A CHLOROPHYLL-PROTEIN COMPLEX

LACKING IN PHOTOSYSTEM I MUTANTS

OF *CHLAMYDOMONAS REINHARDTII*

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ABSTRACT

Sodium dodecyl sulfate gel electrophoresis of unheated, detergent-solubilized thylakoid membranes of *Chlamydomonas reinhardtii* gives two chlorophyll-protein complexes. Chlorophyll-protein complex I (CP I) is blue-green in color and can be dissociated by heat into "free" chlorophyll and a constituent polypeptide (polypeptide 2; mol wt 66,000). Similar experiments with spinach and Chinese cabbage show that the higher plant CP I contains an equivalent polypeptide but of slightly lower molecular weight (64,000). Both polypeptide 2 and its counterpart in spinach are soluble in a 2:1 (vol/vol) mixture of chloroform-methanol. Chemical analysis reveals that *C. reinhardtii* CP I has a chlorophyll *a* to *b* weight ratio of about 5 and that it contains approximately 5% of the total chlorophyll and 8–9% of the total protein of the thylakoid membranes. Thus, it can be calculated that each constituent polypeptide chain is associated with eight to nine chlorophyll molecules. Attempts to measure the molecular weight of CP I by calibrated SDS gels were unsuccessful since the complex migrates anomalously in such gels. Two Mendelian mutants of *C. reinhardtii*, F1 and F14, which lack P700 but have normal photosystem I activity, do not contain CP I or the 66,000-dalton polypeptide in their thylakoid membranes. Our results suggest that CP I is essential for photosystem I reaction center activity and that P700 may be associated with the 66,000-dalton polypeptide.

In green plants and algae, the photosynthetic electron transport chain is bound to the thylakoid membranes and is composed of two photosystems connected by a series of electron carriers (cf. reference 49). The thylakoid membranes can be fractionated by differential centrifugation in the presence of nonionic detergent into two submembrane fragments which are different in their photochemical activities and chemical compositions (cf. reference 11). The light fraction has a high chlorophyll *a* to *b* ratio and is enriched in photosystem I (PS I) activity, whereas the heavy fraction has a low chlorophyll *a* to *b* ratio and is enriched in photosystem II (PS II) activity (cf. reference 11). These experiments indicate that each photosystem may be organized as a specific complex in the membrane.

A more complete solubilization of the thylakoid membranes can be obtained with anionic detergents such as sodium dodecyl sulfate (SDS) or sodium dodecyl benzene sulfonate (SDBS). Ogawa et al. (39) and Thornber et al. (46, 48) reported that short-term electrophoresis of such a detergent extract gave three pigmented bands (39, 46, 48).

Two of these bands are pigment-protein complexes, designated complex I and II, whereas the third band consists of free pigments complexed to the detergent. Pigment analysis showed that complex I is enriched in chlorophyll *a* whereas complex II is enriched in chlorophyll *b* (39, 46, 48). It was inferred from the asymmetric distribution of the chlorophylls that complex I and II are associated with PS I and II, respectively (39, 46). The occurrence of these two complexes has since been confirmed in a wide variety of plants (2, 3, 21, 23-26, 35).

Most of the photochemical activities of complex I and II from higher plants are inactivated by SDS or SDBS (39, 46, 48). In spinach, the involvement of complex I in PS I reaction centers was implied by the presence of a weak light-induced absorbance change at 700 nm (5). On the other hand, Dietrich and Thornber (16) isolated from a blue-green alga *Phormidium luridum* a similar complex which still retains its photochemical activities in spite of the SDS treatment. The blue-green algal complex is able to carry out PS I reaction and is highly enriched in P700, the reaction center chlorophyll of PS I. Accordingly, this complex as well as its counterpart in higher plants was designated as the P700-chlorophyll *a*-protein complex (16). On the basis of amino acid analyses, Thornber (45) postulated that the protein moiety of the complex from *P. luridum* and from higher plants is made up of four identical subunits, each having a molecular weight of 33,000-35,000. In contrast, Anderson and Levine (3) have recently reported that complex I from *C. reinhardtii* contains two polypeptides with mol wt around 60,000. Investigations with a variety of pigment-deficient mutants (1, 47) showed that complex II is not required for photosynthetic electron transport and, presumably, is involved in harvesting light energy for PS II.

We have recently reported the analysis of thylakoid membrane polypeptides of wild-type and mutant strains of *C. reinhardtii* by SDS gradient gel electrophoresis (14). Under our experimental conditions, we have failed to observe any discrete pigment-protein complexes. Since pigment-protein complexes are potentially useful for identifying the functions of membrane polypeptides and investigating nonphotosynthetic mutants, we sought to resolve the differences between our observations and those of others (2, 3, 21, 23-26, 35, 39, 46, 48). We found that complexes I and II could indeed be obtained under our conditions if the SDS-solubilized membranes were not heated before electrophoresis. Upon heating, both complexes are dis-

sociated into free pigments and constituent polypeptides. In this paper, we show that the *C. reinhardtii* complex I has a polypeptide of mol wt 66,000, and that this complex, as well as the constituent polypeptide, is missing from thylakoid membranes of mutants which are deficient in PS I activity.

MATERIALS AND METHODS

Culture Conditions of C. reinhardtii

Cells of the wild-type (137 c, mt⁺) and two mutant strains (F1 and F14) of *C. reinhardtii* were grown in Tris-acetate-phosphate medium under conditions described by Gorman and Levine (19). F1 and F14 were derived from the wild-type (WT) by mutagenesis with methyl methane sulfonate and were selected by virtue of their high fluorescence characteristics (9). These mutants have been shown to lack the light-induced P700 signal (13, 15), indicating that electron flow is blocked at the PS I reaction center.

Preparation of Thylakoid Membranes from C. reinhardtii, Spinach, and Chinese Cabbage

Thylakoid membranes from WT and mutant strains of *C. reinhardtii* were prepared by the flotation procedure described previously (14). Spinach (*Spinacia oleracea*) and Chinese cabbage (*Brassica chinensis*) were obtained from local stores. Chloroplasts from both plants were prepared as described by Izawa and Good (29). Chloroplasts were lysed by gentle homogenization in ice-cold distilled H_2O, and the thylakoid membranes were separated from the stromal enzymes by centrifugation at 12,000 g for 10 min. The membrane pellet was washed once again in distilled H_2O to remove residual soluble proteins. Finally, the membranes were washed twice in 1 mM EDTA (pH 7.5) to release the bound Ca^{++}-dependent ATPase (28).

SDS Gel Electrophoresis of Chlorophyll-Protein Complexes and Membrane Polypeptides

Chlorophyll-protein complexes and membrane polypeptides were analyzed by 7.5-15% acrylamide gradient gel electrophoresis in the presence of 0.1% SDS as described previously (14). Membranes were solubilized in a mixture containing 50 mM Na_2CO_3, 50 mM dithiothreitol (DTT), 2% (wt/vol) SDS, 12% sucrose, and 0.04% bromphenol blue. The final chlorophyll concentration of the preparation was 1 mg/ml giving a sodium dodecyl sulfate (SDS) to chlorophyll weight ratio of 20. The SDS-solubilized membranes were either used directly (nonheated) or incubated at 100°C for 1 min (heated) before electrophoresis. Lipids and photosyn-

thetic pigments were either not removed (unextracted) or removed by extraction at room temperature with either 90% acetone or a 2:1 (vol/vol) mixture of chloroform-methanol (extracted). For acetone extraction, 0.5 ml of thylakoid membranes (750 μg chlorophyll) in 0.1 M $Na_2 \cdot CO_3$-0.1 M DTT was mixed with 4.5 ml of acetone. The acetone-insoluble materials were sedimented by centrifugation at 2,000 g for 10 min and the supernate evaporated to dryness at room temperature under a stream of nitrogen. Both the 90% acetone precipitate and the residue derived from the acetone extract were dissolved in 0.5 ml of the SDS-Na_2CO_3-DTT solution. Extraction with chloroform-methanol was performed by a modification of the Tzagoloff and Akai procedure (50, 42). Thylakoid membranes (750 μg chlorophyll) suspended in 0.5 ml of 0.1 M Na_2CO_3-0.1 M DTT were extracted with 10 ml of a 2:1 (vol/vol) mixture of chloroform-methanol and the precipitate was pelleted by centrifugation as above. The chloroform-methanol extract was evaporated to dryness at room temperature under a stream of nitrogen and the resulting residue as well as the chloroform-methanol precipitate was dissolved in 0.5 ml of the SDS-Na_2CO_3-DTT solution. In other experiments, 50 ml of diethyl ether were added to 10 ml of the chloroform-methanol extract and the mixture was stirred in the cold room for 2 h. The precipitate was pelleted by centrifugation, washed twice in diethyl ether, and finally dissolved in 0.5 ml of SDS-Na_2CO_3-DTT for SDS-gel electrophoresis.

