origin of the carbon atoms in mitochondrial hemes of light-grown and dark-grown, wild-type *Euglena* cells.

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