Plant Physiol. (1986) 80, 429–434
0032-0889/86/80/0429/06/$01.00/0

Isolation and Characterization of a New Minor Chlorophyll *a/b*-Protein Complex (CP24) from Spinach[1]

Received for publication June 13, 1985 and in revised form October 3, 1985

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ABSTRACT

We have identified a new minor chlorophyll *a/b*-protein complex in the thylakoid membranes of spinach (*Spinacia oleracea* L.), which migrates as a green band below CPII on mildly denaturing polyacrylamide gels. This complex, designated CP24, was isolated from octyl glucoside/sodium dodecyl sulfate solubilized spinach grana membrane fractions by preparative gel electrophoresis and has been characterized as to its spectral properties and polypeptide composition. CP24 has a room temperature absorption maximum at 668 nanometers, a chlorophyll *a/b* ratio between 0.8 and 1.2, and contains three or four polypeptides between 20 and 23 kilodaltons. CP24 was also identified in grana membrane preparations from peas (*Pisum sativum*) and barley (*Hordeum vulgare*). We postulate that CP24 functions as a linker component in photosystem II, acting to orient the photosystem II light harvesting components to ensure efficient energy transfer to the reaction center.

Most, and probably all, of the Chl in the thylakoid membranes of green algae and higher plants is noncovalently bound to protein in the form of macromolecular complexes (19). Three major Chl-protein complexes have been identified in thylakoid membranes: a PSI complex which contains the reaction center pigment P_{700}, a PSII reaction center complex containing P_{680}, and the Chl *a/b*-light harvesting complex (Chl *a/b*-LHC) which serves primarily as an antenna for PSII. These CP[2] complexes can be visualized as green bands on SDS polyacrylamide gels. In early studies, only two CP bands were seen; CPI, associated with PSI, and CPII, identified as the Chl *a/b*-LHC (25). However, by using milder conditions for membrane solubilization and electrophoresis, it is now possible to resolve up to 10 pigment-protein complexes (1, 4, 7, 14, 18). The new bands include one or more oligomers of the Chl *a/b*-LHC, one or more high mol wt bands associated with PSI, and one or two bands correlated with PSII. In addition, there are several reports of other Chl *b*-containing complexes associated with either PSII (CP29 or Chl *a/b*-P3; 4, 11, 18) or PSI (LHCI or CPI^{LH}; 6, 17, 22).

We have identified a new minor CP complex from the photosynthetic membranes of spinach, peas, and barley. This new complex, which we call CP24, was isolated from spinach by electrophoresis of octyl glucoside/SDS solubilized membranes on preparative gels under mildly denaturing conditions. We report here on the initial characterization of CP24 from spinach and discuss the possible role of CP24 in photosynthetic membrane function.

MATERIALS AND METHODS

Isolation of CP Complexes. Intact thylakoid membranes were isolated from supermarket spinach using the procedure of Camm and Green (5) with the addition of the protease inhibitors ε-amino-*n*-caproic acid (5 mM) and benzamidine-HCl (1 mM) to all buffers. Grana membrane fractions were isolated from spinach using the procedure of Berthold *et al.* (3) with the modifications described by Dunahay *et al.* (7; see Table I of ref. 7) and with protease inhibitors added to all buffers as above. This preparation will be referred to here as the 'BBY' preparation. (BBY preparations were originally isolated as 'O_2-evolving PSII preparations' and subsequently shown to consist of highly purified grana membrane fragments; 7.) Where indicated, BBY preparations were also obtained from peas or barley grown in the laboratory under a 12 h light/12 h dark light regime and watered with Miracid (Stern's Nurseries, Inc., Geneva, NY). The isolated thylakoids or the BBY membrane fractions were suspended in ice cold 2 mM Tris-maleate pH 7.0 for 10 min, then centrifuged at 25,000*g* for 15 min. The pellet was washed once in the same buffer, then suspended in 0.88% (w/v) octyl glucoside, 0.22% (w/v) SDS, 10% (v/v) glycerol in buffer (octyl glucoside/SDS/Chl = 20/5/1) and stirred for 5 min on ice. Unsolubilized membranes were pelleted by centrifugation at 10,000*g* for 5 min. The dark green supernatant (1.2–1.5 mg Chl per gel) was loaded onto 3.0 mm thick preparative gels (10% acrylamide) prepared according to the method of Kirchanski and Park (13) except that SDS was omitted from the gels. The gels were run at 4°C for 2.5 to 4 h at a constant current of 20 to 30 mamp. The green gels were immediately photographed using a dark purple filter (Corning No. 5113) to improve contrast. To estimate the proportion of Chl associated with each complex, the gels were scanned at 675 nm using a Gelman ACD-18 Densitometer (Gelman Instrument Co., Ann Arbor, MI). The green bands were cut from the gels and stored at −70°C in distilled H_2O.

