Lithium dodecyl sulfate/polyacrylamide gel electrophoresis of thylakoid membranes at 4°C: Characterizations of two additional chlorophyll a-protein complexes

(*Chlamydomonas reinhardtii*/Pisum sativum/photosystem II reaction centers/absorption spectra/immunochemical techniques)

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ABSTRACT Lithium dodecyl sulfate/polyacrylamide gel electrophoresis of *Chlamydomonas reinhardtii* thylakoid membranes at room temperature gave two chlorophyll-protein complexes, CP I and CP II, as had been reported previously. However, when the electrophoresis was performed at 4°C, there was an increase in the amount of chlorophyll associated with CP I and CP II, and in addition, three other chlorophyll-protein complexes appeared. Two of these complexes, designated CP III and CP IV, were characterized and found to be similar in their compositions. Each complex contains four to five molecules of chlorophyll a, one molecule of β -carotene, and one polypeptide chain. The apoprotein of CP III is polypeptide 5 (M_r 50,000) and that of CP IV is polypeptide 6 (M_r 47,000); the two polypeptides are structurally unrelated. Chlorophyll-protein complexes similar to *C. reinhardtii* CP III and CP IV were also detected in higher plants (e.g., *Pisum sativum*). The apoproteins of the higher plant complexes are immunochemically related to those of the *C. reinhardtii* complexes, as shown by crossed immunoelectrophoresis. Absorption spectra of CP III and CP IV at -196°C revealed a component at 682 nm. This observation, together with the previous results on photosystem II mutants [Chua, N.-H. & Bennoun, P. (1975) *Proc. Natl. Acad. Sci. USA* 72, 2175-2179], provides indirect evidence that CP III and CP IV may be involved in the primary photochemistry of photosystem II.

Sodium dodecyl sulfate (NaDodSO₄)/polyacrylamide gel electrophoresis of thylakoid membranes at room temperature yields three pigmented bands (1, 2). Two of these bands have been shown to be chlorophyll-protein (CP) complexes and are designated CP I and CP II, whereas the remaining band consists of free pigments in mixed micelles with NaDodSO₄ (1, 2). CP I accounts for about 10% of the total chlorophyll, has only chlorophyll and β -carotene as pigments, and contains P700, the photochemical chlorophyll of photosystem I (cf. refs. 3 and 4). In contrast, CP II represents about 50% of the total chlorophyll and contains both chlorophyll a and b in addition to carotenoids but lacks photochemical activity (cf. refs. 3 and 4). It is believed to function in harvesting light energy for photosystems I and II (5).

The presence of the free pigment zone raises the possibility that some or all of these pigments may be associated *in vivo* with thylakoid membrane proteins as chlorophyll-protein complexes that are labile under the usual conditions of membrane solubilization and subsequent NaDodSO₄ gel electrophoresis at room temperature. In this paper, we describe a procedure in which thylakoid membranes are solubilized in lithium dodecyl sulfate (LiDodSO₄) and analyzed by polyacrylamide gel electrophoresis in the presence of the same detergent at 4°C. This new procedure minimizes dissociations of chlorophyll-protein complexes during electrophoresis and its use has led to the de-

tection of two additional chlorophyll a-protein complexes whose properties are described in detail here.

MATERIALS AND METHODS

Chlamydomonas reinhardtii (strain 137c) was grown in Tris/acetate/phosphate medium at 25°C under continuous illumination (4000 lux) (6). Thylakoid membranes were purified from exponential-phase cultures by the flotation procedure described previously (7), except that 1 mM phenylmethylsulfonyl fluoride was included in all the buffers used. *Pisum sativum* (var. Alaska) was grown in a greenhouse. About 60 g of leaves in 100 ml of 0.3 M sucrose/25 mM Hepes-KOH (pH 7.5)/1 mM MgCl₂/1 mM phenylmethylsulfonyl fluoride was homogenized in a Waring blender for 30 sec. The homogenate was passed through four layers of Miracloth and the filtrate was centrifuged at $3000 \times g_{\max}$ for 10 min. The pellet was dispersed by homogenization with a Potter homogenizer in 0.3 M sucrose/25 mM Hepes-KOH (pH 7.5)/10 mM EDTA/1 mM phenylmethylsulfonyl fluoride and the homogenate was centrifuged at $50,000 \times g_{\max}$ for 10 min. Thylakoid membranes were purified from the $50,000 \times g_{\max}$ pellet by flotation (7).

