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Events Surrounding the Early Development of *Euglena* Chloroplasts

14. BIOSYNTHESIS OF CYTOCHROME *c*-552 IN WILD TYPE AND MUTANT CELLS[1]

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ABSTRACT

Lack of a suitable assay has thwarted attempts to measure cytochrome *c*-552 in dark-grown wild type cells of *Euglena gracilis* var. *bacillaris* in mutants and in other situations where the concentrations are low. Purification methods are described based on electrofocusing which provide a cytochrome *c*-552 preparation homogeneous enough to elicit a single reactive antibody in rabbits; this antibody is then used as a specific and sensitive assay for cytochrome *c*-552. Dark-grown cells of wild type and of mutants O_1BS, O_2BX, G_1BU and P_1BXL (which make normal sized chloroplasts with abnormal internal structure in the light) have 0.02 to 0.1 $\times 10^{-11}$ micromoles of cytochrome *c*-552 per cell, 10 to 150 times less than light-grown cells. Light-grown cells of these mutants and of wild type show a ratio of chlorophyll to cytochrome of about 300 (mole to mole). Cytochrome *c*-552 is undetectable in dark-grown Y_1BXD, Y_3BUD, and $W_{34}ZUD$ which cannot carry plastid development beyond the proplastid in light; the light-grown cells of these mutants have levels of cytochrome similar to or lower than dark-grown wild type cells. Cytochrome *c*-552 is undetectable in light- and dark-grown mutants in which plastid DNA is undetectable (such as Y_2BUL, W_3BUL, W_8BHL, and $W_{10}BSmL$) consistent with the view, but not proving, that this molecule may be coded, at least in part, in plastid DNA. During light-induced chloroplast development in resting cells, cytochrome *c*-552 formation behaves in all respects like chlorophyll except that the dark-grown cells contain low amounts of the cytochrome *c*-552 but lack chlorophyll. Thus, both cytochrome *c*-552 and chlorophyll show the same lag period even when the length is changed by nutritional manipulation; preillumination largely eliminates the lag in the formation of both molecules, cycloheximide and streptomycin both inhibit the biosynthesis of chlorophyll and cytochrome *c*-552 in the same manner, and the formation of both during chloroplast development is strictly light-dependent. It is shown that chloroplasts isolated from *Euglena* by methods thought to give intact organelles, lack 95% of the cytochrome *c*-552; this and the loss of similar molecules may explain why these isolated chloroplasts are not photosynthetically active.

Cytochrome *c*-552 (Cyt-552) is an electron carrier in photosynthetic electron transport of *Euglena* where it replaces plastocyanin between Cyt *f* and P_{700} (2, 37). Although the biosynthesis of this Cyt during chloroplast development has been studied (5, 23, 31), the lack of a suitable and sensitive assay has hampered its estimation in dark-grown wild type cells, during early light-induced chloroplast development and in mutants, where the levels are close to those of mitochondrial Cyt *c*-556 (23) confounding spectrophotometric estimation. Since the formation of Cyt-552 is sensitive to streptomycin (5), a specific inhibitor of translation on 68S plastid ribosomes (1, 30), there is reason to think that this molecule is synthesized in the plastids and, perhaps, that it is coded in plastid DNA. Inasmuch as Cyt-552 consists of a single polypeptide chain of about 10,000 daltons (7, 24), has been highly purified, is antigenic in rabbits (35), and its amino acid sequence has been determined (24), it is an attractive molecule for further study. In this report we describe the further purification of Cyt-552 to the point where it elicits a single specific antibody and the use of this antibody as a sensitive assay for Cyt-552 during early development of chloroplasts in wild type cells and in various mutants including those in which plastid DNA is undetectable. Brief reports of this work have appeared previously (12, 13, 15).

MATERIALS AND METHODS

Euglena gracilis Klebs var. *bacillaris* Cori, Strain Z Pringsheim and several mutants derived from them were grown at 26 C under constant shaking in light or darkness on Hutner's medium (pH 3.5) (14, 32). For studies of light-induced chloroplast development in nondividing cells, dark-grown cells were transferred to pH 6.8 resting medium (18, 32) and after 3 days the cultures were placed in the light to initiate chloroplast development. Cell counts (38), Chl determinations; (32), and antibiotic treatments (4) were done as described previously.

