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δ-Aminolevulinic Acid Biosynthesis from Glutamate in *Euglena gracilis*[1]

Photocontrol of Enzyme Levels in a Chlorophyll-Free Mutant

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ABSTRACT

Wild-type *Euglena gracilis* cells synthesize the key chlorophyll precursor, δ-aminolevulinic acid (ALA), from glutamate in their plastids. The synthesis requires transfer RNA^{Glu} ($tRNA^{Glu}$) and the three enzymes, glutamyl-tRNA synthetase, glutamyl-tRNA reductase, and glutamate-1-semialdehyde aminotransferase. Nongreening mutant *Euglena* strain $W_{14}ZNaIL$ does not synthesize ALA from glutamate and is devoid of the required $tRNA^{Glu}$. Other cellular $tRNA^{Glu}$s present in the mutant cells were capable of being charged with glutamate, but the resulting glutamyl-tRNAs did not support ALA synthesis. Surprisingly, the mutant cells contain all three of the enzymes, and their cell extracts can convert glutamate to ALA when supplemented with $tRNA^{Glu}$ obtained from wild-type cells. Activity levels of the three enzymes were measured in extracts of cells grown under a number of light conditions. All three activities were diminished in extracts of cells grown in complete darkness, and full induction of activity required 72 hours of growth in the light. A light intensity of 4 microeinsteins per square meter per second was sufficient for full induction. Blue light was as effective as white light, but red light was ineffective, in inducing extractable enzyme activity above that of cells grown in complete darkness, indicating that the light control operates via the nonchloroplast blue light receptor in the mutant cells. Of the three enzyme activities, the one that is most acutely affected by light is glutamate-1-semialdehyde aminotransferase, as has been previously shown for wild-type *Euglena* cells. These results indicate that the enzymes required for ALA synthesis from glutamate are present in an active form in the nongreening mutant cells, even though they cannot participate in ALA formation in these cells because of the absence of the required $tRNA^{Glu}$, and that the activity of all three enzymes is regulated by light. Because the absence of plastid $tRNA^{Glu}$ precludes the synthesis of proteins within the plastids, the three enzymes must be synthesized in the cytoplasm and their genes encoded in the nucleus in *Euglena*.

The phytoflagellate *Euglena gracilis* is a useful model organism in studies of chloroplast differentiation because, like higher plants, it has a light requirement for the synthesis of Chl and development of photosynthetic competence (36). As with plant tissues, *Euglena* cells grown in complete darkness fail to accumulate Chl and, instead, form small quantities of Pchl(ide). Exposure to light brings about the conversion of these pigments to Chl(ide), and in continuous light this is followed by a phase of rapid Chl accumulation (17) and transformation of the developing plastids into photosynthetically functional chloroplasts (37).

The source of plastid tetrapyrrole pigments in *Euglena*, as in higher plants, algae, cyanobacteria, and other oxygenic organisms, is ALA[2] that is formed from glutamate via the tRNA-dependent five-carbon pathway (26, 39). The five-carbon pathway begins with activation of glutamate by ligation to $tRNA^{Glu}$, catalyzed by glutamyl-tRNA synthetase, followed by reduction of the activated glutamate to GSA, catalyzed by glutamyl-tRNA reductase, and transamination of the GSA, catalyzed by GSA aminotransferase, to yield ALA (2).

Unlike other oxygenic organisms, *Euglena* also has the ability to form ALA via the route used by animals, yeasts, and certain bacterial groups, in which the condensation of glycine and succinyl-CoA is catalyzed by ALA synthase (EC 2.3.1.37) (3). ALA that serves as precursor to Chl is synthesized solely by the five-carbon route in *Euglena* (29, 39), whereas ALA that is used for mitochondrial tetrapyrrole synthesis is formed exclusively by the ALA synthase route (39). Certain nongreening *Euglena* mutant strains that do not undergo light-induced plastid differentiation appear to lack the ability to form ALA from glutamate *in vivo*, and these strains rely on the ALA synthase route for all tetrapyrrole precursor biosynthesis (26, 39).

Euglena is known to have two photoregulatory systems. One of these responds to both red and blue light, is localized in the plastids, is apparently absent in certain mutant strains in which plastids are absent or nonfunctional, and has Pchl(ide) as its photoreceptor (10, 36). The other photoregulatory system responds only to blue light and retains its activity in mutant cells lacking functional plastids (32, 36). Neither phytochrome nor phytochrome responses have been reported in *Euglena*.

It was previously shown that, in wild-type *Euglena* cells, all three enzymes and the tRNA required for ALA biosynthesis from glutamate are induced by light and that both red and blue light are effective inducers (25). We have now examined

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[2] Abbreviations: ALA, δ-aminolevulinic acid; GSA, glutamate-1-semialdehyde; tRNA, transfer RNA.

a nongreening *Euglena* mutant strain that forms ALA only by the ALA synthase route *in vivo*. These cells appear to be devoid of the plastid $tRNA^{Glu}$ that is required for ALA synthesis from glutamate. Surprisingly, the cells contain all three enzymes of the five-carbon pathway. The enzymes are active and capable of catalyzing ALA synthesis *in vitro* when supplemented with tRNA obtained from wild-type cells. All three enzyme activities are induced by light, but in contrast to the wild-type cells, enzyme induction in the nongreening cells is promoted by blue, and not by red, light. Portions of this work have been reported previously in brief form (23, 24).

