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Fractionation of Thylakoid Membranes with the Nonionic Detergent Octyl-β-D-glucopyranoside

RESOLUTION OF CHLOROPHYLL-PROTEIN COMPLEX II INTO TWO CHLOROPHYLL-PROTEIN COMPLEXES

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ABSTRACT

The detergent octyl-β-D-glucopyranoside (30 millimolar in 2 millimolar Tris-maleate, pH 7.0) preferentially extracts complexes containing protein and chlorophylls *a* plus *b* (CP) from spinach, leaving a residue highly enriched in CP I (P700-chlorophyll *a* protein). Use of the detergent results in a relatively gentle extraction since little free chlorophyll is formed and since sodium dodecyl sulfate-gel electrophoresis (on 10% acrylamide) of the extract also reveals the presence of two minor chlorophyll *a* complexes (apparent molecular weight, 47,000 and 43,000) instead of the usual single complex. The major complex preserved is CP 64, a chlorophyll *a/b* complex (apparent molecular weight, 64,000) which is an oligomer of another chlorophyll *a/b* complex, CP 27, the light-harvesting complex (apparent molecular weight, 27,000). Dissociation of each complex reveals two polypeptides (molecular weight, 32,000 and 28,000) and limited proteolysis confirms that those of CP 64 have the same structure as those of CP 27. An additional chlorophyll *a/b* complex (apparent molecular weight, 29,000) is clearly separable from CP 27, and differs from it and CP 64 in having a higher chlorophyll *a/b* ratio and a single polypeptide (molecular weight, 29,000) which differs structurally from those of the other complexes.

Considerable information about chloroplast photosynthetic membranes has accumulated from photochemical experiments and electron microscope studies. The need to integrate material from these two approaches into a model of the molecular architecture of the membrane has led to studies involving membrane fractionation using a wide variety of detergents (anionic, nonionic, and zwitterionic). Electrophoresis of the extracts on SDS gels generally reveals two major CP[1] complexes, called CP I (the P700-Chl *a* protein of photosystem I) and CP II (the light-harvesting Chl *a/b* complex) (25). There is often a third major band which appears to be an oligomer of CP II (2, 3, 9, 13, 15, 22). In addition, a number of minor complexes have been reported (2, 10, 12, 17), comprising only a small fraction of the total Chl. It has become clear that, for good preservation of complexes during electrophoresis, it is essential to use low detergent to Chl ratios and to perform all operations at 4 C. Using careful procedures, up to 10 CP complexes have been resolved on gels (12). However, not all these complexes have been characterized and it is not always possible to compare minor complexes in different systems.

The relationship of CP II to its putative oligomer and to the light-harvesting complex isolated by nonelectrophoretic methods (6, 23) is still far from clear. Several workers have suggested that the oligomer is more representative of the native light-harvesting complex because more of the oligomer is obtained when gentle conditions are used (2, 3, 9, 12, 15, 22). The story is complicated by a lack of agreement about the number of polypeptides involved, whether one (9, 22, 23), two (1, 3, 4, 19, 27), or more than two (6, 12).

This paper reports the fractionation of thylakoid membranes from spinach, barley, and wheat, using the nonionic detergent octyl-β-D-glucopyranoside followed by SDS-gel electrophoresis. This technique has allowed the resolution of CP II into two CP complexes with different Chl *a/b* ratios. The putative oligomer of CP II comprises a greater fraction of the total Chl than when membranes are solubilized with SDS. In addition, two Chl *a* complexes instead of one have been resolved in the 40- to 50-kd region of the gel.