For purification of Cyt-552, to the point where it elicits a single reacting antibody, a method modified from that of Pettigrew (24) was employed. Between 600 and 1,000 g of phototrophically grown *E. gracilis* var. *bacillaris*, grown in plastic barrels on the medium described by Lyman and Siegelman (22) and centrifuged in a continuous flow CEPA Schnell centrifuge, was used. This isolation and all further steps were carried out at 4 C unless otherwise stated. The sedimented cells were resuspended in 1 volume of cold 0.1 M Na-phosphate buffer (pH 7.0) and were then passed through a Manton-Gaulin Press at 8,000 p.s.i. previously chilled by passage of ice cubes prepared from the same buffer. The suspension was kept cold during breakage by addition of more buffer ice cubes. The suspension was centrifuged at 15,000*g* for 10 min and the pellets were reextracted with 1 volume of the same buffer for 1 h. After centrifugation, the supernatants were pooled and the pH was adjusted to 5.0 with 2 M acetic acid. The precipitate was removed by centrifugation at 15,000*g* for 10 min. The clear red supernatant fluid was subjected to gel filtration in 700-ml aliquots

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on a column (9 × 40 cm) of Sephadex G-25 (medium grade) in 0.01 M Na-phosphate buffer (pH 7.0). The void volume eluates were pooled and passed through a Whatman DE23 column (4 × 6 cm). The red-brown band at the top of the column was scraped off, resuspended in 100 ml of 0.01 M Na-phosphate buffer (pH 7.0), and the suspension was poured into a plastic column (4 × 6 cm). The column was washed with 750 ml of 0.05 M Na-phosphate buffer (pH 7.0) and the Cyt-552 was eluted with 0.1 M Na-phosphate buffer (pH 7.0).

The pink solution was brought to 60% of saturation of ammonium sulfate and was left for 1 h. The precipitate was centrifuged for 10 min at 15,000*g* and the supernatant was brought to 100% saturation with ammonium sulfate. The precipitate was recovered by centrifugation at 15,000*g* for 10 min and the pellet was resuspended in a minimum volume of glass-distilled H_2O. The solution was then dialyzed in washed tubing (boiled in 5% [w/v] $NaHCO_3$ containing 0.1 mM EDTA, then rinsed in distilled H_2O) against 0.01 M NaCl to remove the ammonium sulfate. The pH of the solution was then brought to 4.0 with 0.1 M acetic acid and the preparation was dialyzed against 0.01 M acetic acid-Na-acetate (pH 4.0) until the conductivity of the sac contents was the same as that of the buffer.

The sac contents were then passed through a column of CM-cellulose (Whatman CM-52, 2 × 10 cm, equilibrated with 0.01 M acetic acid-Na-acetate [pH 4.0] buffer and elution was carried out with 0.02 M acetic acid-Na-acetate buffer (pH 5.0). The main peak fractions containing the red reduced and oxidized Cyt-552 were pooled and dialyzed overnight against 0.01 M Na-phosphate buffer (pH 7.0) followed by dialysis for 1 day against glass-distilled H_2O. The dialyzed solution was concentrated by lyophilization to a concentration of about 2 mg Cyt-552/ml and was stored at −20 C.

This preparation was subjected to analytic isoelectrofocusing in acrylamide gels (16) or to preparative isoelectrofocusing on sucrose columns containing ampholine in the pH range from 3.5 to 10 (20) in the early work. Thereafter preparative isoelectrofocusing was performed as recommended by LKB (36) in a granulated gel bed containing ampholine. The gel bed was prepared with 4 g of Ultrodex (LKB) in a solution of 2.5 ml of ampholine 3.5 to 5 (LKB), 2.5 ml of ampholine 4 to 6 (LKB), and 95 ml of distilled H_2O. This ampholine solution gives a pH range from 3.5 to 5.5. The Cyt-552 (10 mg in 5 ml of gel solution) was placed in a slot cut in the middle of the gel bed and electrofocusing took place for 14 h at 900 v. One M H_3PO_4 was used in the anodic chamber and 1 M NaOH in the cathodic chamber. The two red bands, close to the anode, were carefully cut from the gel; the particles were removed by pouring the gel slurry into a plastic column (0.5 × 5 cm) and eluting Cyt-552 with about 10 ml of distilled H_2O. The pooled Cyt solutions containing both bands were dialyzed against glass-distilled H_2O to remove the ampholyte.

Cyt-552 preparations were subjected to electrophoresis on polyacrylamide gels or on polyacrylamide gels containing SDS as described previously (3, 6, 13, 19) except that no urea at all was used in any solution including the sample buffer.

The highly purified electrofocused Cyt preparations were used for immunization of rabbits as a suspension containing 0.5 mg of Cyt-552 in 1 ml of distilled H_2O and 1.5 ml of complete Freund's adjuvant, subjected to vigorous homogenization. Before immunization, a control bleeding was performed to obtain preimmunization serum. Then each rabbit received the 0.5-mg preparation of Cyt-552 by injection into the toe pad. This was repeated twice more at weekly intervals. Blood was collected every week starting at the 4th week; when the titer decreased a booster was injected. Serum was purified by ammonium sulfate precipitation according to the technique described by Livingston (21). The blood was kept at room temperature until the clot was formed. The serum was then recovered by centrifugation for 10 min at 17,000*g*. This serum was brought to 45% (w/v) of saturation of ammonium sulfate and was left for 0.5 h at 2 C. The precipitate was centrifuged for 10 min at 17,000*g*. The pellet was resuspended in the same volume of 10 mM K-phosphate buffer (pH 6.8). The ammonium sulfate precipitation was repeated. The final pellet was resuspended in a small volume of 10 mM K-phosphate buffer (pH 6.8), dialyzed 1 day against two changes of 2 liters of the same buffer and overnight against 2 liters of 8% NaCl in 10 mM Na-phosphate buffer (pH 7.2). This purified immuneserum was kept at −20 C and used for the experiments to be described.

