Plant Physiol. (1997) 113: 137–147

Conversion of Chlorophyll *b* to Chlorophyll *a* and the Assembly of Chlorophyll with Apoproteins by Isolated Chloroplasts[1]

Tatsuyuki Ohtsuka[2], Hisashi Ito, and Ayumi Tanaka*

Department of Botany, Faculty of Science, Kyoto University, Kyoto 606–01 Japan

The photosynthetic apparatus is reorganized during acclimation to various light environments. During adaptation of plants grown under a low-light to high-light environment, the light-harvesting chlorophyll *a*/*b*-protein complexes decompose concomitantly with an increase in the core complex of photosystem II. To study the mechanisms for reorganization of photosystems, the assembly of chlorophyll with apoproteins was investigated using isolated chloroplasts. When [^{14}C]chlorophyllide *b* was incubated with chloroplasts in the presence of phytyl pyrophosphate, it was esterified and some of the [^{14}C]chlorophyll *b* was converted to [^{14}C]chlorophyll *a* via 7-hydroxymethyl chlorophyll. [^{14}C]Chlorophyll *a* and *b* were incorporated into chlorophyll-protein complexes. Light-harvesting chlorophyll *a*/*b*-protein complexes of PSII had a lower [^{14}C]chlorophyll *a* to [^{14}C]chlorophyll *b* ratio than P700-chlorophyll *a*-protein complexes, indicating the specific binding of chlorophyll to apoproteins in our systems. 7-Hydroxymethyl chlorophyll, an intermediate molecule from chlorophyll *b* to chlorophyll *a*, did not become assembled with any apoproteins. These results indicate that chlorophyll *b* is released from light-harvesting chlorophyll *a*/*b*-protein complexes of photosystem II and converted to chlorophyll *a* via 7-hydroxymethyl chlorophyll in the lipid bilayer and is then used for the formation of core complexes of photosystems. These mechanisms provide the fast, fine regulation of the photosynthetic apparatus during construction of photosystems.

Chlorophyll-protein complexes are major components of the photosynthetic apparatus, which harvests light energy and transfers it to reaction centers (Thornber, 1979). The PSII particle of higher plants is composed of a core complex and light-harvesting complexes. The core complex has D1, D2, CP43, and CP47 (Vermaas, 1993) that are composed of chlorophyll *a* as a photosynthetic pigment. Associated with this core complex is LHCII. The PSI particle is also composed of a core complex, CP1, and LHCI (Golbeck, 1992). For any specific environment, the ratio of the numbers of PSI to PSII and that of light-harvesting complexes to core complexes must be maintained at a constant level.

Each chlorophyll-protein complex is considered to be composed of chlorophyll and its apoprotein in a definite ratio. Kühlbrandt and Wang (1991) have shown that LHCII binds 15 chlorophylls. The chlorophyll to P700 ratio for CP1 isolated by SDS-PAGE was reported to be 120 (Anderson, 1980). Two apoproteins of the PSII core have definite ratios of chlorophyll to apoproteins (Delepelaire and Chua, 1979; Tang and Satoh, 1984). Since all of the chlorophyll molecules bind to apoproteins in thylakoid membranes (Markwell et al., 1979), the ratio of chlorophyll to apoproteins of mature chloroplasts would be constant under any specific environment. In the case of isolated etioplasts, the apoproteins of chlorophyll *a*-protein complexes, which are encoded by the chloroplast genome, accumulate only when chlorophyll is synthesized (Eichacker et al., 1990). Apoproteins of LHCII are synthesized by cytoplasmic ribosomes and imported into chloroplasts. These apoproteins bind chlorophyll *a* and chlorophyll *b* in thylakoid membranes and assemble to PSII core complexes. When the apoproteins do not bind chlorophyll, they are soon degraded (Bennett, 1981). These are possible mechanisms for the stoichiometric regulation between apoproteins and chlorophyll. However, the photosynthetic apparatus shows a dynamic reorganization in response to changes in light intensity and quality. When plants are grown under PSII light, the ratio of PSI to PSII increases to balance the electron flow of PSI and PSII (Chow et al., 1990). When low-light-grown plants are transferred to high light intensity, the amount of LHCII/chlorophyll *a* protein (CP43 and CP47) of the PSII ratio decreases, indicating a reduction in the antenna size of PSII (Lindahl et al., 1995). These findings show that plants reorganize photosystems to achieve efficient photosynthesis by changing the compositions of the chlorophyll-protein complexes. To know how the formation of individual chlorophyll-protein complexes is regulated during greening or reorganization of the photosynthetic apparatus, we must clarify the process of how the chlorophyll and apoproteins are assembled.

Chlorophyll-protein complexes are formed by two mechanisms: by distribution of newly synthesized chlorophyll or by redistribution of chlorophyll. The former is observed during greening of etiolated seedlings (Shimada et al., 1990). Protochlorophyllide is synthesized from glutamate in the stroma and envelope of chloroplasts and reduced to

[1] This work was supported by a grant-in-aid for scientific research from Ministry of Education, Science, and Culture, Japan.

[2] Present address: Green Cross Corp., Osaka 573, Japan.

* Corresponding author; e-mail ayumi@ok-lab.bot.kyoto-u.ac.jp; fax 81–75–753–4257.

