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APPEARANCE AND COMPOSITION OF CHLOROPHYLL-PROTEIN COMPLEXES I AND II DURING CHLOROPLAST MEMBRANE BIOGENESIS IN *CHLAMYDOMONAS REINHARDI* y-1

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SUMMARY

The chlorophyll-protein complexes I and II have been isolated and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis during greening and degreening of *Chlamydomonas reinhardi* y-1. At all stages of membrane formation, the complexes, when present, have a constant composition.

Chlorophyll-protein complex I consists of a major polypeptide(s) of molecular weight 64 000 synthesized in the chloroplast, to which about 29 chlorophyll *a* molecules are bound. The complex is not detected when other polypeptides of chloroplastic origin, related to both Photosystem I and Photosystem II activities, are not synthesized. However, Photosystem I activity can develop in membranes in which chlorophyll-protein complex I is not detectable.

Chlorophyll-protein complex II consists of two polypeptides of cytoplasmic origin, molecular weights 24 000 and 22 000, which bind 12 chlorophylls (*a* and *b*). The chlorophyll-protein complex II can be detected in membranes in which the development of photosystem II activity is prevented. Clipping of a $M_r = 2000$ fragment(s) from the $M_r = 22\,000$ polypeptide following trypsin digestion of membranes, does not affect the complex.

The detection of the complexes is possible only in membranes in which the simultaneous synthesis of both the chlorophyll and the corresponding polypeptides occurs.

The 28 000 dalton polypeptide, reported to be present in the chlorophyll-protein complex II, comigrates with the complex but apparently is not part of the complex itself.

The apparent molecular weight of the chlorophyll-protein complexes I and II are 88 000 and 28 000, respectively. The minimal true value for complex I is 89 000 or 154 000 and for complex II is 56 000.

Abbreviations: DCIP, 2,6-dichlorophenolindophenol, DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethyl urea, SDS, sodium dodecyl sulfate.

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INTRODUCTION

The analysis of the electrophoretic pattern of chloroplast membrane polypeptides in *Chlamydomonas reinhardi* has been widely used as a tool for the identification of specific polypeptides [1–4], their quantitation at different stages of membrane development [1, 5, 6] and their presence in membranes obtained from different mutants [4, 7–9]. This technique has also been useful in the characterization of different membrane fractions [3, 10, 11]. One important result of the use of electrophoretic techniques was the fact that, when using SDS as the membrane solvent, chlorophyll appeared to remain associated with certain membrane polypeptides during migration [9, 12–14].

In the present work an attempt has been made to identify the *C. reinhardi* y-1 chloroplast membrane polypeptides associated with chlorophyll. In addition, the formation of the chlorophyll-protein complexes and their relevance to photosynthetic activity at different developmental stages of the chloroplast membranes, were also studied. The advantage of the use of this organism for such a study stems from the fact that extensive information is available on the development of the photosynthetic apparatus, polypeptide composition of the membranes, and the role of many of these polypeptides in the synthesis, assembly and function of the membranes [15].

METHODS

Preparation of cultures, greening and degreening experiments. C. reinhardi y-1 cells were grown on a mineral medium containing acetate as the sole carbon source, as previously described [16]. Cells were harvested in the logarithmic phase of growth by centrifugation at $5000 \times g$ for 5 min. Degreening was performed by transferring the cells to the dark for 5–6 generations under normal growth condition [16]. For greening experiments, dark-grown cells were washed, resuspended in fresh growth medium at a concentration of about $1 \cdot 10^7$ cells/ml and exposed to light as described [17]. Formation of photosynthetically inactive membranes was obtained by greening the cells in presence of chloramphenicol (200 μg/ml, Abic, Israel) [1]. Repair of photosynthetic activities in these cells was achieved by washing out the chloramphenicol and further incubating the cells either in the dark or in the light with or without addition of cycloheximide [1, 15]. For radioactive labeling of membrane components, sodium [^{3}H]acetate was added to the cell suspension [1].

Preparation of chloroplast membranes and trypsin digestion. Membranes were prepared by breaking cells, suspended in 30 mM Tris (pH 8.0) containing 10 mM KCl (Tris/KCl buffer) (cell concentration of about $5 \cdot 10^8$ cells/ml) in a French press at 7000 lb/inch^2, followed by centrifugation on a discontinuous sucrose gradient (1.0620 g/cm^3 (15 %), 8 ml, 1.1315 g/cm^3 (30 %), 8 ml, 1.2936 g/cm^3 (60 %), 8 ml, in Tris/KCl buffer) at $70\,000 \times g$ for 3 h.

Chloroplast membrane preparations were digested with trypsin (EC 3.4.21.4) (bovine pancreas, type III, Sigma) from either outside or both sides of the thylakoid membranes, as described [18].

Analytical gel electrophoresis. Polypeptide analysis by acrylamide-SDS gel electrophoresis was carried out according to Laemlli [19]. Unless otherwise specified, the membrane samples were not boiled and the chlorophyll and lipids were not

extracted before the run. Subsequently, specific regions could be cut out of the gel and the slices placed, directly or after a heat denaturation, on the stacking gel for a second run. Electrophoresis was then performed as in the first run. The gels were stained by Coomassie Brilliant Blue or Amido Black according to Fairbanks et al. [20]. The apparent molecular weights of polypeptides were estimated according to Neville [21]. Free electrophoretic mobility (M_o) and the retardation coefficient (K_r) were estimated from a Ferguson plot according to Banker and Cotmatn [22]. The amount of each polypeptide was estimated directly on gels stained, with Amido Black, by densitometer tracings at 600 nm. From the amount of protein loaded on the gel and the relative weight of each polypeptide, the amount of individual polypeptides was calculated.

Assays. Methyl viologen and DCIP photoreduction activities were measured on membranes prepared by differential centrifugation and assayed as described [18]. Absorption spectra of pigment-containing bands were recorded directly on gel slices, using an Aminco-Chance double beam spectrophotometer. Chlorophyll was estimated according to Arnon [23] and protein according to Lowry et al. [24]. All reagents used throughout this work were of analytical grade.