For analysis of Cyt-552, cells of *Euglena* (0.5×10^9 cells of light-grown wild type or 2 to 4×10^9 cells of dark-grown wild type or dark or light-grown mutants) were harvested from cultures at the beginning of the stationary phase of growth or at appropriate times from resting cultures undergoing light-induced chloroplast development. Cells were harvested by centrifugation at 1,000*g* for 5 min and the pellets were stored at −20 C. Acetone powders were prepared from these pellets as already described (4). Cyt-552 was extracted from the acetone powders using 4 to 6 ml of glass-distilled H_2O and centrifuging at 17,000*g* for 10 min. This was repeated twice more and the supernatant fluids were combined and lyophilized. The resulting powder was then dissolved in a minimum volume of glass-distilled H_2O. This suspension was then centrifuged at 5,000*g* for 10 min to eliminate any precipitate. In the case of cells containing large amounts of Cyt-552, such as green cells of light-grown wild type, the Cyt-552 can be obtained more rapidly by resuspending the cells in 2 volumes of 10 mM Tris-HCl (pH 8.0) containing 1 mM EDTA, sonicating in a Branson Sonifier for 5 s at 4 amp, and centrifuging for 10 min at 10,000*g*. The supernatant is used directly. For green cells, both techniques gave comparable results.

Ouchterlony immunodiffusion was performed by pouring 3 ml of 1.1% agarose (w/v) dissolved by heating in barbital buffer (0.8 g of barbital and 4.12 g of Na-barbital/l) on a glass plate (2.5 × 7.5 cm). After solidification six wells were punched in a circle around a center well. The distance from the edge of the center to that of the surrounding wells was 2.5 mm, the diameter of the wells was 3 mm. The center well received 20 μl of serum and the surrounding wells 20 μl of the solution to be analyzed. Diffusion took place for 1 day at 26 C.

Quantitative estimations of Cyt-552 were performed by radial immunodiffusion which was found to be more sensitive than rocket immunoelectrophoresis. For this, 11.4 ml of the same agarose solution kept at 50 C was mixed with 0.6 ml of the immuneserum (titer of 502 ± 119 μg antibody protein/ml) and poured on a glass plate of 7 × 9 cm. After solidification 15 wells (diameter 0.5 mm) were punched evenly in the gel bed; each well received 30 μl of extract. Diffusion took place for 24 h at 26 C to allow rings to form. The plates were then photographed and the entire area of the precipitate around the well on the print was cut out and weighed. A linear relationship between the area of the precipitate and the concentration of Cyt-552 was found (Fig. 1).

RESULTS

At the start of this work we began with a method of purification of Cyt-552 which had been improved from a procedure used by others to obtain material homogeneous enough for sequencing purposes (24). This material was reported to yield only two bands on polyacrylamide gel electrophoresis corresponding to the oxidized and reduced forms of Cyt-552. When we immunized rabbits with this preparation, the antibody obtained gave more than one precipitation band in Ouchterlony immunodiffusion against extracts of wild type light-grown *Euglena;* no reaction was obtained with preimmunization serum from the same rabbits. On subjecting these preparations to isolectrofocusing on analytical gels it was found that the Cyt-552 yielded two colored bands at pI values of 4.1 and 3.9 which contained mainly oxidized and reduced Cyt-552, respectively, but faint bands, colorless before staining, were also seen after staining for protein with Coomassie blue. To purify the Cyt-552 further, the preparation from the standard purification was subjected to preparative isoelectrofocusing and the unstained

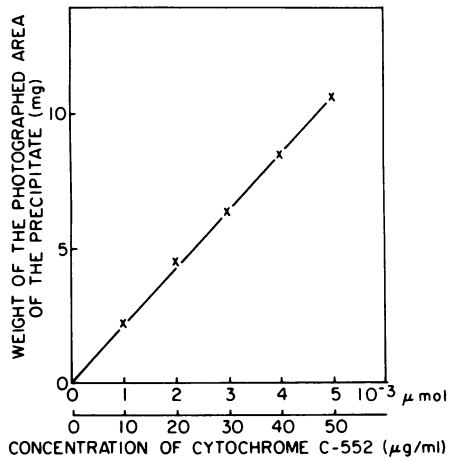

FIG. 1. Relationship between Cyt *c*-552 concentration and surface area of precipitate in radial immunodiffusion determined as weight of photographed area. Radial immunodiffusion was performed as described under "Materials and Methods." Cyt *c*-552 purified by isoelectrofocusing was used as a standard.

colored bands (Fig. 2A) were isolated and used for immunization. The antibody obtained from these rabbits gave only one identical reacting component on Ouchterlony immunodiffusion against all Cyt-552 preparations and against extracts from wild type light-grown or dark-grown cells (Fig. 2C). Ouchterlony immunodiffusion of water extracts from acetone powders of light-grown and dark-grown cells of wild type and mutants having Cyt-552 showed smooth meeting of the single bands indicating that the Cyt-552 antigen is immunologically identical in all cells studied. It is this antibody against highly purified electrofocused Cyt-552 preparations that was used in the experiments to be described in this paper.