To display chlorophyll-protein complexes, nonheated membrane samples were subjected to electrophoresis at 17.5 mA for 3–4 h until the bromphenol blue tracking dye had migrated 4–5 cm from the top of the separation gel. These gels were not stained with Coomassie brilliant blue. For the analysis of membrane polypeptides, the electrophoresis was continued until the dye front had reached the bottom of the separation gel (~20 cm). The gels were stained for proteins as described previously (14).

The free electrophoretic mobilities of membrane polypeptides and standard proteins were determined by the Ferguson plot (6, 12, 37). Electrophoresis was done with SDS-polyacrylamide gels containing single concentrations of acrylamide instead of an acrylamide concentration gradient. The dye front was marked at the termination of the run by punching small holes. Data were plotted according to the Ferguson relationship given by the following equation:

$$\log R_f = Y_o - K_R T,$$

where R_f is the relative mobility of a protein species; Y_o, the free mobility (y intercept); K_R, the retardation coefficient (slope); and T, the percent (wt/vol) concentration of acrylamide plus N,N'-methylene bisacrylamide.

Isolation of Chlorophyll-Protein Complex I

For the large scale isolation of CP I, 4 ml of nonheated membrane samples containing 8 mg chlorophyll were loaded onto a 6-mm slab gel (21 cm × 30 cm). The gel was prepared as described previously except that the separation gel contained 7.5% acrylamide. Electrophoresis was carried out at 75 mA for about 15–16 h. The gel band containing CP I was cut out and the complex eluted from the gel by electrophoresis into a dialysis bag in the presence of 20 mM sodium phosphate buffer (pH 7.0)-0.1% SDS.

Experiments with Mixtures of Wild-Type and Mutant Cells

Mixing experiments using [14C]-labeled wild-type and unlabeled mutant cells were done as follows. Wild-type cells were washed twice in minimal medium (43) and resuspended in the same medium to a density of ~6 × 10^6 cells/ml. [1-[14C]]Sodium acetate (sp act 57.8 mCi/mmol) was then added to a final isotope concentration of 0.5 μCi/ml. After 1 h in the light (intensity 4,000 lx) at 25°C the cells were harvested, washed twice with 0.3 M sucrose-25 mM HEPES-KOH (pH 7.5)-1 mM $MgCl_2$, and resuspended in the same buffer. The chlorophyll concentration of the cell suspension was determined and an aliquot of the [14C]-labeled wild-type cells containing 2–3 mg chlorophyll was mixed with an aliquot of unlabeled mutant cells containing the same amount of chlorophyll. Thylakoid membranes were purified from the mixed cell population as described (14). Radioactivity was measured according to Mans and Novelli (36).

Measurements of Photochemical Reactions and Chlorophyll and Protein Concentrations

Partial photochemical reactions used to assess PS I and PS II activities were performed as described previously (13, 14). Fluorescence induction kinetics were measured with dark-adapted cells at room temperature according to Bennoun (8). Chlorophyll concentrations and chlorophyll a to b ratios were determined by the method of Arnon (4) and protein concentrations by Lowry's procedure (33) using bovine serum albumin as a standard.

Chemicals and Solutions

SDS (sequanal grade) was purchased from Pierce Chemical Co., Rockford, Ill.; Coomassie brilliant blue, bromphenol blue, methyl viologen (MV), dichlorophenol indophenol (DPIP), sodium ascorbate, N-2-hydroxy-ethylpiperazine-N'-2-ethanesulfonic acid (HEPES), ethylenediamine tetraacetic acid (EDTA), and dithiothreitol (DTT) from Sigma Chemical Co., St. Louis, Mo.; p-benzoquinone (PBQ), acrylamide, N,N'-methylene bisacrylamide from Eastman Organic Chemicals Div., Eastman Kodak Co., Rochester, N.Y.; lysozyme, chymotrypsinogen A, catalase, and β-galactosidase from Worthington Biochemical Corp., Freehold, N.J; and bovine serum albumin from Armour Pharmaceutical Co., Chicago, Ill. [1-[14C]]Sodium acetate (sp act 57.8 mCi/mmol) was obtained from New England
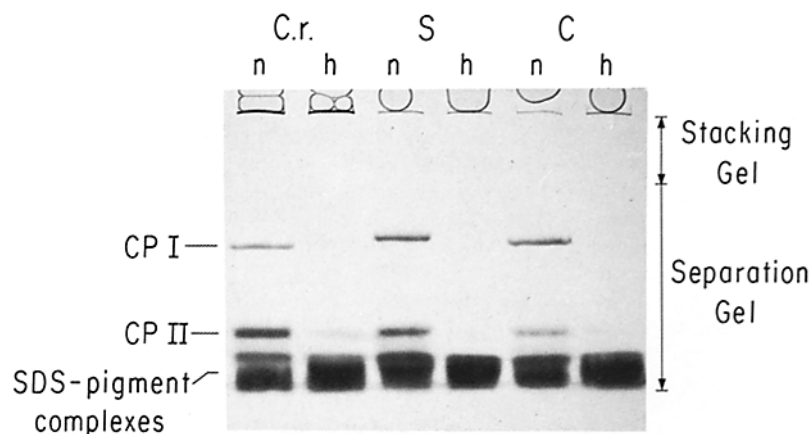
FIGURE 1 Chlorophyll-protein complexes of *C. reinhardtii* (*C.r.*), spinach (*S*), and Chinese cabbage (*C*). Each slot contained heated (*h*) or nonheated (*n*) thylakoid membranes equivalent to 20 µg chlorophyll.

Nuclear, Boston, Mass.; and Cronex 2DC medical X-ray film from Du Pont de Nemours and Co., Wilmington, Del. All stock solutions were passed through 1.2 µm Millipore filters (Millipore Corp., Bedford, Mass.). Stock solutions of acrylamide-*N*,*N*′-methylene bisacrylamide were decolorized with neutral activated charcoal (Sigma) before filtration.

RESULTS

Electrophoresis of Heated and Nonheated Membrane Samples from C. reinhardtii, Spinach, and Chinese Cabbage

In the SDS gel electrophoresis procedure which we reported recently (14), the membrane samples, after having been solubilized in the SDS-Na₂CO₃-DTT mixture, were incubated at 100°C for 1 min before electrophoresis. This heating step was taken to ensure inactivation of any possibly contaminating proteases (51) and to disperse aggregates of polypeptides into their monomeric forms. Under these conditions of sample preparation, some orange-colored materials, presumably carotenoids, were retained at the origin, whereas all the chlorophylls and the rest of the carotenoids migrated as a diffuse zone just behind the bromphenol blue tracking dye. Since the pigments did not form discrete bands coincident with any of the low molecular weight polypeptides, they were presumed to be free of proteins and complexed to SDS only. In the course of establishing the conditions for obtaining specific pigment-protein complexes from thylakoid membranes of *C. reinhardtii* we found that omission of the heating step led to the appearance of two discrete, chlorophyll-con-

taining bands between the origin and the "free" pigment zone (Fig. 1). This heating effect was not unique to *C. reinhardtii* since similar chlorophyll-containing bands could also be obtained in nonheated membranes of spinach and Chinese cabbage (Fig. 1).

The electrophoretic mobilities of the two chlorophyll-containing bands were considerably slower than that of the free pigment zone, indicating that the chlorophyll molecules were complexed with proteins. Furthermore, these two bands also appeared to be similar to the two chlorophyll-protein complexes described by other workers (2, 3, 21, 23-26, 35, 39, 46-48), and therefore they were tentatively designated as chlorophyll-protein complex I and II (CP I and CP II)[1] (Fig. 1).