MATERIALS AND METHODS

Preparation of Washed Chloroplast Membranes. Thirty g of 3- to 4-week-old spinach leaves (*Spinacia oleracea* L., Longstanding Bloomsdale) were homogenized for 4 s in a chilled blender with 90 ml medium containing 0.33 M sorbitol, 2 mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM $Na_4P_2O_7$, 50 mM Hepes-NaOH, pH 6.8 (21). Chloroplasts were sedimented at 6,000*g* for a few seconds and washed once with the above medium. Ribulose 1,5-bisP carboxylase and other soluble proteins were removed with three 10-ml washes with 10 mM $Na_4P_2O_7$-HCl (pH 7.4), followed by three washes with 0.3 M sucrose, 2 mM Tricine (pH 7) to remove the coupling factor (24). If membranes were not washed before treatment with octyl-β-D-glucopyranoside, the soluble proteins were found in the detergent extract and resulted in overstained areas on gels. Washing had no effect on the electrophoretic pattern of CP complexes. Washed membranes were stored at −70 C in 65 mM Tris-HCl (pH 6.8), 0.1% β-mercaptoethanol, or 5 mM DTT and 10% glycerol for up to several months.

Detergent Extraction of Membranes. Following the work of Baron and Thompson (5), washed membranes were extracted with 30 mM octyl glucoside (Sigma) in either 2 or 65 mM Tris-maleate (pH 7.0) at various detergent to Chl ratios from 176:1 to 40:1. The concentration of Chl was varied since detergent concentrations over 30 mM exceed the critical micellar concentration. The best results were obtained with 2 mM buffer and a detergent to Chl ratio of 40, and these conditions were used for subsequent experiments. After incubation for 30 min in the dark at room temperature, the suspension was centrifuged for 30 min at 110,000*g*. The supernatant ("extract") was used directly for SDS-gel electrophoresis after the addition of glycerol to 10%. The residue was solubilized with 2% SDS in 65 mM Tris-HCl (pH 6.8), 0.1%

[1] Abbreviation: CP: chlorophyll-protein (complex).

mercaptoethanol, and 10% glycerol for subsequent electrophoresis.

To compare the octyl glucoside solubilization to the more conventional methods using SDS, washed membranes were taken up in 65 mM Tris-HCl (pH 6.8), 0.1% mercaptoethanol, 10% glycerol, and 20% (w/v) SDS added to give an SDS/Chl ratio of 8. This procedure was carried out at 4 C.

Since breakdown of complexes occurred when octyl glucoside extracts were frozen, even at −70 C, the extracts used for electrophoretic separation of CP complexes were always freshly prepared.

Electrophoresis. The detergent extracts were electrophoresed according to Kirchanski and Park (16) on 10 or 15% polyacrylamide slab gels. Addition of SDS to the octyl glucoside extracts to give a final concentration of 0.7% (w/v) made no difference to the pattern of green bands; presumably there is enough SDS in the running buffer (0.1% w/v) to form SDS complexes. All electrophoretic separations of undissociated complexes were performed in a cold room with prechilled buffer and gel slabs. Apparent mol wt were calculated from Sigma Standards (No. SDS-6) acetylated according to Lane (18).

CP complexes seemed somewhat more stable to freezing in acrylamide gel slices than in detergent solution. Gel slabs were routinely wrapped in foil and frozen at −70 C. Gels frozen at higher temperatures developed bubbles on thawing.

Spectra and Gel Scans. Spectra of CP complexes were determined directly on gel slices, using a Unicam 1750 spectrophotometer. For determination of relative amounts of Chl in the different complexes, lanes were cut from slab gels and scanned at 670 nm with a Gilford gel scanning attachment. The stained gels in Figures 5 and 6 were scanned with a 610-nm filter in a Helena Quick-Scan R and D densitometer.