RESULTS

(1) *Characterization of chlorophyll-protein complexes present in membranes from light-grown cells*

Following electrophoresis of chloroplast membranes obtained from light-

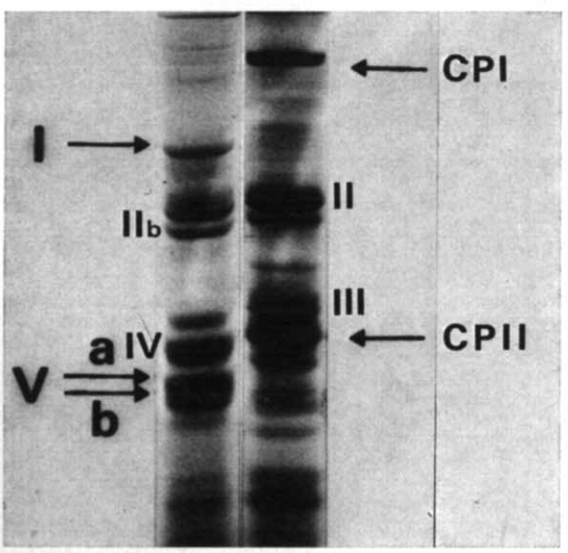

Fig. 1. A comparison between the polypeptide pattern of membranes dissolved in SDS at room temperature or following heat denaturation. Chloroplast membranes were dissolved in sample-buffer containing 2 % (w/v) SDS and 1 % (v/v) β-mercaptoethanol. The membranes were dissolved at room temperature (right) or in boiling water bath for 3 min (left). The gels were stained for polypeptides by Coomassie Brilliant Blue. Roman numerals indicate membrane polypeptides nomenclature. The differences between the patterns are indicated by arrows. CPII and CPI, chlorophyll-containing bands; I, Va and Vb, polypeptide bands that appear or are intensified by heat denaturation.

grown cells dissolved in 2 % (w/v) SDS at room temperature, one obtains three chlorophyll-containing regions. Two of these regions were relatively well defined with apparent molecular weights of 88 000 and 28 000. The third region is diffuse and of a molecular weight lower than 10 000 (Fig. 1). The 88 000 and the 28 000 daltons regions also contained polypeptides and are referred to as chlorophyll-protein complexes I and II, respectively [14].

When the membranes were dissolved in SDS by boiling, all the chlorophyll was released and migrated with the front. Although the polypeptide pattern so obtained is preserved, several changes are observed (Fig. 1). The polypeptides from complex I region are absent in heated membranes, while band I is present only in heated membranes (Fig. 1). In 7.5 % acrylamide gels, band I is resolved into two distinct bands of

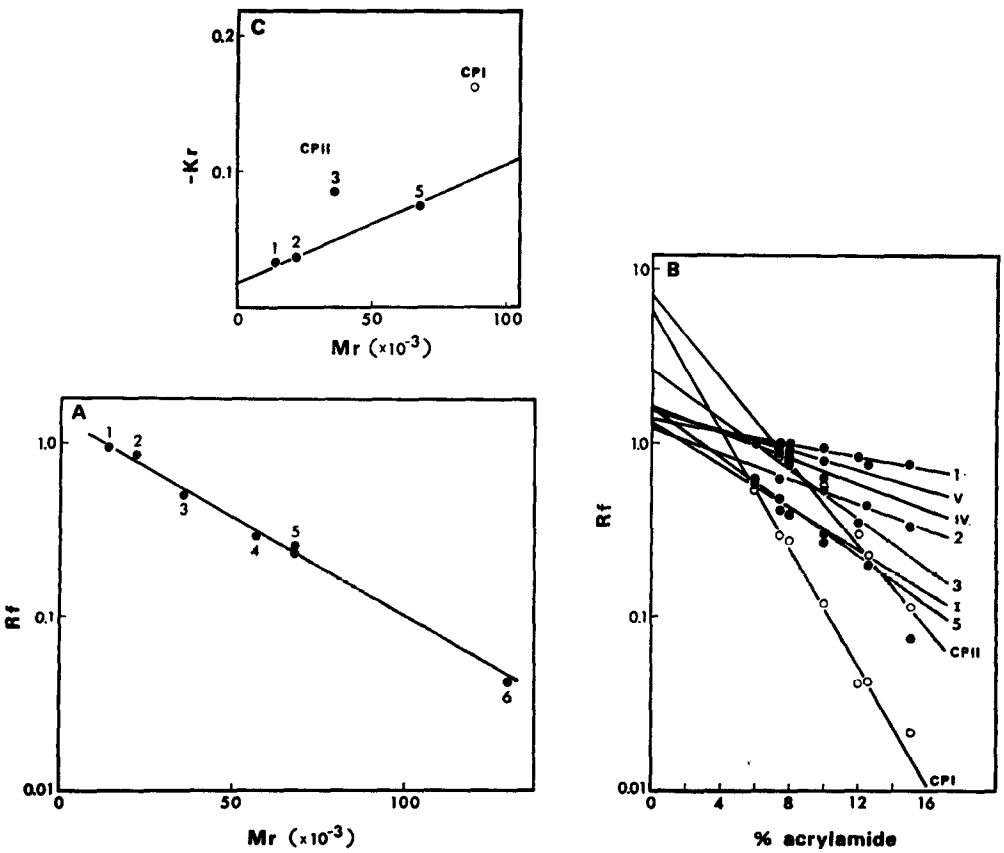

Fig. 2. Estimation of molecular weight (Mr), free electrophoretic mobility (M_o), and the retardation coefficient (K_r) of the chlorophyll-protein complexes and the denatured polypeptides. The molecular weights were estimated on 10 % acrylamide gels according to Neville [21] (A). The M_o values were obtained from the intersect of a Ferguson plot [22] (B) and the K_r values were obtained from the slope of a Ferguson plot [22] (B). The calculated K_r values were also plotted as a function of the estimated molecular weight (C). The markers used for molecular weight and M_o estimation were: 1, lysozyme (14 000); 2, chymotrypsin (22 000); 3, glyceraldehyde dehydrogenase (36 000); 4, pyruvate kinase (57 000); 5, bovine serum albumin (68 000); 6, β-galactosidase (130 000). The roman numerals indicate membrane polypeptides or chlorophyll-protein complexes.