To compare qualitatively the pigment compositions of CP I and CP II from *C. reinhardtii*, absorption spectra of gel bands containing the complexes and of the gel region containing the free pigment zone were taken (Fig. 2). The CP I spectrum has an absorption maximum in the red region of about 676 nm due to chlorophyll *a* but no peak or shoulder around 650 nm, indicating a deficiency in chlorophyll *b*. In contrast, the CP II band is greatly enriched in chlorophyll *b* as indicated by the equal absorption at 652 nm and 670 nm (Fig. 2 *b*). These spectral results are very similar to those reported for CP I and CP II of higher plants (31, 35, 47).

The chlorophyll-protein complexes shown in Fig. 1 were best observed when the tracking dye

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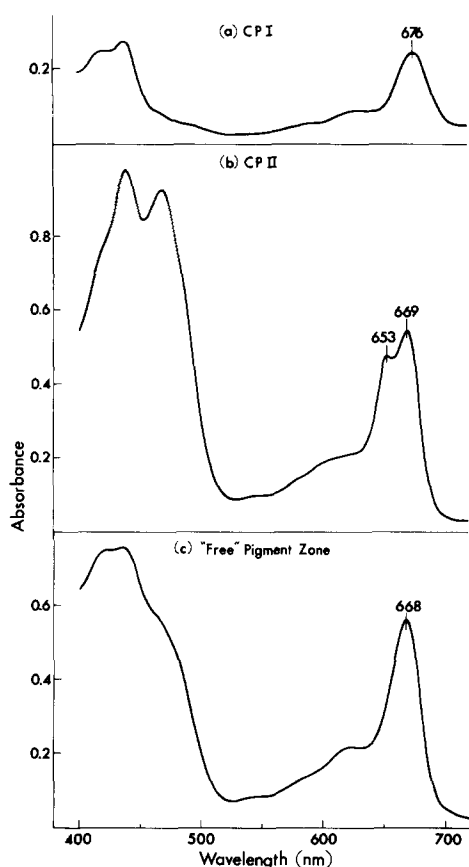[1] It should be noted that CP I and CP II most probably also contain SDS.

FIGURE 2 *In situ* absorption spectra of gel bands containing CP I, CP II, and the free pigment zone of *C. reinhardtii*. Nonheated, SDS-solubilized membranes of *C. reinhardtii* were subjected to short-term electrophoresis as described in Materials and Methods (see also Fig. 1). Absorption spectra were taken according to Thornber and Highkin (47). Gel bands containing CP I, CP II, or the free pigment zone were excised and aligned onto one side of the cuvette. The control cuvette contained a blank gel having the same acrylamide concentration. Absorption spectra were recorded from 400 nm to 720 nm with a Cary 14 recording spectrophotometer.

had migrated only 4-5 cm from the top of the separation gel. However, under these conditions of short-term electrophoresis the membrane polypeptides were not well resolved. To identify the membrane polypeptides associated with these complexes, electrophoresis was continued until the dye front had reached the bottom of the gel (20 cm from the top of the separation gel). We noticed that while CP I appeared relatively stable, CP II progressively lost its pigments during electrophoresis. In *C. reinhardtii*, CP II was finally re-

solved into two faint, pigmented bands coincident with polypeptides 11 and 12 (Fig. 3). Although the CP I band stained for protein, in the heat-treated sample the chlorophyll as well as the protein disappeared from the CP I gel region (Fig. 3). Instead, two new polypeptides (nos. 2 and 8).[2] which were not seen in the gel of nonheated samples, were observed in the gel of the heated sample (Fig. 3, slot 1 vs. slot 2). Since polypeptide 2 has a molecular weight of 66,000 and since there were no higher molecular weight bands except CP I with approximately the staining intensity of polypeptide 2, it seems likely that polypeptide 2 was derived from CP I by heating. Polypeptide 8 (mol wt 36,000), on the other hand, could be derived either from CP I or from other higher molecular weight polypeptides. Similar results were obtained with spinach and Chinese cabbage except that, in these cases, the heat-dissociation of CP I gave rise to a polypeptide of mol wt 64,000 and no polypeptide equivalent to polypeptide 8 was found (Figs. 3 and 4).

Both polypeptide 2 of *C. reinhardtii* and its counterpart in spinach are greatly diminished in membrane samples which had been extracted with a 2:1 (vol/vol) mixture of chloroform-methanol (Fig. 3). Fig. 3 shows that polypeptide 2, as well as several other membrane polypeptides of *C. reinhardtii*, are soluble in chloroform-methanol since they are present in the chloroform-methanol extract (Fig. 3, slot 6) and can be recovered from the latter by precipitation with diethyl ether (Fig. 3, slot 7). On the basis of the intensity of the Coomassie blue stain we estimate the recovery of polypeptide 2 by diethyl ether precipitation to be about 40%. Similar results were obtained for the equivalent polypeptide in spinach (data not shown). Although polypeptide 2 is greatly reduced in the membrane polypeptide profile of the precipitate after extraction with 90% acetone (Fig. 3, slot 3), it is also not found in the acetone extract of thylakoid membranes (Fig. 3, slot 4). Presumably, it is present in the 90% acetone precipitate but because of the acetone treatment, is retained on

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[2] In the nonheated membrane samples (Fig. 3, slot 1; Fig. 10, slot 1) there is a faint polypeptide band (unnumbered) with slightly slower electrophoretic mobility than polypeptide 8. This band is not related to polypeptide 8 since in SDS electrophoretograms which show good separation of the two polypeptides we found that the unnumbered polypeptide is present in roughly equal amounts in heated and nonheated samples.
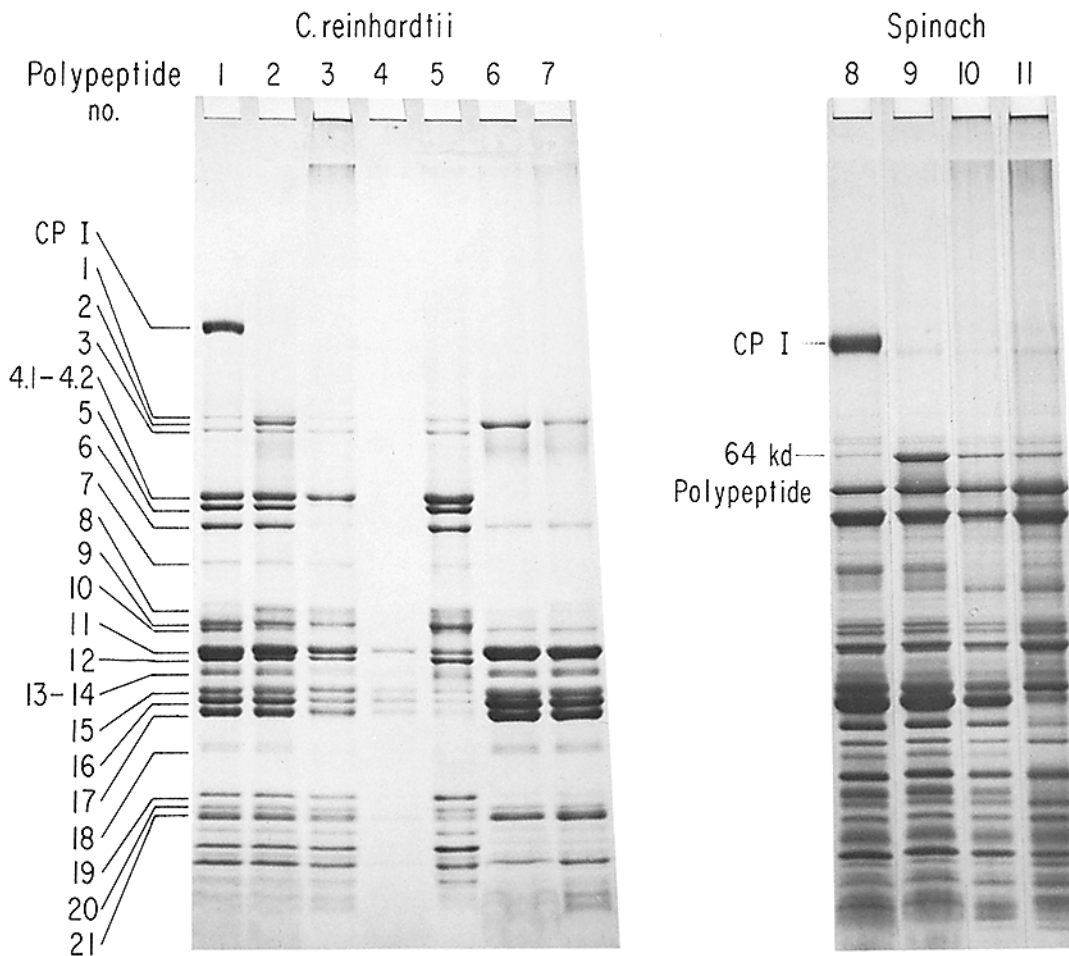
FIGURE 3 Electrophoretogram of chlorophyll-protein complexes and membrane polypeptides of C. reinhardtii and spinach. Slots 1-7 contained samples from the wild-type strain of C. reinhardtii and slots 8-11 contained samples from spinach. 1,8 = unextracted, nonheated membrane preparations (20 μg chlorophyll); 2,9 = unextracted, heated membrane preparations (20 μg chlorophyll); 3,10 = membrane preparations (30 μg chlorophyll) extracted with 90% acetone; 5,11 = membrane preparations (30 μg chlorophyll) extracted with a 2:1 (vol/vol) mixture of chloroform-methanol; 4 = 90% acetone extract of membranes (30 μg chlorophyll); 6 = chloroform-methanol extract of membranes (30 μg chlorophyll); 7 = precipitate recovered by addition of diethyl ether to a chloroform-methanol extract of membranes (30 μg chlorophyll). Membrane samples (slots 1-7) of C. reinhardtii were run in one slab gel whereas those of spinach (slots 8-11) were run in another slab gel. Samples in slots 3-7, 10, and 11 gave the same polypeptide pattern whether heated or not. The molecular weights of the membrane polypeptides were established with proteins of known molecular weights as described previously (14). 64 kd = 64 kilodaltons.

