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POLYPEPTIDES OF CHLOROPLASTIC AND CYTOPLASTIC ORIGIN REQUIRED FOR DEVELOPMENT OF PHOTOSYSTEM II ACTIVITY, AND CHLOROPHYLL-PROTEIN COMPLEXES, IN *EUGLENA GRACILIS* Z CHLOROPLAST MEMBRANES*

MICHAEL GUREVITZ, HANAN KRATZ and ITZHAK OHAD

Department of Biological Chemistry, The Hebrew University of Jerusalem, Jerusalem (Israel)

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SUMMARY

The development of photosynthetic activity and synthesis of chloroplast membrane polypeptides was studied during greening of *Euglena gracilis* Z in alternate light-dark-light cycles. The results show: (a) The development of both Photosystem II and Photosystem I can be dissociated from chlorophyll synthesis. (b) Most of the polypeptides required for development of Photosystem I are already synthesized during the initial light period (10–12 h); the further rise in Photosystem I activity in the dark is not inhibited by cycloheximide nor by chloramphenicol. (c) The development of Photosystem II requires continuous de novo synthesis of polypeptides and is inhibited by chloramphenicol. The water-splitting activity already present at the end of the first light period decays in the presence of chloramphenicol while that of 1,5-diphenylcarbazide oxidation is only partially retained. The activity can be repaired in the absence of chlorophyll synthesis and is correlated with the de novo synthesis of polypeptides of 50 000–60 000 daltons. The synthesis of these polypeptides and associated repair of Photosystem II activity is not inhibited by cycloheximide. (d) The chloroplast membranes can be resolved into about 40 distinct polypeptides, among them several in the molecular weight range 50 000–60 000, 20 000–35 000 and 10 000–15 000, which are major membrane constitutents. (e) The synthesis of two major polypeptides (M_r = 20 000–30 000) required for the formation of chlorophyll-protein complex(es) containing chlorophyll *a* and traces of chlorophyll *b* (CPII?) is light-dependent and cycloheximide-inhibited. It is concluded that the synthesis and addition to the growing membrane of chlorophyll and polypeptides required for the formation of Photosystem II and Photosystem I complexes can be dissociated in time. The H_2O-

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Abbreviations: DCIP, 2,6-dichlorophenolindophenol; DPC, 1,5-diphenylcarbazide; DCMU, 3-(3,4-dichlorophenyl)-1,2-dimethylurea; CPI, chlorophyll-protein complex I; CPII, chlorophyll-protein complex II; SDS, sodium dodecyl sulfate.

* The results of this work have been presented as a preliminary communication at the Annual Conference of the Israeli Biochemical Society, 1975, and the VIIth International Congress on Photobiology, Rome, September 1976. While the manuscript was in preparation, similar results regarding the polypeptide pattern in *Euglena* chloroplasts were reported by Dr. J. Schiff at the conference on the Genetics of Mitochondria and Chloroplasts, Munich, 1976.

splitting enzyme(s) and possibly other components of Photosystem II complex are of chloroplastic origin and turn over in the dark while at least some of the chlorophyll binding polypeptides are of cytoplastic origin and their synthesis is light-controlled.

INTRODUCTION

The process of photosynthetic membrane formation has been extensively studied in a variety of experimental systems since it offers a tool for the elucidation of the mechanism of photosynthesis, as well as for the understanding of membrane biogenesis in general [1, 2]. Many studies have been devoted lately to the resolution of the membrane protein composition and role assignment to the different polypeptides in the formation of the membrane structure and function, as well as the subcellular origin of each specific polypeptide [3–6]. The last point appears to be of special interest in view of the fact that several of the major chloroplast membrane polypeptides involved in the formation of the light-harvesting chlorophyll-protein complex, were found to be synthesized in the cytoplasm and thus have to be specifically transferred across the double chloroplast envelope [2]. On the other hand, polypeptides of chloroplastic origin seem to be required for the development of the photosynthetic reaction centers [7]. The possibility that organelles translate nuclear messages has also been considered and has recently received experimental support [7, 8]. Thus, the interrelations between chloroplast and cytoplasm appear to be of extreme significance in the process of chloroplast membrane development.

The study of this relationship would be facilitated if use could be made of a biological system in which not only the development of the chloroplast membranes could be induced and modulated, but would also allow isolation of intact subcellular organelles. *Euglena gracilis* might be a useful organism for such a study due to the extensive information already accumulated on the process of organelle development [9–11], as well as the possibility to isolate rather intact chloroplasts [12, 13] even at relatively early stages of the greening process [14].

The aim of this work was to identify the major photosynthetic membrane polypeptides in *Euglena* chloroplast and establish their origin and possible function. The information obtained from such a study will serve as a basis for the future investigation of the process of transfer of cytoplasmic polypeptides into the chloroplast, synthesis of the chloroplast membrane polypeptides, and the assembly of these components into a functional membrane in in vitro isolated systems.