Abbreviations: ALA, 5-aminolevulinic acid; CPa, chlorophyll *a*-protein complex of PSII; CP1, P700-chlorophyll *a*-protein complex; LDS, lithium dodecyl sulfate; LHCI, light-harvesting chlorophyll *a*/*b*-protein complexes of PSI; LHCII, light-harvesting chlorophyll *a*/*b*-protein complexes of PSII.

chlorophyllide *a*, which is esterified to chlorophyll *a* in the thylakoid membranes (Wettstein et al., 1995). The newly synthesized chlorophyll assembles with apoproteins of chlorophyll *a*-protein complexes, which are synthesized by chloroplast ribosomes or with those of LHC, which are imported into chloroplasts (Nechushtai et al., 1995). Chlorophyll-protein complexes are also formed by redistribution of chlorophyll previously bound to other apoproteins. Formation of chlorophyll-protein complexes by redistribution of chlorophyll is observed when greening tissues are transferred to darkness (Argyroudi-Akoyunoglou et al., 1982; Tanaka and Tsuji, 1983; Tanaka et al., 1991). CP1 and CPa are formed during dark incubation with a concomitant decrease in LHCII after a short period of illumination. The change in composition of the chlorophyll-protein complex during dark incubation indicates the movement of chlorophyll from LHCII to CP1 and CPa because chlorophyll is not synthesized in the dark in angiosperms. Redistribution of chlorophyll from CPa to LHCII is also observed when intermittently illuminated tissues are incubated in the dark with $CaCl_2$ (Tanaka and Tsuji, 1982; Tanaka et al., 1991, 1995). Thus, chlorophyll, which was once bound to some apoproteins, is reused to form new chlorophyll-protein complexes. However, detailed mechanisms of the distribution and redistribution of chlorophyll are not yet known.

Several studies have elucidated the assembly process of the apoproteins with chlorophylls by isolated chloroplasts or thylakoid membranes. Minami et al. (1986) reported that with isolated chloroplasts the reaction center proteins of PSII (43 and 47 kD) were synthesized, inserted into the membranes, and became bound to chlorophyll. Analysis by using labeled LHCII precursor revealed the assembly process of the chlorophyll with apoproteins. The precursor of the apoproteins of LHCII was inserted into the stroma lamellae and moved to the granal lamellae where apoproteins bind to chlorophyll (Yalovsky et al., 1990). These results also indicate that the newly incorporated apoproteins assembled with preexisting chlorophyll in granal thylakoids because chlorophyll was not synthesized in these experiments. Assembly of chlorophyll with apoproteins was reported with isolated plastids using labeled ALA, a precursor of chlorophyll. Newly synthesized chlorophyll from labeled ALA was assembled into chlorophyll-protein complexes in the presence of chloramphenicol, an inhibitor of protein synthesis by chloroplasts, indicating the assembly of newly synthesized chlorophyll with the preexisting apoproteins (Bhaya and Castelfranco, 1985).

It has been generally accepted that chlorophyll *b* or chlorophyllide *b* is biosynthetically derived from chlorophyll(ide) *a*. The conversion of chlorophyll *b* to chlorophyll *a* had not been thought to occur because reduction of the formyl group to a methyl group is a difficult reaction. However, in previous studies we showed that chlorophyll *b* is converted to chlorophyll *a* via 7-hydroxymethyl chlorophyll by isolated chloroplasts (Ito et al., 1993, 1994, 1996). The conversion of chlorophyll *b* to chlorophyll *a* and the decrease in LHCII accompanied by an increase in CP1 and CPa suggest that chlorophyll *b* of LHCII is released from the apoproteins and converted to chlorophyll *a*, followed by incorporation into CP1 and CPa apoproteins. If chlorophyll *b* of LHCs can be used for the formation of core complexes, this may play an important role in the reorganization of photosystems during adaptation to light quality and intensity. To test this hypothesis, we carried out experiments on the assembly of chlorophyll with apoproteins by isolated chloroplasts or thylakoids. We used [^{14}C]chlorophyllide because, although [^{14}C]chlorophyll can be used to study the assembly by isolated thylakoid membranes or chloroplasts, it is difficult to use since it is lipophilic due to the prenyl side chain and does not dissolve in buffer without detergent. On the other hand, chlorophyllide is soluble in buffer and rapidly esterifies to chlorophyll in thylakoid membranes. [^{14}C]Chlorophyllide *b* was incubated with chloroplasts and the distribution of [^{14}C]chlorophyll *a* and *b* among the various apoproteins was investigated. Chlorophyllide *b* was converted to chlorophyll *a* and incorporated into CP1, but 7-hydroxymethyl chlorophyll, an intermediate molecule of chlorophyll *b* to chlorophyll *a*, did not assemble with apoproteins. These results suggest that chlorophyll *b* was released from LHCII and converted to chlorophyll *a* in thylakoid membranes and then incorporated into CP1.

MATERIALS AND METHODS

Cucumber (*Cucumis sativus* L. cv Aonagajibai) seeds were soaked for about 3 h and grown on moist vermiculite in the dark at 28°C for 4 to 5 d followed by 12 h of continuous light.

Plastid Isolation

Cotyledons were cut from greening seedlings and homogenized with a razor blade blender in an isolation buffer containing 50 mM Hepes-KOH (pH 8.0), 330 mM sorbitol, 0.2% BSA, 5 mM Cys, and 2 mM EDTA. The homogenate was filtered through a nylon mesh and centrifuged at 3000*g* for 3 min. The resultant pellet (crude chloroplasts) was resuspended in 2 mL of the isolation buffer and loaded on a discontinuous gradient of 30, 60, and 90% Percoll (Pharmacia) containing 50 mM Hepes-KOH (pH 8.0), 330 mM sorbitol, 0.2% BSA, 5 mM Cys, and 2 mM EDTA. After the sample was centrifuged at 7000*g* for 10 min, the interface between 60 and 90% Percoll was collected and diluted with the isolation buffer. Intact plastids were pelleted by centrifugation at 5000*g* for 5 min.