Limited Proteolysis. Limited proteolysis was carried out according to the method of Cleveland *et al.* (7) using *Streptococcus aureus* V_8 protease. Green bands excised from preparative gels were soaked in 2% SDS in 65 mM Tris-HCl (pH 6.8), 0.1% mercaptoethanol, and 10% glycerol, dissociated by heating briefly to 100 C, and re-electrophoresed on an 8% slab gel. The polypeptide bands were visualized by immersion of the slab in 4 M sodium acetate for 10 min (14). The bands were cut out, soaked in distilled H_2O for 20 min, then equilibrated in 1 mM EDTA, 125 mM Tris-HCl (pH 6.8), and 0.1% SDS before electrophoresis in the presence of 0.9 to 1.8 μg protease/slot (7). Autoproteolytic fragments originating from the enzyme never appeared as stainable bands.

RESULTS

Electrophoresis of Octyl Glucoside Extract and Residue. When the octyl glucoside extract was electrophoresed on SDS gels, six green complexes were distinguishable (Fig. 1a). The most prominent were two bands with apparent mol wt of 27,000 and 64,000. The former complex is close to the position in which CP II is found when membranes are solubilized with SDS (Fig. 2), and the latter is at the position of the most commonly found oligomer of CP II. It will be referred to here as CP 64, after its apparent mol wt on 10% acrylamide gels. The ratio of CP 64 and CP 27 varied between extractions (compare Fig. 1, a and b). Between these two bands, there were three minor but reproducible green bands with apparent mol wt of 29,000, 43,000, and 47,000. They will be referred to as CP 29, CP 43, and CP 47. Almost no CP I was seen in gels of octyl glucoside extracts (Fig. 1a, lane i). CP I is not destroyed by octyl glucoside, however, since it is the major complex in the residue left after octyl glucoside extraction. Figure 1, lane ii, shows the electrophoretic separation of the residue solubilized in 0.2% SDS. The octyl glucoside-solubilized material does contain a diffuse blue-green, nonfluorescent band at approximately 130 kd, which may represent an oligomeric form of CP I. Free Chl represented about 20% of the Chl in the gels of the octyl glucoside supernatant, indicating that this detergent treatment leads to relatively good preservation of Chl-protein associations

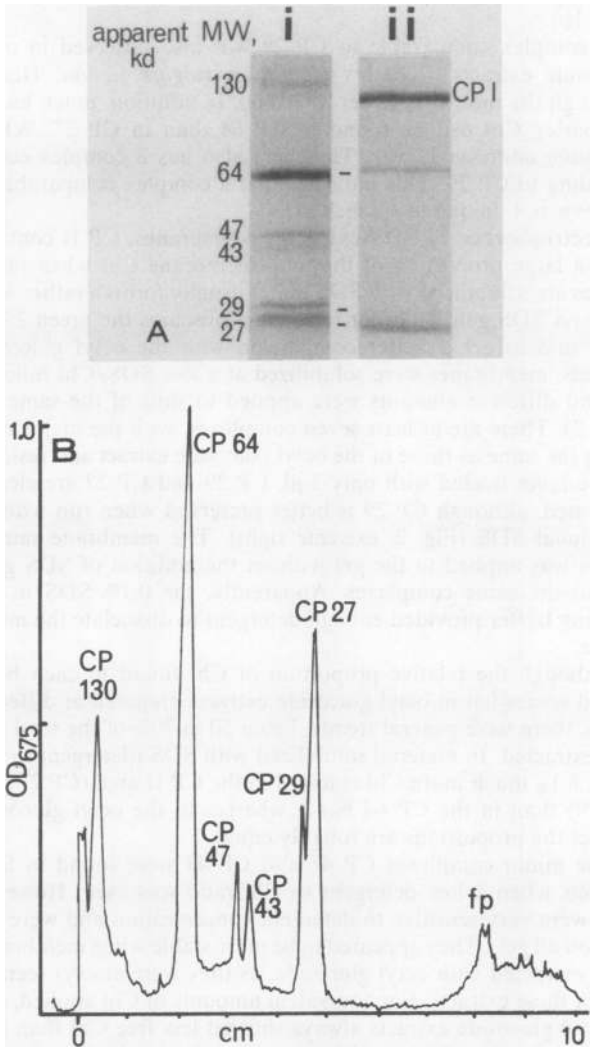

FIG. 1. A: CP complexes of spinach on SDS-gel (i) octyl glucoside extract. ii, residue from octyl glucoside extraction, dissolved in 2% SDS. Unstained gel. MW, mol wt in kilodaltons (kd). B: densitometric scan of unstained SDS-gel of octyl glucoside extract of spinach chloroplasts. fp, free pigment.