MATERIALS AND METHODS

Cell growth and greening procedure. E. gracilis Z was grown in a semi-continuous culture apparatus in the dark [15] for over 3 months. The mineral medium used, containing succinate as the sole carbon source, was prepared according to a modification by Lefort Tran of the basic medium described by Greenblatt and Schiff [16]. Cells were harvested in the logarithmic phase of growth ($1 \cdot 10^6$–$2 \cdot 10^6$ cells/ml) by centrifugation at $400 \times g$ for 1 min at room temperature. For greening, the cells were resuspended in a fresh growth medium at a final concentration of $0.5 \cdot 10^7$–$1 \cdot 10^7$ cells/ml. Under these conditions, cell division was less than 5 % throughout the

experiments, up to 30 h. The cells were exposed to white fluorescent light ($4 \cdot 10^3$–$5 \cdot 10^3$ ergs $\cdot$ cm$^{-2} \cdot$ s^{-1}) at 25 °C and shaken on a rotary shaker (160 cycles/min).

Preparation of chloroplast and chloroplast membrane fractions. Cells were washed twice by centrifugation as above at 4 °C with ice-cold phosphate buffer ($5 \cdot 10^{-2}$ M, pH 7.0) and resuspended in a cold solution containing 0.4 M sucrose and 10^{-2} M NaCl in the same buffer. Intact chloroplasts were obtained by a modification of the procedure described by Katoh and San Pietro [17]. The cells ($3 \cdot 10^8$ cells/ml) were passed through a French pressure cell operated at 2500 lb/inch^2. The disrupted cells were centrifuged first at $200 \times g$ for 5 min. The pellet, containing intact cells and large debris together with many intact chloroplasts, was resuspended and recentrifuged as above, so as to release most of the entrapped chloroplasts. The supernatants from both centrifugations were combined and recentrifuged at $400 \times g$ for 5 min in order to remove intact cells and debris. Chloroplasts were sedimented from this last supernatant by centrifugation at $2500 \times g$ for 10 min. The chloroplast yield was about 20 % as estimated from measurements of chlorophyll content.

Chloroplast membranes were obtained following hypotonic shock of the intact chloroplasts ($2 \cdot 10^{-2}$ M phosphate buffer, pH 7.0) by centrifugation on a discontinuous sucrose gradient containing 5 ml each of: 60, 50, 40, 30 and 15 % (w/v) sucrose in phosphate buffer ($5 \cdot 10^{-2}$ M, pH 7.0). The gradients were centrifuged in a Spinco SW 25 rotor at $24\,000 \times g$ for 2 h. Most of the chloroplast membranes were found at the interface between 30 and 40 %, and 40 and 50 %, respectively. As the chlorophyll content of the cells increased during greening, a shift was obtained in the relative distribution of the membranes towards higher densities. In some experiments a chloroplast membrane-enriched fraction was obtained by disrupting the cells at 8000 lb/inch^2. The same differential centrifugation procedure was followed as above, except that the final centrifugation was at $7500 \times g$ for 10 min.

Measurement of photosynthetic electron transfer activities. Photoreduction of DCIP was measured spectrophotometrically using an Aminco-Chance double beam spectrophotometer. The assay system contained citrate/phosphate buffer, pH 5.0 $5 \cdot 10^{-2}$ M [17]; 10^{-4} M DCIP, and 2.5–3.5 μg chlorophyll/ml. The final volume was 4 ml. The exciting light was filtered through a Schott 6-65 and KG-4 filters with a final intensity of $3.0 \cdot 10^5$ ergs $\cdot$ cm$^{-2} \cdot$ s^{-1}. The photomultiplier was protected by a Corning 4-96 filter. The light minus dark response was recorded at 540 nm. When DPC ($7.4 \cdot 10^{-4}$ M) was used as an electron donor, it was added directly to the reaction mixture and the H_2O-splitting activity of the membranes was not destroyed by treatment with Tris buffer [18]. Photooxidation of methyl viologen in the presence of DCMU (10^{-5} M) was measured polarographically with ascorbate and DCIP as electron donors. The reaction mixture (1 ml) contained: methyl viologen, $2.5 \cdot 10^{-3}$ M; ascorbate, $4 \cdot 10^{-3}$ M; DCIP, $5 \cdot 10^{-5}$ M; and phosphate buffer, $5 \cdot 10^{-2}$ M; pH 6.0.

Radioactive labeling of cells. For radioactive labeling, cells were incubated as described with addition of $Na_2{}^{35}SO_4$ (10 Ci/M, $1.5 \cdot 10^{-3}$ M). Chloroplast membranes were isolated and used for determination of electrophoretic pattern of polypeptides and their radioactive labeling.

Gel electrophoresis. The polypeptide composition was resolved by electrophoresis of isolated chloroplast membranes dissolved in 2 % SDS on 10 % polyacrylamide gels using basically the method of Laemmli [19]. The gels were stained in

Coomassie Brilliant Blue as described by Fairbanks et al. [20]. Usually between 150 and 300 μg of protein were loaded on each gel. The membrane solution was not heated prior to electrophoresis. When radioactively labeled membranes were used, the lipids and chlorophyll were first extracted with 80 % acetone and then twice with ether before solubilization. Determination of the radioactivity distribution in different polypeptides was carried out by freezing the gels and slicing them in 1-mm slices which were further dissolved in H_2O_2 and SDS and counted in a scintillation counter.