To isolate the complexes from the gels, the green bands from several experiments were pooled and homogenized on ice in 30 mM octyl glucoside, 2 mM Tris-maleate (pH 7.0) using a Virtis homogenizer. After removal of the acrylamide by centrifugation, the pale green supernatant was concentrated using an Amicon ultrafiltration device (Amicon Corp., Lexington, MA) equipped with a PM10 filter. To ensure purity of the complexes, CPII* (oligomeric form of Chl *a/b*-LHC) and CP29 were reelectrophoresed on 8% preparative gels, then reisolated and concentrated as above. Except where indicated, CP24 was purified by a single electrophoretic run.

Analytical Electrophoresis. The polypeptide composition of isolated CP complexes was determined using the gel system of

[1] Supported by National Institutes of Health grant GM 22912 and National Science Foundation grant PCM 8118627 to L. A. S.

[2] Abbreviations: octyl glucoside, *n*-octyl-β-D-glucopyranoside; BCA, bicinchoninic acid; CP, chlorophyll-protein; LHC, light harvesting complex.

Laemmli (16) modified by the addition of 4 M urea to the resolving gel. Isolated complexes (30–80 μg Chl per ml) were diluted with an equal volume of 2× solubilization buffer (4% [w/v] SDS, 4% [v/v] mercaptoethanol, 20% [v/v] glycerol, 125 mM Tris-HCl [pH 6.8]), incubated at 37°C for 20 min, and run on 12.5% gels at room temperature. Alternatively, green gel bands were soaked in 2× solubilization buffer, heated as above and placed directly into the wells of analytical gels. The gels were stained with Coomassie brilliant blue R250 and destained by standard procedures, or stained with silver as described by Merril et al. (21).

Spectroscopy. Room temperature absorption spectra and Chl concentrations were determined using a Perkin-Elmer 330 Spectrophotometer. Chl concentrations were determined as described by Arnon (2). The 77K fluorescence spectra were recorded using a Perkin-Elmer MPF-43A fluorescence spectrophotometer adapted for use at liquid N_2 temperature. Spectra were recorded from samples diluted into 50% glycerol (<5 μg Chl per ml) and rapidly frozen onto a glass rod, or from pigmented gel bands soaked overnight in 50% glycerol, then rapidly frozen in liquid N_2.

Protein Determination. The ratio of protein to Chl for CPII*, CP29, and CP24 was estimated using the BCA Protein Assay Reagent from Pierce Chemical Company (Rockford, IL) which is effective in the presence of octyl glucoside and SDS. BSA was used as the standard.

Immunological Techniques. Polyclonal antibodies to the 18 kD polypeptide of the O_2-evolving complex of PSII (10, 15) were provided to us as a generous gift from Dr. David Allred of the University of Colorado and Dr. Michael Seibert of the Solar Energy Research Institute, Golden, CO. Specificity of the antibodies was determined by Western blot analysis (26) using goat anti-rabbit IgG conjugated to horseradish peroxidase (Bio-Rad Laboratories, Richmond, CA).