Preparation of [^{14}C]Chlorophylls

[^{14}C]Chlorophylls were prepared from greening cucumber cotyledons fed with 4-[^{14}C]ALA as described previously (Ito et al., 1994). For the preparation of [^{14}C]7-hydroxymethyl chlorophyll, [^{14}C]chlorophyll *b* and 0.2 mM $NaBH_4$ were dissolved in 0.2 mL of methanol (Holt, 1959). After 5 min the solution was mixed with 0.2 mL of hexane:2-propanol (10:1, v/v) and then added to NaCl-saturated water. The hexane solution was dried under reduced pressure. The dried chlorophylls were stored at −20°C until use.

Preparation of [^{14}C]Chlorophyllides

Thylakoid membranes from garland chrysanthemum leaves were washed with acetone and dried by the methods

of Tanaka (1982) as modified by Ito et al., (1994). The resultant powder was washed twice with 80% (v/v) acetone before use to remove contamination from a small amount of chlorophyll. [^{14}C]Chlorophyll *a*, [^{14}C]chlorophyll *b*, or [^{14}C]7-hydroxymethyl chlorophyll was dissolved in a solution containing 20 mM citrate-NaOH (pH 7.0) and 2 mM sodium ascorbate and incubated with the powder for 1.5 h (Holden, 1960). The incubation was stopped by addition of acetone at a final concentration of 80% (v/v). After the mixture was centrifuged, the green supernatant was stored at −20°C until use.

Incubation of [^{14}C]Chlorophyllide with Chloroplasts

The stock solution of [^{14}C]chlorophyllide was washed twice with hexane to remove chlorophyll from the acetone phase and then combined with 0.5 volume of diethyl ether and 5 volumes of NaCl-saturated water. The ether solution, which contained chlorophyllide, was transferred into an incubation tube and dried under reduced pressure.

Crude or intact chloroplasts isolated as described above were resuspended in an incubation buffer containing 50 mM Hepes-KOH (pH 7.5), 330 mM sorbitol, 0.2% BSA, 10 mM ATP, 5 mM NADPH, 2 mM sodium ascorbate, and 30 mg/mL phytyl PPi, to give a chlorophyll concentration of 0.3 to 0.5 mg/mL. In some experiments sorbitol was omitted from the buffer to incubate chlorophyllide with thylakoid membrane fractions instead of total chloroplasts. The mixture corresponding to 0.25 or 0.50 mg of chlorophyll was transferred into a tube containing [^{14}C]chlorophyllide and then incubated in the dark at 28°C. Chlorophyll concentrations were determined as described by Arnon (1949).

Fractionation of Thylakoid Membranes by Triton X-100 Suc Density Gradient Centrifugation

After incubation with [^{14}C]chlorophyllide, the chloroplast suspension was diluted with 5 mM EDTA (pH 8.0) and centrifuged at 10,000*g* for 10 min. The pellet (washed membranes) was diluted with distilled water to give a chlorophyll concentration of 0.40 mg/ml. Next, 20% Triton X-100 (w/v) was added to the suspension to give a final concentration of 0.5%. After the mixture was centrifuged at 40,000*g* for 15 min, the green supernatant was loaded on a linear 0.1 to 1.0 M Suc density gradient containing 0.1% Triton X-100 and centrifuged at 40,000 rpm in a swing rotor (RPS56T, Hitachi, Tokyo, Japan) for 14 h at 4°C. These procedures resulted in the resolution of four green bands, as shown in Figure 1A.

Purification of LHCII and CP1

For the purification of LHCII, bands I and II were collected from Triton X-100 Suc density gradients, and $MgCl_2$ and KCl were added to the final concentrations of 10 and 100 mM, respectively (Burke et al., 1978). The solution was centrifuged at 50,000 rpm in an angle rotor (RP70T, Hitachi) at 4°C for 3 h, resulting in sedimentation of LHCII. To purify CP1, the washed membranes were resuspended in a buffer containing 300 mM Tris-HCl (pH 8.8) and 1.0% (w/v) LDS. After the sample was centrifuged at 40,000*g* for 15 min, the supernatant was subjected to Suc density gradient centrifugation as described above, except that 0.15% (w/v) LDS was included in the Suc density gradient instead of Triton X-100. These procedures resulted in the resolution of two green bands on the gradient (Fig. 4A), the lower one of which was shown to contain exclusively CP1 (Fig. 4B).

Analysis of Chlorophyll by HPLC and Determination of Their Radioactivities

Chlorophylls were extracted with acetone from Suc solutions of green bands on the Suc gradient and subjected to HPLC on an octadecyl silica column (6 mm [i.d.] × 150 mm) using methanol as the elution agent at a flow rate of 1.5 mL/min at room temperatures (Shioi et al., 1983). Chlorophylls were monitored by their A_{663} and quantified from the chromatographic response with known quantities of the relevant pigment. Eluates were collected every 40 s and the radioactivity of each fraction was measured in a liquid scintillation counter (LSC 900, Aloka, Tokyo, Japan). Quenching was corrected for by the external standard method. Counting efficiency for ^{14}C varied between 87.5 and 92.5%.

Analysis of Chlorophyll-Protein Complex or Protein Compositions of Green Bands on Suc Density Gradients

To determine the chlorophyll-protein complex compositions of bands I to IV, Suc solutions of the bands were subjected to disc SDS-PAGE under nondenaturing conditions according to the method of Tanaka et al. (1987). After electrophoresis at 6 mA in a cold room for about 50 min, the gels were then subjected to densitometric scanning at 675 nm with a dual-wavelength spectrophotometer (no. 556, Hitachi). For analysis of protein compositions of the two bands on the LDS Suc density gradient, the proteins of each band were pelleted by centrifugation and subjected to SDS-PAGE according to the method of Laemmli (1970).

