

Formation of Chlorophyll-Protein Complexes during Greening. 2. Redistribution of Chlorophyll among Apoproteins

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The formation of Chl-protein complexes (CPs) in cucumber cotyledons during a dark period after a brief illumination was studied. SDS-PAGE analysis showed that the P700-Chl *a*-protein complex (CP1) and Chl *a*-protein complex of the PS II core (CPa) increased, with a concomitant decrease in the light-harvesting Chl *a/b*-protein complex of PS II (LHCII), during 24-h dark incubation of cotyledons after 6 h of continuous illumination. In agreement with these results, curve analysis revealed that spectral components characteristic of CP1 and CPa increased while those of Chl *b* decreased during the dark incubation. Since Chl is not synthesized in the dark, Chl must be released from LHCII and re-incorporated into CP1 and CPa. The amounts of apoproteins of CP1 and 43 kDa protein (one of the apoproteins of CPa) increased during the dark incubation, and the increase could be inhibited by chloramphenicol (CAP). CP1 did not increase in the dark when tissues were incubated with CAP which inhibited the synthesis of apoproteins of CP1, indicating that CP formation by Chl redistribution needs newly synthesized apoproteins. The decrease in LHCII apoproteins during dark incubation was inhibited by CAP probably because Chl was not removed from LHCII by apoproteins of CP1 and CPa, whose synthesis was blocked by the presence of CAP. When intermittently-illuminated cotyledons containing a little LHCII were incubated with CaCl₂ in the dark, Chl *b* and LHCII apoproteins accumulated with the disappearance of 43 kDa protein; Chl of 43 kDa protein may be utilized for LHCII formation. We concluded that Chl molecules once bound with their apoproteins are redistributed among the apoproteins.

Key words: Chlorophyll-protein complex — Cucumber — Curve analysis — Greening.

As well as by distribution of newly synthesized Chl (Shimada et al. 1990, Tanaka and Tsuji 1985), CPs are formed by redistribution of Chl (Tanaka and Tsuji 1982, 1983). CP formation by redistribution of Chl was observed when greening tissues were transferred to darkness. LHCII was formed during dark incubation of cucumber cotyledons with CaCl₂ after intermittent illumination (Tanaka and Tsuji 1982). Since only CP1 and CPa were formed during intermittent light and no Chl was synthesized in the subsequent dark period, the dark formation of LHCII is probably due to migration of Chl from CP1 and/or CPa into LHCII apoproteins. On the other hand, when cucumber seedlings were placed in the dark after continuous illumination, CP1 increased with a concurrent

decrease in LHCII (Tanaka and Tsuji 1983). With bean leaves, Akoyunoglou et al. (1982) also reported the formation of CP1 and CPa in the dark after continuous light. These results indicate that Chl migrates from LHCII into CP1 and CPa and vice versa. Such redistribution was also observed with isolated chloroplasts. Schmidt et al. (1981) showed that LHCII apoproteins imported into isolated chloroplasts assembled in the thylakoid lipid and became bound to Chl in the dark. Bhaya and Castelfranco (1985) showed incorporation of newly synthesized Chl into CPs in isolated plastids under light, which was not inhibited by CAP. These observations support the idea that CPs are formed through the exchange of Chl among apoproteins. However, when the incorporation of Chl into apoproteins takes place in the dark, it is not clear whether Chl is incorporated into apoproteins that have been accumulated in the preceding light period or those synthesized in the dark period.

Exact determination of the amounts of CPs in thyla-

Abbreviations: CAP, chloramphenicol; CP, chlorophyll-protein complex; CPa, chlorophyll *a*-protein complex of photosystem II; CP1, P700-chlorophyll *a*-protein complex; LHCII, light-harvesting chlorophyll *a/b*-protein complex of photosystem II.

koid membranes using PAGE has been difficult due to the release of some Chl from apoproteins during electrophoretic procedures. In order to study the quantitative changes in CPs, we developed a method for separating CPs with a minimal amount of free Chl. A promising alternative method is curve analysis of the absorbance spectrum of Chl in tissue homogenates which does not include procedures leading to liberation of Chl from apoproteins or loss of particular portions of CPs; it is used as a reliable method for analyzing changes in the CP composition in green tissues (French et al. 1972).

In the present study, we showed that CPs were formed through redistribution of Chl in the absence of net synthesis of Chl, using the improved PAGE method and curve analysis of the absorbance spectrum of Chl in tissue homogenates. The findings suggest that Chl molecules may be redistributed among apoproteins according to their differential affinity for Chl *a*.

Materials and Methods

Plant materials—Cucumber seeds were germinated in the dark at 28°C for 5 days (Shimada et al. 1990). Seedlings were illuminated with white fluorescent light continuously or intermittently at an intensity of 5,000 lux at 28°C.

Treatments—After illumination, cotyledons were excised from cucumber seedlings and incubated with water, 200 µg/ml CAP or 100 mM CaCl₂ in the dark for 24 h as described previously (Shimada et al. 1990).

Chl determination—Chl was extracted with 80% acetone under a safety green light, and determined using a fluorescence spectrophotometer (Hitachi 650-10S). Calibration was performed with purified Chl *a* and *b* as described previously (Shimada et al. 1990).

SDS-PAGE of CPs—Cucumber cotyledons were homogenized in a medium containing 0.5 M sucrose, 50 mM Na-Tricine (pH 8.0) and 5 mM EDTA with a blender, and then with a mortar and pestle. The homogenate was filtered through a layer of Miracloth and centrifuged at 8,000 × *g* for 15 min. The pellet was washed twice with 5 mM EDTA (pH 8.0) and solubilized with 0.5% Triton X-100 (0.15 mg Chl/ml). This was done by mixing the sample gently with a glass homogenizer in an ice bath. The solution was loaded on a 20% sucrose cushion and centrifuged at 15,000 × *g* for 30 min. A large amount of insoluble non-pigmented matter was pelleted at the bottom and the green supernatant remained above the sucrose cushion. The supernatant was loaded on a 0.1–1.0 M sucrose gradient (4 ml, 6-cm long) containing 0.08% Triton X-100 and centrifuged at 40,000 rpm for 14 h in a swing rotor (Hitachi 56T). The single green band obtained on the gradient was taken and diluted 1:1 with the solubilizing buffer described below. CPs were separated by SDS-

PAGE according to Anderson et al. (1978) with the following modifications. To minimize the amount of free Chl, concentrations of SDS were lowered to 0–1% in the solubilizing buffer and 0.005 or 0.02% in the reservoir buffer. SDS was not included in the gel, and stacking gel was omitted. Electrophoresis was done in a cold room for about 20 min. After electrophoresis, the gel was scanned at 675 and 650 nm with a gel scanner attached to a Hitachi 556 spectrophotometer.