M_r = 63 000 and 65 000. However, in 10 % acrylamide gels, although occasionally resolved into two bands, it generally appears as a single diffuse band of M_r = 64 000. In addition, in the heated membranes, bands Va and Vb are slightly intensified, although polypeptide IV is still present in complex II region (Fig. 1). The estimated M_r of bands IV, Va and Vb are 28 000, 24 000 and 22 000, respectively (Fig. 2A). The free electrophoretic mobility of all of these polypeptides is 1.2–1.6 (Fig. 2B) and their K_r values are proportional to their estimated M_r (Fig. 2C). The estimated molecular weight values for both complex I and complex II may be apparent, since both complexes exhibit high free electrophoretic mobility (6–7) and K_r values (Fig. 2).

The chlorophyll content and composition of the chlorophyll-protein complexes were analysed. It was found that complex I contained 15 % of the chlorophyll loaded on the gel. This consisted of chlorophyll *a* only and the weight ratio chlorophyll/protein (estimated spectrophotometrically) was 0.45. Complex II contained 28 % of the total chlorophyll and consisted of equal amounts of chlorophyll *a* and *b*. All the chlorophyll *b* present in the membranes was found to be located in this band, which had a weight ratio chlorophyll/protein of 0.09 (cf. also ref. 14).

To identify the polypeptide composition of the complexes, the chlorophyll-containing bands were sliced from the gels and rerun either directly or following heat denaturation. The results are shown in Fig. 3. Complex I migrated in the second run

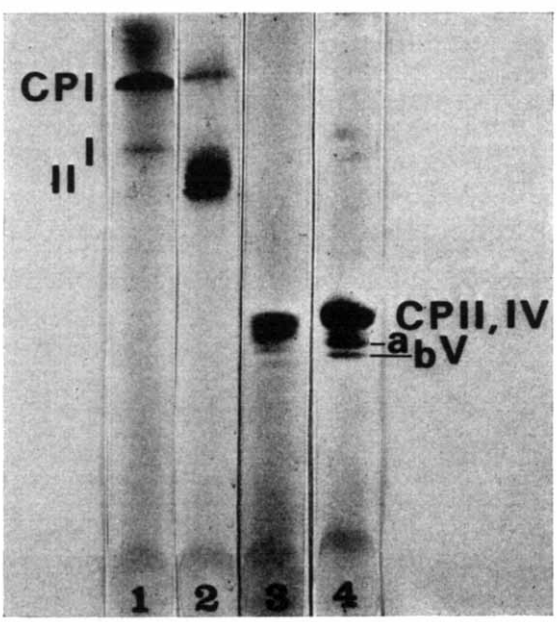

Fig. 3. Polypeptide composition of isolated chlorophyll-protein complexes. Chlorophyll-containing regions were cut off from gels and were analyzed for their polypeptide composition, directly or after heat denaturation for 2–5 min by a second, identical SDS-polyacrylamide gel electrophoresis. The gels were stained for polypeptides by Coomassie Brilliant Blue, only after the second run. 1, chlorophyll-protein complex I, directly resolved; 2, chlorophyll-protein complex I, after heat denaturation; 3, chlorophyll-protein complex II, directly resolved; 4, chlorophyll-protein complex II, after heat denaturation. Chlorophyll migrated with the polypeptide bands only in the directly resolved samples, 1 and 3.

with the same mobility as in the first, and only a negligible amount of chlorophyll migrated with the front. Staining of this gel revealed a major polypeptide band coinciding with complex I and traces of polypeptides II (50 000) and IIb (46 000). Following heat denaturation, all the chlorophyll was released and migrated with the front. Although the resolution obtained in the second run was poorer than in the first, polypeptide bands at the molecular weight range of polypeptides II and IIb could be observed. The major band migrated as a $M_r = 64\,000$ polypeptide(s), with a mobility similar to band I found in electrophoretograms of heat-denatured membranes.

Complex II retained its mobility during the second run, and most of the chlorophyll (*a*, *b*) was still associated with this band. Staining of this gel revealed a major $M_r = 28\,000$ polypeptide band. Following heat denaturation, all the chlorophyll was released and three distinct polypeptide bands were revealed. These were bands IV (28 000), Va (24 000) and Vb (22 000).

The chlorophyll can also be released from the complexes by adding SH reagents, such as dithiothreitol, mercaptoethanol, dithioerythritol and *N*-ethylmaleimide, or reducing agents, such as $NaBH_4$, to membranes dissolved in SDS at room temperature. Complex I appeared to be more sensitive to dithiothreitol and dithioerythritol, while complex II appeared to be preferentially affected in samples treated with mercaptoethanol (Fig. 4). At high concentrations (> 0.5 M), all these reagents caused dissociation of the complexes.

When purified chlorophyll or chlorophyll-lipid micelles were sonicated together

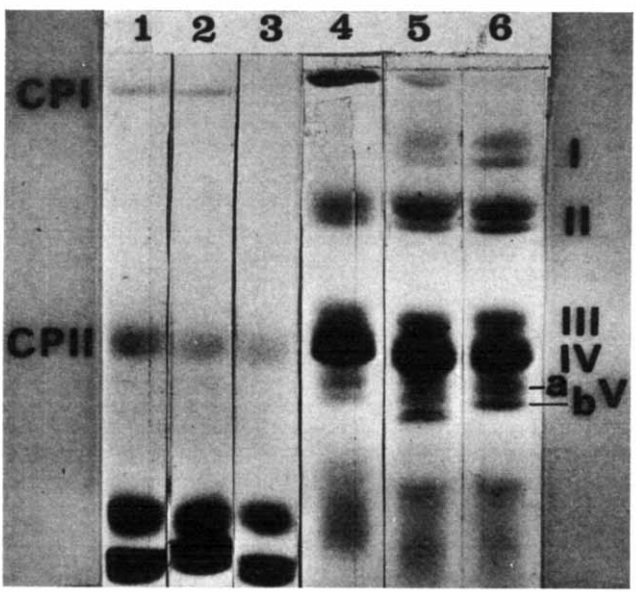

Fig. 4. Dissociation of the chlorophyll-protein complexes by S-S reducing agents. Chloroplast membranes were dissolved at room temperature, in buffer containing 2 % (w/v) SDS and different concentrations of S-S reducing agents. Chlorophyll-protein complexes are shown on gels before staining (gels 1–3), and the polypeptide composition is shown after Coomassie Brilliant Blue staining (gels 4–6). Gels 1 and 4, no S-S reducing agents were added; 2, 5 % (v/v) mercaptoethanol; 3, 0.5 M dithioerythritol; 5, 0.2 M dithiothreitol; 6, 0.4 M dithiothreitol.