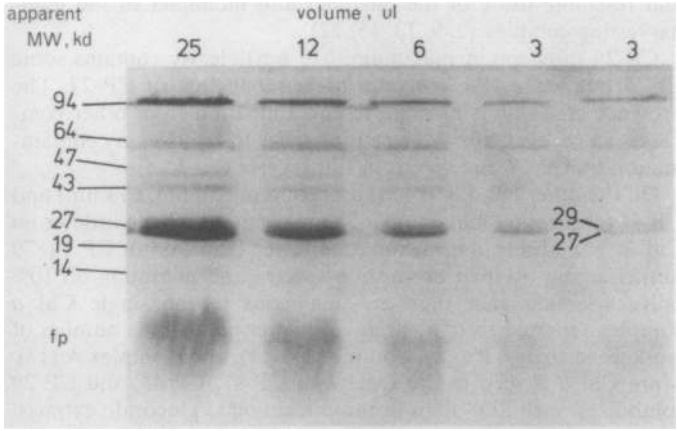

FIG. 2. SDS-electrophoresis of aliquots of SDS-solubilized thylakoids (detergent to Chl ratio, 8:1). No SDS was added to the sample on the extreme right; the running buffer and gel supplied enough. The gel was not stained. fp, free pigment; mw, mol wt in kilodaltons (kd).

(Fig. 1b).

A complex comparable to CP 29 was also observed in octyl glucoside extracts of barley (*Hordeum vulgare* L. var. Herta), although the mol wt is closer to 30,000. In addition, much less of the barley Chl can be found in CP 64 than in CP 27. Wheat (*Triticum aestivum* L. var. Thatcher) also has a complex corresponding to CP 29. This indicates that a complex comparable to CP 29 is not unique to spinach.

Electrophoresis of SDS-extracted Membranes. CP II contains such a large proportion of the total membrane Chl when membranes are solubilized with SDS that it usually forms a rather wide band on SDS gels. In order to see if it obscures the green 29-kd band and to get a better comparison with the octyl glucoside extracts, membranes were solubilized at a low SDS/Chl ratio (8:1), and different amounts were applied to slots of the same gel (Fig. 2). There are at least seven complexes, with the major ones being the same as those of the octyl glucoside extract and residue. In the lanes loaded with only 3 μl, CP 29 and CP 27 are clearly separated, although CP 29 is better preserved when run without additional SDS (Fig. 2, extreme right). The membrane sample which was applied to the gel without the addition of SDS gave rise to the same complexes. Apparently, the 0.1% SDS in the running buffer provided enough detergent to dissociate the membrane.

Although the relative proportion of Chl found in each band varied somewhat in octyl glucoside extracts prepared at different times, there were general trends. From 50 to 70% of the total Chl was extracted. In material solubilized with SDS (detergent to Chl ratio, 8:1), much more Chl is found in the CP II area (CP 27 plus CP 29) than in the CP 64 band, whereas in the octyl glucoside extract the proportions are roughly equal.

The minor complexes CP 47 and CP 43 were found in SDS extracts when a low detergent to Chl ratio was used. However, they were very sensitive to detergent concentration and were not seen on all gels. They appeared to be more stable when membranes were extracted with octyl glucoside, as they were always seen on gels of these extracts. For equivalent amounts of Chl applied, gels of octyl glucoside extracts always showed less free Chl than gels of SDS extracts. With both methods, the use of relatively low detergent to Chl ratios and electrophoresis in the cold minimized the release of free Chl.