MATERIALS AND METHODS

Growth of Cells

Axenic cultures of *Euglena gracilis* Klebs var Pringsheim wild-type strain Z and nongreening mutant strain W_{14}ZNalL were grown in glucose-based heterotrophic medium in the light or complete darkness as described previously (3). Cells referred to as dark grown had been subcultured in continuous darkness for at least 1 year before experiments.

For experiments in which the effects of light intensity and spectral composition were being studied, cells were grown as described (25). Light intensity measurement and the use of colored filters to provide light of restricted wavelength ranges were done as described before (25).

Cell Extraction for Enzyme Preparation

Cell cultures in the exponential growth phase were thoroughly chilled on ice under the light conditions in which they were grown. All subsequent operations were performed at 0 to 4°C. Cells were harvested by centrifugation, washed, resuspended, and disrupted by sonication as described previously (25). Cell debris and unbroken cells were removed by centrifugation for 10 min at 10,000*g*, and the supernatant was treated as described previously (25). The clarified supernatant was fractionated by differential $(NH_4)_2SO_4$ precipitation between 35 and 60% of saturating concentration of $(NH_4)_2SO_4$, in the presence of 5 mM EDTA and 0.004% PMSF, desalted by passage through Sephadex G-25 that was preequilibrated and eluted with column buffer (1.0 M glycerol, 50 mM Tricine, 15 mM $MgCl_2$, 1.0 mM DTT, 20 μM pyridoxal-5-phosphate [pH 7.9]). The protein-containing effluent was supplemented with 0.004% PMSF and stored at −75°C. In some cases, where indicated, the differential $(NH_4)_2SO_4$ precipitation step was omitted.

Enzyme Fractionation by Affinity Chromatography

Fractionation of extracts from light-grown wild-type cells on Reactive Blue 2-Sepharose was done as previously described to yield a fraction, designated Blue-Sepharose-unbound, which contains GSA aminotransferase activity but is essentially devoid of glutamyl-tRNA synthetase and glutamyl-tRNA reductase (42).

Cell Extraction for tRNA

RNA was prepared by phenol and chloroform-isoamyl alcohol extraction of high-speed supernatant obtained from cells sonicated in RNA extraction medium (100 mM NaCl, 10 mM Tris-HCl, 10 mM Mg-acetate, 10 mM β-mercaptoethanol, 5 mM EDTA [pH 7.5]), and isolated by DEAE-cellulose chromatography as described previously (11, 25, 40).

Colorimetric Assay for *in Vitro* ALA Formation

Assays were carried out by modifications of a previously described method (40). Incubation was for 60 min at 30°C in 0.25 to 1.0 mL of assay medium (1.0 M glycerol, 50 mM Tricine, 15 mM $MgCl_2$, 5 mM ATP, 5 mM levulinate, 1.0 mM NADPH, 1.0 mM glutamate, 1.0 mM DTT, 20 μM pyridoxal-5-phosphate [pH 7.9]). Incubations contained 10 to 12 A_{260} units of RNA and 2.0 to 5.0 mg of enzyme protein per mL of reaction volume. Reactions were terminated by addition of 1/10 volume of 1.0 M citric acid and one volume of 10% (w/v) SDS followed by heating for 3 min at 95°C. ALA was isolated on Dowex 50W-X8 (Na), ethylacetoacetate was added, and the solutions were heated to 95°C for 15 min to form 1-methyl-2-carboxyethyl-3-propionic acid pyrrole (22). The product was quantitated spectrophotometrically after reaction with an equal volume of Ehrlich-Hg reagent (38), using a Cary model 219 spectrophotometer (Varian Instruments, Palo Alto, CA). The A_{553} of unincubated control samples was subtracted from those of incubated samples to determine net A_{553} values, and ALA was calculated from a standard curve with samples containing known amounts of ALA.

Chl Determination

Chl was determined by measurement of A_{665} in methanol extracts of cells, and using an absorption coefficient of 6.66×10^4 L mol^{-1} cm^{-1} for Chl *a*, derived from the data in ref. 20. Cell pellets were extracted with 10 volumes of ice-cold methanol by incubating on ice for 5 min in the dark. Cell debris was removed by centrifugation, and serial dilutions were examined by absorption spectroscopy. Fully corrected fluorescence emission and excitation spectra of the samples were obtained with a Fluorolog double-grating dual-monochromator spectrofluorometer (Spex Industries, Metuchen, NJ).