The presence of chlorophyll-protein complexes was detected by carrying out the electrophoresis before delipidation of the membranes and using only 1 % SDS for membrane solubilization. Freshly prepared membranes were used in this case.

Absorption spectra of the pigmented regions of the gels were determined directly on gel slices using an Aminco-Chance double beam spectrophotometer.

Determination of the apparent molecular weight of polypeptides following SDS-polyacrylamide gel electrophoresis was carried out according to Weber and Osborn [21].

Analytical procedures. Protein was determined according to Lowry et al. [22] following solubilization of the material in 0.1 M NaOH and 1 % SDS using bovine serum albumin as a standard. Chlorophyll was measured in 80 % acetone extracts according to Arnon [23]. Cell concentration was determined by counting in a hemocytometer. All reagents used throughout this work were of analytical grade.

RESULTS

Development of photosynthetic activity during alternate light-dark cycles

The kinetics of the greening process of *Euglena* in continuous light is well documented [9, 10]. The process consists of a lag period of about 3–8 h followed by an increase in the rate of chlorophyll synthesis until the chlorophyll content is similar to that of light-grown cells (48–72 h) (cf. also Fig. 1).

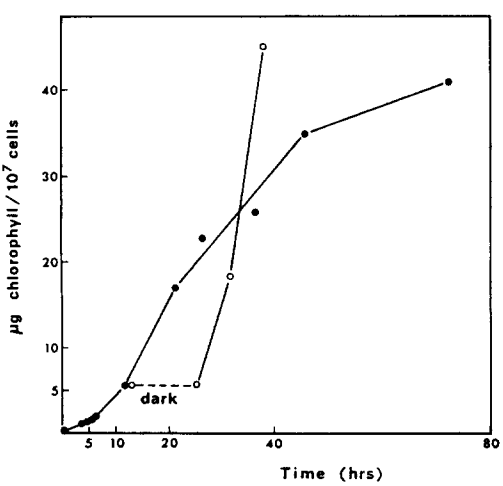


Fig. 1. Time course of chlorophyll synthesis. Dark-grown cells were suspended in fresh growth medium and incubated in continuous light (●—●) or transferred to the dark after 12 h and incubated in the dark for 11 h (○- - -○) and then reexposed to the light (○—○).

Holowinsky and Schiff [10] have shown that brief illumination followed by dark incubation of 12 h eliminates the lag in chlorophyll synthesis during a second light period. The pre-illumination triggers a series of complex synthetic processes which result in the potentiation of the system towards a fast response to the second light exposure. In our work we have taken advantage of this procedure, but extended the first illumination from 8 to 12 h (L_1), so as to obtain a chloroplast-containing membrane with sufficient chlorophyll to enable detection of photosynthetic activity already at the end of the first light period (Fig. 1). It was assumed that during the dark incubation following L_1, synthesis and assembly of some of the polypeptides required for membrane development will continue, as indicated from a structural study reported by Salvador [24]. If this were the case, one would expect to detect the addition of such components from measurable changes in the photosynthetic activity or detection of incorporation of radioactive precursors in specific membrane components. Furthermore, during a second illumination period (L_2) chlorophyll and eventually protein synthesis and incorporation into the membrane will proceed and result in additional changes in the organization and activity of the membrane. This procedure could resolve in time the addition of different polypeptides to the membranes and serve as a basis for the identification of their possible function. In addition, the subcellular site of their synthesis could be established by use of appropriate protein synthesis inhibitors.

As shown in Table I, photosynthetic electron transfer by both Photosystems II and I is present at rather high specific activities already at the end of L_1 when the chlorophyll content of the cells is below 2.5 μg chlorophyll/10^7 cells. During the dark incubation, both activities increase and Photosystem II activity almost doubles in absence of chlorophyll synthesis. When the cells are re-exposed to light (L_2), chlorophyll synthesis proceeds as expected. The specific activity on a chlorophyll basis of Photosystem II is reduced 4-fold during the L_2 period, while that of Photosystem I is reduced to about one-half. The total Photosystem II activity per cell increases by about 80 %, while the total activity of Photosystem I per cell increases over 3-fold, indicating that components involved in this activity are preferentially integrated into the membranes during the L_2 period (Table I).

In experiments in which optimal conditions were sought for the increase in

TABLE I

DEVELOPMENT OF PHOTOSYSTEMS I AND II ELECTRON TRANSFER ACTIVITY DURING ALTERNATE LIGHT-DARK-LIGHT INCUBATION

Dark-grown cells were washed, resuspended in fresh growth medium at a final concentration of 10^7 cells/ml, and incubated as shown. Specific activities are given as μmol substrate reduced/mg chlorophyll per h, and total activity as μmol substrate reduced/10^7 cells per h. Photosystem II activity was measured with H_2O as an electron donor. For additional details see Materials and Methods.

Treatment	μg chlorophyll per 10^7 cells	Photosystem I		Photosystem II	
		Specific activity	Activity per 10^7 cells	Specific activity	Activity per 10^7 cells
10 h light (L_1)	2.2	227	499	56	124
12 h dark (D)	2.2	330	726	121	266
12 h light (L_2)	14.1	155	2185	35	493

photosynthetic activity during the dark incubation and for chlorophyll synthesis during the second light exposure, it was found that best results were obtained when the chlorophyll content at the end of L_1 did not exceed 2.5 μg chlorophyll/10^7 cells (8–12 h light), while the minimal length of the dark period should not be shorter than 12 h.