The highly purified electrofocused Cyt-552 gave only one band when analyzed by electrophoresis on polyacrylamide gels (Fig. 2B, slot 2); on SDS-polyacrylamide gels it yielded a single band corresponding to a polypeptide of 10,000 daltons in agreement with previous estimates of the mol wt (Fig. 2B, slot 3) (7, 24). Occasionally, one or two minor bands were detected on polyacrylamide gels but they may represent aggregates of the Cyt-552 as suggested by others (Fig. 2B, slot 1) (35).

Using the specific antibody prepared against electrofocused Cyt-552, the amount of this protein in wild type *Euglena* and in various mutants thought to involve plastid DNA (28) was determined. Table I shows that dark-grown cells of wild type *bacillaris* and Z contain comparable low amounts of Cyt-552. Dark-grown cells of mutants which contain normal proplastids and which form full sized chloroplasts on light induction (Table I, group II) contain low amounts of Cyt-552 comparable to dark-grown wild type cells. Cyt-552 is not detectable in dark-grown cells of Y_1 and Y_3 (Table I, group III) mutants in which the plastids do not develop significantly beyond the proplastid stage on light induction (27, 28). Similarly, mutant W_{34} which does not accumulate Chl in the light (Table I, group IV) does not form detectable Cyt-552 in darkness. Cyt-552 is not detectable in mutants of *Euglena* in which plastid DNA, Pchl(ide), and other plastid constituents are not detectable (Table I, group V).

Light-grown cells of wild type contain about 100 times more Cyt-552 than dark-grown cells (Table I) representing the Cyt-552 formed during chloroplast development. The mutants of group II which form appreciable amounts of Chl, in some cases comparable to wild type, also form comparable amounts of Cyt-552 although there is some variation among the various mutants. Although mutants of group III develop little beyond the proplastid, small amounts of Cyt-552 comparable to dark-grown wild type cells are formed. These mutants form low amounts of Chl. Mutant W_{34}, which forms no detectable Chl, forms very small amounts of Cyt-552. Again, the mutants of group V fail to form detectable Cyt-552 in the light.

That the amounts of Cyt-552 in light-grown wild type and mutants of group II are comparable to the amount of Chl formed