with chloroplast membranes, the chlorophyll content of such membranes, reisolated on sucrose density gradient, was considerably increased. The buoyant density of these membranes decreased from 1.23 to 1.18 g/cm^3 and examination by electron microscopy showed no free micelles, indicating that the chlorophyll was incorporated into the membranes. When radioactive chlorophyll was used in such experiments, less than 1 % exchange between the added labelled chlorophyll and the chlorophyll complexed with protein was detected. Even when the chlorophyll micelles were extensively incubated with the membranes (up to 3 h), this exchange did not increase.

(2) *Effect of mild trypsin digestion of chloroplast membranes on the chlorophyll-protein complexes*

It has been previously demonstrated, that treatment of isolated chloroplast membranes from *C. reinhardi* with trypsin at 10 °C results in the preferential digestion of specific membrane polypeptides, as well as the loss of certain photosynthetic activities [18, 25]. In membranes obtained from light-grown cells, trypsin digestion removed a fragment of polypeptide Vb (22 000), resulting in a distinct increase in its electrophoretic mobility. Polypeptide IV (28 000) was only slightly digested and polypeptide I (64 000) was unaffected [25].

It was, therefore, determined whether trypsin digestion has any effect on the presence and properties of the chlorophyll-protein complexes. Following trypsin

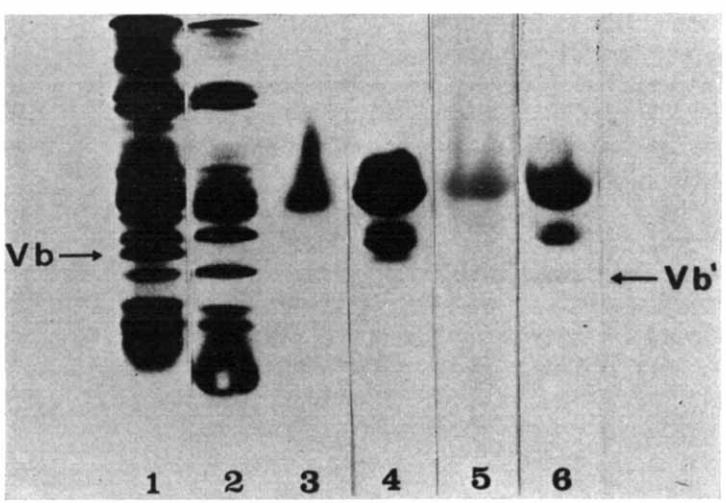

Fig. 5. Polypeptide composition of chlorophyll-protein complex II obtained from digested membranes. Chloroplast membranes were digested by trypsin (100 μg/ml, see Methods). After the digestion, the membranes were dissolved in SDS at room temperature and were separated by polyacrylamide gel electrophoresis (1–2). Chlorophyll-protein II complexes were cut off, and the slices were analyzed by a second identical run of gel electrophoresis, directly or after heat denaturation (3–6). For polypeptides detection, Coomassie Brilliant Blue was used. 1, untreated membranes dissolved at room temperature; 2, digested membranes dissolved at room temperature; 3, chlorophyll-protein complex II of untreated membranes, directly resolved; 4, chlorophyll-protein complex II of untreated membranes after heat denaturation; 5, chlorophyll-protein complex II of digested membranes, directly resolved; 6, chlorophyll-protein complex II of digested membranes after heat denaturation. Arrow indicates polypeptide Vb, which regains higher mobility by trypsin digestion (Vb').

digestion of membranes obtained from light-grown cells, both complexes could still be separated by SDS gel electrophoresis. When the gel regions containing the complexes were sliced and run a second time without heat denaturation, each complex retained its original mobility. Staining of these gels did not disclose any polypeptide bands exclusive of the chlorophyll-containing bands. If the second run was carried out after heat denaturation of the complexes, the chlorophyll was released, as expected. Complex I released mostly polypeptide I and complex II released polypeptides IV and Va. Polypeptide Vb was also released from complex II but exhibited a higher mobility (20 000) (Fig. 5). The amount of chlorophyll in complex I was not changed upon trypsin digestion. However, a 30 % reduction in the amount of chlorophyll found in complex II was observed, due to a somewhat preferential loss of chlorophyll *b* (Fig. 5).

(3) *Formation of chlorophyll-protein complexes and their relation to photosynthetic activity, during development of chloroplast membranes*

The relative content of chlorophyll and specific membrane polypeptides, as well as the photosynthetic activity, can be altered, in *C. reinhardi* y-1, under different conditions, such as degreening (dark-grown), greening (light exposure) or greening with chloramphenicol followed by repair [15]. Thus, one could take advantage of these systems to test whether correlation exists between changes in the above-mentioned parameters and the presence of chlorophyll-protein complexes.