Effect of protein synthesis inhibitors on development of electron transfer activity in the dark

It is currently accepted that chloramphenicol and cycloheximide specifically inhibit the synthesis of proteins by organelle and cytoplasmic ribosomes, respectively [25]. The origin of the proteins required for the increase in photosynthetic activity in the dark period (following pre-illumination) was identified by use of these drugs which were added to the incubation medium at the time of transferring to the dark. The cells were then incubated for 12 h, membrane fractions prepared as described, and activity of both Photosystems II and I measured. The results (Table II) show that in cells incubated in the presence of chloramphenicol, the water-splitting activity of Photosystem II, developed already in the L_1 period, was lost completely. When DPC was used as a donor, a residual activity of 20–40 % was still present. Both chloramphenicol and cycloheximide did not prevent the increase in the activity of Photosystem I in the dark (Table II). These data could indicate that components synthesized in the organelle and involved in the activity of H_2O-splitting system turn over in the dark quite rapidly.

The loss of Photosystem II activity in the presence of chloramphenicol was less prominent when the initial chlorophyll content of the cells was higher than 2.5–3 μg chlorophyll/10^7 cells. In such cells the increase in photosynthetic activity in the dark was less pronounced, although it was still inhibited by chloramphenicol. Both chloramphenicol and cycloheximide had no effect on the chlorophyll content of the cells which remained constant throughout the dark incubation period.

Since it was possible to obtain cells in which Photosystem II activity was differentially lost, one could use them in order to find out the origin of the polypeptides

TABLE II

EFFECT OF PROTEIN SYNTHESIS INHIBITORS ON THE DEVELOPMENT OF PHOTOSYNTHETIC ELECTRON TRANSFER IN THE DARK

Dark-grown cells were washed and resuspended in fresh growth medium at a final concentration of 10^7 cells/ml. The suspension was exposed to the light for 9 h (chlorophyll content 2 μg/10^7 cells), then further incubated in the dark for 12 h with or without addition of chloramphenicol or cycloheximide. No change in the chlorophyll content occurred during this period. Activities are given as μmol substrate reduced/mg chlorophyll per h.

Measured parameter		Activity at end of first light period	Activity at end of incubation in the dark with addition of:		
			None	Chloram-phenicol	Cyclo-heximide
Photosystem I	Ascorbate+DCIP → methyl viologen	192	298	273	287
Photosystem II	H_2O → DCIP	42	80	0	40
	Diphenylcarbazide → DCIP	75	132	61	63

TABLE III

REPAIR OF PHOTOSYSTEM II ELECTRON TRANSFER ACTIVITY LOST DURING INCUBATION OF THE CELLS IN THE DARK IN PRESENCE OF CHLORAMPHENICOL

Same experimental procedure as in Table II. The cells incubated in the dark in the presence of chloramphenicol were washed free of the drug, resuspended in fresh growth medium at a concentration of 10^7 cells/ml, and further incubated in the light for 9 h with or without addition of chloramphenicol or cycloheximide. The activities are given as μmol DCIP reduced/mg chlorophyll per h; the data are an average of five experiments.

Additions	Incubation conditions:				
	Dark			Light	
	Activity with electron donor		Δμg chlorophyll per 10^7 cells	Activity with electron donor	
	H_2O	DPC		H_2O	DPC
None	40±15	55±15	13±4	60±20	90±25
Cycloheximide	70±20	70±20	2±1	85±20	150±30
Chloramphenicol	0	18±10	8±3	0	25±10

required for reestablishing this activity. When cells pre-illuminated for 8 h and then incubated in the dark in the presence of chloramphenicol for 12 h were washed and further incubated, either in dark or light, it was found that the Photosystem II activity, measured with H_2O as electron donor, is reestablished; whereas that measured with DPC as an electron donor increases significantly. The repair of activity was completely blocked by chloramphenicol but not by cycloheximide (Table III). The total activity calculated per cell increased more in the light when chlorophyll was synthesized. However, the specific activity on a chlorophyll basis was higher in systems in which chlorophyll synthesis was prevented either by dark incubation or incubation in the presence of cycloheximide in the light.

Electrophoretic pattern and radioactive labeling of membrane polypeptides during development or repair of photosynthetic activity

The electrophoretic pattern of isolated chloroplast membrane polypeptides obtained from light-grown cells is shown in Fig. 2. One can distinguish several major polypeptides in the molecular weight range of 50 000–60 000, 20 000–35 000 and 10 000–15 000, as well as many minor bands. The electrophoretic pattern of membranes isolated from cells which were pre-illuminated for 10 h and then incubated in the dark for 12 h is also shown in Fig. 2. One can see that some of the major bands in the molecular weight range of 20 000–35 000 and 10 000–15 000 are missing or are reduced and appear only as faint diffuse bands (arrows).