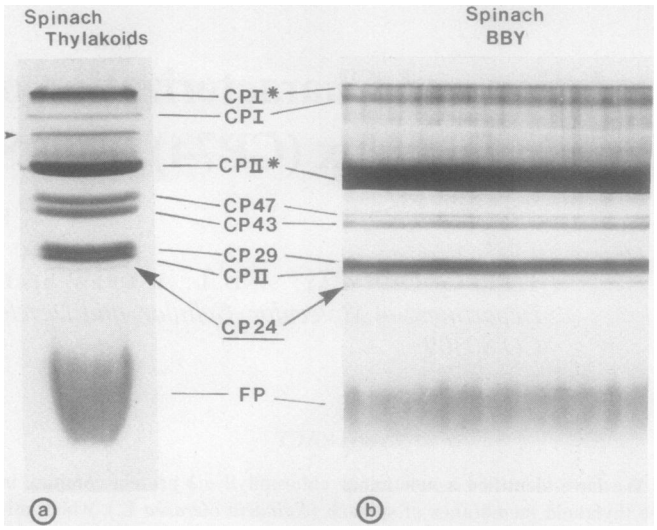

FIG. 1. a, Unstained, mildly denaturing polyacrylamide gel showing the CP complexes of spinach thylakoid membranes. CPI* and CPI represent two forms of the PSI complex (6). CPII and CPII* are monomeric and oligomeric forms of the Chl *a/b*-LHC (20); the band marked with an arrowhead is probably also an oligomer of this same complex. CP47 and CP43 have been correlated respectively with the PSII reaction center and with a PSII internal antenna (24), and CP29 is a Chl *a/b*-protein complex also proposed to function as a PSII antenna (4, 11). CP24 is just detectable as a faint band below CPII. The nomenclature is modified from Camm and Green (4) and McDonnel and Staehelin (20). FP, free pigment. b, Section of an unstained preparative gel as in (a) showing the CP composition of a grana membrane fraction (BBY preparation) from spinach. Note the enrichment of CP24 in this preparation.

RESULTS

Figure 1a shows the CP complexes of spinach thylakoid membranes resolved as green bands on a mildly denaturing polyacrylamide gel. Under the conditions used here (see "Materials and Methods"), we often saw a very faint green band migrating below CPII which has not previously been described; this new band can just be detected in Figure 1a. Based on the fact that this new band migrates below CPII (also called CP27; 4), and in keeping with the 'CP' nomenclature (6) of CP bands on gels, we call this complex 'CP24'. We found that the chance of seeing CP24, and the proportion of Chl associated with this band, was increased if a purified grana membrane fraction (BBY) was used as starting material (Fig. 1b). For subsequent experiments, CP24 was isolated from preparative gels of octyl glucoside/SDS solubilized BBY preparations. The yield of CP24 was very low; even in BBY preparations, less than 2% of the Chl migrated with CP24 (based on densitometer scans of the green gels) and less than 0.5% of the Chl loaded onto the preparative gels was isolated as CP24.

CP24 was characterized as to its spectral properties and polypeptide composition to determine if it was a unique CP complex or merely a breakdown product of other complexes in the membrane. The room temperature absorption spectrum of CP24 is shown in Figure 2. CP24 exhibits a red absorption maximum at 668 nm; the presence of Chl *b* is indicated by shoulders at 650 nm and 470 nm. Figure 2 also includes the spectra of the two other major Chl *b*-containing complexes that are enriched in grana thylakoid membranes, the Chl *a/b*-LHC (isolated here from gels as CPII*), and CP29, which has been postulated to function as an internal antenna for PSII (4, 11). The red absorption maximum is 672 nm for CPII* and 674 nm for CP29. The Chl *a/b* ratio of CP24 ranged from 0.8 to 1.2 (depending on the season and the source of the spinach), in contrast to 1.1 and 1.8 to 2.0 for CPII* and CP29, respectively.

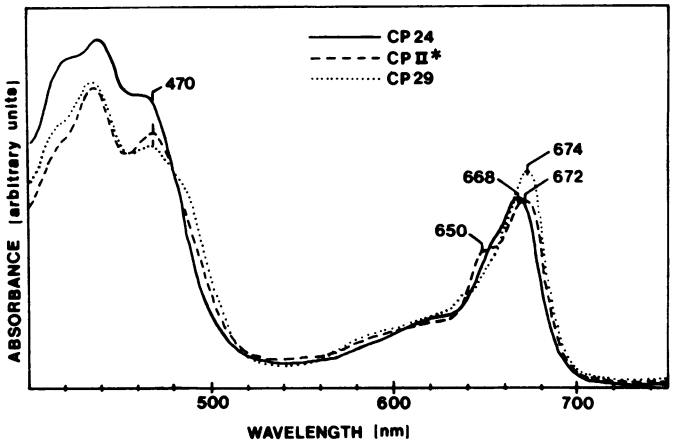

FIG. 2. Room temperature absorption spectra of the CP complexes recorded directly from the green bands excised from mildly denaturing gels.