FIGURE 4 Electrophoretogram of isolated chlorophyll-protein complex 1 from C. reinhardtii, spinach, and Chinese cabbage. Slots 1, 7, and 11 contained unextracted, nonheated membrane preparations (25 μg chlorophyll). Slots 2, 8, and 12 contained unextracted, heated membrane preparations (25 μg chlorophyll). In slots 4, 10, and 14 gel strips containing CP I band from electrophoresis of unheated membrane preparation (50 μg chlorophyll) were cut out, placed in the slots, and re-electrophoresed without further treatment. In slots 3, 9, and 13 gel strips containing CP I band obtained as above were heated in boiling upper reservoir buffer (0.04 M boric acid-0.41 Tris-0.1% SDS, pH 8.65) for 15 s before re-electrophoresis. In slots 5 and 6, aliquots of CP I (2.05 μg chlorophyll) isolated from C. reinhardtii as described in Materials and Methods were electrophoresed with (slot 5) or without (slot 6) prior incubation at 100°C for 1 min. n, nonheated; h, heated; 64 kd, 64 kilodaltons. Samples in slots 1-14 were all run in the same gel.
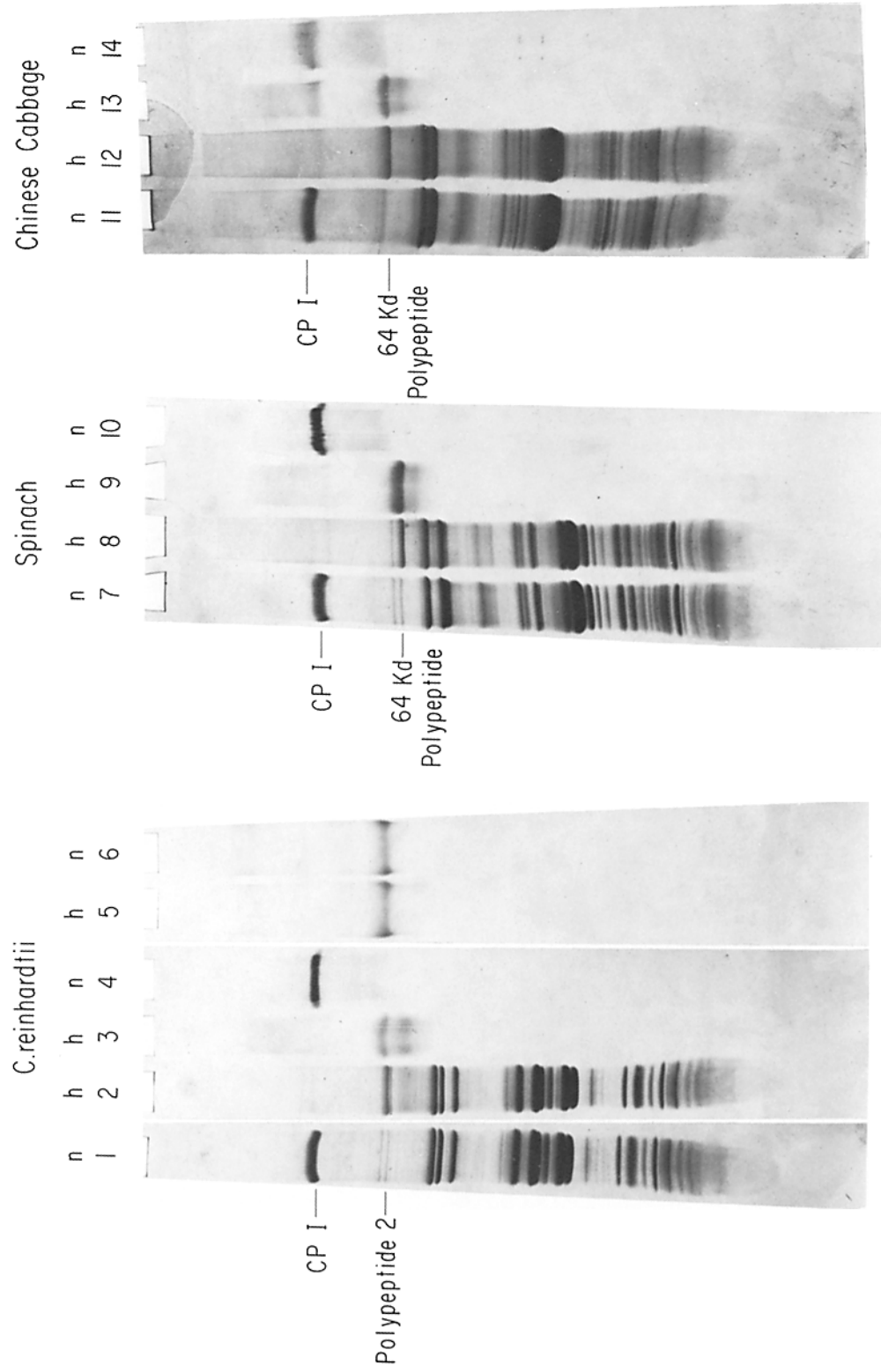
top of the stacking gel (Fig. 3, slot 3). Similar results were also obtained with the 64,000-dalton polypeptide of spinach (data not shown). It should be noted that several workers (18, 27, 30, 32) have used 90% acetone for the delipidation of thylakoid membranes.

Some Chemical Properties of CP I and the Identification of its Constituent Polypeptide

Results in the previous section suggest that the protein moiety of CP I consists of a polypeptide whose molecular weight is between 64,000 and 66,000. However, because of the large number of polypeptides in the membrane any minor polypeptides that were released from CP I by heat might have escaped detection. Furthermore, in the case of *C. reinhardtii* there is the possibility that polypeptide 8 may also be a constituent of the complex. In order to obtain direct information on this point, two identical gel strips containing CP I of *C. reinhardtii* were placed directly in adjacent slots and re-electrophoresed with and without prior heating. As shown in Fig. 4, CP I remained intact in the unheated sample but upon heating the pigments were released and moved just behind the tracking dye (slot 3), whereas the remaining protein moiety migrated as a single band in the position of polypeptide 2. Although no discrete bands were found elsewhere in the gel, there was always a slight smear of protein materials moving just in front of polypeptide 2.