Complexes I and II were still present after the first cell division in the dark,

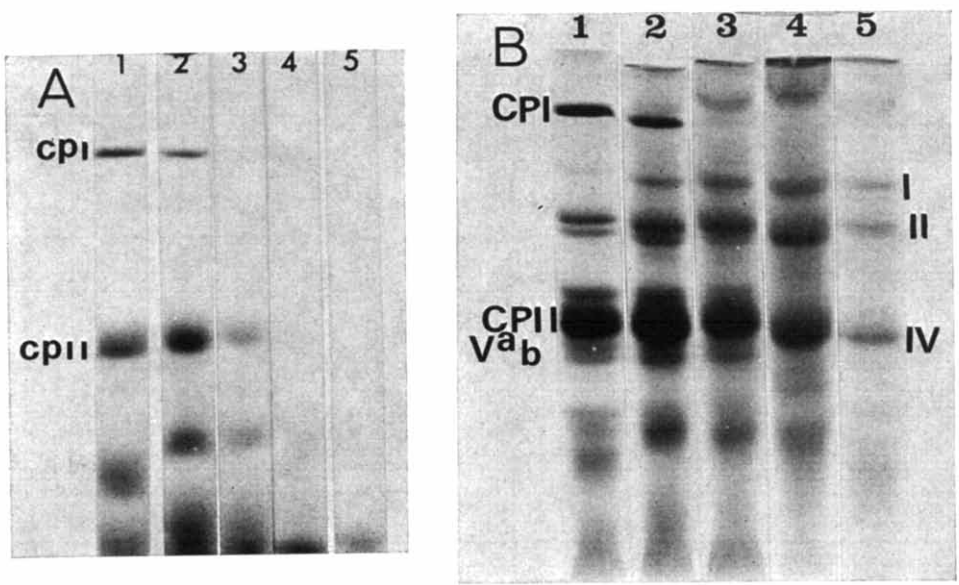

Fig. 6. Chlorophyll-protein complexes and polypeptide composition of membranes following growth in the dark (degreening). Chloroplast membranes were obtained from cells grown in the dark under dividing conditions. The membranes were analyzed for chlorophyll-protein complexes by SDS-polyacrylamide gel electrophoresis (A), while the same gels were stained later by Coomassie Brilliant Blue for polypeptide composition (B). Numbers indicate generations of growth in the dark.

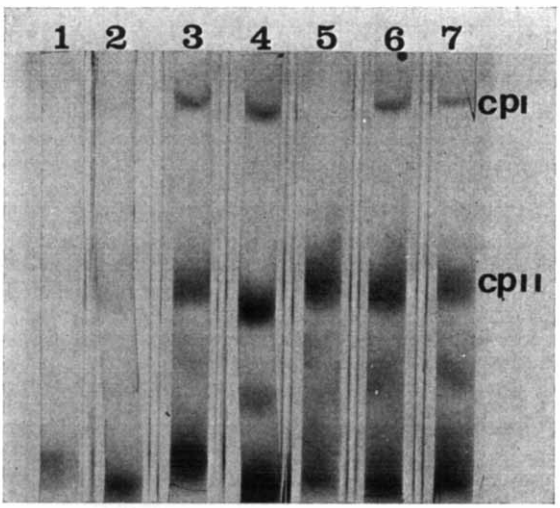

Fig. 7. Appearance of chlorophyll-protein complexes during chloroplast development. Chloroplast membranes were obtained from cells at different stages of the greening process, including development of photosynthetically inactive chloroplasts in the presence of chloramphenicol, or repair of its activity under different conditions (see Methods). Chlorophyll-protein complexes were detected by SDS gel electrophoresis. 1–4, normal development (0.8, 2.5, 6.6 and 9.7 μg chlorophyll/10^7 cells, respectively); 5, development in the presence of chloramphenicol (4.2 μg chlorophyll/10^7 cells) or repair in the dark (no further chlorophyll synthesis); 6, repair in the light (6.1 μg chlorophyll/10^7 cells); 7, repair in the presence of cycloheximide (0.5 μg/ml) (4.8 μg chlorophyll/10^7 cells).

when chlorophyll synthesis does not take place. Complex I was lost at the second division, and complex II disappeared between the third and fourth divisions in the dark (Fig. 6A). Staining of the gels showed that polypeptide I (64 000), very lightly stained in electrophoretograms of membranes obtained from light-grown cells, gradually appeared during division in the dark, concomitant with the disappearance of complex I and the M_r = 88 000 polypeptide band (Fig. 6B). However, following heat denaturation, polypeptide I is always detected. Polypeptides Va (24 000) and Vb (22 000) are present in membranes from light-grown cells and disappear gradually during the degreening. These polypeptides are completely lost between the third and fourth generations, concomitant with the disappearance of complex II (Fig. 6). At this stage, polypeptides Va and Vb cannot be detected even in heat-denatured membranes. The M_r = 28 000 polypeptide band was, however, not lost at any stage of degreening. During the greening process both complexes reappeared, and could be detected when the chlorophyll level was only 2.5 μg/10^7 cells (Fig. 7). The normal chlorophyll content of these cells when grown in the light is 15–25 μg/10^7 cells. When greening was carried out in the presence of chloramphenicol, only complex II was detected, although the chlorophyll content (6 μg/10^7 cells) was sufficient to allow detection of complex I, if present. Following repair of these cells in the dark without chlorophyll synthesis, complex I did not appear even though photosynthetic activities of both photosystems were regained (Table I). Complex I reappeared, however, when the repair of photosynthetic activity of these cells occurred under condition allowing chlorophyll synthesis (Fig. 7). Measurements of photosynthetic activity during

TABLE I

CHLOROPHYLL CONTENT, PHOTOSYNTHETIC ACTIVITIES AND PRESENCE OF CHLOROPHYLL-PROTEIN COMPLEXES, IN MEMBRANES DEVELOPED UNDER DIFFERENT CONDITIONS

Unless otherwise specified, *C. reinhardi* y-l cells were used. Photosynthetic activities in μmol/mg chlorophyll per h were measured as light-dependent electron flow. For Photosystem II we measured DCIP reduction, using H_2O as an electron donor. For Photosystem I we measured O_2 uptake when DCIP plus ascorbate donated electrons to methyl viologen (see Methods). The concentration of chloramphenicol was 200 μg/ml and that of cycloheximide was 0.5 μg/ml. Arrows indicate transfer of the cells to new incubation conditions. Numbers in parentheses indicate increase in chlorophyll. Complexes presence (+) or absence (−) were detected by SDS-polyacrylamide gel electrophoresis.