Absorption Spectra. Figure 3 shows the visible absorption spectra of the complexes solubilized by octyl glucoside and separated on SDS gels. Spectra were obtained from complexes embedded in polyacrylamide, using a clear gel slice as a blank. The spectra of CP 64 and CP 27 are very similar (absorption maxima, 673 nm) and resemble those of the oligomer and monomer of the light-harvesting complex (2, 9, 13, 15, 22).

CP 29 (absorption maximum, 675 nm) clearly contains some Chl *b* but has a Chl *a*/*b* ratio higher than that of CP 27. The presence of Chl *b* is not due to contamination from other complexes since, even after re-electrophoresis to remove any contamination by CP 27, the spectrum still reveals Chl *b*.

On the other hand, CP 43 (absorption maximum, 673 nm) and CP 47 (absorption maximum, 672 nm) appear to have little or no Chl *b*. The absorption maxima are lower than that of CP I (679 nm). Judging by their absorption spectra and migration on 10% polyacrylamide gels, they are analogous to the single Chl *a* complex reported from a variety of higher plants by a number of workers, such as CP a (2), Complex IV (11), and Complex A (13). More Chl *b* is seen in the spectra of CP 47, CP 43, and CP 29 solubilized with SDS than in those from octyl glucoside extracts. This is probably due to contamination by continuing breakdown of CP 64 to CP 27 during electrophoresis.

Polypeptides of CP 64, CP 29, and CP 27. There is still considerable controversy about the number of polypeptides associated with the light-harvesting complex, and no general agreement about whether CP II in SDS extracts represents a naturally occurring subunit of this complex (26). To learn the relationship of CP 27 to its putative oligomer, CP 64, and to determine their relationship to CP 29, the green bands were cut out of gels and re-electrophoresed on a second gel in the cold. CP 27 and CP 29 gave green bands at the expected position (Fig. 4). A large fraction of CP 64 broke down to give a green band migrating at the position of CP 27. This suggests that CP 64 may be an oligomer of CP 27.

When gel slices containing complexes were heat-dissociated in the presence of 2% SDS before electrophoresis, CP 64 and CP 27 both generated polypeptides of 32 and 28 kd. CP 29, on the other hand, generated a single polypeptide of 29 kd (Fig. 4).