In another series of experiments, aliquots of CP I, cut out from the preparative gel and eluted as described in Materials and Methods, were electrophoresed in the same slab gel used for the above experiments. Only *one* polypeptide with mobility similar to that of polypeptide 2 was obtained in both the heated and nonheated sample (Fig. 4), indicating that the complex was already dissociated under the isolation conditions used. From these results we conclude that the protein moiety of CP I from *C. reinhardtii* consists of only the 66,000-dalton species (polypeptide 2) and polypeptide 8 is not a constituent of the complex. The nature of the smear obtained only in the heated gel strip (Fig. 4, slot 3) and in the heated thylakoid membrane preparation (Fig. 4, slot 2) is not clear. Since this smear was not seen with isolated CP I (Fig. 4, slots 5 and 6; Fig. 5), we suggest that it is due to degradation of polypeptide 2.

Similar results were obtained with re-electrophoresis of gel strips containing CP I's of spinach

and Chinese cabbage (Fig. 4). In both plants, the heat-dissociation of CP I resulted in free chlorophyll and a polypeptide of mol wt 64,000. The CP I of Chinese cabbage, however, was less stable and was partially dissociated even without heating.

To confirm the results obtained with organic solvent extractions of thylakoid membranes from *C. reinhardtii*, isolated CP I was extracted with 90% acetone as well as a 2:1 (vol/vol) mixture of chloroform-methanol. Fig. 5 shows that the constituent polypeptide of CP I is not extracted by
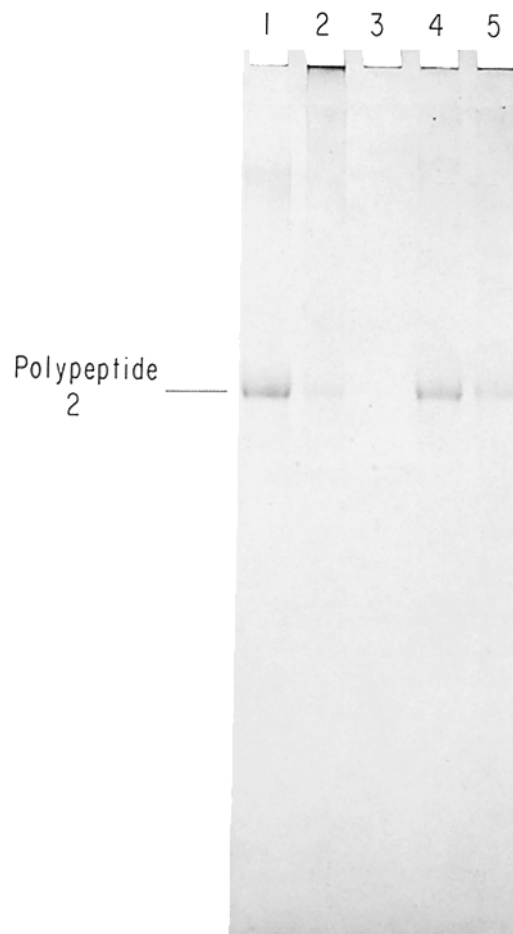

FIGURE 5 Effects of organic solvent extraction on the constituent polypeptide of CP I from *C. reinhardtii*. (1) Unextracted CP I; (2) 90% acetone precipitate of CP I; (3) 90% acetone extract; (4) chloroform-methanol (2:1; vol/vol) extract of CP I; (5) diethyl ether precipitate of (4). All slots contained equivalent amounts (2.8 μg chlorophyll) of CP I. Procedures for extractions with organic solvents were similar to those used for thylakoid membranes as detailed in Materials and Methods.

TABLE I

Some Chemical Properties of Thylakoid Membranes and CP I from C. reinhardtii

	chla/chlb (wt/wt)	protein/chl (wt/wt)	% total chl	% total protein
Thylakoid membrane	2.26	5	100	100
	(2.10–2.44)	(4.8–5.2)		
CP I	5.27	8.8	5	8.8
	(5.06–5.57)	(7.3–10.5)	(4.5–5.6)	(7.3–10.5)

Thylakoid membranes and CP I were isolated from *C. reinhardtii* as described in Materials and Methods. Each value is the mean of three independent experiments. Values given within brackets indicate the range of variations among experiments.

chl, chlorophyll.

90% acetone (slot 3) but as a result of the acetone treatment about 50% of the Coomassie blue-stained material was retained at the origin (slot 2) leading to a decrease in the amount of polypeptide 2 in the separation gel. In contrast, this polypeptide is completely soluble in chloroform-methanol (Fig. 5, slot 4) since no precipitate was obtained upon addition of the organic solvent mixture to CP I. Approximately 50% of polypeptide 2 in the chloroform-methanol extract could be recovered by precipitation with diethyl ether (Fig. 5, slot 5). These results show conclusively that the delipidated polypeptide 2 obtained by organic solvent extraction of CP I has the same electrophoretic mobility as the polypeptide 2 liberated by heat treatment of the same complex.

Table I gives some of the chemical properties of CP I from *C. reinhardtii* isolated and eluted as described in Materials and Methods. In general, the chlorophyll *a* to chlorophyll *b* (wt/wt) ratio and the protein to chlorophyll (wt/wt) ratio are very similar to those obtained with comparable complexes of higher plants (45, 46, 48). Using this value and assuming that the constituent polypeptide has a molecular weight of 66,000 we can calculate that there are approximately eight to nine chlorophyll molecules per polypeptide chain.

For an accurate estimation of the molecular weights of CP I and its constituent polypeptide by SDS gel electrophoresis, it is necessary to ascertain that their free electrophoretic mobilities (6, 12, 37) are close to those of the protein standards (β-galactosidase, bovine serum albumin, catalase, chymotrypsinogen A, and lysozyme) used for molecular weight calibration.

Ferguson plots of the relative mobilities of CP I, polypeptide 2, and protein standards indicated that polypeptide 2 and all but one marker protein had nearly equal free mobilities (y intercept, Fig. 6).
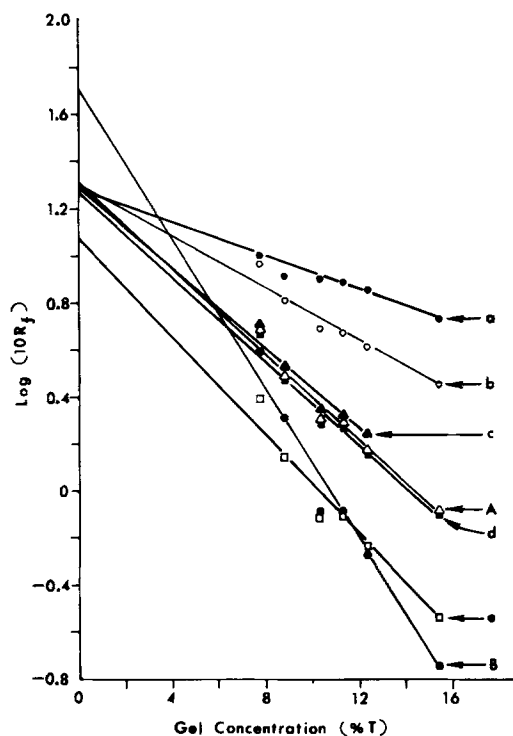

Figure 6 Plots of log (10 R_f) vs. T for CP I, polypeptide 2, and molecular weight standards. (A) polypeptide 2; (B) CP I; (a) lysozyme; (b) chymotrypsinogen A; (c) catalase; (d) bovine serum albumin; (e) β-galactosidase. Experiments were done as described in Materials and Methods. T refers to the percent (wt/vol) concentration of acrylamide plus N,N'-methylene bisacrylamide.

The free mobility of CP I, however, was more than 30% larger than that of the standards. These results demonstrate that CP I behaves anomalously in SDS gels and that its apparent molecular weight is therefore a function of acrylamide concentration. This anomalous migration rate is remi-

	Rate of reaction (μmol O_2 evolved or consumed/mg chlorophyll/h)			
Strain	PBQ-Hill	Ferri-cyanide-Hill	MV-Hill	DPIPH$_2$ $\rightarrow$ MV
Wild-type	210	90	75	495
F1	74	77	<3	20
F14	64	100	8	33

The PBQ-Hill reaction was carried out in a reaction mixture containing whole cells, 15 μg chlorophyll/ml; potassium phosphate buffer (pH 7.0), 10 mM; and p-benzoquinone, 2 mM. For the ferricyanide-Hill reaction the mixture contained chloroplast fragments, 30 μg chlorophyll/ml; HEPES-KOH (pH 7.0), 40 mM; KCl, 20 mM; MgCl$_2$, 2.5 mM; NH$_4$Cl, 2 mM; and potassium ferricyanide, 5 mM. The reaction mixture for the MV-Hill reaction contained chloroplast fragments, 30 μg chlorophyll/ml; HEPES-KOH (pH 7.0), 40 mM; KCl, 20 mM; MgCl$_2$, 2.5 mM; NH$_4$Cl, 2 mM; MV, 0.2 mM; and NaN$_3$, 1 mM. The photoreduction of MV with DPIP-ascorbate couple as the electron donor was performed with a reaction mixture containing chloroplast fragments, 7.5-15 μg chlorophyll/ml, HEPES-KOH (pH 7.0), 40 mM; KCl, 20 mM; MgCl$_2$, 2.5 mM; NH$_4$Cl, 2 mM; MV, 0.2 mM; DPIP, 0.1 mM; sodium ascorbate, 3 mM; NaN$_3$, 1 mM; and DCMU, 10 μM.

niscent of that observed with the major glycoprotein from the human erythrocyte membrane (6) and emphasizes the need of determining the molecular weight by techniques (44) other than SDS gel electrophoresis.