In experiments for Table 1, a simple and rapid method of PAGE was used which allowed us to start from a small amount of tissues. Ten cotyledons were homogenized with a glass homogenizer in 5 ml of the same grinding medium as above. The homogenates were passed through a layer of nylon mesh and centrifuged for 10 min at 15,000 × *g*. The pellet was suspended with 5 mM EDTA (pH 8.0) and centrifuged for 10 min at 10,000 × *g*, and washed again with the same procedure. The pellet was dissolved with 0.3 ml of solubilizing medium containing 50 mM Tris-HCl (pH 8.8), 10% glycerol and 1% SDS. After centrifugation of the sample, CPs were separated with PAGE according to Anderson et al. (1978).

Isolation of PS I particles—For preparation of samples used for curve analysis, PS I particles were isolated from spinach chloroplasts according to Mullet et al. (1980). Thylakoid membranes were solubilized with Triton X-100 and loaded on a sucrose gradient. PS I was obtained as a lower dark green band on the gradient after centrifugation.

Antisera—Washed membranes from spinach chloroplasts (Tanaka and Tsuji 1982) were solubilized without heating and electrophoresed on a slab gel according to Laemmli (1970). The green band corresponding to CP1 was excised. The gel section was homogenized in Laemmli's solubilizing medium and centrifuged. The supernatant was heated and reelectrophoresed by the same system. After staining the gel with Coomassie Brilliant Blue R-250, the band containing CP1 apoprotein was excised. For purification of 43 kDa peptide, PS II membranes from spinach leaves (Kuwabara and Murata 1982) were electrophoresed. After staining, the band of 43 kDa peptide was excised and reelectrophoresed. The purified peptides were used as antigens to raise antibody in rabbits. Antiserum to LHClI apoproteins was obtained as described previously (Shimada et al. 1990).

Immunoblotting—After electrophoresis of whole protein extract from cucumber cotyledons, peptides on the gel were electrophoretically transferred to nitrocellulose filter and incubated with antisera. Immune complexes were made visible (Shimada et al. 1990).

Measurement of absorbance spectra and curve analysis—Cotyledons were homogenized with 50 mM Tris-HCl (pH 8.0) with a glass homogenizer in an ice bath. Absorbance spectra of the homogenates were measured at liquid ni-

trogen temperatures in a Hitachi 556 spectrophotometer (Tanaka and Tsuji 1985). Absorbance spectrum of suspensions of PS I particles from spinach chloroplasts was measured in the same way.

The absorbance was read at intervals of 1 nm along the curves on the recorder chart. The data were stored in a computer and the wavelength was transformed into the wavenumber. The derivative of a curve was obtained by computing differences between the readings along the curve. Higher order derivatives were obtained by repeating the differentiating process. For curve fitting, the peak wavenumbers obtained from the fourth derivative spectrum of fully greened cotyledons and the half widths which were arbitrarily determined were input as initial conditions (French et al. 1972). The steepest descent method was employed for least squares fitting of component Gaussian bands to spectral data.

Results

Redistribution of Chl to CP1 and CPa—Five-day-old etiolated cucumber seedlings were illuminated for 6 h and then incubated in the dark for 24 h. Redistribution of Chl among CPs in thylakoid membranes during the dark incubation was studied. In order to compare CP compositions before and after the dark incubation exactly, liberation of Chl from CP during analytical procedures should be minimized. Therefore, we developed an improved method of PAGE which is suitable for separating CPs from such greening tissues with very little free Chl, and inserted a step for purification of Triton-solubilized thylakoid membranes by sucrose density gradient centrifugation before the electrophoresis (cf. Materials and Methods). Figs. 1 and 2 show patterns of PAGE separation of CPs of purified thylakoid membranes from 6-h illuminated cotyledons before and after the dark incubation, respectively. In either case, PAGE was done with various concentrations of SDS. Without SDS in solubilizing buffer, CPs were separated with very little free Chl (Fig. 1A and 2A). With increasing concentration of SDS in solubilizing buffer, however, free Chl increased though a better resolution of CPs was obtained (Figs. 1B and C, Figs. 2B–E). Comparison of the peak height of each CP is possible between electrophoretic patterns with little free Chl. The peak positions are determined from those of sharp peaks obtained with increasing concentration of SDS in the solubilizing buffer. With 6-h illuminated cotyledons, two peaks of CP1 and LHCII were evident (Fig. 1A). However, after the subsequent 24 h of dark incubation, a prominent CPa peak appeared in addition to the increased CP1 peak, LHCII being recognized as only a small bulge at the region of its oligomeric form (Fig. 2A). These results show that when CP1 and CPa were formed during the dark incubation, LHCII disappeared, suggesting that Chl migrated

from LHCII to CP1 and CPa.

We then examined whether newly synthesized apoproteins are required for the CP formation, using CAP which inhibits the synthesis of chloroplast-encoded proteins like apoproteins of CP1 and CPa. Table 1 shows that the increase in CP1 during 24-h dark incubation after 4 h in light was inhibited when CAP was present during the dark incubation. This indicates that CP1 formation in the dark needs synthesis of its apoproteins.

Table 2 shows the changes of Chl content in cotyledons during 24-h dark incubation after 4 h of continuous illumination. When cotyledons were incubated with water in the dark, Chl *b* decreased but Chl *a* did not. The decrease in Chl *b* could be inhibited by CAP and by CaCl₂, although the latter also caused a decrease in Chl *a*.