When cells were labeled in the dark period with ^{35}S, and the pattern of radioactivity distribution in membrane polypeptides was analyzed (Fig. 3), it was found that the radioactivity matches quite closely the absorbance patterns, thus indicating that most of the polypeptides present continue to be synthesized in the dark. However, when the labeling was carried out in the presence of chloramphenicol, the radioactivity of several bands in the molecular weight range of 50 000–60 000 was drastically

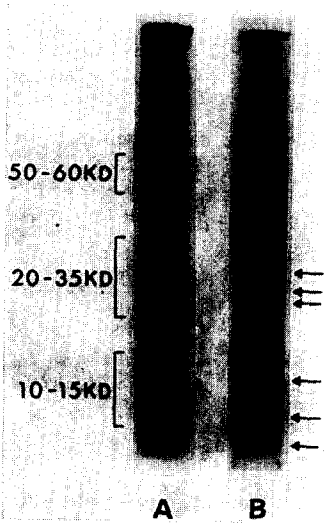


Fig. 2. Electrophoretic pattern of isolated chloroplast membranes. Chloroplasts were obtained from (A) dark-grown cells exposed to continuous light for 36 h (chlorophyll content 35 $\mu g/10^7$ cells); (B) cells exposed to the light for 10 h (chlorophyll content 2 $\mu g/10^7$ cells) and then further incubated in the dark for 11 h. Arrows indicate polypeptides, the synthesis of which is inhibited by cycloheximide in light (cf. Figs. 3 and 4).

reduced, concomitantly with the reduction in the corresponding absorbances of the stained bands (arrow) and loss of the Photosystem II activity (cf. Table II).

When cells, incubated in the dark in the presence of chloramphenicol, were washed free of the drug and further incubated in the light without or with addition of chloramphenicol or cycloheximide (repair conditions), it was found that incorporation of ^{35}S in the presence of chloramphenicol was very low in the polypeptides of molecular weight range 50 000–60 000 (arrow) but significant in the control cells or those incubated in the presence of cycloheximide (Fig. 4). On the other hand, a high incorporation was detected in the bands of molecular weight range 20 000–35 000, in both control cells incubated in the presence of chloramphenicol (asterisk) but not in the cells incubated in the presence of cycloheximide (Fig. 4). The high radioactivity incorporation in these bands was correlated with a significant increase in the absorbance of the corresponding stained bands.

When the membranes were dissolved in SDS without heating or extraction of lipids, one could easily detect on the non-stained gel a chlorophyll-containing band in the molecular weight range of 130 000 and two additional faint and diffuse chlorophyll-containing regions in the molecular weight range of 20 000–35 000. Most of the chlorophyll loaded on the gel, however, runs as a heavy diffuse band with the front. The chlorophyll-containing bands were sliced off the gel and their absorption spectra in the red region recorded. The high molecular weight band (Fig. 5A) showed the presence of chlorophyll *a* with a maximal absorption at 675 nm. The lower bands (Figs. 5B and 5C) showed a maximal absorption at 675 nm, in addition, a shoulder at 662 nm (possibly chlorophyll *b*) was present. The chlorophyll found at the front (free pigment) was mostly chlorophyll *a* ($\lambda_{max} \simeq$ 668–670 nm).