Chlorophyll-Protein Complexes, Thylakoid Membrane Polypeptides, and Photosynthetic Properties of C. reinhardtii Mutants Deficient in PS I Activity

To determine the relationship between CP I and photosystem I in *C. reinhardtii*, we examined the chlorophyll-protein complexes and thylakoid membrane polypeptides in two PS I mutants, F1 and F14. These mutants were previously shown to lack P700 (13, 15). The data in Table II confirm earlier reports (13, 15) that the two mutants are greatly deficient in PS I activity but are able to perform PS II reactions.

Because of the lower rates of the p-benzoquinone (PBQ)-Hill reaction in these mutants com-

pared to the WT (Table II), fluorescence induction kinetics were measured to ensure that the PS II reaction centers were not affected in these mutants. Similar results were obtained for F1 and F14 but only those of F14 will be presented here.

The fluorescence induction kinetics of WT and F14 were first studied in the absence of DCMU. WT cells show a biphasic fluorescence rise followed by a decline to a steady-state level (Fig. 7). The fluorescence decline is attributed to the reoxidation of the plastoquinone pool by PS I (20). Cells of F14, on the other hand, show only the biphasic fluorescence rise with no subsequent decline in fluorescence yield (Fig. 7). These results demonstrate that electron flow from H$_2$O through PS II to the plastoquinone pool is normal but the reoxidation of reduced plastoquinone is blocked. The area circumscribed by the fluorescence rise curve and the maximum level of fluorescence represents the amount of oxidizing equivalents between PS II reaction center and the mutant block point (Fig. 8 a).

The above experiments were repeated in the presence of DCMU which inhibits the reoxidation of Q, the primary electron acceptor of PS II (17). In the presence of this inhibitor, the area circumscribed by the fluorescence rise curve and the maximum level of fluorescence (Fig. 8 b) represents the number of active PS II reaction centers (8, 14). With this method it was found that F14 has the same number of active PS II reaction centers as WT (Fig. 8 b and c). Furthermore, by comparing the fluorescence rise curve in the presence and absence of DCMU (Fig. 8 b and a) we estimated that there are approximately 14-15 electron equivalents between PS II and the block point in F14. This finding is consistent with the fact that electron flow in F14 is blocked at the level of P700 (13). These results also show that the slower rate of the PBQ-Hill reaction in F1 and F14 is not due to a deficiency of PS II reaction centers but is probably
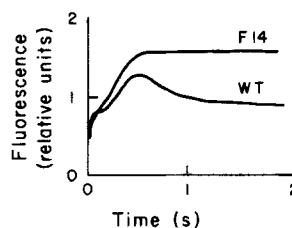

FIGURE 7 Fluorescence induction kinetics of wild-type and F14. Experiments were done with dark-adapted cells as described in Materials and Methods.

due to the fact that in WT cells PBQ is reduced at a faster rate by PS I as compared to PS II.

Fig. 9 shows that both F1 and F14 lack the CP I band but still retain the CP II band and the free pigment zone. Comparison of the mutant membrane polypeptides with those of the WT revealed that polypeptide 2 is completely missing in heated membrane preparations of mutants (Fig. 10).
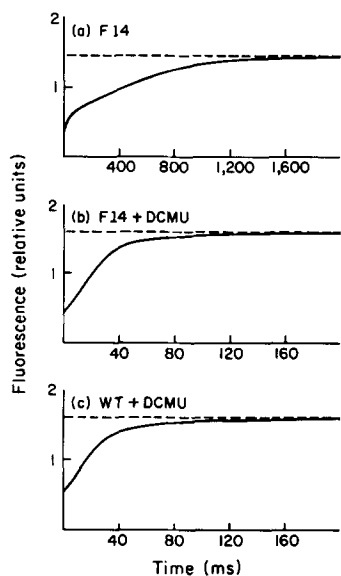

FIGURE 8   Fluorescence induction kinetics of wild-type and F14 in the presence of DCMU. Experiments were done with dark-adapted cells as described in Materials and Methods. (b) and (c) contained 10 μM DCMU. Note that the time scale in (a) is 10 times that in (b).

Furthermore, polypeptide 2 is also absent from chloroform-methanol (2:1, vol/vol) extracts of mutant membranes although it is present in the corresponding WT extracts (Fig. 11). The absence of polypeptide 2 in the mutants is reinforced by results obtained with mixing experiments in which thylakoid membranes were isolated from a mixture of ^{14}C-labeled WT and unlabeled mutant cells. Fig. 10 shows that both CP I and polypeptide 2 reappeared in thylakoid membranes prepared from a mixture of F1 and ^{14}C-labeled WT (slots 4, 9) as well as a mixture of F14 and ^{14}C-labeled WT (slots 5, 10), as assessed by both polypeptide stain and autoradiography. In some preparations of F1 and F14, there is an extra polypeptide migrating just ahead of polypeptide 7, and some preparations of F14 have slightly diminished amounts of polypeptides 4.1 and 4.2 relative to other polypeptides. The recovery of polypeptide 7 in WT as well as the mutants is highly variable (cf. Fig. 3, slot 1; Fig. 4, slot 1; Fig. 10, slot 1) as has been noted before (14). Since F1 and F14 lack P700 (13, 15) these results strongly suggest that CP I and polypeptide 2 are essential for the activity of the PS I reaction center in C. reinhardtii.