As another approach to studying the changes in CPs during dark incubation after illumination, we used curve

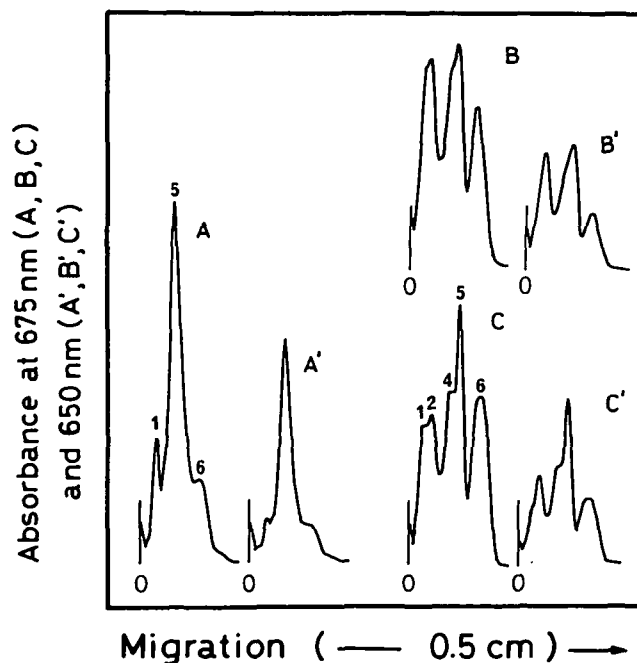


Fig. 1 Densitometric tracing of CPs separated by SDS-PAGE from 6-h illuminated cucumber cotyledons. A single green band obtained on the sucrose gradient was electrophoresed (cf. Materials and Methods). CPs were separated with various concentrations of SDS in the solubilizing and the reservoir buffers, respectively, as follows: A, A', 0% and 0.02%; B, B', 0.3% and 0.005%; C, C', 0.5% and 0.005%. The gels were scanned at 675 nm (A, B and C) and 650 nm (A', B' and C'). CP separation patterns with the least free Chl were obtained when the lowest concentration of SDS was used in the electrophoretic system (A, A'). Peaks were identified by comparison with those obtained with higher concentrations of SDS. 1, CP1; 2, oligomeric form of LHCII; 4, unidentified; 5, monomeric form of LHCII; 6, free Chl.

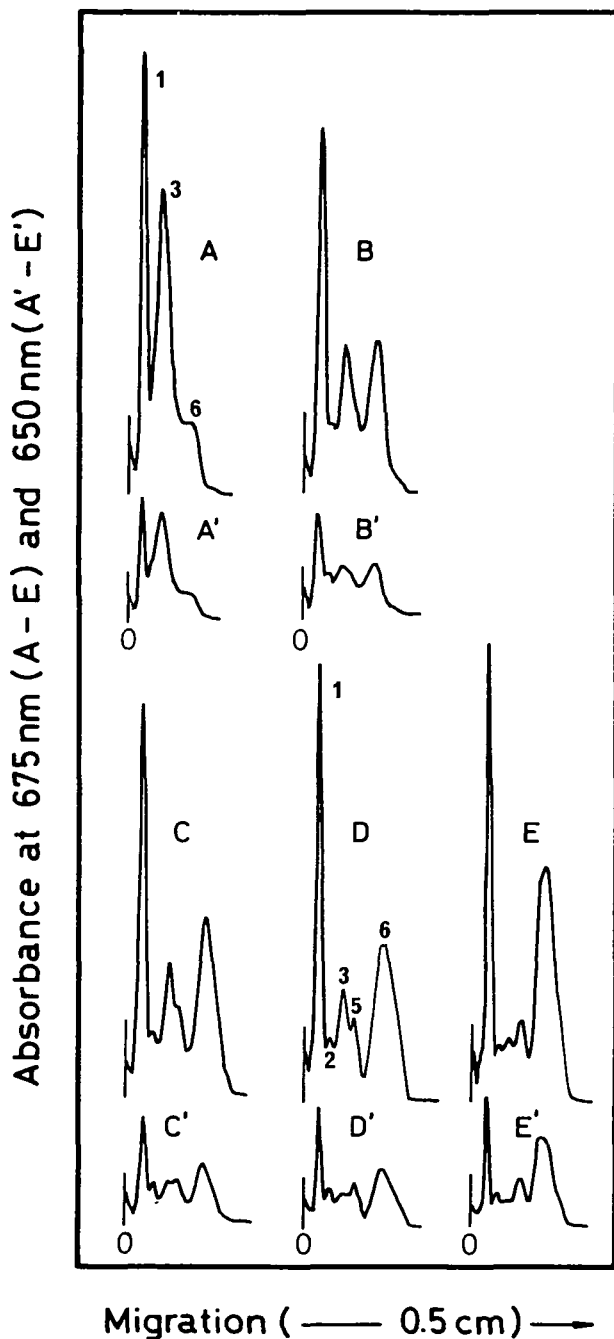


Fig. 2 Densitometric tracing of CPs separated by SDS-PAGE from cucumber cotyledons incubated in the dark after illumination. Etiolated seedlings were illuminated continuously for 6 h and then incubated in the dark for 24 h. The concentrations of SDS in the solubilizing buffer were 0% (A, A'), 0.1% (B, B'), 0.3% (C, C'), 0.5% (D, D') and 1.0% (E, E'). The reservoir buffer was 0.02% SDS. The gels were scanned at 675 nm (A, B, C, D and E) and 650 nm (A', B', C', D' and E'). 1, CP1; 2, oligomeric form of LHCII; 3, CPa; 5, monomeric form of LHCII; 6, free Chl. Other explanations are as given in Fig. 1.