Assay for Glutamate-Accepting Ability of tRNA

[^{14}C]Glutamyl-tRNA formation from [^{14}C]glutamate was measured by the appearance of TCA-precipitable radioactivity in reaction mixtures (26). Incubation was carried out for 30 min at 30°C in 250 μL of reaction medium similar to the assay medium for ALA formation. NADPH and levulinate were omitted from this medium, and the glutamate was present at a concentration of 100 μM and contained 840,000 cpm of [1-^{14}C]glutamate (the final specific radioactivity was 34 cpm pmol^{-1}). Reactions were terminated by the addition of 1.0 mL of 84% (v/v) aqueous acetone containing 12.5% (w/v) TCA, and precipitated radioactivity was quantitated as described before (25). Zero time controls had fewer than 200 cpm in the final pellets, and all duplicates agreed within 15%.

Glutamyl-tRNA Synthetase Assay

A tritium-based filter-binding assay similar to that previously described was used (34). Reactions were carried out in 500 μL of column buffer containing 5 mM ATP, 25 A_{260} units of tRNA isolated (as described above) from light-grown wild-type cells, 100 μM final glutamate concentration having 5.5×10^7 cpm of [3,4-^{3}H]glutamate (the final specific radioactivity was 1100 cpm pmol^{-1}), and enzyme extract (400–500 μg of protein). Enzyme extract was prepared as a 35 to 60% differential $(NH_4)_2SO_4$ precipitate (as described above) from mutant cells grown in the light or dark. The reactions were initiated by addition of enzyme that was prewarmed to 30°C, incubation was for 5 min at 30°C, and the reactions were terminated by the application of 15-μL samples onto glass fiber filters, which were processed as described previously (25). Radioactivity was determined by liquid scintillation spectroscopy in a Beckman LS-5000 TD instrument, with Cytoscint liquid scintillant. ^{3}H-counting efficiency was approximately 50%. Counting times were sufficient to achieve a counting error of not more than 0.2%. Control incubations consisted of boiled enzyme extracts incubated with complete assay mixture.

Isolation of [^{3}H]Glutamyl-tRNA for Use as Enzyme Substrate

For determination of the ability of extracts from light- and dark-grown cells to form glutamyl-tRNA that can serve as a substrate for ALA formation, the ^{3}H-based glutamyl-tRNA synthetase assay reaction, as described above, was terminated by the addition of 2 mL of aminoacyl-tRNA buffer (100 mM Mes, 100 mM glutamate, 10 mM $MgCl_2$ [pH 5.8]). The mixture was shaken with 3 mL of phenol (preequilibrated with aminoacyl-tRNA buffer), and glutamyl-tRNA was isolated as described before (25).

For preparation of larger quantities of glutamyl-tRNA for use as substrate in the assay of glutamyl-tRNA reductase activity, 250 A_{260} units of tRNA extracted from light-grown wild-type cells was precipitated with 2.5 volumes of absolute ethanol at −75°C for 1 h in the reaction tube. The precipitate was sedimented by centrifugation at 13,000*g* for 30 min. The reaction mixture (1.0 mL) contained the redissolved tRNA, 5 mM ATP, 100 μM glutamate having 5×10^8 cpm of [3,4-^{3}H] glutamate (the final specific radioactivity was 5000 cpm pmol^{-1}), and extract from light-grown wild-type cells (1.8 mg of protein). The reaction was initiated by addition of glutamate, allowed to proceed for 30 min at 30°C, and terminated by addition of 2 mL of aminoacyl-tRNA buffer. Immediately before addition of the termination buffer, 5 μL of incubation mixture was removed and transferred to a glass fiber filter disc for determination of glutamyl-tRNA product. The reaction mixture and the filter were processed as described previously (34). The dried glutamyl-tRNA was dissolved in half-strength aminoacyl-tRNA buffer at a concentration of 500 A_{260} units per mL, and aliquots were stored at −75°C.

In Vitro Conversion of [^{3}H]Glutamyl-tRNA to ALA

Incubation was for 60 min at 30°C, in 250 μL of column buffer containing 5 mM levulinate, 2.0 mM Mes, 1.0 mM NADPH, 1.0 mM unlabeled glutamate, enzyme extract from light-grown mutant cells (500 μg of protein), and 2.5 A_{260} units of [^{3}H]glutamyl-tRNA that was precharged by the enzyme being tested (as described above) and containing 1.0 to 1.4×10^5 cpm of ^{3}H (the specific radioactivity of the glutamate in [^{3}H]glutamyl-tRNA was 1100 cpm pmol^{-1}). ALA was isolated as described above, and the ALA pyrrole was extracted into ethyl ether and counted by liquid scintillation as described before (1, 25).

Glutamyl-tRNA Reductase Assay

Glutamyl-tRNA reductase activity was quantitated by measuring the ability of varying amounts of an enzyme extract to form ALA in the presence of constant levels of both precharged [^{3}H]glutamyl-tRNA (the specific radioactivity was 5000 cpm pmol^{-1}) and GSA aminotransferase as described previously (25). Constant GSA aminotransferase activity was achieved by supplementing the incubation mixtures with adjusted amounts of Blue-Sepharose-unbound fraction obtained from light-grown wild-type cells by affinity fractionation as described before (42).