When the polypeptide profiles of the heated and nonheated samples of the mutants were compared it is clear that, in spite of the absence of CP I, polypeptide 8 could be obtained in the heated samples (Fig. 10).[2] This observation supports the earlier conclusion that polypeptide 8 is not a constituent of CP I and must be derived from the heat-induced dissociation of some other high molecular weight band in the nonheated sample.
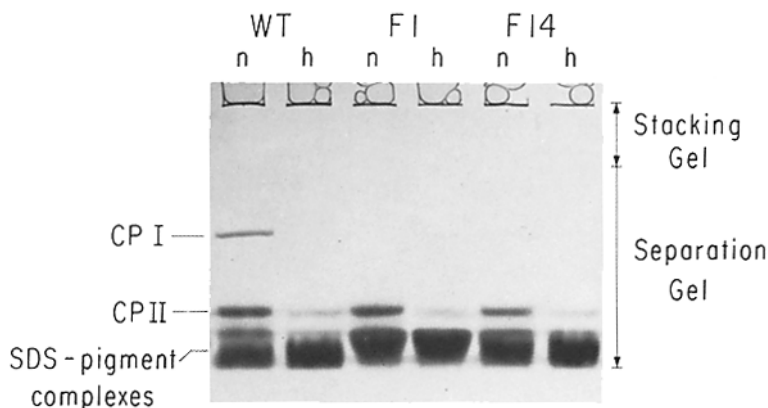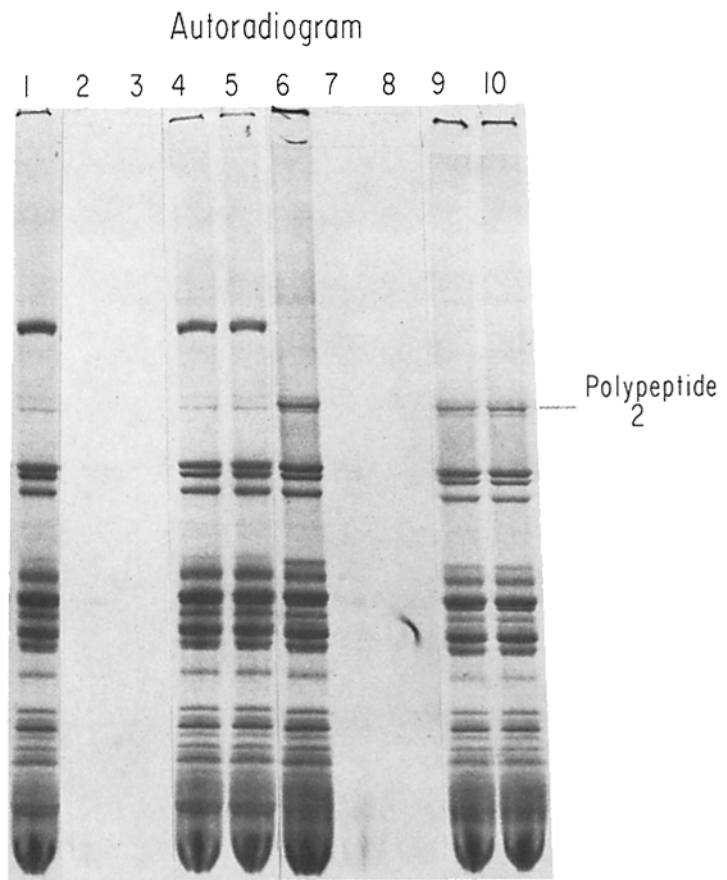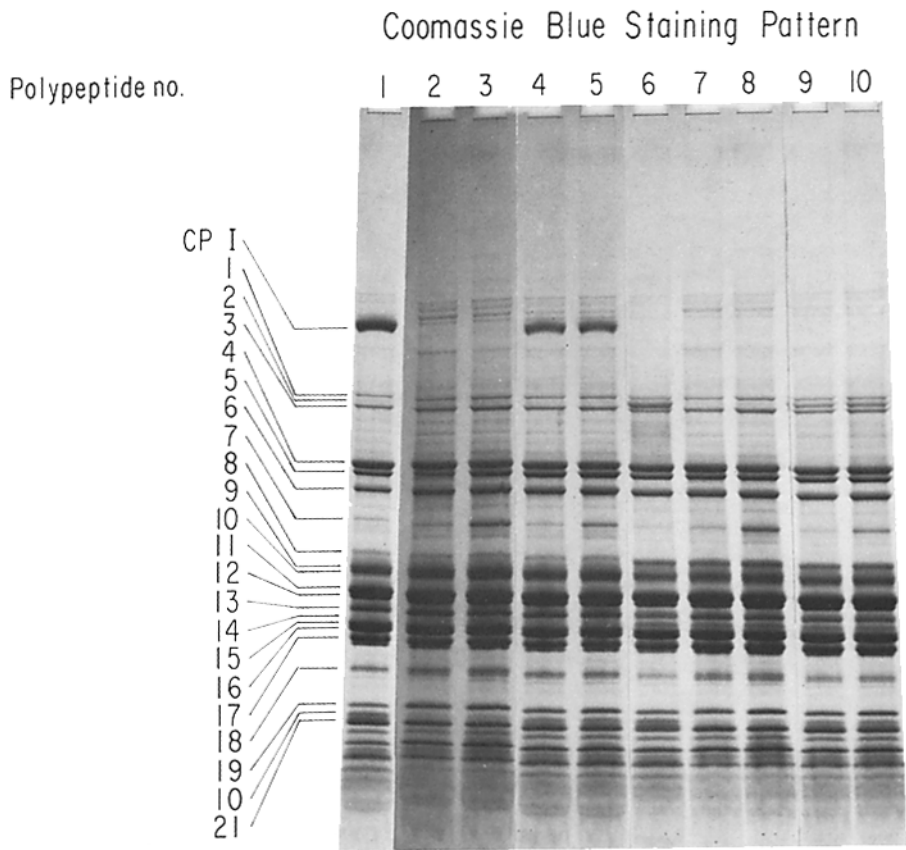

FIGURE 9   Chlorophyll-protein complexes in wild-type and mutant strains of C. reinhardtii. Each slot contained heated (h) and nonheated (n) thylakoid membrane preparations equivalent to 20 μg chlorophyll.

Coomassie Blue Staining Pattern

Polypeptide no.

1 2 3 4 5 6 7 8 9 10

CP I
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
10
21

Autoradiogram

1 2 3 4 5 6 7 8 9 10

Polypeptide 2

DISCUSSION

Although the existence of CP I has been known for some time (39, 46) the nature of its protein constituents has not been unambiguously estab-

lished. Thornber (45) proposed that CP I is made up of four identical subunits, each of which has a 35,000-dalton polypeptide and 4–5 chlorophyll molecules, thus giving a molecular weight of 150,000 for the entire complex. This value was
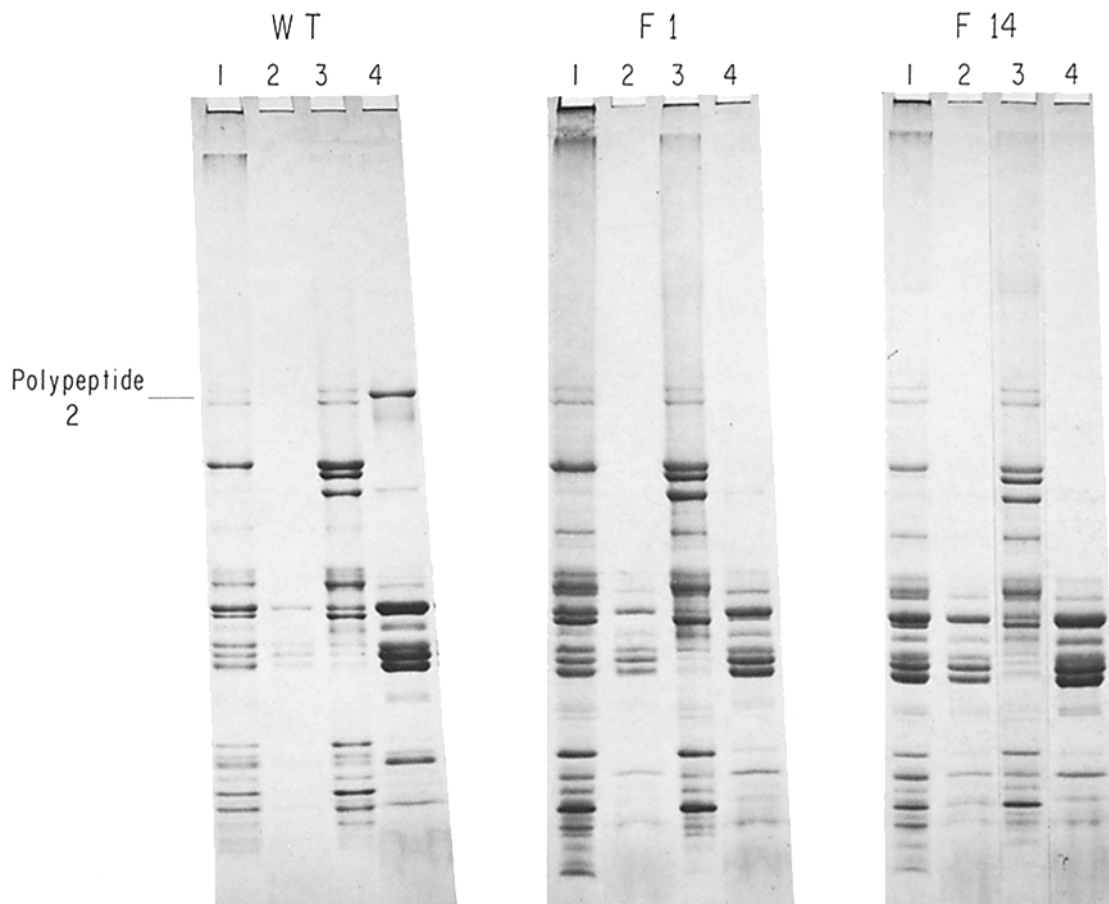
W T   F 1   F 14

1 2 3 4   1 2 3 4   1 2 3 4

Polypeptide 2

FIGURE 11   Effects of organic solvent extraction on the thylakoid membrane polypeptides of wild-type and mutant strains. 1, membrane samples (30 µg chlorophyll) extracted with 90% acetone; 2, 90% acetone extract; 3, membrane samples (30 µg chlorophyll) extracted with a 2:1 (vol/vol) mixture of chloroform-methanol; 4, chloroform-methanol extract. Equivalent amount of membrane sample was loaded in each slot. For other details see Materials and Methods.