Cells	Chlorophyll content ($\mu g/10^7$ cells)	Photosystem I activity	Chlorophyll-protein complex I	Photosystem II activity	Chlorophyll-protein complex II
Light grown	30.0	185	+	160	+
Dark grown					
1 generation	15.0	300	+	145	+
2 generations	7.0	400	−	200	+
4 generations	1.5	150	−	15	−
5 generations	0.6	30	−	1	−
Greening cells					
2 h light	2.0	250	−	60	−
6 h light	6.0	400	+	500	+
12 h light	10.0	180	+	200	+
12 h light+ chloramphenicol	6.0	10	−	12	+
6 h light+ chloramphenicol ↓ 6 h light	9.0 (3)	194	+	350	+
6 h light+ chloramphenicol ↓ 6 h light+cycloheximide	7.0 (1)*	150	+*	300	+
6 h light+chloramphenicol ↓ 6 h dark	6.0 (0)	130	−	270	+
T_4**					
Light-grown 25 °C	20.0	200	+	120	+
Light-grown 37 °C	23.0	200	−	0	+
37 °C growth ↓ 25 °C non-dividing 10 h	23.0 (0)	200	−	100	+

* In several experiments cycloheximide prevented almost completely the increase in chlorophyll content during the second incubation. In these experiments cycloheximide → chlorophyll-protein complex I was not detected.

** For comparison, data are shown for *C. reinhardi* T_4 temperature sensitive mutant, to be published elsewhere [29].

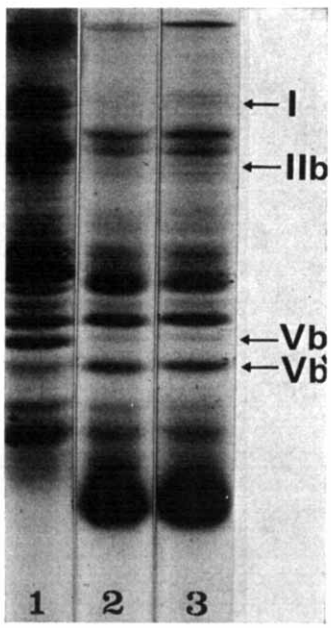

Fig. 8. Polypeptide pattern of membranes obtained from cells greened in the presence of chloramphenicol and digested by trypsin. Chloroplast membranes were obtained from dark-grown cells greened in the presence of chloramphenicol (200 μg/ml) for 12 h. The membranes were digested by trypsin (100 μg/ml) with (3) or without (2) sonication (see Methods). Membranes were dissolved in SDS at room temperature, and the polypeptide composition was observed following Coomassie Brilliant Blue staining of the polyacrylamide gels. 1, untreated membranes; 2, digestion without sonication; 3, digestion after sonication for 30 s at 0 °C. Arrows indicate polypeptides which are extensively digested, I, IIb and Vb.

degreening, greening and repair showed no positive correlation between the presence of chlorophyll-protein complexes I or II and the electron transfer activities of Photosystem I or Photosystem II (Table I).

In trypsin-digested membranes obtained from cells greened in the presence of chloramphenicol, complex II could still be detected. Analysis of polypeptide pattern of such membranes (Fig. 8) showed normal amounts of polypeptide Va (24 000). Polypeptide Vb exhibited a higher mobility (20 000) and the amount of polypeptide IV (28 000) was significantly reduced (cf. also ref. 25). Chlorophyll-protein complex II isolated from such membranes could still be resolved upon heat denaturation into polypeptides Va (24 000), normal amounts of modified Vb (20 000), but considerably reduced amounts of IV (28 000).

DISCUSSION

The polypeptide composition of the complexes and their relevance to photosynthetic activity

The presence of chlorophyll-protein complexes in photosynthetic membranes of higher plants and algae, including *C. reinhardi*, is well established [14, 26].

The polypeptide composition of the complexes in *C. reinhardi* is still controversial. Anderson and Levine [27] reported that complex I contained two distinct polypeptides in the molecular weight range of 60 000. According to Picaud and Acker [8], the complex consisted of 56 000 and 44 000 dalton polypeptides. Chua et al. [9], have claimed that complex I contained a single polypeptide of $M_r = 66\,000$ which binds about eight chlorophylls.

Complex II is considered by Kan and Thornber [13] to have an apparent molecular weight of 29 000, and consist of a single polypeptide of 29 000, to which three molecules each of chlorophyll *b* and chlorophyll *a* are bound. On the other hand, Anderson and Levine [27] have claimed that this complex contains two polypeptides, IIb and IIc, of 24 000 and 22 000 daltons, respectively.

It is well established that, during degreening and greening of *C. reinhardi* y-l, different membrane polypeptides, including those reported to be part of the chlorophyll-protein complexes, are differentially lost or synthesized. Moreover, the relative composition of the membranes, in terms of specific polypeptides and chlorophyll, can be modulated quite extensively by use of appropriate protein synthesis inhibitors [15]. Thus, it was considered that a study of the fate of complexes I and II during the process of membrane degradation and biogenesis would provide useful information, as to what is the minimal polypeptide composition required for the formation and/or maintenance of these complexes, and their relation to the photosynthetic activity of Photosystems I and II.

The results presented in this work support several conclusions, discussed in detail below.

(1) *Chlorophyll-protein complex I.* The complex I in *C. reinhardi* has an apparent molecular weight of 88 000. Following either heat denaturation or extensive reduction of S-S bonds, it released chlorophyll *a* and a major polypeptide(s) of 64 000 daltons, synthesized in the chloroplast [3]. The weight ratio chlorophyll/protein in this complex was found to be 0.45. Thus, one can calculate a molar ratio of about 29 chlorophylls per polypeptide of 64 000 daltons, or, if the complex contains two polypeptides (63 000 and 65 000), the molar ratio would be about 15 chlorophylls per polypeptide (Table II). A highly purified Photosystem I reaction center preparation from spinach has been shown to contain about 20 chlorophyll *a* molecules [28].