RESULTS

Fractionation of Thylakoid Membranes by Suc Density Gradient Centrifugation

At the last step of chlorophyll biosynthesis, chlorophyllide is esterified and incorporated into chlorophyll-protein complexes. Exogenous chlorophyllide is also esterified when incubated with etioplast membranes (Ito et al., 1993, 1994) or with membrane fractions from isolated etioplasts (Rüdiger et al., 1980; Benz and Rüdiger, 1981). In this study we first investigated whether exogenous chlorophyllide is incorporated into chlorophyll-protein complexes after conversion to chlorophyll when incubated with thylakoid membrane prepared from greening cucumber tissues in which chlorophyll-protein complexes were actively synthesized. [^{14}C]Chlorophyllide was used instead of nonlabeled chlorophyllide *a* to distinguish chlorophyll synthesized in vitro from preexisting chlorophyll. After the incubation of [^{14}C]chlorophyllide, thylakoid membranes were solubi-

lized in 0.5% Triton X-100 and then fractionated by a Suc density gradient to separate photosynthetic particles.

Figure 1A shows a resolution profile of photosynthetic particles on a Suc density gradient. Four green bands (I–IV) were separated and subjected to SDS-PAGE under nondenaturing conditions to determine the composition of their chlorophyll-protein complexes (Fig. 1B). Band I contained free chlorophylls and monomeric forms of LHCII with a small amount of oligomeric forms of LHCII. Free chlorophylls were abundant in the upper part of band I and LHCII was abundant in the lower part. Some of the free chlorophyll would have been released from the chlorophyll-protein complexes during the treatment with Triton X-100 or with SDS. Band II contained a large amount of oligomeric LHCII with a small amount of monomeric LHCII. The chlorophyll *a* to *b* ratio for band II was between 1.7 and 2.2, which is higher than that for the major species of LHCII, the ratios of which were estimated to be between 1.1 and 1.3 (Dainese and Bassi, 1991; Kühlbrandt and Wang, 1991). Band II would have contained small amounts of CPa in addition to LHCII, although CPa was not found on the nondenaturing gel. CP1 was found in bands III and IV. The chlorophyll *a* to *b* ratio for band III was between 10 and 11, whereas that for band IV was between 24 and 26. The difference in the chlorophyll *a* to *b* ratio between bands III and IV is due to the different contents of LHCI. Free chlorophyll on gels for bands II, III, and IV would be generated during electrophoresis.

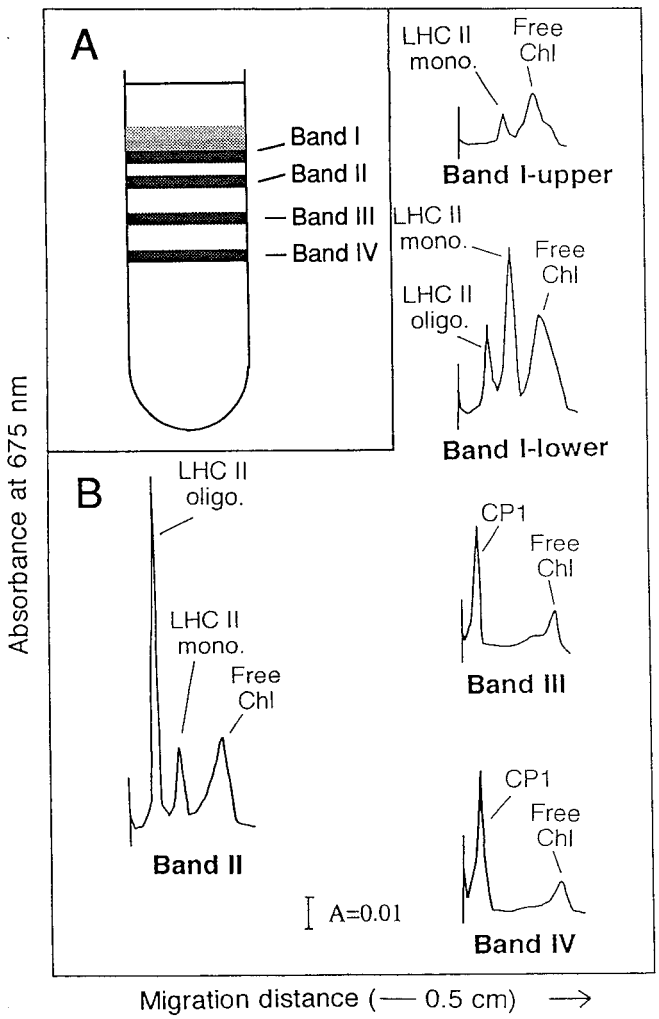

Figure 1. Fractionation of thylakoid membranes into photosynthetic particles by Triton X-100 Suc density gradient centrifugation. A, Thylakoid membranes were isolated from 12-h illuminated cotyledons and solubilized in 0.5% Triton X-100. The solution was loaded on a linear 0.1 to 1.0 M Suc density gradient containing 0.1% Triton X-100 and centrifuged at 40,000 rpm for 14 h at 4°C. B, Green bands obtained by Suc density gradient centrifugation were subjected to SDS-PAGE under nondenaturing conditions. After electrophoresis, the gels were densitometrically scanned at 675 nm. The upper and lower regions of band I were separately collected for SDS-PAGE. Mono, Monomeric form of LHCII; oligo, oligomeric form of LHCII; Chl, chlorophyll.