FIGURE 10   Electrophoretogram and autoradiogram of thylakoid membranes prepared from wild-type, F1, F14, a mixture of F1 and ^{14}C-labeled wild-type, and a mixture of F14 and ^{14}C-labeled wild-type. Membrane samples in slots 1–5 were not heated, whereas those in slots 6–10 were incubated at 100°C for 1 min after solubilization in SDS. 1,6 = ^{14}C-labeled wild-type, 20 µg chlorophyll (13,800 cpm); 2,7 = F1, 20 µg chlorophyll; 3,8 = F14, 20 µg chlorophyll; 4,9 = mixture of F1 and ^{14}C-labeled wild-type, 20 µg chlorophyll (6,660 cpm); 5,10 = mixture of F14 and ^{14}C-labeled wild-type, 20 µg chlorophyll (6,760 cpm). All samples were run in the same slab gel. The gel was dried down on a piece of Whatman 3MM chromatography paper and the dried gel placed in contact with an X-ray film for ~4 wk. For other experimental details see Materials and Methods.

later modified to 100,000 by Kung and Thornber (31). Anderson and Levine (2) reported a similar mol wt value (110,000) for the *C. reinhardtii* CP I but they found that this complex has two polypeptides around 60,000 daltons. The results reported in this paper show that the protein moiety of CP I from *C. reinhardtii* consists of one polypeptide (polypeptide 2; mol wt = 66,000). This polypeptide can be obtained either by heat treatment of CP I or by delipidation of the complex with a 2:1 (vol/vol) mixture of chloroform-methanol followed by precipitation with diethyl ether. In both cases, the polypeptides obtained have the same electrophoretic mobility in SDS gels. Our attempts to determine the molecular weight of CP I by SDS gel electrophoresis were unsuccessful because the complex shows an anomalous migration rate. Its molecular weight, therefore, cannot be measured by calibrated SDS gels, as was attempted by Kung and Thornber (31) and Anderson and Levine (2). A similar anomalous behavior on SDS gels has been reported for subunit I of yeast cytochrome oxidase (40).

Chemical analysis shows that in CP I each polypeptide 2 is associated with eight to nine chlorophyll molecules. This number represents only those chlorophyll molecules that are tightly bound to the polypeptide in the presence of high concentrations of SDS (SDS-chlorophyll molar ratio of 62.5:1). It is conceivable that additional chlorophyll molecules may be associated with the polypeptide in vivo. Chlorophyll is an amphipathic lipid since it has a long hydrophobic tail (phytol) and a polar head group (porphyrin ring) (10). It seems reasonable to assume that the chlorophyll molecules are bound to the polypeptide primarily by hydrophobic forces involving the phytol tails and hydrophobic sites on the polypeptide. The same hydrophobic sites are probably implicated in the binding of SDS. If this were the case, CP I should bind less SDS on a weight basis compared to the other membrane polypeptides and this deficiency in SDS binding may be one of the several factors responsible for its anomalous behavior in SDS gels.

In heated membranes and heated gel bands containing CP I, there is always a slight smear of protein material migrating ahead of polypeptide 2 (cf. Fig. 3). Two lines of evidence suggest that this smear is due to a degradation of polypeptide 2: (a) the intensity of the smear; as assessed by Coomassie blue staining, varied from one experiment to another (see Fig. 3, slot 2; Fig. 4, slots 2 and 3; Fig. 10, slot 6); and (b) re-electrophoresis of CP I eluted from preparative gels gave only one band, corresponding to polypeptide 2 (Figs. 3 and 5). This tendency of polypeptide 2 to undergo partial degradation may account for the observation of Anderson and Levine (2) that *C. reinhardtii* CP I has two polypeptides with very similar molecular weights.

Klein and Vernon (30) detected a 63,000-dalton polypeptide in subchloroplast fragments, TSF I and TSF IIa, which are enriched in chlorophyll *a*, and concluded that this polypeptide is involved in binding chlorophyll *a*. The 63,000-dalton polypeptide is missing from HP 700 particles which are highly enriched in P700 (30), and therefore appears to be different from the constituent polypeptide of CP I. Furthermore, their membrane preparations were extracted with acetone before electrophoresis. We found that in samples extracted with either 90% acetone or a chloroform-methanol mixture (2:1, vol/vol) polypeptide 2 or its equivalent is missing or present only in small amounts in the SDS electrophoretograms (Fig. 3). Similarly, we believe that the CP I constituent polypeptide is absent or is present in reduced amounts in SDS electrophoretograms of PS I-enriched fractions prepared by Nolan and Park (38), since these authors delipidated their preparations by at least seven extractions with a 1:2 (vol/vol) mixture of chloroform-methanol (cf. Fig. 1 of Henriques et al. [22] and Fig. 3 of this paper).

Dietrich and Thornber (16) showed that CP I from *P. luridum* is highly enriched in P700, the reaction center component of PS I. Since light-harvesting chlorophyll molecules are also present in this complex, these workers (16) concluded that CP I is a P700-chlorophyll *a*-protein complex. Recently, Shiozawa et al. (41) have purified a photochemically active P700-chlorophyll *a*-protein complex from Triton X-100 extract of higher plant chloroplasts and showed that CP I can be derived from this complex by SDS treatment. Our results support and extend the conclusions of Dietrich and Thornber (16) and Shiozawa et al. (41). We found that CP I from *C. reinhardtii* and two higher plants contains a polypeptide of mol wt 64,000–66,000 in association with chlorophylls. Both CP I and its constituent polypeptide (polypeptide 2) are missing from the thylakoid membranes of two *C. reinhardtii* mutant strains, F1 and F14, which lack light-induced P700 signal (13, 15)

but have the same number of PS II reaction centers as the WT (cf. Results). The absence of polypeptide 2 from the mutant membranes has been demonstrated by three independent experiments: (a) polypeptide 2 is missing from heated membrane preparations of mutants but reappears in membranes prepared from a mixture of ^{14}C-labeled WT and unlabeled mutant cells (Fig. 10); (b) chloroform-methanol (2:1, vol/vol) extract of WT membranes contains polypeptide 2 but the corresponding extracts of mutant membranes do not (Fig. 11); (c) rabbit antiserum directed against CP I forms a precipitin line with heated, SDS-solubilized membranes of WT in double-diffusion assays but gives no reaction with similar membrane preparations of the mutants (unpublished results). The results obtained with the PS I mutants, taken together with those of Dietrich and Thornber (16) and Shiozawa et al. (41), strongly suggest that P700 is associated with polypeptide 2 or its higher plant equivalent.

In addition to CP I and polypeptide 2, F1 and F14 are also greatly deficient in two low molecular weight polypeptides (nos. 20 and 21), but since these polypeptides are not part of CP I, as shown by the results presented in Figs. 4 and 5, they are probably not involved in the binding of P700. However, polypeptides 20 and 21 may be close neighbors of CP I in the membrane and their insertion into the latter may be essential for the incorporation of polypeptide 2 (or CP I) and vice versa. Although there is a possibility that these low molecular weight polypeptides may be required for PS I activity, our results with the PS I mutants clearly show that they are not required for the transfer of electrons from H_2O to the plastoquinone pool. The CP I band has previously been reported to be missing in a P700-less mutant of *Scenedesmus* (21) and a plastome mutant of *Antirrhinum majus* which is impaired in PS I reaction (23). Whether or not these mutants are also deficient in the constituent polypeptide of the complex remains to be established.

Genetic analyses of F1 and F14 show that they segregate in a Mendelian fashion (unpublished observations) and, presumably, the mutations are located in the nucleus. It is not known, however, whether they are allelic or not. Work is in progress to determine whether these mutations are coding for structural or regulatory components.

After the completion of the work described in this paper, Bengis and Nelson (7) showed that in

Swiss chard chloroplasts the P700 signal is associated with a 70,000-dalton polypeptide and Machold (34) reported that the protein moiety of *Vicia faba* CPI consists of one polypeptide of mol wt 69,400. Our results concur with the observations of these workers.

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