In addition to the 64 000 daltons major polypeptide, several minor bands are released from isolated complex I following complete denaturation. These polypeptides, with mobilities in the molecular weight range of 46 000–50 000, all originate in the chloroplast [3], and some of them are essential for Photosystem II activity [3, 4, 29]. Moreover, the 46 000 molecular weight region can be further resolved into several polypeptides, two of which are missing in a *C. reinhardi* temperature-sensitive mutant T_4 when grown at 37 °C [29]. The membranes obtained from such cells do not exhibit Photosystem II activity, and although they have a normal Photosystem I activity, complex I cannot be detected in such membranes [29]. Thus, one cannot simply identify complex I with Photosystem I, as implied by reports showing that *Fl*, Fl_4 [9] and Fl_5 [8] *C. reinhardi* mutants, a *Scenedesmus* mutant 8 [30] and higher plant mutants [3], are all deficient in Photosystem I activity and do not exhibit complex I. In addition, during degreening of y-l cells, complex I is lost at the second division, while Photosystem I specific activity is actually higher than at the onset of the degreening (cf. also ref. 32). Furthermore, Photosystem I activity develops normally

TABLE II

MOLAR RATIO OF CHLOROPHYLL BOUND TO MEMBRANE POLYPEPTIDES ASSOCIATED WITH ISOLATED CHLOROPHYLL-PROTEIN COMPLEXES OR ACTIVE PHOTOSYSTEM PREPARATIONS

The molecular weights are calculated from relative mobilities of the polypeptide following heat denaturation of the membranes. For the calculation of the amount of each polypeptide see Methods. The number of mol per polypeptide band was calculated on the basis of its amount and molecular weight. The polypeptide composition of the chlorophyll-protein complexes was estimated by slicing the complex-containing region of the gels and rerunning them following heat denaturation. The data concerning the polypeptide composition of active preparations of both photosystems are given for comparison [3]. The values for the molar ratio of chlorophyll to protein in the complexes were calculated from direct spectrophotometric measurements of chlorophyll in the complexes and relating them to the molar content of the corresponding polypeptides. For the calculations of chlorophyll bound to each polypeptide, see Discussion.

Polypeptide	Mol. wt. $\times 10^{-3}$	% by wt.	nmol / band	Presence in isolated fractions				Bound chlorophyll/protein	
				CPI	PSI	CPII	PSII	Weight	Molar
I	64±2	4.4	0.21	+	+	−	−	0.45	29
II	50±2	7.6	0.46	±	+	−	−		
IIb	46±2	3.9	0.25	±	−	−	+		
III	32±1	9.2	0.86	−	±	−	+		
IV	28±1	15.4	1.65	−	±	+	+	0.09	
Va	24±0.5	15.4	1.92	−	−	+	+		12 [ ] 6.6
Vb	22±0.5	10.2	1.39	−	−	+	+		

CPI, chlorophyll-protein complex I; CPII, chlorophyll-protein complex II; PSI, Photosystem I; PSII, Photosystem II.

without a parallel development of complex I when y-1 cells, greened in the presence of chloramphenicol, were repaired under conditions which do not allow chlorophyll synthesis. Measurements of photosynthetic efficiency in such membranes indicate that the light-harvesting system of Photosystem I is present.

Thus, while the polypeptides required for Photosystem I activity are also essential for the stabilization of complex I [8, 9, 30, 31] they are not sufficient, and several additional polypeptides are required for the detection of complex I. The discrepancy between the fact that complex I and thus chlorophyll *P*-700 [14], are missing while Photosystem I activity can be detected, may be explained in the light of the data published by Rurainsky [33], who claimed that chlorophyll *P*-700 is not essential for linear electron flow through Photosystem I.

(2) *Chlorophyll-protein complex II.* The complex II with a molecular weight of 28 000, appeared to contain a single polypeptide band. Upon denaturation by heat or S-S reducing agents, this complex released chlorophyll and revealed the presence of three major polypeptides of 28 000, 24 000, and 22 000 daltons. The direct measurement of chlorophyll/protein weight ratio in this complex yields a value of 0.09. This ratio was calculated for the non-dissociated complex superimposed on polypeptide IV, and the molar ratio of chlorophyll to a complex containing all three polypeptides would be 6.6. Since the weight ratio of polypeptides Va+Vb to the total protein in the complex II region is 0.52, the corrected value for the molar ratio chlorophyll/polypeptides Va+Vb would be 12 (Table II).

Based on the data presented here, as well as elsewhere [8, 9, 13, 27], it can be concluded that the 28 000 dalton polypeptide* is a major contaminant of the complex which comigrates with it but does not bind chlorophyll per se. The chlorophyll-binding polypeptides of the complex II region are apparently the 24 000 and 22 000** dalton polypeptides. This conclusion is based on the following arguments: (A) Complex II shows an anomalous mobility. Following complete denaturation one can expect a change in the mobility of the released polypeptides. This is the case for the 24 000 and 22 000 dalton polypeptides, but not for the 28 000 dalton polypeptide (cf. also ref. 9, 13 and 27). These three polypeptides do not exhibit anomalous mobility and preliminary results indicate that the 28 000 dalton polypeptide can be resolved from complex II at different acrylamide concentrations. (B) Complex II is lost, during degreening of the y-1 cells, between the third and fourth division. At this time the relative amounts of the 22 000 and 24 000 dalton polypeptides are very much reduced, while polypeptide IV (28 000) is still present in substantial amounts. The complex reappears as soon as the depleted polypeptides and chlorophyll are synthesized de novo during greening. (C) Although trypsin digestion in situ clipped a 2000 dalton fragment(s) from polypeptide Vb (cf. also ref. 25), one can still find the modified polypeptide upon dissociation of the isolated, otherwise unmodified complex. One can assume that, either the trypsin-sensitive segment is not involved in chlorophyll binding, or, if it is, it is stabilized within the complex by S-S bonds and hydrophobic association. (D) Extensive digestion of polypeptide IV (28 000) can be achieved in membranes formed in presence of chloramphenicol (cf. also ref. 25) without parallel loss or change in the complex. (E) One can experimentally alter the amount of chlorophyll found in the complex by varying the SDS concentration or the duration of the electrophoresis. When the chlorophyll content of the complex is lower, the amount of free polypeptides Va (24 000) and Vb (22 000) detected in the stained gel is greater, while the isolated residual complex, when fully denatured, reveals lower amounts of these polypeptides during the second run. The amount of polypeptide IV (28 000) remains constant and independent of the chlorophyll content of the complex.