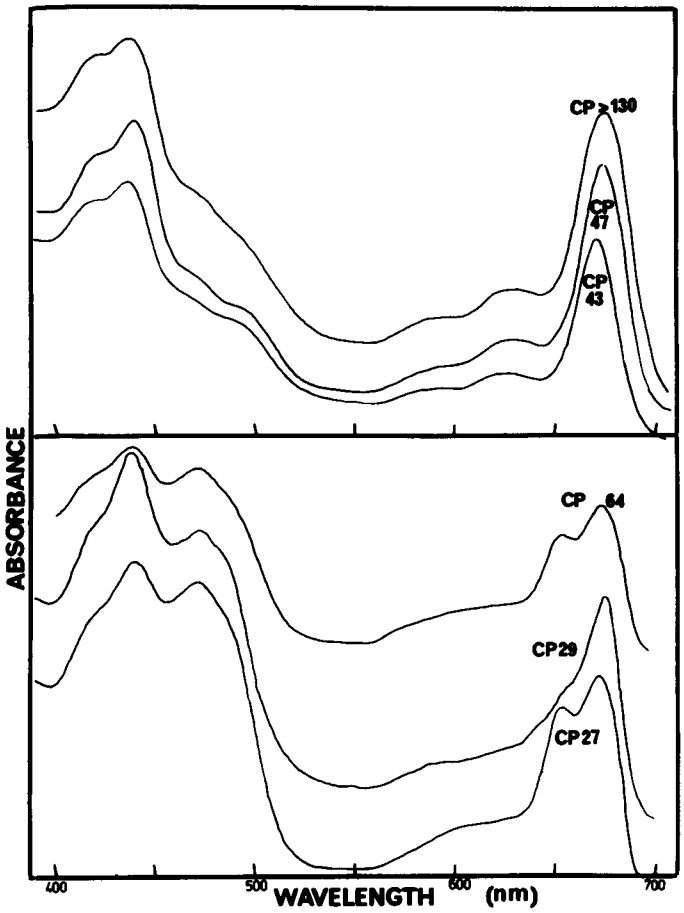

FIG. 3. Visible spectra of CP complexes in gel slices. Top, Chl *a*-containing complexes; bottom, Chl *a* + *b* = containing complexes.

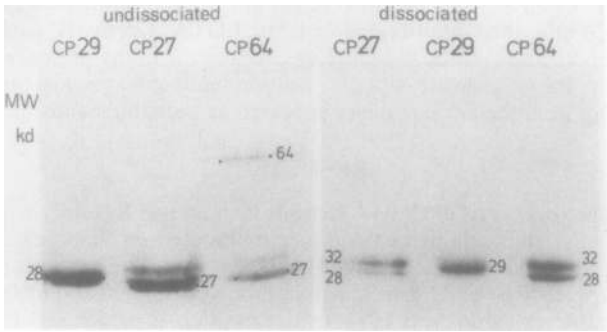

FIG. 4. SDS-electrophoresis of isolated CP complexes in 10% acrylamide. Complexes on the right were heated in 2% SDS before re-electrophoresis. Dots show the position of green bands before the Coomassie blue stain. MW, mol wt in kilodaltons (kd).

The complexes of barley were examined briefly. The complex corresponding to CP 29 yielded only one polypeptide (approximate mol wt, 30,000). In contrast to spinach, dissociated CP 64 from barley has only one polypeptide (approximate mol wt, 28,000). Dissociated CP 27 has a major polypeptide (approximately mol wt, 28,000) and a few other minor bands that are probably co-electrophoresing contaminants since they are lacking from dissociated CP 64.

The polypeptides of CP 64, CP 27, and CP 29 from spinach were examined by limited proteolysis. Similar patterns of stainable fragments were generated from the 32-kd polypeptides of CP 27 and CP 64 (Fig. 5a), indicating considerable, if not complete, structural similarity. In addition, the patterns generated by limited proteolysis of the 28-kd polypeptides from these two CP complexes also indicated considerable similarity (Fig. 5b).

Figure 6 shows the patterns from CP 29 and CP 27 polypeptides run on the same gel. The patterns generated by the two CP 27 polypeptides showed strong similarities. Each proteolysis yielded two major bands and a cluster of two or more low mol wt bands. Additional enzyme merely caused digestion of the higher mol wt bands and an increase in the lower mol wt bands. Considerable structural similarity is suggested.

The proteolysis pattern from the CP 29 apoprotein is quite different from the above. There is a high mol wt doublet and three lower mol wt bands, the lowest of which has a similar mobility to the low mol wt cluster of the CP 29 polypeptides. However, the differences in pattern indicate considerable if not complete structural dissimilarity.