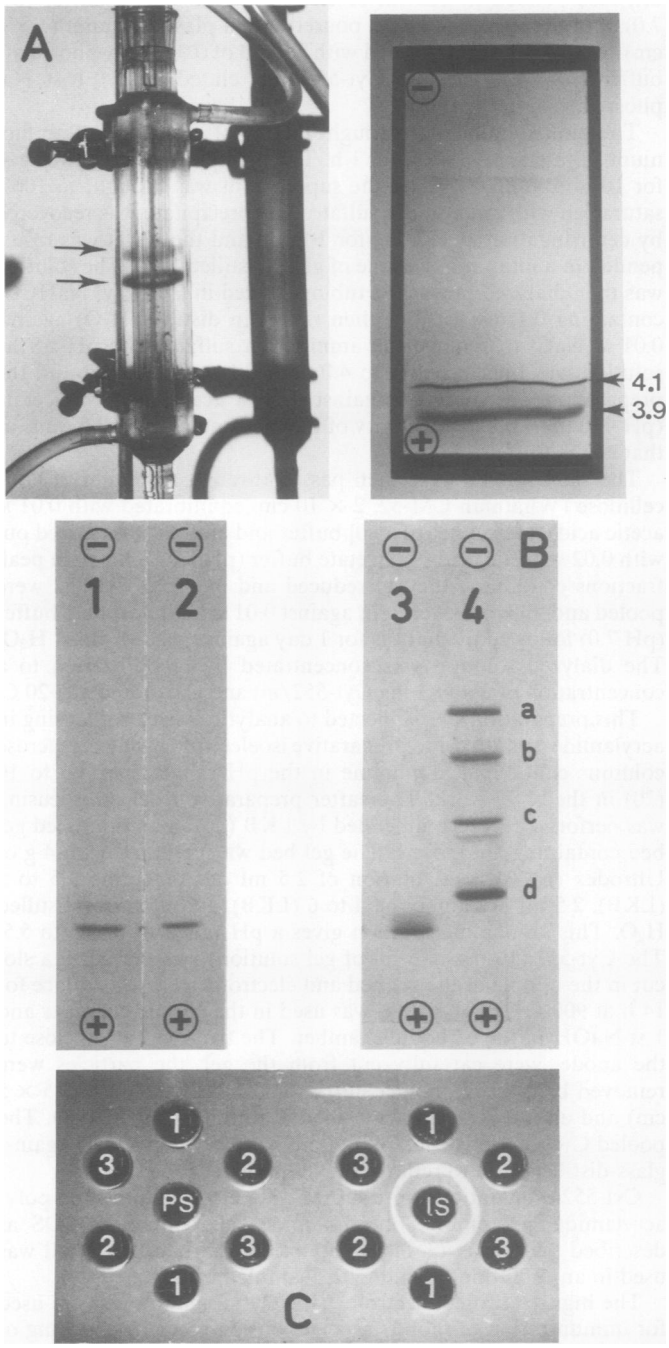

FIG. 2. Purification of Cyt *c*-552 and specificity of antibody. A: separation of purified Cyt *c*-552 by isoelectrofocusing. Cyt *c*-552 obtained after passage through the CM-cellulose column was subjected to isoelectrofocusing on a sucrose gradient or on a flat bed gel as described under "Materials and Methods." Bands are the unstained colored Cyt *c*-552. B: electrophoretograms of purified Cyt *c*-552. 1: Electrophoresis of Cyt *c*-552 on polyacrylamide gel before isoelectrofocusing; 2: electrophoresis of Cyt *c*-552 on polyacrylamide gel after isoelectrofocusing; 3: electrophoresis of Cyt *c*-552 on SDS-polyacrylamide gel after isoelectrofocusing; 4: electrophoresis of standard proteins on SDS-polyacrylamide gel. Mol wt standards are: BSA (a), 67,000; ovalbumin (b), 43,000; chymotrypsinogen (c), 25,000; Cyt *c* from horse (d), 12,400. C: Ouchterlony immunodiffusion of electrofocused Cyt *c*-552 (1) and acetone powder extract from light-grown cells (2) or dark-grown cells (3) against preimmunization serum (PS) and immune serum (IS).

TABLE I. Amount of cytochrome c-552 in Euglena gracilis

Cells were grown on glutamate-malate pH 3.5 medium at 26 C either in the dark (DG) or in the light (LG) (14). Cells were harvested at the beginning of the stationary phase (around 3 x 10^6 cells/ml) and cytochrome c-552 was extracted and its concentration measured as described in Materials and Methods.

Group	Strains	Characteristics[a]	cytochrome c-552 cellular content[b,c] DG	LG	ratio LG/DG	chlorophyll[b,c,d]	CHL/CYT[b] moles/moles
I	Wild type bacillaris	Plastid DNA present; proplastids and protochlorophyll(ide) present in the dark; chloroplasts and chlorophyll present in the light.	0.094 ± 0.023	6.840 ± 3.455	10-140[e]	2252 ± 882	385 ± 180
	Wild type Z		0.040	4.858	121	1607	328
II	O_2BX	Plastid DNA present; proplastids and protochlorophyll(ide) present in the dark; chloroplasts and lower amounts of chlorophyll present in the light.	0.048	1.674	35	980	393
	O_1BS		0.016	3.018	189	711	243
	P_1BXL		0.058	1.642	28	454	268
	G_1BU		0.039	1.593	41	276	226
III	Y_1BXD	Plastid DNA present; proplastids and protochlorophyll(ide) present in the dark; slightly developed proplastids and very low amounts of chlorophyll present in the light.	not detectable[f]	0.088	-	0.2	2
	Y_3BUL		not detectable[f]	0.020	-	0.6	30
IV	W_{34}ZUD	Chloroplast rRNAs present indicating the presence of at least part of the plastid DNA, slightly developed proplastids when DG cells are placed in the light, chlorophyll not detectable under normal illumination.	not detectable[f]	0.010	-	not detectable	-
V	Y_2BUL	Plastid DNA undetectable; abnormal proplastids in the dark and in the light; protochlorophyll(ide) and chlorophyll undetectable.	not detectable	not detectable	-	not detectable	-
	W_3BUL		not detectable	not detectable	-	not detectable	-
	W_8BHL		not detectable	not detectable	-	not detectable	-
	W_{10}BSmL		not detectable	not detectable	-	not detectable	-

[a] For more details concerning the properties of the mutants see refs. 9, 17, 26, 28, 32.

[b] For wild type bacillaris each value reported represents the mean of eight experiments ± the 95% confidence interval of the mean. This confidence interval was calculated as follows: The standard deviation of the measurements (s) was calculated in the usual way. The standard error of the mean ($s_{\bar{x}}$) was calculated from $s_{\bar{x}} = s/\sqrt{N}$ and was multiplied by t_{95} for the appropriate number of degrees of freedom to yield the 95% confidence interval.

[c] Values are expressed X 10^{-11} μmoles per cell using a molecular weight of 10,000 for cytochrome c-552 and 900 for chlorophyll.

[d] Cells of Group V also lack protochlorophyll(ide) in dark-grown cells.

[e] Represents the ratio of the smallest value for light-grown cells to the largest value for dark-grown cells (10) and the largest value for light-grown cells to the smallest value for dark-grown cells (140).