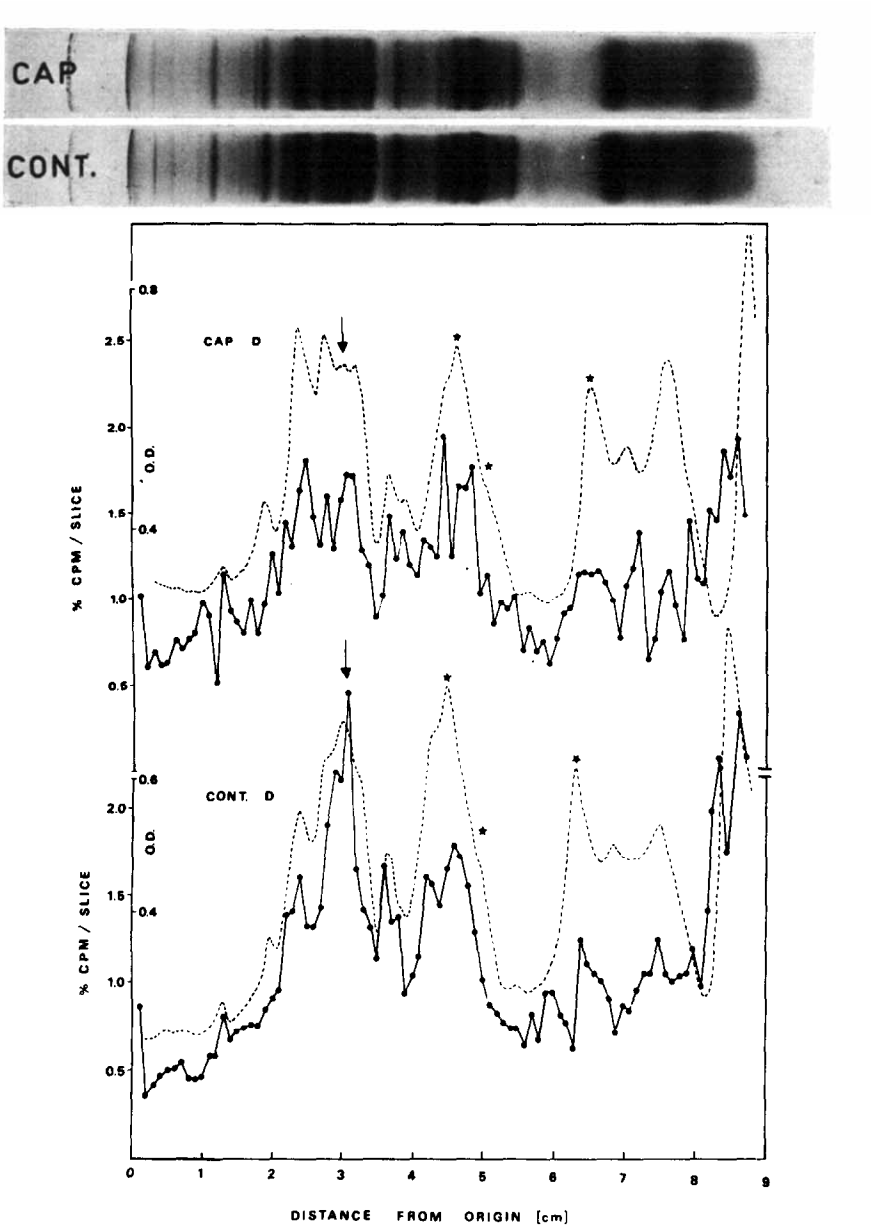
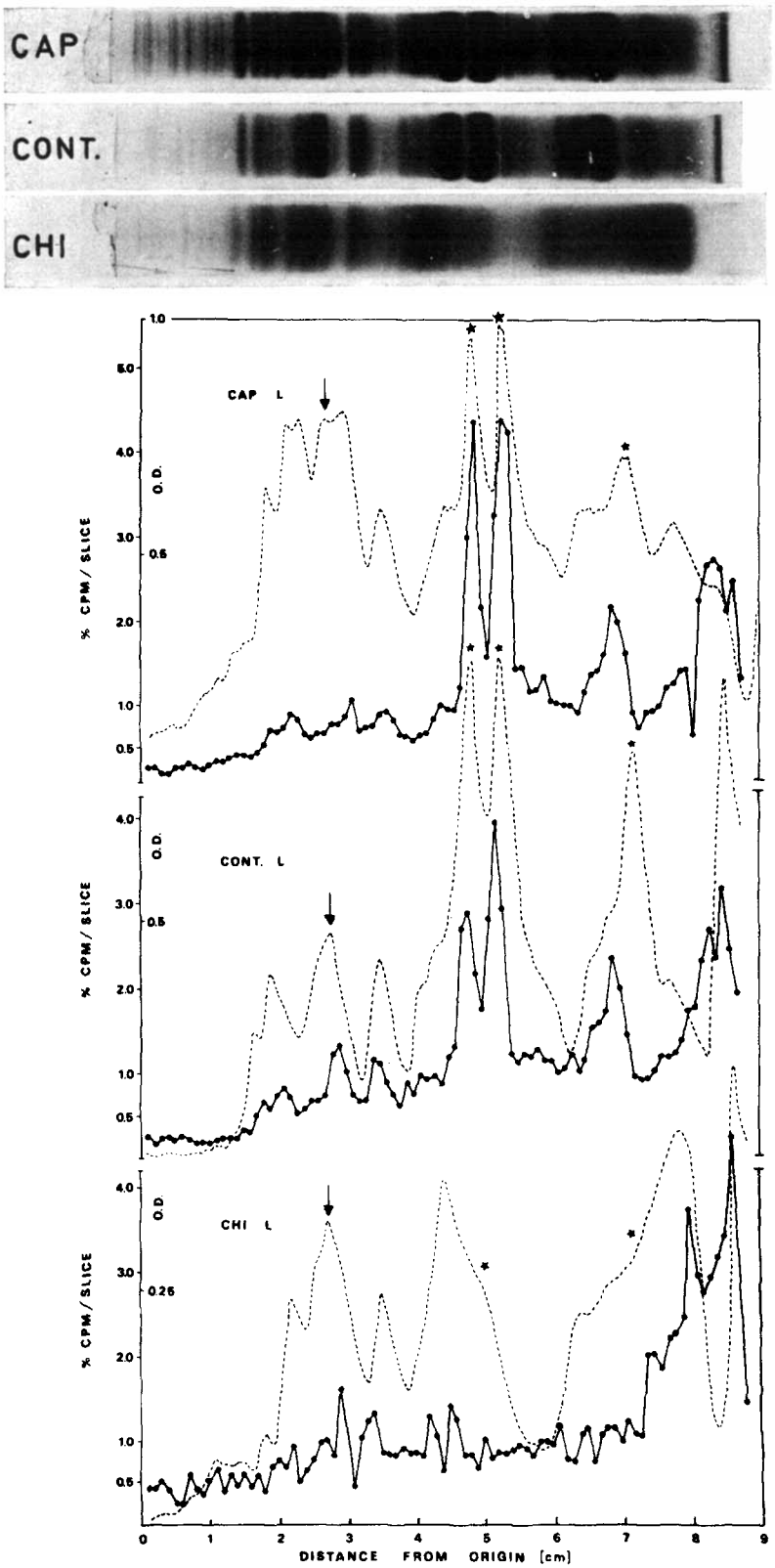