The 77K fluorescence spectra of CP24, CPII*, and CP29 were determined for the isolated complexes as described in "Materials and Methods" (spectra not shown). All three complexes produce a single sharp fluorescence maximum, but the wavelength of the maximum shifts somewhat for CP24 and CPII* depending on whether the spectrum is recorded for complexes in gels or diluted into glycerol. CP24 produces a fluorescence maximum at 682 nm in the gel, but the peak shifts to 678 nm for the diluted material. CPII* fluoresces at 684 nm in the gel, and at 680 nm in glycerol. CP29 produces a 77K fluorescence peak at 683 nm under both conditions.

The polypeptide compositions of the BBY preparation and the isolated CP, as determined under fully denaturing conditions, are shown in Figure 3. When the gels are stained with Coomassie blue, two major polypeptides at 18 and 23 kD, plus a diffusely staining region at 22 kD, are detectable in CP24 (Fig. 3a). When the same gel is subsequently stained with silver (Fig. 3b), a fourth band at 20 kD becomes visible and the diffuse material (possibly a doublet) at 22 kD becomes more prominent. In contrast, CPII* is composed of two major polypeptides with apparent mol wt of 25 and 26 kD plus a minor component at 24.5 kD. CP29 contains a prominent band at 25.5 kD, which sometimes migrates as a doublet; this protein was shown by partial protease digestion to be unique from the components of CPII* (4). In contrast to CP24, silver staining does not reveal any additional bands in either CPII* or CP29.

Occasionally, CP24 complexes were isolated which were lacking or reduced in the 18 kD polypeptide. This result suggested that the 18 kD component might be a comigrating contaminent rather than an integral component of CP24. We decided to test whether the 18 kD polypeptide isolated with CP24 might actually be the 16 to 18 kD polypeptide believed to be a component of the PSII O_2-evolving complex (10); this polypeptide will be designated here as 18-O_2. Western blot analysis showed that polyclonal antibodies to 18-O_2 which specifically recognize the 18 kD polypeptide of the O_2-evolving apparatus, also cross-react very strongly with the 18 kD polypeptide of CP24.

18-O_2 can be removed from BBY-type membrane fragments (i.e., grana membrane fractions with the lumenal surface exposed to the medium; 7) by washing the membrane fragments with either alkaline Tris buffer or high salt (15). We found that we could treat the BBY membranes by either procedure and still isolate CP24 complexes on preparative gels as described above. These CP24 complexes from washed membranes were 'normal' based on spectral properties and Chl *a/b* ratios, but did not contain the 18 kD polypeptide (Fig. 3c). Thus, in subsequent experiments, CP24 was isolated from spinach BBY membranes which were first washed to remove 18-O_2.

The analysis of the polypeptide composition of the complexes also indicated that the ratio of protein to Chl in CP24 is higher than that of the other CP complexes. For example, it was necessary to load approximately four to six times more CPII* than CP24 per well (based on Chl concentration) in order to see similar amounts of stainable protein (Fig. 3). To quantitate this observation, the ratio of protein to Chl was estimated as described in "Materials and Methods." For CPII* and CP29, the ratio of protein to Chl (w/w) was approximately 4.5 and 7.8, respectively; for CP24, the ratio was between 10 and 11.