GSA Aminotransferase Assay

GSA aminotransferase activity was measured by assaying the conversion of chemically synthesized GSA to ALA by cell extracts as described previously (25). GSA was prepared from 4-amino-5-hexenoic acid (vinyl-γ-aminobutyric acid) by the method of Gough *et al.* (14).

Other Procedures

Cell population densities were determined with a Coulter Counter (model ZBI, Coulter Electronics). Protein concentrations were determined by the dye-binding method of Bradford (6) using BSA as the standard. Pancreatic RNase A (Sigma type I-AS) was dissolved in RNase buffer (1.0 M Tris-HCl, 15 mM NaCl [pH 7.5]), heated for 8 min at 100°C, cooled slowly, and stored at −20°C (21).

Chemicals

4-Amino-5-hexenoic acid was a generous gift from Merrell Dow Research Institute, Cincinnati, OH. L-[3,4-^{3}H]Glutamate and L-[1-^{14}C]glutamate were purchased from Du Pont-New England Nuclear, Wratten filters from Eastman Kodak Co., DEAE-cellulose DE-23 from Whatman, glass fiber filter discs from Schleicher & Schuell, Cytoscint liquid scintillation cocktail from ICN Biomedicals, and Miracloth from Calbiochem-Behring. All other chemicals were from Sigma, Fisher, and Research Organics.

RESULTS

Chl Content of Cells

Chl content was measured in methanol extracts of wild-type strain Z and mutant W_{14}ZNalL cells that were grown in the light and harvested in the late exponential growth phase. Chl content was determined from the A_{665} of diluted methanol extracts. Wild-type cells had 7 nmol of Chl per 10^6 cells, which is equal to 66 nmol per mL of culture at the cell density of

9.6×10^6 per mL at the time of harvest. The Chl content of mutant cells (harvested at approximately the same cell density), as calculated from the A_{665} of their undiluted methanol extract, was less than 2×10^{-5} of these values. However, the absorption spectrum of the mutant cell extract did not exhibit a peak at 665 nm, and the absorption at that wavelength appeared to be due entirely to tailing from the carotenoid region of the spectrum.

To measure the Chl content of the mutant cells more sensitively, the fluorescence spectra of their undiluted methanol extract was determined and compared to the fluorescence spectrum of a 50,000-fold diluted methanol extract of wild-type cells. With excitation at 443 nm, the wild-type extract had an emission peak at 675 nm. The undiluted mutant extract had no detectable emission peak at 675 nm. The minimal detectable peak was <1% of the value for the 50,000-fold diluted wild-type extract. Therefore, the Chl content in the mutant cells was no more than 2×10^{-7} of the content in wild-type cells.

The mutant cell extract was also examined for the presence of Pchl(ide) by absorption and fluorescence spectroscopy, and no Pchl(ide) was detected. Although the lower limit for detection of Pchl(ide) was not quantitatively determined, the limit was probably in the same range as that for Chl.

Content of tRNA that is Capable of Supporting ALA Formation

tRNA extracted from light- and dark-grown wild-type and mutant cells was tested for the ability to support ALA formation in an enzyme extract of light-grown wild-type cells that had been depleted of endogenous tRNA by RNase predigestion. After the further action of RNase was blocked by the addition of human placental RNase inhibitor, incubation was done in the presence and absence of added tRNA.

About three times more ALA was made in the incubation with added tRNA from light-grown wild-type cells than in the incubation with an equal amount of added tRNA from dark-grown wild-type cells (Table I). Stimulation of ALA formation by added tRNA from light- or dark-grown mutant cells was below the limit of detection.

Previous results indicated a slight stimulation of ALA formation in wild-type cell extracts by RNA extracted from mutant cells (26). In the earlier experiments, endogenous RNA was not removed from the wild-type cell extracts before the addition of the tested RNA. The endogenous RNA was active in ALA formation and the apparent stimulation by the added RNA derived from the mutant cells was probably caused by a protective effect of the added RNA on the endogenous RNA due to competition for the action of endogenous RNases. In the current experiments, in which endogenous RNA was removed from the wild-type cell extracts before the addition of RNA from the mutant cells, no stimulation of ALA formation was observed.

The inability of tRNA from mutant cells to stimulate ALA formation in enzyme extract of wild-type cells was not due to the inability of the enzyme extract to charge the tRNA with glutamate. tRNA from light-grown wild-type and mutant cells were both charged by enzyme extract from light-grown wild-type cells (Table I).

ALA Formation from Glutamate in Mutant Cell Extracts

Extract from light-grown mutant cells was tested for the ability to form ALA *in vitro*, in the presence or absence of glutamate and tRNA extracted from light-grown wild-type cells. tRNA from wild-type cells and glutamate were both absolutely required for enzyme activity (Table II). Incubation of extracts in the absence of either substrate yielded values for total ALA formation that did not differ significantly from those of the unincubated control.