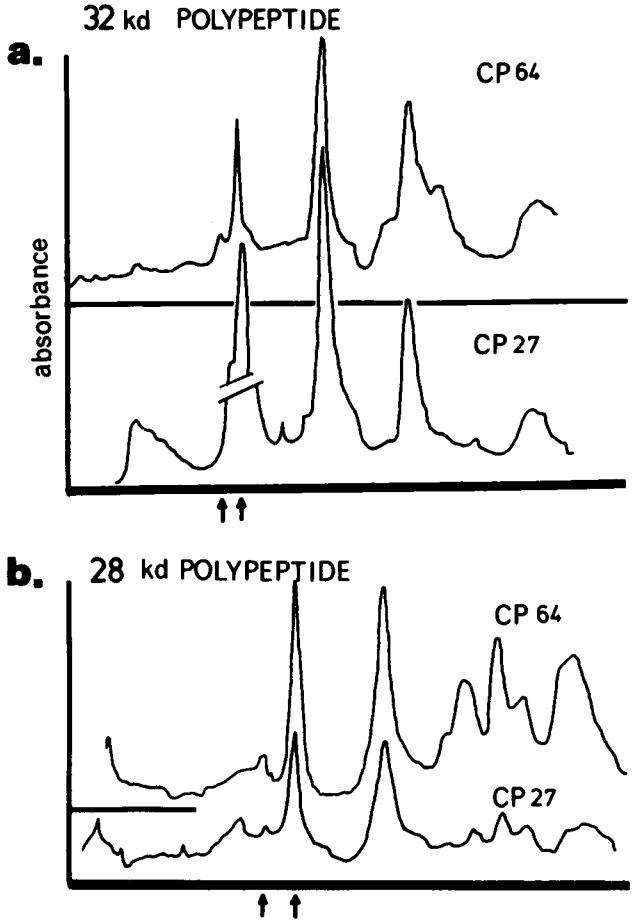

FIG. 5. Densitometric scan of Coomassie-stained limited proteolysis patterns of polypeptides from CP 64 and CP 27 on 15% acrylamide. a, the 32-kd polypeptide from CP 27 and CP 64; b, the 28-kd polypeptide from CP 27 and CP 64. Arrows indicate the bands corresponding to the parent polypeptide and the *S. aureus* protease.

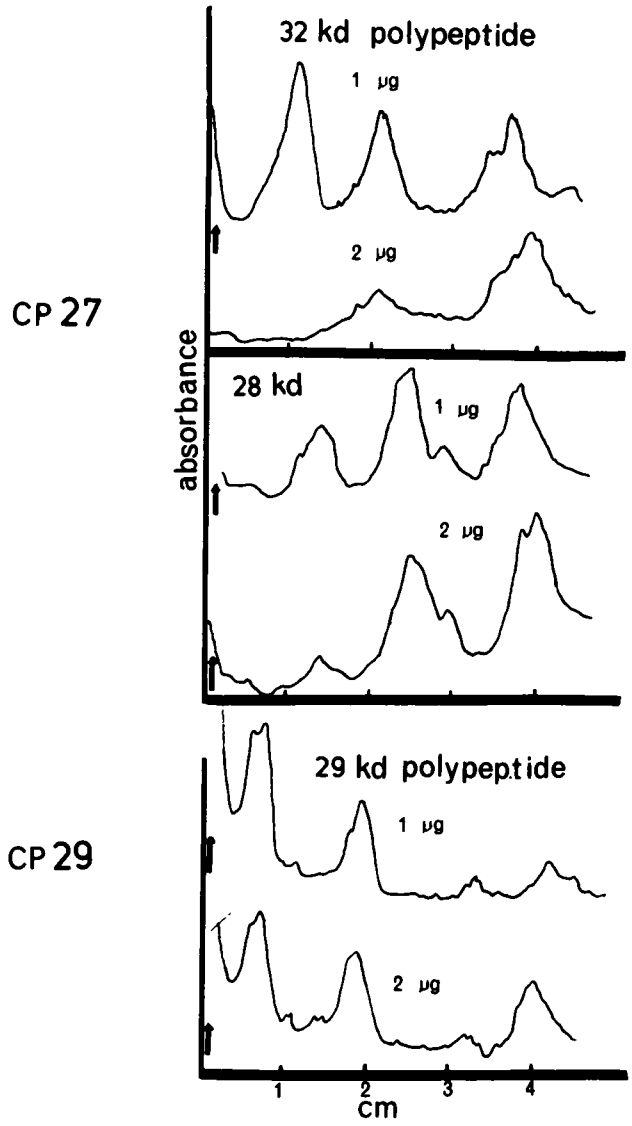

FIG. 6. Densitometric scan of Coomassie-stained limited proteolysis patterns of polypeptides from CP 27 and CP 29. Arrows indicate the bands corresponding to the parent polypeptide. The upper scan of each pair was from a digestion with 1.0 µg protease; the lower, with 2.0 µg. All lanes are from the same gel.