[f] Ouchterlony double immunodiffusions of water extracts from the acetone powders prepared from these strains gave a faint precipitate line. However, due to the high protein concentration needed, the precipitate obtained may not be specific.

is evident from an inspection of Table I where the ratio of Chl to Cyt-552 is about 300, in the range of commonly accepted sizes of the photosynthetic unit. Mutants of group III form little Chl compared with formation of Cyt-552 leading to the low ratios shown in Table I.

When dark-grown resting cells of wild type *Euglena* are placed in the light, Cyt-552 and Chl are formed with similar kinetics (Fig. 3), but the synthesis of Cyt-552 may start more slowly at the beginning of the lag phase. The length of the lag phase in Chl synthesis can be varied by changing the previous nutritional history of the cells (10). Table II shows that the lag phase for Cyt-552 is similar to the one for Chl even when the length of the lag for Chl is changed nutritionally. The ratio of Chl to Cyt-552 (Fig. 3) is low at the beginning of chloroplast development due to the

Table II. Correlation of length of lag period in chlorophyll and cytochrome c-552 synthesis

Dark-grown cells were transferred to pH 6.8 resting medium either in the middle of log phase (young cells) or at the beginning of stationary phase of growth (old cells), incubated for three days in darkness and then illuminated. The length of the lag phase was measured as described previously (10).

Stage of cells	Length of the lag phase (hours)	
	Chlorophyll	Cytochrome c-552
Young cells	4	4.5
Old cells	10	11
	10	9.5
	10	10

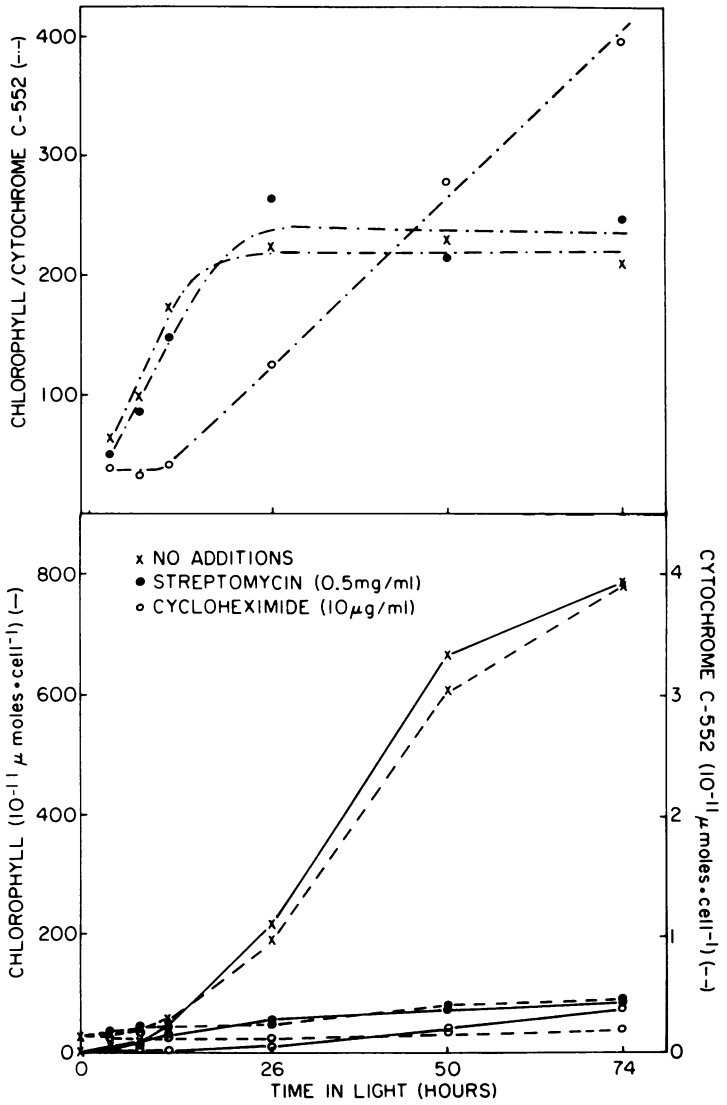

FIG. 3. Effect of cycloheximide and streptomycin on synthesis of Chl and Cyt *c*-552 during light-induced chloroplast development in resting dark-grown wild type cells. Antibiotics were present during entire period of chloroplast development. Cells grown in the dark were transferred to pH 6.8 resting medium, incubated 3 days in darkness, and then divided into three aliquots: streptomycin (0.5 mg/ml) was added to one, 16 h before being placed in the light (●); cytoheximide (10 μg/ml) was added to another one on being placed in the light (○); and a third was illuminated without additions (×) (18).

presence of Cyt-552 in dark-grown cells (Table I) and increases during chloroplast development to reach values found in light-grown cells.

Streptomycin, a specific inhibitor of translation on plastid ribosomes (1, 30) and cycloheximide, a specific inhibitor of translation on 87S cytoplasmic ribosomes (1, 11) added at the beginning of chloroplast development block Cyt-552 formation as well as Chl biosynthesis (Fig. 3). As found previously for Chl (5), inhibition of Cyt-552 formation by cycloheximide is immediate; streptomycin inhibition commences only at around 12 h of chloroplast development.