Incorporation of Newly Synthesized Chlorophyll into Chlorophyll-Protein Complexes

[^{14}C]Chlorophyllide *a* was incubated with thylakoid membrane and fractionated by Triton X-100 Suc density gradient centrifugation. Chlorophylls from each green band on the gradient were subjected to HPLC using an octadecyl silica column to separate chlorophyll *a* and chlorophyll *b*, and their radioactivities were determined (Fig. 2). Chlorophyll *a* from bands I to IV was radioactive, suggesting that [^{14}C]chlorophyllide *a* was esterified and incorporated into LHCII and PSI. When [^{14}C]chlorophyllide *a* was incubated with intact chloroplasts, its distribution among bands I to IV was the same as in the thylakoid membrane fraction (data not shown), indicating that incorporation of newly synthesized chlorophyll into chlorophyll-protein complexes does not require stromal factors. Chlorophyll would be incorporated into preexisting chlorophyll-protein complexes rather than become assembled with newly synthesized proteins because these apoproteins could not be synthesized during incubation in our experiments. Chlorophyll *b* did not accumulate when chlorophyllide *a* was incubated with thylakoid membranes or intact chloroplasts, indicating that chlorophyll *a* to chlorophyll *b* conversion had not occurred in our systems.

Conversion of Chlorophyll *b* to Chlorophyll *a* and Their Incorporation into Chlorophyll-Protein Complexes

Previously we reported that chlorophyll *b* was converted to chlorophyll *a* by isolated etiochloroplasts as well as by etioplasts (Ito et al., 1993, 1994, 1996). This time we examined whether chlorophyll *a* converted from chlorophyll *b* is incorporated into chlorophyll-protein complexes. After incubation of [^{14}C]chlorophyllide *b* with etiochloroplasts, thylakoid membranes were fractionated by Suc density gradient centrifugation and chlorophylls were extracted from each green band. Figures 3 and 4 show HPLC separation profiles of chlorophylls from bands I to IV and the radioactivities of the fractionated eluates measured by liquid scintillation counting. Fractions of chlorophyll *a* and chlorophyll *b* had radioactivities indicating that not only chlorophyll *b* but also chlorophyll *a* converted from chlorophyll *b* had been incorporated into the chlorophyll-

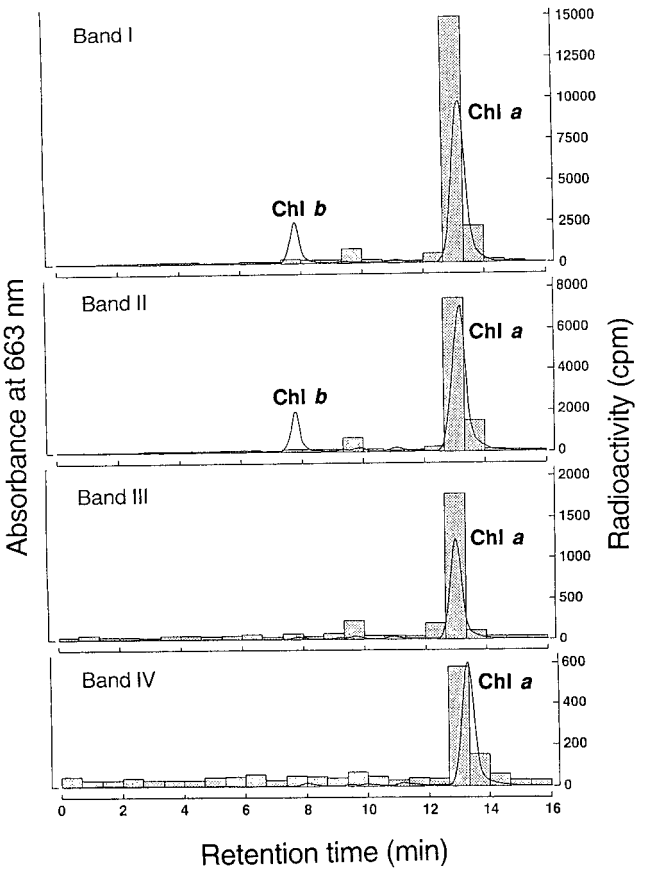

Figure 2. Esterification of chlorophyllide *a* and its incorporation into photosynthetic particles. After the incubation of [^{14}C]chlorophyllide *a* with thylakoid membrane fraction (0.25 mg of chlorophyll) isolated from 12-h illuminated cotyledons, the membranes were fractionated by Triton X-100 Suc density gradient centrifugation as described in Figure 1A. Chlorophylls extracted from bands I to IV were subjected to HPLC using an octadecyl silica column. Eluates were collected every 40 s and the radioactivity of each fraction was measured by liquid scintillation counting (shown by columns). Traced lines show HPLC elution profiles monitored by measuring A_{663}. For band I, 40% of the chlorophylls collected from the Suc gradient were analyzed. Chl, Chlorophyll.

protein complexes of LHCII and PSI. Radioactivities of the fractions of 5'40" to 7'20" were higher than the background radioactivity. These would be due to accumulation of [^{14}C]chlorophyll *b* esterified with geranylgenaniol, dihydrogeranylgeraniol, or tetrahydrogeranylgeraniol. These results indicate that chlorophylls can be complexed with apoproteins before their prenyl moieties are fully hydrogenated, as found by Maloney et al. (1989).

The distribution of radioactivity among bands I to IV is shown in Figure 4B. More than 90% of the total radioactivity was found in bands I and II and less than 10% was found in bands III and IV. Specific radioactivity of chlorophyll for bands I and II ([^{14}C]chlorophyll/chlorophyll) was much higher than those for bands III and IV (Fig. 4A). Since band II contained mainly LHCII, and bands III and IV contained exclusively PSI (Fig. 1B), the distribution of specific radioactivities indicates that chlorophyll synthesized from exogenous chlorophyllide was incorporated preferentially into LHCII rather than into PSI. Two possible explanations can be postulated: (a) LHCII has more unoccupied sites for chlorophyll binding than CP1 or (b) LHCII is a peripheral complex of PSII, whereas CP1 is embedded in the peripheral proteins, and chlorophyll molecules are more accessible to LHCII than CP1.