CONCLUSIONS

Octyl glucoside preferentially extracts the light-harvesting complex from thylakoid membranes and helps maintain it in an oligomeric form. In addition, it is possible to eliminate CP I entirely from extracts, although an oligomer may be present. This selectivity is an advantage in the investigation of complexes of PS II.

In addition, octyl glucoside results in well preserved complexes, and the amount of Chl solubilized during extraction is low. Dunkley and Anderson (9) found increased stabilization of complexes with another nonionic detergent and suggested that the detergent might preferentially replace the boundary lipids surrounding a complex, thus shielding it from dissociation by SDS in the electrophoretic buffer. As a consequence of improved preservation, the oligomer CP 64 is present in higher concentration and there is less of the monomer CP 27 than after SDS extraction. On

electrophoretic gels of the octyl glucoside extracts, CP 27 is not overloaded and CP 29 is clearly visible. This new complex corresponds very well in electrophoretic position and relative Chl *a/b* ratio to the complex described from *Vicia* by Machold and Meister (20). We confirm and extend their finding that the dissociated polypeptide of CP 29 is different from those of CP 27 and CP 64. We can demonstrate a difference in mol wt between the CP 29 polypeptide and the CP 27 polypeptides even without using urea during electrophoresis. Limited proteolysis shows a clear structural difference.

Complexes corresponding to CP 29 are probably widespread in higher plants, as CP 29 is present in octyl glucoside extracts of grass as well as spinach thylakoids. In addition, published scans of unstained gels sometimes show shoulders of higher mol wt than the main LHC band, *e.g.* tobacco (ref. 22; Fig. 1) and cowpea (13). We postulate that the variability observed by other workers across the band representing the light-harvesting Chl *a/b* complex (with higher Chl *a* concentration near the trailing edge) (25) can be accounted for by partial co-electrophoresis of the two complexes.

We have shown that CP 27, the monomer of the light-harvesting complex, contains only two polypeptides. This is in contrast to the material prepared by cation precipitation from Triton X-100 extracts of pea, which has three polypeptides (6). The two polypeptides from spinach CP 27 probably have structural similarities. This is in agreement with work on *Acetabularia* (3) which showed that the two polypeptides of the Chl *a/b*-protein complex had similar immunological properties, and similar (although not identical) tryptic fingerprints. However, in the absence of serological evidence, structural homology between the two spinach polypeptides is not proven.

With the use of octyl glucoside, two Chl *a* complexes are recovered instead of the usual single complex (2, 11, 13). The improved preservation afforded by the use of octyl glucoside is probably responsible for the detection of two Chl *a* complexes in our system since two such complexes have been detected in extracts from thylakoid membranes of *Acetabularia* (B. R. Green and J. Van Houten, in preparation), and *Chlamydomonas* and pea (8). Analysis of *Chlamydomonas* mutants deficient in PS II reaction center suggests that these complexes are part of the reaction center in *Chlamydomonas* and pea (8). The relative stability of CP 47 and CP 43 after octyl glucoside extraction will facilitate further characterization in spinach.

In summary, we have shown the value of octyl glucoside in extracting and preserving labile CP complexes, such as CP 47 and CP 43. We have shown by comparison of spectra, by limited proteolysis patterns and by the partial breakdown of CP 64 during re-electrophoresis that CP 64 is an oligomer of CP 27 only. Our data suggest that CP 64 represents a less denatured form of the complex. The new Chl *a/b* complex, CP 29, is unrelated in Chl *a/b* ratio and in polypeptide composition to CP 27 or CP 64. The function of this complex is still unknown.

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