Table 1 Effect of CAP on the change in CP1 content during dark incubation after continuous illumination

Treatment	Chl of CP1 (% of total Chl)
4L	11.4
4L+D.W.	36.1
4L+D.CAP	17.7

Cotyledons were excised from 4-h illuminated cucumber seedlings (4L) and incubated with water (4L+D.W) or 200 $\mu\text{g}/\text{ml}$ CAP (4L+D.CAP) in the dark for 24 h. CPs were separated with SDS-PAGE and the gel was scanned at 675 nm. The area under the peak generated by CP1 band is expressed as the percentage of the whole area under the densitogram tracing.

analysis of the absorbance spectrum. After the seedlings had been illuminated for 4 h, the cotyledons were excised and incubated with water, CAP and CaCl_2 for 24 h in the dark. Absorbance spectra of their homogenates were analyzed (Fig. 3) and the areas of the components obtained from the curve analysis were listed in Table 3. Cb649 and Cb640, which are spectral components of Chl *b* (Brown and Schoch 1891, Brown 1983), decreased during the dark incubation. This decrease agrees with that in the Chl *b* content of tissues measured with the acetone extract (Table 2). Ca688 is considered to be a spectral component of CP1, and Ca683 to be that of CPa, based on the analysis of purified complexes (Van Dorssen et al. 1987, Satoh and Butler 1978) and mutants lacking specific components (Simpson 1988). It was confirmed that Ca683 is not a spectral component of PS I: Curve analysis of PS I particles isolated from spinach chloroplasts showed the absence of the Ca683 component in this preparation (data not shown). Ca688 and Ca683 increased during the dark incubation. However, their increase as well as the decrease in Cb640 and Cb649 was inhibited when CAP or CaCl_2 was present during the dark incubation. These findings indicate that

Table 2 Changes in Chl contents during dark incubation after illumination with continuous light

Treatment	Chl <i>a</i>	Chl <i>b</i>	Chl <i>a</i> + <i>b</i>	Chl <i>a</i> / <i>b</i>
	(μg/cotyledons)			
4L	3.01	0.72	3.73	4.2
4L+D.W	3.46	0.20	3.66	17.6
4L+D.CAP	3.38	0.61	3.98	5.6
4L+D.Ca	2.44	0.65	3.09	3.8

4L+D.Ca: 4-h continuous light followed by 24-h dark incubation with 100 mM CaCl_2 . Conditions for other treatments are as described in Table 1.

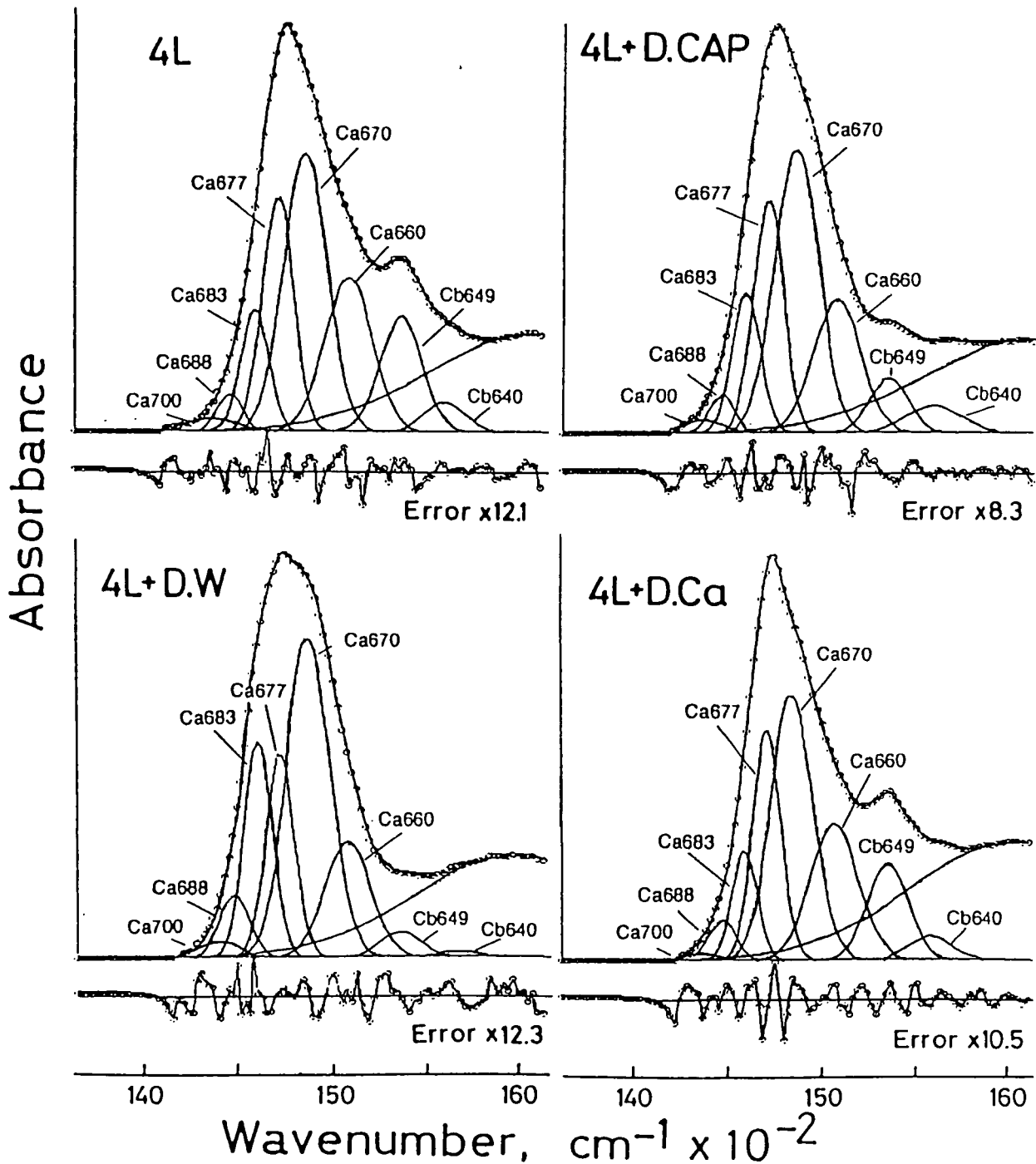


Fig. 3 Curve analysis of the low-temperature spectra of Chl in homogenates of cucumber cotyledons incubated in the dark after continuous illumination. The observed data were plotted as circles, with the line through them being the sum of the component curves. 4L, 4-h continuous light; 4L+D.W, 4-h continuous light followed by 24-h dark incubation with water; 4L+D.CAP, 4-h continuous light followed by 24-h dark incubation with CAP; 4L+D.Ca, 4-h continuous light followed by 24-h dark incubation with CaCl₂. Concentrations of CAP and CaCl₂ were 200 $\mu\text{g}/\text{ml}$ and 100 mM , respectively.