The ratio of Chl to Cyt-552 is similar for the control cells and

for the streptomycin-treated cells, indicating that in these cells Chl and Cyt-552 are inhibited to the same extent. The behavior of this ratio during illumination is very different for cycloheximide-treated cells. The increase observed indicates that Cyt-552 formation is inhibited more than Chl synthesis, but we are dealing here with ratios based on very small amounts of Chl and Cyt-552.

Results obtained when antibiotics are added later during chloroplast development are shown in Figure 4. In this case cycloheximide blocks the formation of Chl and Cyt-552 almost completely while streptomycin inhibits both Chl and Cyt-552 by only 40% after 8 h, the time for complete permeation. These observations are in agreement with others (4, 8, 11) indicating that antibiotics which affect chloroplast translation have less effect when they are added later during chloroplast development. Although no satisfactory explanation has been advanced for this phenomenon, the fact that Cyt-552 and Chl behave in the same manner once again suggests that the factors affecting their synthesis are very similar.

Preillumination for 2 h followed by a 12-h dark period (prior) to exposure of the cells to continuous light results in potentiation or elimination of the lag phase in Chl formation (18). Under these conditions there is no synthesis of Cyt-552 during the dark period but the synthesis starts rapidly on exposure of the cells to continuous light (Fig. 5). The lag phase is greatly reduced, but due to the limitations imposed by the immunological assay, it is difficult to say whether the lag phase is entirely abolished as it is for Chl. Cyt-552 synthesis continues to be light-dependent later in development since Cyt formation stops when nonpotentiated cells are returned to darkness after 21 h of illumination (Fig. 4).

Although various methods exist for the preparation of chloroplasts from *Euglena* (*e.g.* see 25 and 29) which appear reasonably intact in the electron microscope and incorporate radioactive amino acids into proteins, none of these preparations show significant photosynthetic O_2 evolution or CO_2 fixation. Table III shows that such chloroplast preparations contain very low levels of Cyt-552. The leakage of Cyt-552 out of the chloroplast occurs early during isolation, perhaps during the breaking of the cells. This loss of about 95% of the Cyt-552 is probably symptomatic of the leakage of still other plastid constituents during isolation and may be one of the reasons that these plastid preparations fail to carry out photosynthetic CO_2 fixation. It may also explain why the efficiency of amino acid incorporation in these plastids is lower than that from higher plants which show good rates of photosynthesis.

DISCUSSION AND CONCLUSIONS

The use of immunological techniques for the measurement of Cyt-552 has permitted a demonstration of its presence or absence

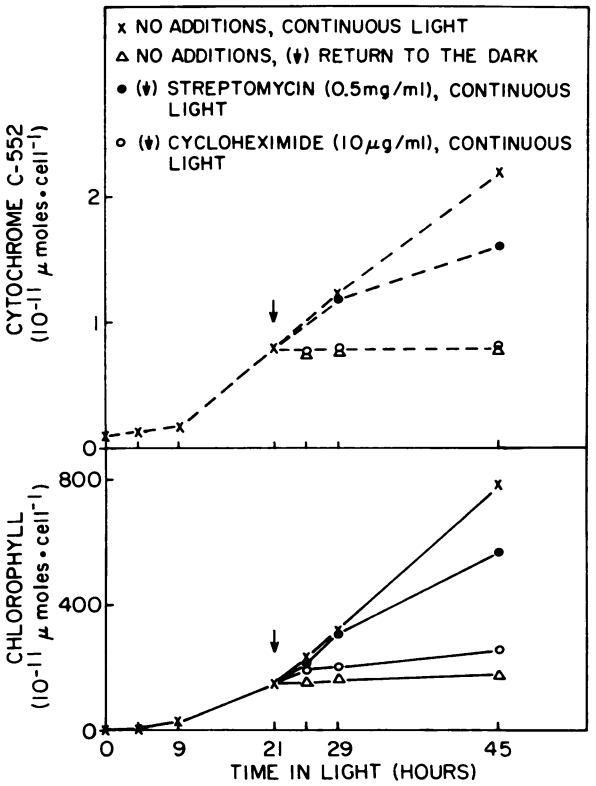

FIG. 4. Effect of cycloheximide and streptomycin on synthesis of Chl and Cyt c-552 during light-induced chloroplast development in resting dark-grown wild type cells. Antibiotics were added after 21 h of illumination. Cells grown in the dark were transferred to pH 6.8 resting medium, incubated 3 days in darkness, and then illuminated for 21 h. The culture was then divided into four aliquots: one was returned to darkness without additions (□), the other three remained in the light; one of these received streptomycin (0.5 mg/ml) (●); another received cycloheximide (10 μg/ml) (○); and the last one served as a control (×).