Samples for electrophoresis were prepared by solubilization of purified thylakoid membranes at 4°C in 50 mM Na₂CO₃/50 mM dithiothreitol/12% sucrose/2% LiDodSO₄ to a final chlorophyll concentration of 1 mg/ml and a chlorophyll/LiDodSO₄ (wt/wt) ratio of 1:20. Polyacrylamide gel electrophoresis was carried out according to Laemmli (8) with the following modifications: NaDodSO₄ was omitted from the stacking gel, the resolving gel, and the upper and lower reservoir buffers; the stacking gel (1 mm $\times$ 20 mm $\times$ 300 mm) contained 5% acrylamide whereas the resolving gel (1 mm $\times$ 200 mm $\times$ 300 mm) contained a 7.5-15% linear gradient of acrylamide stabilized by a 5-17.5% sucrose gradient (7); and LiDodSO₄ and EDTA were added to the upper reservoir buffer to final concentrations of 0.1% and 1 mM, respectively. Electrophoresis was performed at 4°C in the dark at a constant power setting of 4 W/mm width for approximately 12 hr.

Monospecific antibodies to *C. reinhardtii* polypeptides 5 and 6 were prepared as described previously (9). IgG fractions were purified from the antisera according to Harboe and Ingild (10). Ouchterlony double diffusion assays were performed at room temperature for 24 hr in 1% (wt/vol) agarose containing 50 mM Tris-HCl (pH 8.6), 0.1 NaCl, and 1% Triton X-100 (9). Two-dimensional crossed immunoelectrophoresis was carried out by a modification (9) of the method described by Converse and Papermaster (11).

Absorption spectra at 25°C and -196°C were recorded with an Aminco DW 2 spectrophotometer. Carotenoid composition of chlorophyll-protein complexes was analyzed by thin-layer

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Abbreviations: NaDodSO₄, sodium dodecyl sulfate; LiDodSO₄, lithium dodecyl sulfate; CP I-IV, chlorophyll-protein complexes I-IV.

chromatography (12). Chlorophyll concentrations were measured according to Arnon (13) and protein concentrations according to Lowry *et al.* (14), with bovine serum albumin as a standard.

LiDodSO₄ was purchased from Gallard-Schlesinger, Carle Place, NY. All other chemicals were of reagent grade.

RESULTS

It has been reported (2) that NaDodSO₄ gel electrophoresis of unheated thylakoid membranes of *C. reinhardtii* gives two chlorophyll-protein complexes, designated CP I and CP II. Whereas CP I is stable, CP II progressively loses its pigments during electrophoresis at room temperature (15). Because the interaction between chlorophyll and protein is expected to be predominantly hydrophobic, we reasoned that the stability of the chlorophyll-protein complex may be enhanced at low temperatures. To examine this possibility, thylakoid membranes of *C. reinhardtii* were solubilized in LiDodSO₄ at 4°C and the electrophoresis was carried out at the same temperature. In these experiments, LiDodSO₄ was used instead of NaDodSO₄ because the latter precipitates in the cold. Fig. 1 shows that there is indeed a dramatic increase in the amounts of chlorophyll associated with CP II at 4°C (slot 2) as compared to 25°C (slot 1). Furthermore, in the low temperature sample, three new chlorophyll-protein complexes appear between CP I and CP II. We have designated these complexes as CP III, CP IV, and CP V, in the order of increasing electrophoretic mobility in the 7.5–15% gradient gel system.