Table 3 Curve analysis of the absorbance spectra of Chl in homogenates of cucumber cotyledons illuminated with continuous light and then incubated with CaCl₂ or CAP in the dark

Treatment	Ca700	Ca688	Ca683	Ca677 (% of total Chl <i>a</i>)	Ca670	Ca660	Cb649	Cb640
Fully greened	8	9	10	18	32	23	15	5
4L	2	3	11	23	38	23	15	4
4L+D.W	2	6	18	17	41	16	3	1
4L+D.CAP	2	3	13	22	40	20	7	5
4L+D.Ca	1	4	10	22	40	23	14	4

Incubation conditions are as described in Table 2. The area of each component is expressed as the percentage of the sum of the areas of Chl *a* components.

CP1 and CPa were formed with a concomitant loss of LHCII in the dark, these changes being inhibited by CAP or CaCl₂.

Next, we examined changes in the levels of apoproteins of CP1, CPa, and LHCII during dark incubation with CAP and CaCl₂ after 4 h in light. The CP1 and 43 kDa apoproteins increased and LHCII apoproteins decreased during 24 h of dark incubation with water after 4 h of illumination (Fig. 4). These changes were inhibited by CAP. In the presence of CaCl₂, LHCII apoproteins remained unchanged during dark incubation. The 43 kDa protein disappeared, but the CP1 apoproteins never decreased, suggesting that CPa releases its Chl but CP1 does not. The above results show that synthesis of CP1 and 43 kDa apoproteins continued during dark incubation after continuous illumination. Therefore, we concluded that Chl molecules released from LHCII became bound with the newly synthesized apoproteins, thus being reutilized to form CPs in the dark.

Chl redistribution to LHCII—In order to study redistribution of Chl from Chl *a*-protein complexes to LHCII, we examined CP changes using curve analysis and immunoblotting. Intermittent light was employed, because it accumulates Chl *a* with very little Chl *b*, allowing

us to easily show the formation of Chl *b* and LHCII during the subsequent dark incubation with CaCl₂ (Tanaka and Tsuji 1982). Absorbance spectra and their components obtained by curve analysis are shown in Fig. 5 and Table 4. Cb640 and Cb649 were very small in intermittently illuminated cotyledons, in agreement with the lack of Chl *b* in these tissues. No obvious changes in the amounts of spectral components were observed during dark incubation of intermittently illuminated cotyledons. This is probably due to the lack of LHCII which could serve as a source of Chl for the formation of CP1 and CPa. CAP had no effect on the amounts of the spectral components. However, Cb640 and Cb649 increased when cotyledons were incubated with CaCl₂ in the dark. The proportion of Ca683 to total Chl *a* components decreased to some extent (Tables 3 and 4). The decrease in the actual amount of this component was clear, considering that Chl *a* per cotyledon decreased by 20% (Table 2). Therefore, CPa may be one of the candidates for the source of Chl supply for the formation of LHCII.

Next, changes in levels of LHCII apoproteins and 43 kDa protein during dark incubation of intermittently illuminated cotyledons were examined (Fig. 6). A small amount of LHCII apoproteins accumulated at the end of

Table 4 Curve analysis of the absorbance spectra of Chl in homogenates of cucumber cotyledons illuminated with intermittent light and then incubated with CaCl₂ or CAP in the dark

Treatment	Ca700	Ca688	Ca683	Ca677 (% of total Chl <i>a</i>)	Ca670	Ca660	Cb649	Cb640
IL	3	5	18	16	40	18	2	0
IL+D.W	2	6	17	18	39	18	1	0
IL+D.CAP	2	4	20	16	41	15	1	0
IL+D.Ca	3	7	16	19	38	17	4	1

Cotyledons were excised from cucumber seedlings illuminated with 10 times of intermittent light. They were incubated with water (IL+D.W), 200 µg/ml CAP (IL+D.CAP) or 100 mM CaCl₂ (IL+D.Ca) in the dark for 24 h. The amount of each component is expressed as in Table 3.

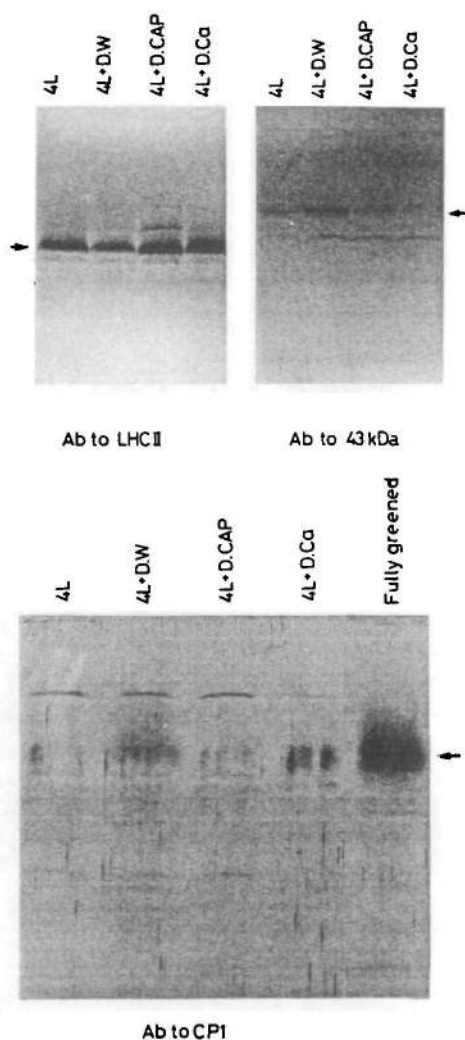


Fig. 4 Immunoblot analysis of CP1 apoproteins, 43 kDa peptides and LHCII apoproteins from cucumber cotyledons incubated with CAP or CaCl_2 in the dark for 24 h after 4 h of illumination. Whole proteins from cucumber cotyledons were electrophoresed on 14% acrylamide gel and blotted onto nitrocellulose filter. Antisera (Ab) against 43 kDa peptide of PS II (upper right), LHCII apoproteins (upper left) and CP1 apoproteins (lower) were used as the immunological probes. Notations for treatments are as given in Fig. 3.

intermittent illumination, but decreased during the subsequent dark incubation with water. On the other hand, a considerable amount of 43 kDa protein accumulated during intermittent illumination, followed by a further increase during the subsequent dark incubation. However, when cotyledons were incubated with CaCl_2 in darkness after intermittent illumination, a large amount of LHCII apoproteins accumulated and the 43 kDa protein disappeared, indicating that Chl bound to 43 kDa protein served as a source of Chl supply for the formation of LHCII.