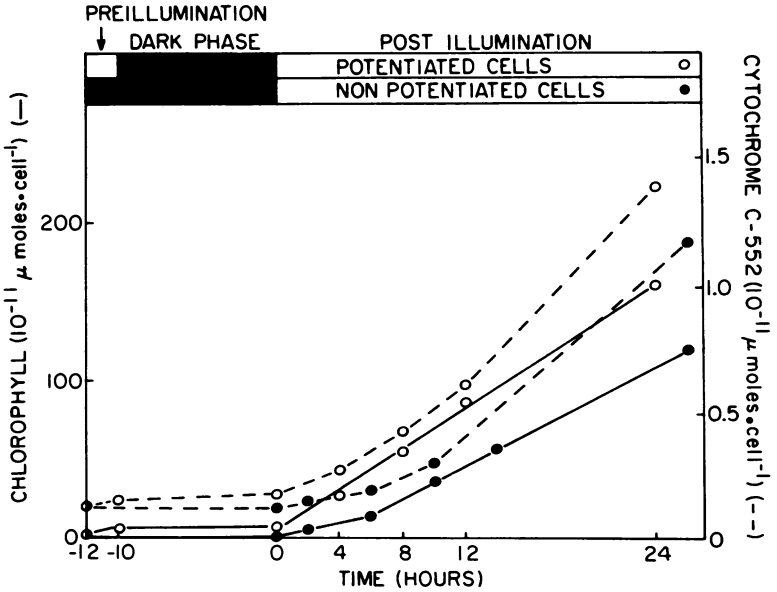

FIG. 5. Effect of preillumination on synthesis of Chl and Cyt c-552. Cells grown in the dark were transferred to pH 6.8 resting medium and were incubated for 3 days in darkness. Conditions for preillumination, dark phase, and postillumination were as described by Holowinsky and Schiff (18).

Table III. Cytochrome c-552 content of isolated chloroplasts

Cytochrome c-552 was extracted from intact cells or isolated chloroplasts as described in Materials and Methods and was estimated by radial immunodiffusion of the water extracts from acetone powders.

Material used		Ratio Chlorophyll/Cytochrome c-552 (Moles/Moles)	Percentage of cytochrome c-552[a]
Intact cells		532	100
Method I	Crude chloroplasts	9780	5.4
	Once-washed chloroplasts	12584	4.2
Method II		13883	3.8

Method I. Chloroplasts were prepared by differential centrifugation as described by Schwartzbach et al. (29).

Method II. Chloroplasts were prepared by purification on ludox gradients as described by Salisbury et al. (25).

[a] Calculated from the ratio cytochrome c-552/chlorophyll; the value obtained for intact cells corresponds to 100%.

and a measure of its concentration in situations where this could not be done before, and where data were lacking, inferential, or confined to periods of plastid development when the concentration of Cyt-552 was high (5, 23, 31, 34). The picture that emerges is that Cyt-552 formation is closely related to the formation of Chl. During normal development the length of the lag phase in Chl synthesis and Cyt-552 formation are closely related. The syntheses of both are strictly light-dependent and cease during the dark period of potentiation or after placing the cells in darkness after 1 day of illumination. Both show an accelerated formation after preillumination and a dark period and the ratio of Chl to Cyt-552 in the major period of plastid development is fairly constant, as it is during treatment with streptomycin, implying that photosynthetic units of constant composition are being formed. This is also suggested by the constancy of the ratio in various mutants which form Chl and Cyt-552.

Chl [as well as Pchl(ide)] and Cyt-552 are undetectable in mutants in which plastid DNA in undetectable. This would indicate that Cyt-552 and certain enzymes of Chl synthesis are coded in plastid DNA or that their formation is highly repressed in the absence of plastid DNA and other constituents of the normal wild type plastid. Both Chl synthesis and Cyt-552 formation are blocked by streptomycin and cycloheximide indicating a need for protein synthesis in both the cytoplasmic and plastid compartments for the formation of both constituents. The formation of Cyt-552 requires at least three steps: the synthesis of heme, the formation of the polypeptide, and the assembly of both into the mature Cyt-552. The protein synthesis required for these three steps may be divided between the plastid and the cytoplasmic ribosomes. Chl synthesis, like that of heme, requires many enzymes; some of these may be synthesized on plastid ribosomes and others on cytoplasmic ribosomes. This would explain the similar action of the inhibitors on the synthesis of Cyt-552 and Chl.

Another area of explanation is possible as well. It appears that the plastid thylakoid membrane constituents of *Euglena* regulate each other's synthesis, perhaps to insure the proper sequence of assembly of the membrane. For example, inhibition of carotenoid or Chl biosynthesis blocks the formation of membrane constituents and their assembly (33). It is possible that the reason that Chl and Cyt-552 formation appear to be similarly controlled is that the inhibition of the formation of any one constituent, say Chl or carotenoid, brings about an inhibition of the synthesis of the others, such as Cyt-552 (or, for that matter, any of the other 35 odd polypeptides that constitute the mature plastid thylakoid (3; and Bingham and Schiff, in preparation). Further work is necessary to sort out these explanations and to determine where all of these plastid thylakoid membrane constituents are coded and synthesized.

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