As a first step toward detailed characterization of CP III and CP IV, we optimized the yield of the two complexes by varying systematically the concentration of LiDodSO₄ used in the

solubilization of thylakoid membranes and in the electrophoresis buffer. We found that the best results were obtained when 0.1% and 2% LiDodSO₄ were used in the upper reservoir buffer and in the solubilization of thylakoid membranes (1 mg of chlorophyll per ml of 50 mM Na₂CO₃/50 mM dithiothreitol), respectively. Lower concentrations of the detergent in both cases led to a trailing of CP II that masked the other complexes.

To isolate and purify large amounts of the two complexes for analysis, a thylakoid membrane sample (4 mg of chlorophyll) was electrophoresed in a preparative slab gel (2 × 250 × 300 mm). After electrophoresis, the gel band containing the appropriate complex was excised, homogenized in 50 mM Tris/0.4 M glycine/1 mM phenylmethylsulfonyl fluoride, and the gel pieces in the homogenate were removed by centrifugation at 50,000 × *g*_{max} for 20 min. The resulting supernatant, which contained the eluted chlorophyll-protein complex, was then analyzed by LiDodSO₄/polyacrylamide gel electrophoresis at 4°C. Fig. 1 shows that CP III (slot 3) and CP IV (slot 5) isolated by this procedure are still contaminated by small amounts of CP II. The latter contaminant, however, could be eliminated by a second preparative slab gel electrophoresis to yield pure CP III (Fig. 1, slot 4) and CP IV (Fig. 1, slot 6).

To identify the polypeptide constituent(s) of the purified CP III and CP IV, we made use of the previous observation that chlorophyll-protein complexes can be readily dissociated by incubation at 100°C for 1 min (16). We therefore compared the polypeptide patterns of heated and nonheated samples of the two complexes by LiDodSO₄/polyacrylamide gel electrophoresis. Fig. 2 shows that the nonheated sample of CP III (slot 5) contains polypeptide 5 in addition to the chlorophyll-protein band. Upon heating there is a dissociation of the complex and a concomitant increase in the amount of polypeptide 5 (Fig. 2,

FIG. 1. Chlorophyll-protein complexes from *C. reinhardtii* and *P. sativum*. Analysis was by LiDodSO₄/polyacrylamide gel electrophoresis at 4 and 25°C. Slots 1 and 2, thylakoid membranes (20 µg of chlorophyll) of *C. reinhardtii* analyzed at 25 and 4°C, respectively; slots 3 and 4, *C. reinhardtii* CP III after purification by one and two cycles of preparative gel electrophoresis, respectively; slots 5 and 6, *C. reinhardtii* CP IV after purification by one and two cycles of preparative gel electrophoresis, respectively; slots 7 and 8, thylakoid membranes (20 µg of chlorophyll) of *P. sativum* analyzed at 25 and 4°C, respectively. Gels were not stained. Pictures of gels were taken with an Oriol blue filter (G 774-4450) to increase the intensity of the chlorophyll-protein complexes.

FIG. 2. Polypeptide patterns of CP III and CP IV from *C. reinhardtii*. Analysis was by LiDodSO₄/polyacrylamide gel electrophoresis at 4°C. Gels were stained with Coomassie brilliant blue R250. Slots 1 and 2, thylakoid membranes (20 µg of chlorophyll); slots 3 and 4, CP III after purification by one cycle of preparative gel electrophoresis; slots 5 and 6, CP III after purification by two cycles of preparative gel electrophoresis; slots 7 and 8, CP IV after purification by one cycle of preparative gel electrophoresis; slots 9 and 10, CP IV after purification by two cycles of preparative gel electrophoresis. nH, Nonheated sample; H, heated at 100°C for 1 min.