Thus all the above results obtained from curve analysis and immunoblotting suggest the migration of Chl from CPa to LHCII during the dark incubation with CaCl_2 after intermittent light.

Discussion

When CPs of thylakoid membranes from 6-h illuminated cucumber cotyledons were analyzed by the revised SDS-PAGE method, which minimizes the liberation of Chl from CP by using a low concentration of SDS, almost all the Chl was found in the forms of CP1 and LHCII on the gel. However, it was difficult to determine whether CPa was present or not in 6-h illuminated cotyledons, because it could not be clearly separated from other CPs overlapping with the dense band of monomeric LHCII when low concentrations of SDS were used. The amount of CPa should be small, if it is present, at that stage of greening. However, CP1 and CPa accumulated when 6-h illuminated cotyledons were placed in the dark for 24 h. Therefore, Chl molecules bound to LHCII apoproteins were reutilized for the formation of CP1 and CPa.

Curve analysis offers the advantage of being able to monitor CP changes without loss of particular portions of CPs during preparation when the whole homogenate of tissues is used. The results obtained by this method agree with those obtained by PAGE that CP1 and CPa increased with a concurrent decrease in LHCII during dark incubation of 6-h illuminated cotyledons.

Kreuz et al. (1986) reported that the concentration of CP1 apoproteins in etiolated barley seedlings was below the limit of detection, though their mRNA accumulated to a considerable level. Klein and Mullet (1987) showed that etioplasts of dark-grown barley do not synthesize the two apoproteins each of CP1 and CPa. In the present experiments, however, etiolated barley leaves accumulated CP1 apoproteins and 43 kDa protein in the dark after 4 h of illumination. This indicates that these apoproteins are synthesized in the dark after light exposure. It has been reported that translation of CP1 apoproteins was inhibited by some factors in etioplasts (Klein and Mullet 1987).

CP1 did not accumulate in the dark after light exposure when the synthesis of its apoproteins was inhibited by CAP (Table 1), indicating that formation of CP1 in the dark requires synthesis of its apoproteins. Newly synthesized apoproteins of CP1 and CPa may take Chl *a* away from LHCII and use it to form their CPs. This Chl transfer is thought to occur because apoproteins of CP1 and CPa have a higher affinity for Chl *a* than those of LHCII (Shimada et al. 1990). Chl-depleted LHCII apoproteins may become unstable and subject to degradation. However, when the synthesis of apoproteins of CP1 and CPa was inhibited by CAP, Chl of LHCII was not removed and hence this CP remained stable.

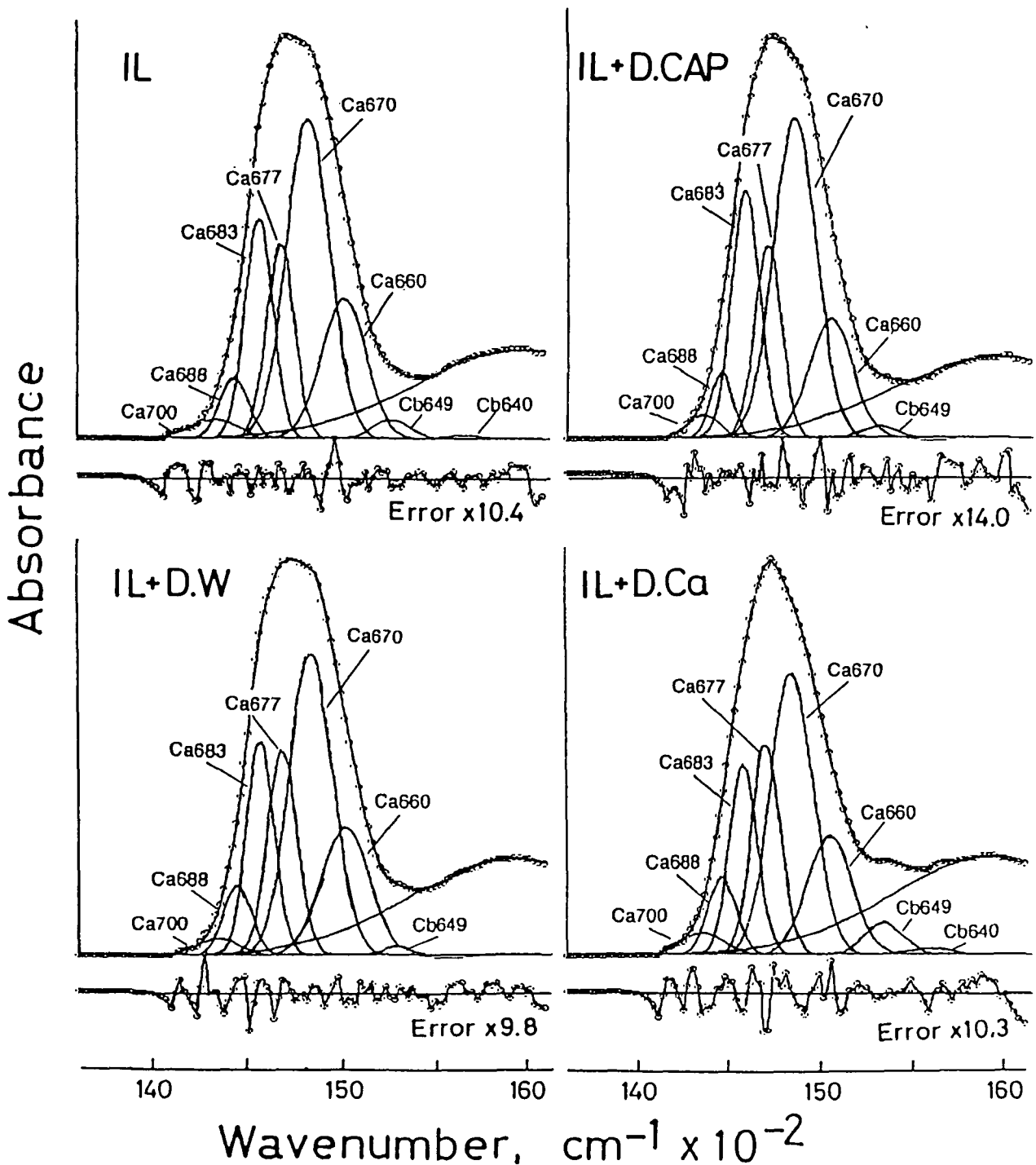


Fig. 5 Curve analysis of the low-temperature spectra of Chl in homogenates of cucumber cotyledons incubated in the dark for 24 h after 10 times of intermittent light. Other explanations are as described in Fig. 3.