CPII* and CP29, isolated from 10% gels, were reelectrophoresed on 8% gels to ensure purity of the complexes prior to further analysis. Under these conditions, CPII* migrates as CPII* plus the monomeric form CPII (both bands contain the three Chl *a/b*-LHC polypeptides and exhibit identical spectra; 4, 20), and CP29 migrates as a single CP band. In contrast, when nondenatured CP24 is rerun on 12.5% mildly denaturing gels the complex migrates as four distinct green regions above the free pigment zone (Fig. 4). These bands, designated 24-1, 24-2, 24-3, and 24-4, each contain a specific subset of CP24 polypeptides (Fig. 5). Room temperature absorption spectra were recorded for 24-2 and 24-3 (24-1 and 24-4 were too faint to produce detectable spectral readings). Both 24-2 and 24-3 exhibit absorption spectra similar to that of CP24 in that both have red absorption maxima at 668 to 669 nm and contain Chl *b* (data not shown). However, each time the experiment was repeated (4×), the heights of the 470 and 650 nm Chl *b* peaks varied relative to the 669 nm Chl *a* peak, indicating variable Chl *a/b* ratios in the complexes. This result suggests that the CP24 complex and its subcomplexes are unstable and dissociate unpredictably during storage and reelectrophoresis.

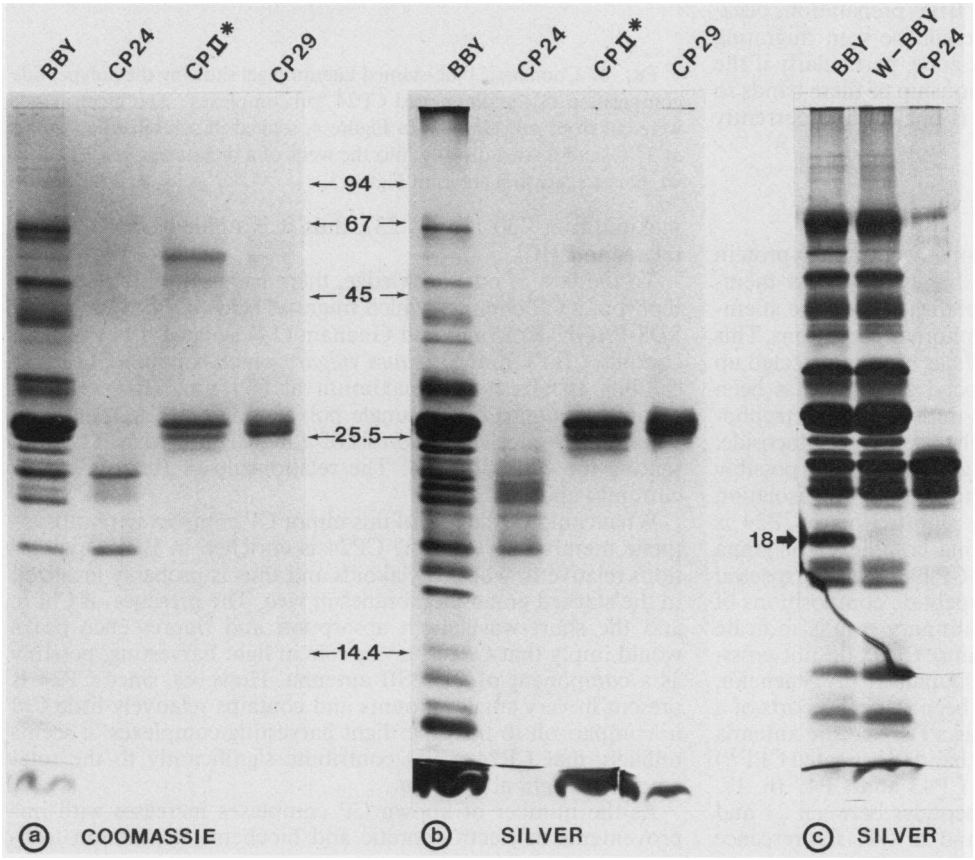

FIG. 3. Polypeptide composition of a BBY preparation and of the isolated CP complexes as determined by SDS-PAGE under fully denaturing conditions. a, Stained with Coomassie blue; b, the same gel as in (a) subsequently stained with silver. The lanes were loaded as follows: BBY, 12 μg Chl; CP24, 0.7 μg Chl; CPII*, 3 μg Chl, CP29, 1 μg Chl. The high mol wt bands in CPII* are aggregates of the CPII* polypeptides induced by heating the sample prior to electrophoresis. The mol wt standards are marked with arrows and represented in kD; the protein standards used were (from top to bottom) phosphorylase *a*, ovalbumin, BSA, chymotrypsinogen, lysozyme. c, Polypeptide composition of a normal BBY preparation from spinach (BBY) and of the same sample following a wash with 300 mM NaCl (W-BBY) to remove the 18 kD polypeptide associated with the O_2-evolving complex of PSII (large arrow). The third lane shows the composition of CP24 isolated from NaCl-washed membranes. Note the loss of the 18 kD component from CP24.