FIG. 3. Absorption spectra of CP III and CP IV from *C. reinhardtii*. The chlorophyll-protein complexes were purified by two cycles of preparative gel electrophoresis at 4°C. (A) CP III and (B) CP IV. Figure shows absorption spectra at 25°C, whereas *Insets* show absorption spectra at -196°C. Solid line, nonheated sample; broken line, heated sample.

slot 6); no other polypeptide is detected. These results provide conclusive evidence that the apoprotein of CP III is polypeptide 5. The small amount of polypeptide 5 detected in the nonheated sample (Fig. 2, slot 5) is due to a partial dissociation of CP III during electrophoresis even at 4°C. By using similar techniques, it can be shown that the apoprotein of CP IV is polypeptide 6 (Fig. 2, slots 9 and 10). It should be noted that in both cases the complex obtained after one cycle of preparative gel electrophoresis is still contaminated by other membrane polypeptides, including those of CP II, although there is always an increase in the amounts of polypeptide 5 or 6, as the case may be, upon heating (Fig. 2, slots 3, 4, 7, and 8). The intensification of the stains in polypeptides 5 and 6 is also evident by comparing the polypeptide profiles of heated and nonheated thylakoid membrane samples (Fig. 2, slots 1 and 2).

Fig. 2 also shows that the native CP III and CP IV have a faster electrophoretic mobility in the 7.5–15% gradient gel system as compared to their respective apoproteins. This anomalous migration of the two complexes was further confirmed by analysis of heated and nonheated thylakoid membrane polypeptides in gels containing 8, 9, 10, 11, 12, and 15% acrylamide. Ferguson plots (16) shows that polypeptides 5 and 6 have free electrophoretic mobilities similar to those of marker proteins, whereas the free electrophoretic mobilities of CP III and CP IV are much higher (data not shown). From these results, we concluded that it is possible to determine the molecular weights of polypeptides 5 and 6, but not those of the chlorophyll-protein complexes, by LiDodSO₄ polyacrylamide gel electrophoresis. The apparent molecular weights of polypeptides 5 and 6 are estimated to be 50,000 and 47,000, respectively.

Table 1. Some chemical properties of CP III and CP IV

	Molar ratio		% of total chlorophyll
	Chlorophyll a/ protein	Chlorophyll a/ β -carotene	
CP III	4.1 $\pm$ 0.3	4.05 $\pm$ 0.4	1
CP IV	5.1 $\pm$ 0.3	5.5 $\pm$ 1.5	1

Values represent averages from three independent preparations, $\pm$ SD. The molecular weights of polypeptides 5 and 6 are taken to be 50,000 and 47,000, respectively. Chlorophyll was measured according to Arnon (13) after extraction of the gel band containing the appropriate complex.

Fig. 3 shows the absorption spectra of pure CP III and CP IV at two different temperatures. At room temperature, CP III displays one peak at 674 nm, which is shifted to 670 nm upon heating, and a poorly resolved shoulder around 680 nm, which is heat sensitive (Fig. 3A). CP IV shows a similar peak at 671 nm and a shoulder at 680 nm that is not detected in the heated sample (Fig. 3B). More fine structures were revealed when the spectra were recorded at -196°C . CP III shows primarily an asymmetrical peak at 673 nm and a small heat-sensitive peak at 642 nm. CP IV, on the other hand, has a major peak at 671 nm and two heat-labile peaks at 682 nm and 641 nm. The spectral characteristics suggest that the two complexes contain chlorophyll a and carotenoids but no chlorophyll b. These results are confirmed by direct pigment analysis presented in Table 1.

Because of the similarity between CP III and CP IV in their pigment compositions (Table 1) there is a possibility that the latter is derived from the former by limited proteolysis, which could have occurred during cell fractionation. To examine this possibility the two complexes were tested with monospecific antibodies against polypeptides 5 and 6 by double diffusion and were found to be immunologically unrelated (Fig. 4). On the basis of this criterion, we conclude that CP III and CP IV are distinct entities.