In chlorophyll-deficient mutants, complex II was not detectable and polypeptides of 24 000 and 22 000 dalton were lacking in the membranes [27]. These chlorophyll-deficient mutants, as well as the ac-5 mutant of *C. reinhardi*, which also lacks these two polypeptides [7], exhibit significant levels of Photosystem II activity. In Euglena, chlorophyll *b* and complex II are not detected, and Photosystem II is active. The two polypeptides, however, are major components of the chloroplast membrane [34].

That complex II is not essential for Photosystem II activity is the basic argument for its identification as a light-harvesting chlorophyll-protein complex [14]. In addition to the fact that complex II is not essential for Photosystem II activity, the present data show that the detection of complex II does not necessarily imply that Photosystem II is active, as is the case in y-1 membranes formed in presence of chloramphenicol. Similar results were obtained with 37 °C grown T_4 mutant [29]. Thus, it can be concluded that the polypeptides required for the establishment of Photosystem II activity and those required for the stabilization of complex II, are not identical.

* IV in our nomenclature, IIb in Hoober's [2] and Levine's [11, 27] and 11–12 in Chua and Bennoun's [4].

** Va, Vb in our nomenclature, IIc in Hoober's [2] and Levine's [27] and 15–17 in Chua and Bennoun's [4].

Estimation of the molecular weight of the chlorophyll-protein complexes

The estimation of the true molecular weights of the chlorophyll-protein complexes by SDS-polyacrylamide gel electrophoresis is not possible. The existence of chlorophyll-protein complexes following dissolution in SDS implies, a priori, that the specific tertiary structure of the polypeptide is, at least partially, unmodified [35]. Thus, these complexes would not bind the theoretical amount of SDS (1.4 g/g protein) to form the rodlike shape ascribed to a true SDS-protein complex [36]. Therefore, the mobility of the complexes would not be a unique function of their molecular weights [36]. Indeed, we have found that the free electrophoretic mobility of both complexes is considerably higher (6–7) than that of their completely denatured polypeptide components (1.2–1.6). Similar results were previously reported for complex I [9]. The increase in the free electrophoretic mobility implies that the charge per unit mass of the complex is higher, the shape of the complex is less asymmetric and/or the size of the complex is smaller than expected for a true protein-SDS complex of similar mass. That the K_r (retardation coefficient) values obtained for both complexes are higher than expected from their apparent molecular weight (Fig. 2C) indicates that the complexes are large and/or more asymmetric than expected. The high free electrophoretic mobility is, therefore, a result of higher charge per unit mass and the anomalous mobility exhibited by the complexes would be due to these three unusual parameters. Thus, all measurements or estimates of the molecular weight of the complexes by SDS-polyacrylamide gel electrophoresis should be considered as apparent only.

The true minimal molecular weight of complex I is either 90 000 or 154 000, depending on whether it contains one 64 000 dalton polypeptide or one 63 000 dalton polypeptide and one 65 000 dalton polypeptide per 29 chlorophyll *a* molecules. For complex II, the minimal true dalton would be 57 000, assuming it consists of one 22 000 dalton polypeptide, one 24 000 dalton polypeptide, and six chlorophyll *a* and six chlorophyll *b* molecules.

De novo formation of the complexes

From the results of the greening experiments reported in this work, it appears that the complexes can be formed only when chlorophyll is synthesized simultaneously with the polypeptide moiety of the complex. Complex I appears during the repair of chloramphenicol-treated cells only when chlorophyll, even in very small amounts, is synthesized de novo. The relatively high amounts of preexisting chlorophyll are not utilized for the formation of the complex when only the polypeptide moiety is synthesized during the repair process. In addition, no complex is formed when chlorophyll is synthesized without de novo synthesis of the corresponding polypeptides. Thus, during greening in presence of chloramphenicol which prevents the de novo synthesis of polypeptides I (64 000), II (50 000) and IIb (46 000), complex I is not formed, even though chlorophyll *a* and chlorophyll *b* are synthesized. Complex II is always formed under conditions allowing chloroplast membrane formation in *C. reinhardi* y-1. It has been reported for higher plants, however, that complex II is not formed in the presence of cycloheximide [37, 38]. For complex I results similar to ours were obtained [37, 38]. This behaviour of the complexes indicates very tight association of the chlorophyll with the corresponding polypeptide. This is also inferred from the following: (1) stability in SDS solution; (2) stability of the polypeptides towards extraction by guanidine [35], and their co-extraction with lipids and

pigments by organic solvents (Bar-Nun, S., unpublished, cf. also ref. 9); (3) lower turnover rate of complexed chlorophyll, relative to free chlorophyll [39]; (4) low exchange rates between bound and free chlorophyll; (5) digestion of polypeptide I (64 000) by trypsin only in membranes developed in the presence of chloramphenicol, in which complex I is missing (cf. also ref. 25).

It has been suggested that chlorophyll synthesis requires availability of acceptor-protein [40] and that chlorophyll is required for the occurrence of chlorophyll-binding proteins in chromatophore membrane of green bacteria [41]. It is attractive to speculate that the association between the chlorophyll and the polypeptide occurs prior to or during the insertion of the chlorophyll-protein complexes into the developing membrane and results in a thermodynamically more stable molecular conformation of the polypeptide.

While the synthesis of the polypeptides of complex I may occur on thylakoid-bound ribosomes within the chloroplast, the synthesis of the 24 000 and 22 000 dalton polypeptides is carried out by cytoplasmic ribosomes and is light dependent. The association of chlorophyll with the corresponding polypeptides may possibly play a role in the transport of these polypeptides across the chloroplast envelope.

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