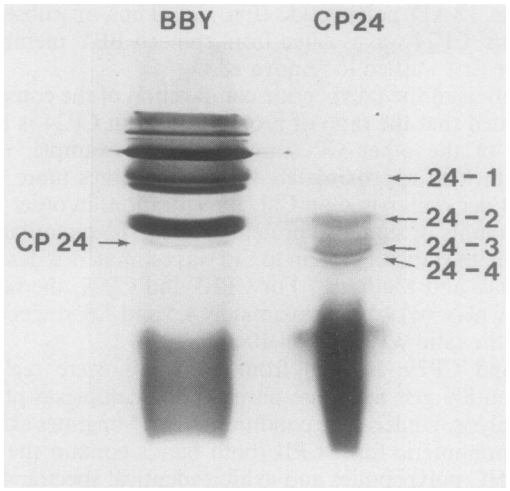

FIG. 4. Unstained, mildly denaturing 12.5% polyacrylamide gel showing the pigmented bands seen upon reelectrophoresis of CP24 isolated from spinach BBY preparations on 10% gels. 24-1 and 24-2 are apparently larger complexes than CP24 based on migration distance in the gel and possibly represent aggregates or oligomers of CP24 components (see Fig. 5).

To test for the presence of CP24 in other plant species, BBY preparations were also isolated from pea and barley leaves and analyzed on mildly denaturing gels as described for spinach. As shown in Figure 6, a and b, a band migrating in the position of CP24 was present in both peas and barley. The room temperature absorption spectra of these complexes are similar to that of CP24 from spinach, and CP24 from both peas and barley also contain several polypeptides between 18 and 23 kD (Fig. 7).

One other interesting observation is shown in Figure 6c. When intact thylakoid membranes from spinach were electrophoresed on preparative gels as described for the BBY preparation, occasionally up to four faint green bands could be seen migrating below CPII and above the free pigment zone, particularly if the gels were very heavily loaded. The relationship of these bands to CP24, and to the CP24 subcomplexes, is unclear and currently under investigation.

DISCUSSION

We have demonstrated the existence of a new Chl *a/b*-protein complex (CP24) in spinach, pea, and barley thylakoid membranes using octyl glucoside/SDS solubilization of the membranes in combination with mild electrophoretic conditions. This complex is apparently very unstable and has been undetected up to this time. The nonionic detergent octyl glucoside has been shown to stabilize minor CP complexes under some electrophoretic conditions (4, 6, 7). It is likely that the use of octyl glucoside, the use of grana membranes as starting material, and possibly the inclusion of the two protease inhibitors in the isolation buffers, allowed us to isolate CP24 as reported here. CP24 is distinct from the other Chl *b*-containing complexes of grana membranes, CPII* (Chl *a/b*-LHC) and CP29, based on spectral characteristics, Chl *a/b* ratios, and polypeptide compositions of the three complexes. In addition, preliminary results indicate that polyclonal antibodies prepared against CPII* do not cross-react with the CP24 polypeptides (TG Dunahay, LA Staehelin, unpublished data). Recently there have been several reports of a new Chl *b* complex, LHCI, which acts as a PSI-specific antenna (6, 17, 22, 23). LHCI migrates as a green band (designated CPI^{LH}) in approximately the same position as CP43 and CP47 (6, 17, 22). CPI^{LH} contains three or four polypeptides between 21 and 24 kD, has a Chl *a/b* ratio of 3.7, and a 77K fluorescence