Fig. 3. Electrophoretic pattern and radioactive labeling of chloroplast membrane polypeptides during development of photosynthetic activity in the dark. Dark-grown cells were pre-illuminated for 10 h (chlorophyll content 2 $\mu g/10^7$ cells) and then transferred to the dark and further incubated for 11 h with (CAP D) or without (CONT D) addition of chloramphenicol. Labeling with ^{35}S was started at onset of dark incubation. —, radioactivity; ····, absorbance. Arrows indicate polypeptides which are not labeled in the presence of chloramphenicol. Stars indicate polypeptides formed during the pre-illumination, but poorly labeled in the dark. For comparison, pictures of the stained gels are also shown. The photosynthetic activity of Photosystem II (μmol DCIP reduced/mg chlorophyll per h) at the end of the pre-illumination period was 60 and 120 with H_2O and DPC as electron donors, respectively; 137 and 194 at the end of the dark incubation in the control, and 40 and 60 for the chloramphenicol-treated cells.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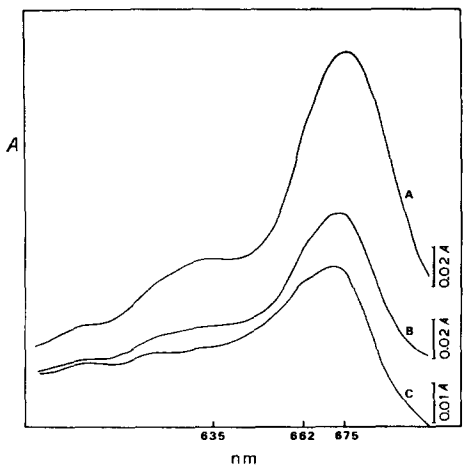

Fig. 5. Absorption spectra in the red region of chlorophyll-containing bands, separated by electrophoresis of isolated chloroplast membranes on SDS-polyacrylamide gels. Chloroplasts were obtained from dark-grown cells incubated in the light for 36 h (chlorophyll content 30 $\mu g/10^7$ cells). The membranes were dissolved in 1 % SDS solution (25 °C), and electrophoresis carried out without addition of β-mercaptoethanol. The sharp chlorophyll containing band in the region of 130 000 daltons (chlorophyll protein complex I, A), and the diffuse bands (B), (30 000–35 000 daltons) and (C), (20 000–25 000) daltons were cut from the gels and spectra obtained by scanning the gel slices as described in Materials and Methods.

DISCUSSION

The light-dependent development of the chloroplast in *Euglena* consists of several superimposed phenomena: transformation of the proplastid into a differentiated organelle containing a functional protein-synthesizing machinery; synthesis of soluble enzymatic complexes required for the dark cycle of carbon fixation; synthesis of components and assembly of the photosynthetic membrane.

In the course of the greening of these cells in continuous light it is experimentally difficult, if not impossible, to separate these processes. Holowinsky and

Fig. 4. Electrophoretic pattern and radioactive labeling of chloroplast membrane polypeptides during repair of photosynthetic activity of chloramphenicol-treated cells. Dark-grown cells were pre-illuminated for 10 h (chlorophyll content 2 $\mu g/10^7$ cells) and then transferred to the dark with addition of chloramphenicol and further incubated for 11 h. The cells were then washed free of the drug, resuspended in fresh growth medium, and exposed to the light for 9 h without (CONT L) or with addition of chloramphenicol (CAP L) or cycloheximide (CHI L); ^{35}S was added at onset of the second illumination period. The final chlorophyll content was 17.8, 13.9, and 3.3 μg cells in the control, chloramphenicol- and cycloheximide-treated cells, respectively. Initial photosynthetic activity (μmol DCIP reduced/mg chlorophyll per h) at onset of illumination was 40 and 60 with H_2O and DPC as electron donors (cf. Fig. 3); at the end of the incubation in the light the respective activities were: 141 and 174 for the control cells; 0 and 61 for the chloramphenicol-treated cells; and 180 and 323 for the cycloheximide-treated cells. Arrows indicate polypeptides which are not labeled in the presence of chloramphenciol. Stars indicate polypeptides which are not labeled in the presence of cycloheximide but are synthesized in the light-incubated cells in the presence of chloramphenicol. —, radioactivity; ···, absorbance.

Schiff [10] have demonstrated that a short illumination triggers the process of initial plastid differentiation which can continue in the dark, thus setting the stage for a fast subsequent development of photosynthetic membranes in the light.

In the present work we have modified this procedure so as to be able to analyze the process of membrane development separate from the initial differentiation. Using this system it was expected that it will be possible to establish whether the synthesis of all or some of the photosynthetic membrane polypeptides is light controlled, what is the subcellular origin of membrane polypeptides, and whether one can ascribe to them specific functions and eventually obtain information on the assembly process.