These results contrast with a previous report in which it was concluded that extracts of light-grown mutant cells were inactive in converting glutamate to ALA, even when supplemented with RNA from wild-type cells (26). The difference can be ascribed to the higher sensitivity of the current assay

Table I. *Ability of tRNA to Accept Glutamate and Support ALA Formation with Enzyme Extract from Light-Grown Wild-Type Cells*

ALA formation was assayed as described in the text, using cell extract that was pretreated with RNase to remove endogenous tRNA. Incubations contained 1.2 mg of protein extracted from light-grown wild-type cells and 5 A_{260} units of the indicated RNA. [^{14}C]Glutamyl-tRNA formation was assayed by incubating extract of light-grown wild-type cells with [^{14}C]glutamate plus tRNA isolated from light-grown wild-type or mutant cells. Incubations contained 1.03 mg of protein extracted from light-grown wild-type cells and 5 A_{260} units of the indicated tRNA.

Source of Added tRNA	ALA Formation		Glutamyl-tRNA Formation	
	nmol mg^{-1} protein	*%*	*pmol mg^{-1} protein*	*net increase (%)*
Light-grown wild-type cells	2.58	100	235	100
Dark-grown wild-type cells	0.89	34	ND[a]	
Light-grown mutant cells	0.10	4	144	61
Dark-grown mutant cells	0.11	4	ND	
None (endogenous tRNA removed)	0.06	2	ND	
None (endogenous tRNA present)	ND		67	
None, unincubated control	ND		6	

[a] Not determined.

Table II. *tRNA and Glutamate Requirements for ALA Formation by Enzyme Extract from Light-Grown Mutant Cells*

Cell extract (1.3 mg of protein) from light-grown mutant cells was incubated for 60 min at 30°C in 500 μL of assay buffer with or without 1 mM glutamate and with or without 6.25 A_{260} units of tRNA extracted from light-grown wild-type cells.

Incubation Content	ALA Formation	
	nmol mg^{-1} protein	%
Complete	1.10	100
−tRNA	0.04	0
−Glutamate	0.05	1
Unincubated control	0.04	0

resulting from increased cell extraction efficiency, the consistent use of PMSF to prevent proteolysis, partial purification and enrichment for activity by differential $(NH_4)_2SO_4$ precipitation, and the use of fresh DTT in the assay medium.

Effect of Growth in Light or Dark on *in Vitro* ALA-Forming Activity

Extracts from mutant cells that were grown in the light or dark for many generations were tested for the ability to form ALA from glutamate *in vitro* when supplemented with tRNA extracted from light-grown wild-type cells. The activities and their light to dark ratio are tabulated on a per mg protein basis and also on the basis of equivalent mass of extracted cells (Table III). Small differences in the activity ratios for the two comparison bases are due to small differences in the quantities of proteins extracted from the light- and dark-grown cells.

Extracts from cells that were grown in the dark had little or no ALA-forming activity. In contrast, extracts from cells grown in the light for many generations had high levels of *in vitro* ALA-forming activity when supplemented with the required tRNA that was derived from light-grown wild-type cells. Incubations containing combined extracts from both light- and dark-grown cells had about two thirds the ALA-forming activity, per mg of light-grown cell extract, of the activity of the incubation containing only light-grown cell extract, indicating that the lower activity in the extract from dark-grown cells, compared with the light-grown cell extract, was not due to the presence of an inhibitor (data not shown).

Time Course for Induction of ALA-Forming Activity by Light

Nongreening mutant cells that had been grown in complete darkness for many generations were allowed to continue growing in the dark or transferred to the light at specific time intervals ranging from 12 to 72 h before harvest. A control sample consisted of cells that were light adapted.

After 12 h of growth in the light, the extractable ALA-forming activity level was only slightly above the level measured in extract of dark-adapted cells (Fig. 1). After 72 h of continuous exposure to light culture conditions, the cells had extractable enzyme activity levels approaching those of light-adapted cells. In all subsequent studies of the effects of incident light intensity and spectral composition on extractable ALA-forming activity, dark-adapted cells were illuminated for 48 h before harvest.

Effect of Light Intensity on Induction of *in Vitro* ALA-Forming Activity

Nongreening mutant cells that had been growing in complete darkness for many generations were transferred to light of different incident intensities and allowed to continue growing for 48 h before harvesting.