The ratio of [^{14}C]chlorophyll *a* to [^{14}C]chlorophyll *b* for total thylakoids was 1:3, whereas that for band II and bands III and IV was 1.4 and 1.1, respectively (Fig. 4B). This indicates that chlorophyll *b* was preferentially incorporated into LHCII. These observations are consistent with the fact that the chlorophyll *a* to chlorophyll *b* ratio for PSI is higher than that for LHCII. Thus, newly synthesized chlorophyll *a* and

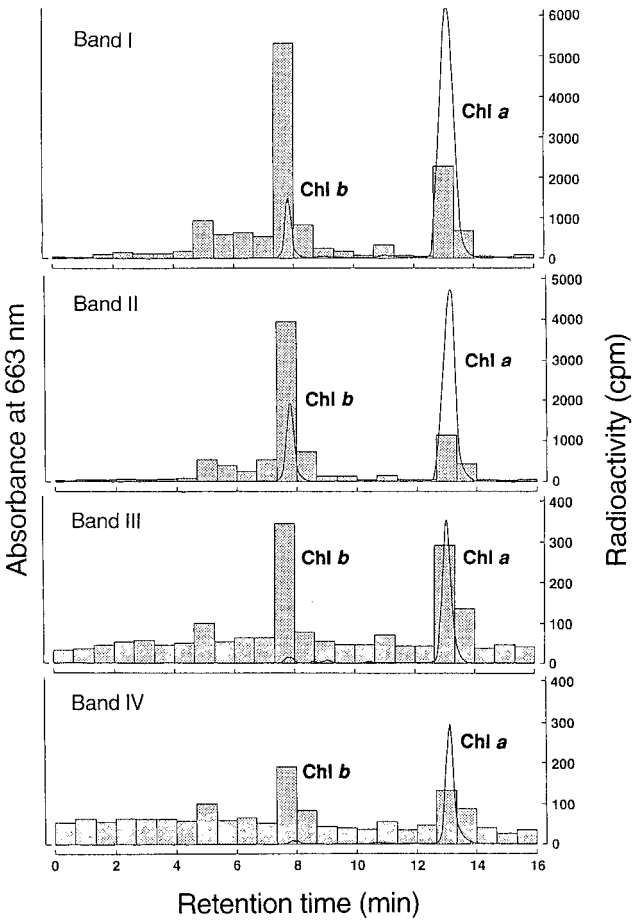

Figure 3. Synthesis of chlorophyll *a* and chlorophyll *b* from chlorophyllide *b* and their incorporation into photosynthetic particles. After incubation of [^{14}C]chlorophyllide *b* with intact chloroplasts (0.25 mg of chlorophyll) isolated from 12-h illuminated cotyledons, thylakoid membranes were fractionated by Triton X-100 Suc density gradient centrifugation as described in Figure 1A. Chlorophylls from bands I to IV were subjected to HPLC using an octadecyl silica column. Eluates were collected every 40 s and the radioactivity of each fraction was measured by liquid scintillation counting (shown by columns). Traced lines show HPLC elution profiles monitored by measuring A_{663}. For band I, 40% of the chlorophyll collected from the Suc gradient was analyzed here because band I had a lot of chlorophyll. Chl, Chlorophyll.

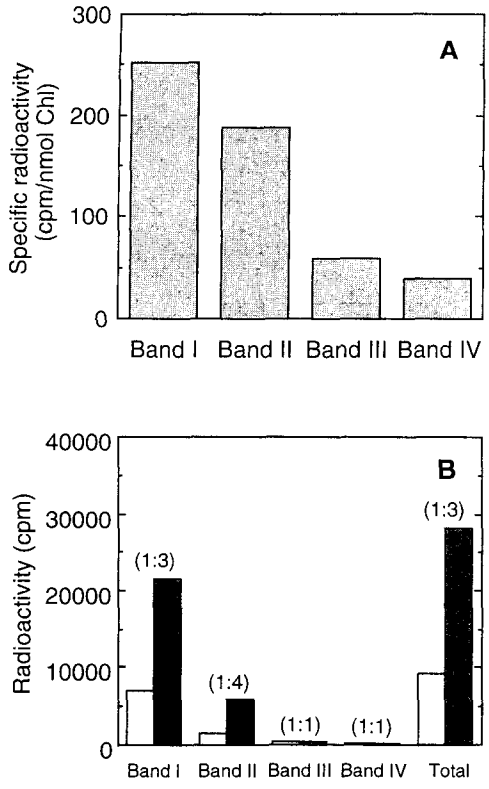

Figure 4. Distribution among photosynthetic particles of chlorophyll *a* and chlorophyll *b* synthesized from chlorophyllide *b*. The radioactivities and chlorophyll amounts were calculated from Figure 3. A, Specific radioactivities of bands I to IV. Specific radioactivities of chlorophylls (cpm/nmol) were determined from the radioactivities of chlorophyll-containing fractions and the amounts of chlorophylls. Chlorophylls were quantified from the chromatographic response with known quantities of the relevant pigments. B, Distribution of [^{14}C]chlorophyll. Open columns, Chlorophyll *a*; filled columns, chlorophyll *b*. The ratios of [^{14}C]chlorophyll *a* to [^{14}C]chlorophyll *b* (cpm/cpm) are shown in parentheses. The data for the sum of bands I to IV are also shown (total).

chlorophyll *b* from exogenous chlorophyllide *b* were distributed to photosynthetic particles in isolated etiochloroplasts as they are distributed in vivo. During the early period of incubation, the [^{14}C]chlorophyll *a* to [^{14}C]chlorophyll *b* ratio is very low, because esterification of [^{14}C]chlorophyllide rapidly proceeds compared with the chlorophyll *b* to chlorophyll *a* conversion, resulting in predominant accumulation of [^{14}C]chlorophyll *b* (Ito et al., 1994). A large amount of [^{14}C]chlorophyll *b* would be incorporated into LHCII and PSI during the early periods of incubation, which would be one reason why LHCII and PSI had a lower [^{14}C]chlorophyll *a* to [^{14}C]chlorophyll *b* ratio than their chlorophyll *a* to chlorophyll *b* ratio.