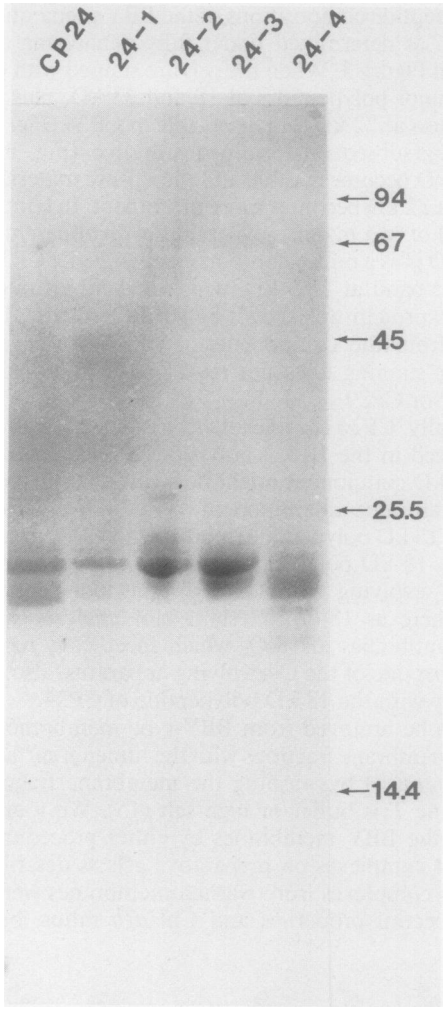

FIG. 5. Coomassie blue-stained Laemmli gel showing the polypeptide composition of the pigmented CP24 'subcomplexes'. The green bands were cut from gels like that in Figure 4, soaked in solubilization buffer at 37°C, and loaded directly into the wells of a denaturing gel. The mol wt markers (arrows) are as in Figure 3.

maximum at 730 nm (6, 23), thus it is unlikely that CP24 is related to LHCI.

To the best of our knowledge, there has been only one other report of a CP complex which migrates below CPII. Using mild SDS-PAGE, Krishnan and Gnanam (14) isolated a low mol wt complex ('IId') from *Sorghum vulgare* which contains Chl *b* and exhibits an absorption maximum at 669 nm. However, this complex contains only a single polypeptide of 15 kD, and can be isolated by reelectrophoresis of a higher mol wt band representing the Chl *a/b*-LHC. The relationship of IId to CP24 is currently unclear.

What could be the role of this minor CP complex in photosynthetic membrane function? CP24 is enriched in BBY preparations relative to whole thylakoids and thus is probably localized in the stacked grana membranes *in vivo*. The presence of Chl *b*, and the short wavelength absorption and fluorescence peaks would imply that CP24 plays a role in light harvesting, possibly as a component of the PSII antenna. However, since CP24 is present in very small amounts and contains relatively little Chl in comparison to the other light harvesting complexes, it seems unlikely that CP24 could contribute significantly to the total amount of light absorption.

As the number of known CP complexes increases with improvements in electrophoretic and biochemical isolation tech-