The results of this work show that one can resolve the membrane polypeptides into about 40 distinct bands. Among these, several in the molecular weight range of 50 000–60 000, 20 000–35 000 and 10 000–15 000 are major membrane components. Based on the changes in the intensity of the stained bands, as well as incorporation of ^{35}S, one can conclude that major polypeptides in the molecular weight range of 20 000–30 000 and possibly one in the range of 15 000 are synthesized only in the light, concomitant with chlorophyll synthesis. Their synthesis is inhibited by cycloheximide but not by chloramphenicol, indicating that they are products of cytoplasmic translation. In this respect these polypeptides are similar to those found in *Chlamydomonas* [3, 26]. In *Chlamydomonas* some of these polypeptides are associated with the formation of the chlorophyll-protein complex CPII which contains equal amounts of chlorophyll *a* and *b* [27, 28]. In *Euglena* it is very difficult to demonstrate the presence of this complex by the usual procedures. This can be explained if one considers that the presence of chlorophyll *b* is essential for the detection of the CPII complex [28–30] and that, under our experimental conditions, *Euglena* chloroplasts contain only traces of chlorophyll *b*. Indeed, small amounts of chlorophyll appear to be present in non-stained gels in the region corresponding to the membrane polypeptides of cytoplasmic origin, and the presence of small amounts of chlorophyll absorbing around 662 nm in addition to chlorophyll absorbing at 675 nm could be detected. Thus, one can tentatively identify the major polypeptides in the molecular weight range of 23 000–30 000 as being similar to the polypeptide binding chlorophyll *a* and *b* and forming the CPII complex in other organisms [27, 28, 31, 32]. The molecular weight of the chlorophyll-containing bands are only apparent. The true values cannot be estimated since the complexes usually exhibit higher retardation coefficients and free mobilities than expected for an SDS-protein complex [27].

The development of photosynthetic activity of the reaction centers of Photosystems I and II once initiated was shown to continue in the dark in absence of chlorophyll and cytoplasmic protein synthesis, as reported earlier also for *Chlamydomonas* [9, 26]. However, inhibition of 70 S translation by chloramphenicol not only blocked the further development of the H_2O-splitting activity, but also allowed the already present activity to decay, indicating that eventually under our experimental conditions some of the membrane polypeptides required for the H_2O-splitting activity of Photosystem II might turnover in the dark. This is further supported by the fact that incorporation of ^{35}S into the polypeptides of molecular weight range 50 000–60 000 is inhibited by chloramphenicol as compared with the control untreated cells. The lost activity can be repaired either in dark or light in the presence of cycloheximide. The repair of activity is accompanied by labeling polypeptides in the molecular weight range of 50 000–60 000, as well as in the range of 20 000–35 000.

However, the synthesis of the latter is not essential for the regaining of activity and can be inhibited by cycloheximide, which does not prevent the synthesis of the polypeptides in the molecular weight range of 50 000–60 000.

Thus, one can identify the polypeptides in the molecular weight range 50 000–60 000 as being of chloroplast translation. Their synthesis is correlated to the repair of Photosystem II activity. The presence of polypeptides of chloroplastic origin required for Photosystem II activity in the same range of molecular weight was demonstrated also in *Chlamydomonas* [8, 33, 34].

The decay of Photosystem II activity in the cells incubated in the dark in presence of chloramphenicol is more pronounced for the H_2O-splitting activity. This activity is not very stable in *Euglena* chloroplast and it is easily lost by treating the membranes at pH 8.0, not only with Tris as reported for other chloroplasts [18], but also with other buffers which have not been shown to remove the Mn^{2+} required for this activity. Results based on calculations of parameters derived from measurements of fluorescence induction [35] indicate that the reaction centers of Photosystem II are still present, but their connection to the rest of the chain seems to be impaired (Cahen, D., Gurevitz, M. and Ohad, I., unpublished). This is in agreement with the fact that DPC oxidation is still detectable in the chloroplast following incubation in presence of chloramphenicol.

Development of Photosystem I in *Euglena* is not inhibited in our experimental condition either by chloramphenicol or by cycloheximide. The rise in Photosystem I activity during dark incubation can be explained either, if one assumes that already existing components are utilized and become integrated into the membranes, or by a change in the membrane organization. In this respect *Euglena* chloroplasts differ from *Chlamydomonas*, in which it was shown that development of Photosystem I activity requires continuous chloroplast translation [2]. On the basis of the present data one cannot identify the subcellular origin of the membrane polypeptides required for Photosystem I activity.

During the second light incubation, when the chlorophyll content of the cells increases 7-fold, the photosynthetic activity calculated on a chlorophyll basis decreases for both Photosystem I and Photosystem II. However, when the calculation is done on a cell basis, one can demonstrate that both activities increase 2–3-fold. This can be explained if one assumes that a large fraction of the newly synthesized chlorophyll is not connected with the formation of active centers, but with that of the light-harvesting chlorophyll complexes. Indeed, as expected, in this case a large increase occurs in the staining intensity of 20 000–30 000-dalton polypeptides which are highly labeled by ^{35}S.

The experimental system described in this work permits an independent modulation of the polypeptide composition and the chlorophyll content of the membrane, as well as the functions resulting from their presence and integration. More information on the changes occurring in the membrane organization and activity during the alternate light-dark-light exposure can be obtained by use of additional measurements such as flash yield, quantum yield and fluorescence induction. Such studies are now in progress in our laboratory.

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