Chlorophyll can bind nonspecifically to proteins other than the apoproteins of chlorophyll-protein complexes. Bands I and II have many colorless proteins and bands III and IV have many peripheral proteins assembling with CP1. To confirm that chlorophyll is assembled with the apoproteins, we purified the chlorophyll-protein complexes and determined the amount of radioactive chlorophyll. After incubation of [^{14}C]chlorophyllide *a* or [^{14}C]chlorophyllide *b* with chloroplasts, thylakoid membranes were fractionated by Suc density gradient centrifugation, and LHCII was purified by magnesium precipitation from bands I and II according to the method of Burke et al. (1978). Chlorophylls from the purified LHCII were subjected to HPLC and liquid scintillation counting. After incubation with [^{14}C]chlorophyllide *a*, [^{14}C]chlorophyll *a* was detected but [^{14}C]chlorophyll *b* was not in purified LHCII (Fig. 5A). After incubation with [^{14}C]chlorophyllide *b*, [^{14}C]chlorophyll *b* and small amounts of [^{14}C]chlorophyll *a* were found (Fig. 5B). These results indicate that chlorophyll was incorporated into LHCII apoproteins.

We also showed that chlorophyll *a* synthesized from chlorophyll *b* was incorporated into PSI particles (Fig. 4), but this did not indicate that chlorophyll *a* was incorporated into CP1, because PSI particles isolated by Triton X-100 Suc density gradient centrifugation contained many proteins in addition to CP1 apoproteins. To show that chlorophyll *a* was actually incorporated into CP1, thylakoid membranes were treated with 1.0% LDS, resulting in dissociation of peripheral proteins from CP1.

When LDS-solubilized thylakoid membranes were fractionated by LDS Suc density gradient centrifugation, two green bands were resolved (Fig. 6A). Protein compositions of the two bands were analyzed by SDS-PAGE (Fig. 6B). Most of the proteins of thylakoid membranes were found in the upper green band, whereas most of the proteins in the lower band were CP1 apoproteins. The protein bands with slow mobilities on the gel were identified as aggre-

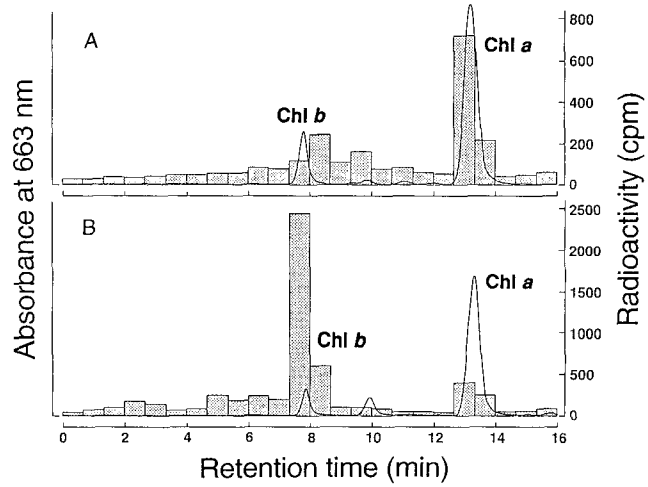

Figure 5. Incorporation of chlorophyll into LHCII. After the incubation of [^{14}C]chlorophyllide *a* (A) or [^{14}C]chlorophyllide *b* (B) with chloroplasts (0.25 mg of chlorophyll) isolated from 12-h illuminated cotyledons, thylakoid membranes were fractionated by Triton X-100 Suc density gradient centrifugation as described in Figure 1A. Bands I and II were collected and LHCII was purified as described in "Materials and Methods." The chlorophyll extracted from LHCII was subjected to HPLC using an octadecyl silica column. Eluates were collected every 40 s and the radioactivity of each fraction was measured by liquid scintillation counting (shown by columns). Traced lines show HPLC elution profiles monitored by measuring A_{663}. Chl, Chlorophyll.

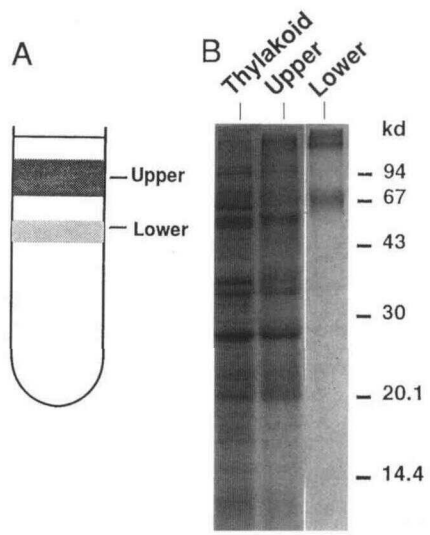

Figure 6. Isolation of CP1 by LDS Suc density gradient centrifugation. A, Thylakoid membranes were solubilized in 1.0% LDS. The solution was loaded on a linear 0.1 to 1.0 M Suc density gradient containing 0.15% LDS and centrifuged at 40,000 rpm for 14 h at 4°C. B, Proteins from the upper or lower band were subjected to SDS-PAGE. Proteins from thylakoid membranes were also electrophoresed. Gels were stained with Coomassie brilliant blue after electrophoresis.

gated forms of CP1 apoproteins by immunoblot analysis (data not shown). These results indicate that CP1 was purified by LDS Suc density gradient centrifugation.