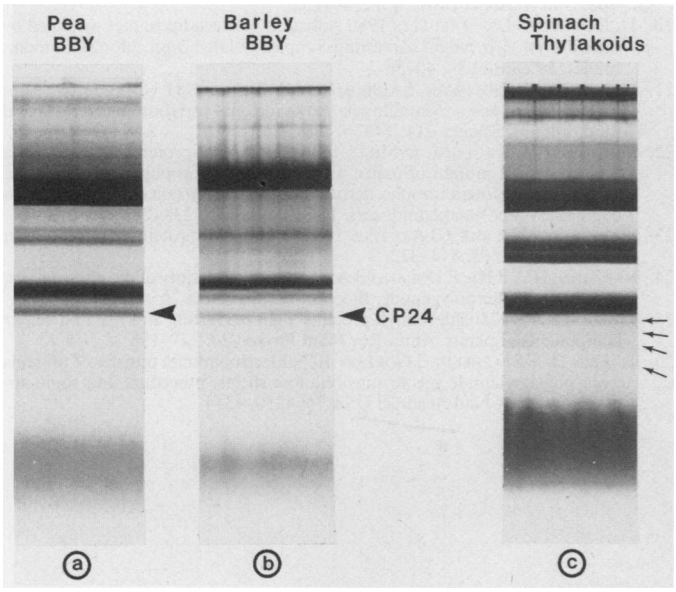

FIG. 6. CP composition of BBY preparations from pea (a) and barley (b) as resolved on mildly denaturing 10% gels. CP24 bands are present in both samples. Note that these samples were *not* washed with NaCl to remove the 18 kD polypeptide prior to solubilization. c, Preparative mildly denaturing gel showing the CP composition of intact thylakoids from spinach. Up to four faint green bands migrating between CPII and the free pigment zone (marked here with arrows) can often be seen on gels of this type, particularly when the gels are very heavily loaded.

niques, it becomes apparent that the light harvesting systems of higher plants may be much more complicated than has previously been believed. We speculate here that the situation in higher plants may actually be analogous to that found in cyanobacteria and red algae. These organisms do not contain Chl *b*. Light collection is accomplished by a peripheral membrane complex called the phycobilisome. (For comprehensive reviews of phycobilisome structure and function, see refs. 8 and 9.) Phycobilisomes are macromolecular aggregates of colored proteins (biliproteins) joined by pigmented and nonpigmented linker proteins into precise linear arrays. The orientation of the biliproteins results in extremely efficient transfer of excitation energy from the antenna molecules to the reaction center pigments. The data presented here suggest that CP24 could function in the PSII antenna apparatus of higher plants in a capacity similar to that of the pigmented linker polypeptides in the phycobilisomes of cyanobacteria. CP24 could precisely link and orient the accessory PSII antenna complexes such as CP43, CP29, or the Chl *a/b*-LHC to promote efficient energy transfer to the reaction center. Indirect support for this idea comes from a recent report by Ish-Shalom and Ohad (12). These investigators showed that the PSI-specific antenna in *Chlamydomonas,* CP0, is composed of a peripheral antenna and a connecting pigment-protein complex required for efficient energy transfer from the peripheral antenna to the PSI reaction center. It is possible that CP24 serves an analogous role as a linker between the PSII reaction center and one of its antenna complexes.

Acknowledgments—We would like to thank Dr. Sallie G. Sprague and Ms. Trish J. Moore for critical reading of the manuscript.

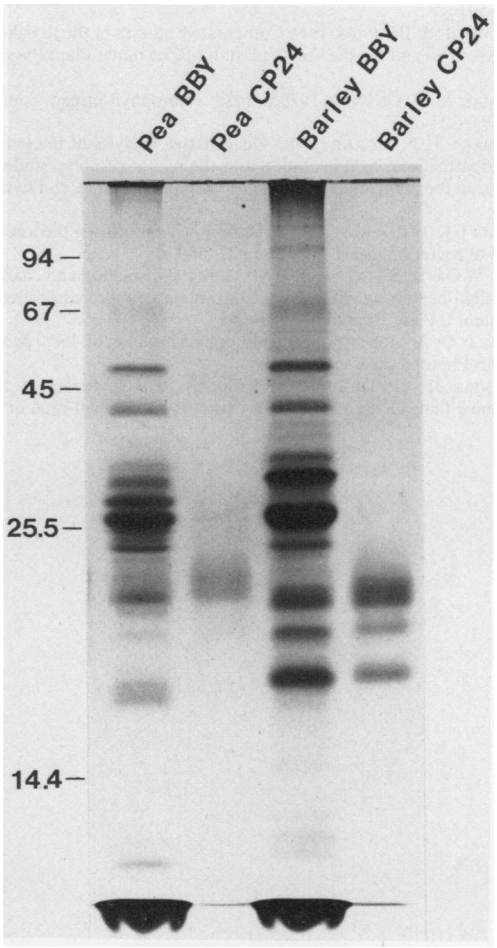

FIG. 7. Laemmli gel showing the polypeptide composition of BBY preparations and CP24 complexes from peas and barley; stained with silver. These complexes were isolated from BBY preparations which had not been washed to remove the 18 kD polypeptide; note that this protein is present in barley CP24 but lacking in this sample of CP24 from peas, supporting our finding that the 18 kD polypeptide is not an integral component of the CP24 complex.

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