The finding of CP III and CP IV in *C. reinhardtii* raises the question whether similar chlorophyll-protein complexes are also present in higher plants. To this end, thylakoid membranes were prepared from *P. sativum* and examined by Li-DodSO₄/polyacrylamide gel electrophoresis at 4°C and 25°C. Comparison of the gels at these two temperatures (Fig. 1, slots

FIG. 4. Immunodiffusion of *C. reinhardtii* CP III and CP IV with antisera against polypeptides 5 and 6.

FIG. 5. Two-dimensional crossed immunoelectrophoresis of chlorophyll-protein complexes and thylakoid membrane polypeptides of *P. sativum* with antisera against *C. reinhardtii* polypeptides 5 and 6. In the first dimension, thylakoid membranes ($10 \mu\text{g}$ of chlorophyll) of *P. sativum* were analyzed by LiDodSO₄/polyacrylamide gel electrophoresis at 4°C . (A) The antibody gel contained 0.272 mg cm^{-2} of IgG against *C. reinhardtii* polypeptide 5. (B) The antibody gel contained 0.375 mg cm^{-2} of IgG against *C. reinhardtii* polypeptide 6.

7 and 8) shows the presence of two chlorophyll-protein complexes that are detected at 4°C but not 25°C . Spectral analysis of the complexes after purification by preparative slab gel electrophoresis showed that they contain chlorophyll a but not chlorophyll b (data not shown). By analogy to the two *C. reinhardtii* complexes, these two new chlorophyll-protein complexes in *P. sativum* are also designed as CP III and CP IV in the order of increasing electrophoretic mobilities.

The pairwise relationships between the complexes of *C. reinhardtii* and of *P. sativum* were established by crossed immunoelectrophoresis (9, 11), using monospecific antibodies directed against polypeptides 5 and 6. Fig. 5 shows that, in both cases, two completely fused peaks are obtained, one corresponding to the chlorophyll-protein complex itself and the other to the apoprotein (*P. sativum* polypeptides 6 or 7). *P. sativum* CP III reacts with antibodies against *C. reinhardtii* polypeptide 5, whereas (Fig. 5A) *P. sativum* CP IV reacts with antibodies against *C. reinhardtii* polypeptide 6 (Fig. 5B).

DISCUSSION

In this paper, we demonstrate that chlorophyll-protein complexes derived from thylakoid membranes can be stabilized during polyacrylamide gel electrophoresis in LiDodSO₄ at 4°C . The application of this technique has led to the discovery, in *C. reinhardtii*, of several chlorophyll-protein complexes that have hitherto escaped detection in similar experiments carried out at room temperature (15, 16). Two of these complexes, designated CP III and CP IV, have been purified and found to contain chlorophyll a but no chlorophyll b. It should be pointed out that chlorophyll a-protein complexes other than CP I have been reported recently in two higher plants (17, 18); however, in both cases, only one complex was seen, and in no instance was the complex well characterized.

We have developed a preparative procedure for the isolation and purification of CP III and CP IV in amounts that allow their characterizations in considerable detail. The electrophoretic mobility of the purified CP III or CP IV is identical to that of the corresponding complex in the starting material (thylakoid

membrane sample) (Fig. 1, slots 2, 4, and 6), indicating that the composition of the complexes has not been altered during their purification. Chemical analysis shows that CP III and CP IV are very similar in their compositions. Each complex consists of four to five molecules of chlorophyll a, one molecule of β -carotene, and one copy of a polypeptide in the 45,000–50,000 dalton range. Direct measurements of chlorophylls in the various chlorophyll-protein complexes show that CP III and CP IV each represents about 1% of the total chlorophyll. Assuming that the size of a photosynthetic unit is 400–500 chlorophyll molecules (19), it is evident that there is only one copy of each complex per photosynthetic unit.