Chl *a* released from CPs appears to be reutilized for the formation of other CPs. The question remains of how Chl *b* is metabolized. Chl *b* has been considered to be synthesized from Chl *a*, but not vice versa. However, there is no reliable evidence that Chl *b* cannot be converted to

Chl *a* (Castelfranco and Beale 1983). Kupke (1963) observed an increase in Chl *a* with a concomitant decrease in Chl *b* in the dark, suggesting the conversion of Chl *b* to Chl *a*. Rudoi et al. (1981) reported that etiolated wheat leaves synthesized Chl *a* when incubated with Chlide *b* in

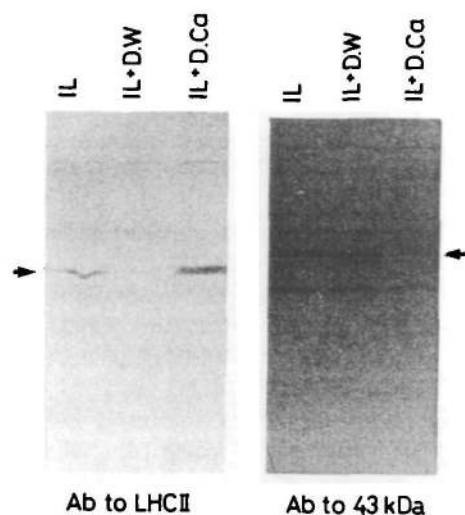


Fig. 6 Immunoblot analysis of 43 kDa peptides and LHCII apoproteins from cucumber cotyledons incubated with or without CaCl_2 in the dark after 10 times of intermittent light. See legends to Fig. 5 for treatments. Other explanations are as described in Fig. 4.

the dark. We also observed that Chl *a* increased in the dark while the total amount of Chl *a* and Chl *b* remained unchanged (Tanaka and Tsuji 1981). At present, the possibility can not be excluded that Chl *b* is released from LHCII and converted to Chl *a* to be used for the formation of CP1 and CPa.

In a previous paper, analysis of CP composition by PAGE showed that Chl was transferred from other CPs to LHCII when intermittently illuminated cotyledons were incubated with CaCl_2 in the dark (Tanaka and Tsuji 1982). These results were substantiated with those obtained by curve analysis (Fig. 5, Table 4) and immunoblotting (Fig. 6). The present experiments further suggest that the Chl used for LHCII formation comes from CPa. CPa probably releases its Chl, as suggested by disappearance of 43 kDa protein during dark incubation with CaCl_2 after continuous or intermittent illumination (Figs. 4 and 6). CaCl_2 treatment may remove Chl *a* from CPa, producing free Chl *a* molecules which are available for the formation of other CPs (Shimada et al. 1990). Some of these Chl *a* molecules may be converted to Chl *b*, which is then incorporated into LHCII apoproteins together with Chl *a*. However, when cotyledons were incubated with CaCl_2 in the presence of CAP, liberated Chl *a* was utilized for LHCII formation preferentially, because of the absence of newly synthesized apoproteins of CP1 and CPa, which otherwise would compete with LHCII apoproteins for Chl *a*. This is why CAP enhanced the CaCl_2 effect in inducing LHCII formation in the dark (Shimada et al. 1990). It is not known whether Chl *a* molecules liberated by CaCl_2

treatment are free in the lipid bilayer or loosely attached to some proteins.

The formation of CP by redistribution of Chl can be shown only in the dark after a brief period of illumination. When Chl is being synthesized, it is difficult to distinguish between CPs formed by this mechanism and those formed by the distribution of newly synthesized Chl. However, both the Chl redistribution and the distribution of newly synthesized Chl may occur while Chl is being actively synthesized in greening tissues.

To form the functional photosystems composed of different CPs in a definite ratio, the following conditions are required (a) The rates of Chl and apoprotein syntheses must be coregulated; (b) the Chl *a* to *b* conversion must be regulated to provide the Chl *a* and Chl *b* required for the synthesis of Chl *a/b*-protein complexes; (c) the exchange of Chl among different apoproteins must play an important role in these processes. A proposed scheme for CP formation by distribution of newly synthesized Chl and redistribution of Chl is shown in Fig. 7. Pchl *a* is photoreduced into Chl *a* and then phytylated to become Chl *a*. Newly synthesized Chl *a* is incorporated into apoproteins of CP1 and CPa. Some of the Chl *a* is converted to Chl *b* and incorporated into LHCII apoproteins together with Chl *a*. However, when the rate of Chl synthesis is low, almost all the Chl *a* is preferentially used for the formation of CP1 and CPa, because the affinity of apoproteins of CP1 and CPa for Chl *a* is higher than that of LHCII apoproteins. Such preferential formation of CP1 and CPa is observed during intermittent illumination (Argyroudi-Akoyunoglou and Akoyunoglou 1979). The preferential accumulation of CP1 is also observed when tissues are in the lag period for Chl synthesis under continuous light (Tanaka and Tsuji 1985). When Chl is synthesized in a sufficient amount or synthesis of apoproteins of CP1 and CPa is limited, Chl can be used for the forma-

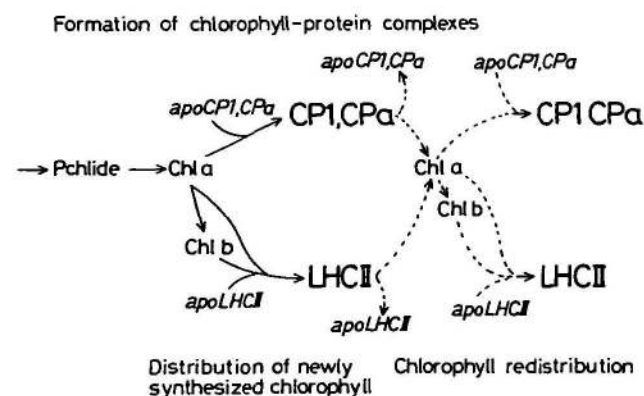


Fig. 7 A proposed scheme for CP formation by distribution of newly synthesized Chl and by redistribution of Chl. See text for explanation.