Extracts of cells that had been grown in the dark for long periods had relatively little ALA-forming activity (0.14 nmol ALA formed/mg protein). Extract of cells grown for 48 h in light of 2 μE m^{-2} s^{-1} intensity formed 0.59 nmol ALA/mg protein, and extract of cells grown between 4 and 64 μE m^{-2} s^{-1} intensity all formed between 0.96 and 1.02 nmol ALA/mg protein. In subsequent studies of the effects of spectral

Table III. *Effects of Growth in the Light or Dark on ALA-Forming Activity and the Activities of the Three Enzymes Required for Transformation of Glutamate to ALA in Extracts of Mutant Cells*

Assays for ALA formation contained 1 mg of protein and 6.25 A_{260} units of tRNA (from light-grown wild-type cells) and were incubated for 60 min. Assays for glutamyl-tRNA synthetase contained 400 to 500 μg of protein and 25 A_{260} units of tRNA (from light-grown wild-type cells) and were incubated for 5 min. Assays for glutamyl-tRNA reductase contained 12 μg of protein, 12 mg of Blue-Sepharose unbound protein fraction obtained from light-grown wild-type cells, and 210 nM glutamyl-tRNA and were incubated for 5 min. Assays for GSA aminotransferase contained 1 mg of protein (prepared from cell extracts with the differential $(NH_4)_2SO_4$ precipitation step omitted) and 33 μM GSA and were incubated for 20 min.

Cell Extract	ALA Formation	Glutamyl-tRNA Synthetase	Glutamyl-tRNA Reductase	GSA Aminotransferase
		nmol mg^{-1} protein h^{-1}		
Light grown	3.05	49.32	0.24	5.22
Dark grown	0.07	35.04	0.08	0.39
Light to dark activity ratio	43.57	1.41	2.89	13.38
		nmol g^{-1} cells h^{-1}		
Light grown	12.24	280.36	1.35	55.71
Dark grown	0.22	154.90	0.36	4.56
Light to dark activity ratio	55.64	1.81	3.75	12.22

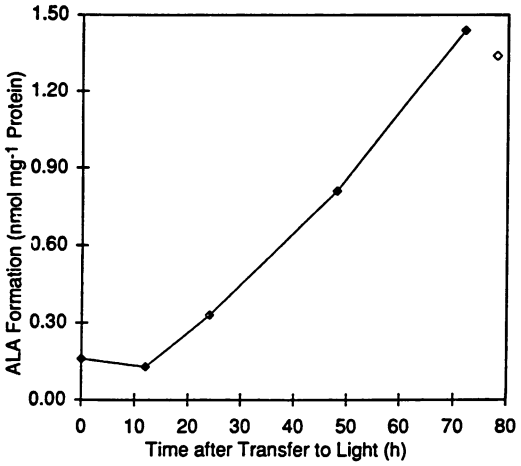

Figure 1. ALA-forming activity in extracts of dark-grown mutant cells at various times after transfer to white light of 13 μE m^{-2} s^{-1} intensity (◆). Incubations (1 mL) contained 2.25 mg of protein (prepared from cell extracts with the differential $(NH_4)_2SO_4$ precipitation step omitted) and 10 A_{260} units of tRNA isolated from light-grown wild-type cells. Incubation was for 60 min at 30°C. The activity in extract of fully light-adapted cells is shown near the right border (◇).

composition on extractable ALA-forming activity, dark-adapted cells were exposed to light for 48 h at an incident intensity of 13 μE m^{-2} s^{-1}.

Effect of Light Wavelength on Induction of *in Vitro* ALA-Forming Activity

Mutant cells that had been growing in complete darkness for many generations were transferred to white or colored light and allowed to continue growing for 48 h. All total incident light intensities were equalized to 13 μE m^{-2} s^{-1} by combining layers of Miracloth with the colored filters and/or by supplementing the standard mixture of cool-white and red fluorescent lights with incandescent light.

Blue light (400–480 nm) was comparable to white light in its ability to induce extractable ALA-forming activity, but red light (650–700 nm) was ineffective in inducing enzyme activity levels above those of cells grown in the dark (Table IV).

Effect of Growth in Light or Dark on Extractable Glutamyl-tRNA Synthetase, Glutamyl-tRNA Reductase, and GSA Aminotransferase Activities

Extracts of mutant cells that had been growing continuously in the light or dark for many generations were examined for the activities of the three enzymes required for the transformation of glutamate to ALA. The activities and their light to dark ratios are tabulated on a per mg protein basis and also on the basis of equivalent mass of extracted cells (Table III). As discussed above, small differences in the activity ratios for the two comparison bases are due to small differences in the quantities of proteins extracted from the light- and dark-grown cells. Because the three enzymes were assayed under different incubation conditions using extracts at different stages of purification, and because of differential extraction efficiency and stability of the enzymes, comparisons of the relative *in vitro* activities of the three enzymes is not warranted. However, because each enzyme was assayed identically in extracts of light- and dark-grown cells, the relative activities of any one enzyme in extracts of light- *versus* dark-grown cells are meaningfully comparable.