One interesting feature of the two chlorophyll-protein complexes is their anomalous migration rates in LiDodSO₄/polyacrylamide gels. In the 7.5–15% gradient gel system, each complex has a faster electrophoretic mobility compared to its apoprotein. This observation, together with the observations reported earlier for CP I (16), reemphasizes the danger of estimating the molecular weights of chlorophyll-protein complexes by dodecyl sulfate gel electrophoresis, as has been done by others (15). However, from the composition of the two complexes, it can be calculated that the minimum weights of CP III and CP IV should be 54,500 and 52,500 daltons, respectively.

The finding of CP III and CP IV raises the question as to their functions. Two lines of indirect evidence strongly suggest that the two complexes are related to the photosystem II reaction center. (i) Similar to CP I, CP III and CP IV each contain only chlorophyll a and β -carotene and are present in the concentration of one copy per photosynthetic unit. However, the absorption maxima of the two complexes are at a shorter wavelength compared to that of CP I. One striking feature of the low temperature absorption spectra is the presence of a shoulder at 682 nm (more distinct in CP IV than in CP III) and a minor peak at 641–642 nm, both of which disappear upon heating (Fig. 3). Döring and Witt* have reported that the spectrum of P680, the photochemical chlorophyll of photosystem II, contains two peaks in the red region: a major one at 682 nm and a minor one at 640 nm. Therefore, it seems likely that the 682 nm and the 641–642 nm bands in the spectra of the two complexes could be due to the presence of P680. However, attempts to demonstrate photochemical activity in these complexes have been unsuccessful so far. (ii) A mutant (F34) of *C. reinhardtii* that possesses high fluorescence yield with no fluorescence induction has been isolated. This mutant is completely devoid of photosystem II activity (7). Analysis of the mutant thylakoid membranes showed a complete absence of polypeptide 6, the apoprotein of CP IV, and a strong deficiency in polypeptide 5, the apoprotein of CP III. Spectral analysis reveals that small amounts of chlorophyll absorbing at 680 nm and 670 nm are missing from this mutant (unpublished data). Furthermore, a suppressor of F34 has been isolated in which photosystem II activity has been restored to 50% of the wild-type level. Thylakoid membranes of this suppressor (F34SU1) contain 50% of polypeptide 6 and almost all of polypeptide 5 as compared to the wild type (7).

In addition to occurring in *C. reinhardtii*, the complexes CP III and CP IV are present in *P. sativum* thylakoid membranes. However, due to the instability of the higher plant complexes and consequently their low yields, it has not been possible to purify them in quantities sufficient for detailed analysis. Nevertheless, we have established by crossed immunoelectrophoresis that *P. sativum* CP III and CP IV contain polypeptides immunochemically related to *C. reinhardtii* polypeptides 5 and

* Döring, G. & Witt, H. T. (1972) *Proceedings of the 2nd International Congress of Photosynthesis Research*, pp. 39–45.

6, respectively. On the basis of immunochemical techniques, thylakoid membrane polypeptides homologous to *C. reinhardtii* polypeptides 5 and 6 have also been identified in spinach (9), corn, barley, and *Euglena gracilis* (unpublished data). Therefore, it is likely that CP III and CP IV are ubiquitous in higher plants and green algae. Interestingly, *C. reinhardtii* polypeptides 5 and 6 have been shown to be synthesized on chloroplast ribosomes (20) and a non-Mendelian mutant of *C. reinhardtii*, Thm-u-1, that synthesizes a variant form of polypeptide 5 has been isolated (21).

The procedure of polyacrylamide gel electrophoresis at 4°C in the presence of LiDodSO₄ described in this paper may be useful for the analysis of membrane systems other than thylakoid membranes. As exemplified by the chlorophyll-protein complexes investigated here, we expect that membrane proteins that contain ligands bound by hydrophobic forces—e.g., certain cytochromes and bacteriochlorophyll-protein complexes—may still retain their ligands during LiDodSO₄ gel electrophoresis in the cold. Furthermore, this procedure should allow the detection of certain membrane protein complexes whose polypeptide subunits are not dissociated by dodecyl sulfate at 4°C (22).

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