tion of LHCII. In the case of Chl redistribution, Chl is released from LHCII and incorporated into apoproteins of CP1 and CPa. This is observed when the greening tissues are transferred to darkness (Fig. 2). The redistribution also proceeds based on the differential affinity of apoproteins for Chl *a*. Redistribution of Chl from 43 kDa protein to LHCII apoproteins is observed when intermittently illuminated cotyledons are incubated with CaCl₂ in the dark (Tanaka and Tsuji 1982). Thus, the factors which determine the rate of formation of each CP are the rates of syntheses of Chl and its apoprotein and the affinity of the apoprotein for Chl *a*. Further study is needed to clarify the details of the mechanisms of Chl distribution.

References

- Anderson, J. M., Waldrom, J. G. and Shorne, S. W. (1978) Spectral characterization of six complexes resolved by an improved electrophoretic procedure. *FEBS Lett.* 92: 227–233.
- Argyroudi-Akoyunoglou, J. H. and Akoyunoglou, G. (1979) The chlorophyll-protein complexes of the thylakoid in greening plastids of *Phaseolus vulgaris*. *FEBS Lett.* 104: 78–84.
- Argyroudi-Akoyunoglou, J. H., Akoyunoglou, A., Kalosakas, K. and Akoyunoglou, G. (1982) Reorganization of the photosystem II unit in developing thylakoids of higher plants after transfer to darkness. Changes in chlorophyll *b*, light-harvesting chlorophyll protein content, and grana stacking. *Plant Physiol.* 70: 1242–1248.
- Bhaya, D. and Castelfranco, P. A. (1985) Chlorophyll biosynthesis and assembly into chlorophyll-protein complexes in isolated developing chloroplasts. *Proc. Natl. Acad. Sci. USA* 82: 5370–5374.
- Brown, J. S. (1983) A new evaluation of chlorophyll absorption in photosynthetic membranes. *Photosynth. Res.* 4: 375–383.
- Brown, J. S. and Schoch, S. (1981) Spectral analysis of chlorophyll-protein complexes from higher plant chloroplasts. *Biochim. Biophys. Acta* 636: 201–209.
- Castelfranco, P. A. and Beale, I. B. (1983) Chlorophyll synthesis: Recent advances and areas of current interest. *Annu. Rev. Plant Physiol.* 34: 241–278.
- Van Dorssen, R. J., Plijter, J. J., Den Ouden, A., Amesz, J. and Van Gorkom, H. J. (1987) Spectroscopic properties of chloroplast grana membranes and of the core of Photosystem II. *Biochim. Biophys. Acta* 890: 134–143.
- French, C. S., Brown, J. S. and Lawrence, M. C. (1972) Four universal forms of chlorophyll *a*. *Plant Physiol.* 49: 421–429.
- Klein, R. R. and Mullet, J. E. (1987) Control of gene expression during higher plant chloroplast biogenesis. Protein synthesis and transcript levels of *psbA*, *psaA-psaB*, and *rbcL* in dark-grown and illuminated barley seedlings. *J. Biol. Chem.* 262: 4341–4348.
- Kreuz, K., Dehesh, K. and Apel, K. (1986) The light-dependent accumulation of the P700 chlorophyll *a* protein of the photosystem I reaction center in barley. *Eur. J. Biochem.* 159: 459–467.
- Kupke, D. W. (1963) Chlorophyll *a* appearance in the dark in higher plants. *Science* 140: 49–51.
- Kuwabara, T. and Murata, N. (1982) Inactivation of photosynthetic oxygen evolution and concomitant release of three polypeptides in the photosystem II particles of spinach chloroplasts. *Plant Cell Physiol.* 23: 533–539.
- Laemmli, O. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227: 680–685.
- Mullet, J. E., Burke, J. J. and Arntzen, C. J. (1980) Chlorophyll proteins of photosystem I. *Plant Physiol.* 65: 814–822.
- Rudoi, A. B., Vezitskii, A. Yu. and Shlyk, A. A. (1981) Study of the enzymatic system transforming chlorophyllide into chlorophyll in etiolated leaves, using exogenous substrates. *Biochimiya* 47: 733–739.
- Satoh, K. and Butler, W. L. (1978) Low temperature spectral properties of subchloroplast fractions purified from spinach. *Plant Physiol.* 61: 373–379.
- Schmidt, G. W., Bartlett, S. G., Grossman, A. R., Cashmore, A. R. and Chua, N.-H. (1981) Biosynthetic pathways of two polypeptide subunits of the light-harvesting chlorophyll *a/b* protein complex. *J. Cell. Biol.* 91: 468–478.
- Shimada, Y., Tanaka, A., Tanaka, Y., Takabe, T., Takabe, T. and Tsuji, H. (1990) Formation of chlorophyll-protein complexes during greening 1. Distribution of newly synthesized chlorophyll. *Plant Cell Physiol.* 31: 639–647.
- Simpson, D. J. (1988) Low temperature absorption spectroscopy of barley mutants. Gaussian deconvolution and fourth derivative analysis. *Carlsberg Res. Commun.* 53: 343–356.
- Tanaka, A. and Tsuji, H. (1981) Changes in chlorophyll *a* and *b* content in dark-incubated cotyledons excised from illuminated seedlings. The effect of calcium. *Plant Physiol.* 68: 567–570.
- Tanaka, A. and Tsuji, H. (1982) Calcium-induced formation of chlorophyll *b* and light-harvesting chlorophyll *a/b*-protein complex in cucumber cotyledons in the dark. *Biochim. Biophys. Acta* 680: 265–270.
- Tanaka, A. and Tsuji, H. (1983) Formation of chlorophyll-protein complexes in greening cucumber cotyledons in light and then in darkness. *Plant Cell Physiol.* 24: 101–108.
- Tanaka, A. and Tsuji, H. (1985) Appearance of chlorophyll-protein complexes in greening barley seedlings. *Plant Cell Physiol.* 26: 893–902.