Extracts from both light- and dark-grown cells were able to charge the tRNA, and the extract from dark-grown cells was about two thirds as active as the extract from light-grown cells. The [^{3}H]glutamyl-tRNA produced in these incubations was isolated and tested for its ability to be used as a substrate for conversion to ALA by extract of light-grown mutant cells. Incubation conditions in the ALA formation assay were chosen so that free [^{3}H]glutamate, which might arise by hydrolysis of [^{3}H]glutamyl-tRNA, could not be effectively converted to ALA. With tRNA derived from light-grown wild-type cells as substrate, glutamyl-tRNA formed by enzyme extracts of both light- and dark-grown mutant cells was effective as a substrate for subsequent ALA formation by enzyme extract from light-grown mutant cells. During the ALA-forming incubation, between 10 and 21% of the glutamyl-tRNA was converted to ALA (data not shown).

Extract from light-grown mutant cells had approximately three to four times as much glutamyl-tRNA reductase activity as did an equivalent amount of extract from dark-grown cells. Net GSA aminotransferase activity of the extracts from dark-grown mutant cells was approximately 7% of the activity in extracts from light-grown cells. The tabulated values for GSA aminotransferase activity are the net activities, after subtraction of the contribution from nonenzymatic conversion of GSA, which was determined in control incubations containing heat-denatured enzyme.

DISCUSSION

Euglena is unique among organisms in having the ability to form ALA by both the tRNA-dependent five-carbon pathway and by ALA synthase-catalyzed condensation of glycine with succinyl-CoA. *In vivo*, the five-carbon pathway is the sole source of Chl precursors in *Euglena* (29, 39), and this pathway is inactive in dark-grown cells (25, 39). The ALA

Table IV. *Induction of in Vitro ALA-Forming Activity in Dark-Grown Mutant Cells by Light of Various Wavelength Ranges*

Dark-grown cells were kept in the dark or transferred to light having the indicated wavelength range and allowed to continue growing heterotrophically for 48 h before harvesting. All light intensities were equalized to a total fluence rate of 13 μE m^{-2} s^{-1}. ALA formation was measured in 1-mL incubations containing 3 to 5 mg of protein (prepared from cell extracts with the differential $(NH_4)_2SO_4$ precipitation step omitted) and 10 A_{260} units of tRNA derived from light-grown wild-type cells.

Light	Wavelength Range	ALA Formation	
	nm	*nmol mg^{-1} protein*	%
White	400–700	0.77	100
Red	650–700	0.18	23
Blue	400–480	0.67	87
Dark control		0.16	21

synthase route, on the other hand, is the sole source of mitochondrial tetrapyrrole precursors in *Euglena* and may also supply other cellular tetrapyrrole end products in the dark (39). ALA synthase activity is much higher in extracts of dark-grown cells than in light-grown cell extracts, and the extractable activity declines precipitously within the first few hours after transfer of dark-grown cells to the light (7, 12). Certain nongreening mutant *Euglena* strains appear to lack the capacity to form ALA from glutamate *in vivo* (39), and in these strains, ALA synthase activity remains high in the light or dark (3). We have examined mutant W_{14}ZNalL, a nongreening *Euglena* strain, for the presence of the tRNA and three enzymes needed for ALA synthesis from glutamate and for the effects of light on the cellular content of these macromolecules.

Before the cells were further characterized, it was desirable to establish whether they were totally devoid of Chl or were, instead, only partially deficient in this pigment, as has been reported for other nongreening *Euglena* strains (32). It was determined with a sensitive fluorometric assay that strain W_{14}ZNalL cells contain no more than 2×10^{-7} of the Chl content of wild-type strain Z cells. This extremely low value corresponds to <850 Chl molecules per cell, or <70 molecules per plastid, assuming that there are 12 plastids per cell (19). Within the limits of detectability, strain W_{14}ZNalL is completely devoid of Chl and Pchl(ide).

Assay of the tRNA that is needed for ALA synthesis was based on the ability of an RNA fraction isolated from the mutant cells to reconstitute ALA formation in an enzyme extract from wild-type cells that was depleted of endogenous tRNA by predigestion with RNase. No stimulation of ALA formation by tRNA derived from the mutant cells was detected in this assay. This was true whether the tRNA was derived from light- or dark-grown mutant cells. Other cellular glutamate-accepting tRNAs present in the mutant cells, even when charged with glutamate, did not support ALA synthesis. Within the limits of detectability, strain W_{14}ZNalL is completely devoid of the tRNA that is required for ALA formation from glutamate.

Because the mutant cells do not form Chl or glutamate-derived ALA and lack the tRNA needed for ALA synthesis from glutamate, it was surprising to discover that they contain the enzymes of the five-carbon pathway and that, moreover, the activities of these enzymes are induced by light. When supplemented with tRNA derived from wild-type cells, enzyme extracts from light-grown mutant cells were active in catalyzing ALA formation from glutamate. The rate of ALA formation catalyzed by extract of light-grown mutant cells was approximately 30% of the rate catalyzed by extracts of similarly grown wild-type cells, and this rate was significantly higher than that catalyzed by extracts of dark-grown wild-type cells (25).

Although ALA-forming activity was present in extracts of light-grown cells, little or no activity was found in dark-grown cell extracts. Mixing experiments indicated that the absence of activity in dark-grown cell extracts was not attributable to the presence of an inhibitor.

The time course and light intensity dependence for induction of ALA-forming enzymes in the mutant cells resembled those for induction of activity in wild-type *Euglena* (25). However, the wavelength requirements for the light induction were different in the two cell types. The mutant cells responded only to blue light, whereas wild-type cells responded to both red and blue light (25).

Two well-documented photoreceptor systems exist in *Euglena*. One is the red/blue system, which is localized in the plastids and uses Pchl(ide) as the photoreceptor (10, 36). The other is the blue system, which seems to be localized outside of the plastids and whose photoreceptor has not been unequivocally defined (32, 36). Induction of the ALA-forming system by red light in wild-type cells indicates that the red/blue system can mediate the induction. Because both photoreceptor systems respond to blue light, it was not possible to ascertain whether the blue system can independently mediate the induction in wild-type cells. However, because the mutant cells respond only to blue light and are insensitive to red light, presumably because they lack the red/blue photoreceptor, it can be concluded that induction of the activity of the ALA-forming enzymes by blue light in the mutant cells is mediated by the blue system alone.

The activities of all three enzymes of the five-carbon pathway were higher in extracts of cells that were grown in the light than in dark-grown cell extracts. In their relative responses to light, the induction pattern of the three enzyme activities in the mutant cells resembles that of wild-type cells (25). Total cellular glutamyl-tRNA synthetase was the activity that was least affected by light, with a light to dark ratio of about 1.5. Glutamyl-tRNA reductase activity was about 3 times higher in the extract of light-grown cells than in the dark-grown cell extract. GSA aminotransferase was the most acutely affected activity, with a light to dark ratio of >12. No one of the light to dark ratios of enzyme activities can alone account for the light to dark ratio of ALA-forming activity of the extracts, which is about 50. Because all three enzyme reactions are functionally linked in the reaction sequence by supplying a substrate and/or consuming a product of the other enzymes, it is possible that each of the individual activities has some additive influence on the overall rate of conversion of glutamate to ALA.

In all cases examined, the tRNA that is required for ALA synthesis from glutamate is $tRNA^{Glu(UUC)}$ (4, 27, 28, 34, 35). It is of interest that only one plastid $tRNA^{Glu}$ gene has been reported in *Euglena* (15). The single plastid $tRNA^{Glu}$ must therefore be required for both plastid ALA formation and plastid protein synthesis. The absence of the tRNA that supports ALA formation in the mutant cells implies that they are devoid of plastid $tRNA^{Glu}$ and that they are therefore incapable of synthesizing plastid-encoded proteins. The presence of the three enzymes of the five-carbon pathway for ALA biosynthesis in cells that cannot carry out plastid protein synthesis indicates that the enzymes are encoded on nuclear genes and synthesized in the cytoplasm.

Strain W_{14}ZNalL was previously reported to lack detectable plastid DNA, as determined by CsCl density gradient analysis (32). However, the cells apparently contain some proplastid-like membrane-bound inclusions (33). Other nongreening *Euglena* strains that were earlier considered to be aplastidic were later found to contain some plastid gene sequences (18). The apparent absence of plastid $tRNA^{Glu}$ in strain W_{14}ZNalL indicates that, even if the cells do contain some plastid DNA,

protein-coding genes of this DNA cannot be translated in the cells.

Whether or not other plastid functions are retained in mutant W_{14}ZNalL is currently unknown. In particular, it remains to be determined whether the cells are capable of removing N-terminal transit peptides from cytoplasmically synthesized proteins that are targeted for entry into plastids. The enzymes of the five-carbon pathway are normally localized within the plastids (13, 31). The presence and induction by light of active forms of several other enzymes that are normally localized in chloroplasts have been reported previously for other nongreening *Euglena* strains (5, 9, 16). It will be of interest to determine whether these enzymes are correctly processed in the mutant cells or whether they are enzymatically active in the unprocessed form, as has been reported for one other plastid-localized enzyme (8). In this connection, it is of interest that, in preliminary studies, no ALA-forming ability or GSA aminotransferase activity was detected in extracts of another nongreening mutant *Euglena* strain, W_{10}BSmL (S. M. Mayer and S. I. Beale, unpublished data). Unlike W_{14}ZNalL cells, W_{10}BSmL cells were reported to be completely devoid of plastid remnants (30).

In summary, nongreening mutant *Euglena* strain W_{14}ZNalL lacks Chl and plastid tRNA capable of supporting ALA synthesis from glutamate via the tRNA-dependent five-carbon pathway. However, the cells contain all three enzymes of this pathway. As in wild-type cells, activities of the enzymes are induced by growth of the cells in the light. However, unlike in wild-type cells, red light is ineffective in inducing the enzymes in the mutant cells. The apparent absence of plastid $tRNA^{Glu}$ suggests that the mutant cells cannot translate plastid-encoded genes and that the enzymes of the five-carbon pathway are encoded in the nucleus.

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