Radioactive chlorophyll of purified CP1 was analyzed by HPLC and scintillation counting (Fig. 7). Total thylakoid membranes had almost the same radioactive chlorophyll *a* as chlorophyll *b* (Fig. 7A), whereas most of the radioactive pigment of purified CP1 was chlorophyll *a* (Fig. 7B). The radioactivity of the fraction from 7′20″ to 8′00″ from isolated CP1 would be derived from [^{14}C]chlorophyll *a* esterified with geranilgeranyol and not from [^{14}C]chlorophyll *b* esterified with phytol. This is consistent with our observation that a small amount of chlorophyll *a* esterified with geranilgeranyol accumulated when chlorophyllide *b* was incubated with etioplasts. These results indicate that chlorophyll *a* can be incorporated into CP1 after conversion from chlorophyll *b*.

Distribution of 7-Hydroxymethyl Chlorophyll in the Thylakoid Membrane

7-Hydroxymethyl chlorophyll is an intermediate molecule between chlorophyll *b* and chlorophyll *a* and accumulates when chlorophyllide *b* is incubated with etioplasts (Ito et al., 1996). To investigate the localization of 7-hydroxymethyl chlorophyll during the conversion of chlorophyll *b* to chlorophyll *a*, [^{14}C]7-hydroxymethyl chlorophyll was incubated with intact chloroplasts or thylakoid membranes, and the membranes were fractionated by Triton X-100 Suc density gradient. Chlorophylls from bands I to IV were subjected to HPLC and liquid scintillation counting (Fig. 8). Small amounts of [^{14}C]7-hydroxymethyl chlorophyll and large amounts of [^{14}C]chlorophyll *a* accumulated when [^{14}C]7-hydroxymethyl chlorophyllide was incubated with intact chloroplasts (Fig. 8A), whereas large amounts of [^{14}C]7-hydroxymethyl chlorophyll with only a trace of [^{14}C]chlorophyll *a* accumulated when the incubation was carried out with thylakoid membranes (Fig. 8B). The activity of 7-hydroxymethyl chlorophyll reductase, which catalyzes the conversion of 7-hydroxymethyl chlorophyll to chlorophyll *a*, was much lower in the thylakoid membrane fraction than in that of intact chloroplasts. These results are consistent with the observation that chlorophyll *b* to chlorophyll *a* conversion requires both the soluble and membrane fractions of etioplasts (Ito et al., 1993, 1994).

As shown in Figure 8A, when [^{14}C]7-hydroxymethyl chlorophyllide was incubated with intact chloroplasts, [^{14}C]chlorophyll *a* was found in bands I to IV, whereas almost all of the [^{14}C]7-hydroxymethyl chlorophyll was found in band I, with only trace amounts in band II (Fig. 8A, open columns). This suggests that almost none of the [^{14}C]7-hydroxymethyl chlorophyll was incorporated into LHCII or CP1.

The possibility that 7-hydroxymethyl chlorophyll exists as a free pigment is also supported by the experiment with thylakoid membrane fractions (Fig. 8B), in which [^{14}C]7-hydroxymethyl chlorophyll accumulated at a much higher level than with intact chloroplasts (compare with Fig. 8A). The amounts of [^{14}C]chlorophyll *a* distributed to bands I (LHCII monomer) and II (LHCII oligomer) came to 67 and 33%, respectively (Fig. 9B), although the total amounts of

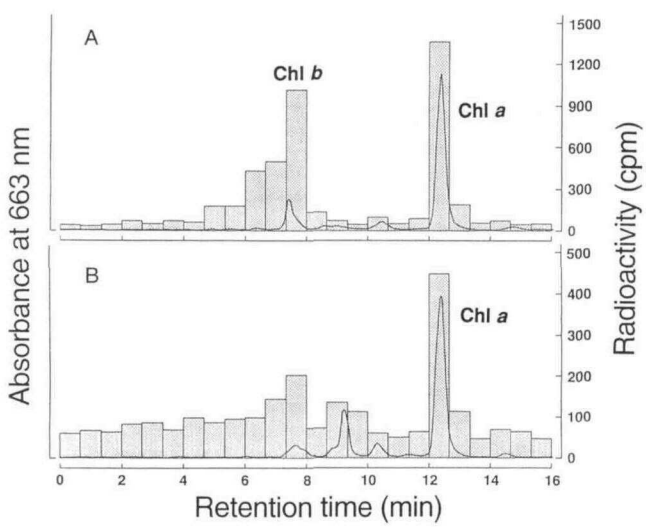

Figure 7. Incorporation of chlorophyll into CP1. After the incubation of [^{14}C]chlorophyllide *b* with intact chloroplasts (0.50 mg of chlorophyll) isolated from 12-h illuminated cotyledons, the reaction mixture was separated into two aliquots so that the large and small aliquots had 95 and 5% volumes of the original mixture, respectively. Thylakoid membranes from the large aliquot was fractionated by LDS Suc density gradient centrifugation to purify CP1, as described in Figure 6. Chlorophyll from the small aliquot (A) and isolated CP1 (B) were subjected to HPLC using an octadecyl silica column. Eluates were collected every 40 s and the radioactivity of each fraction was measured by liquid scintillation counting (shown by columns). Traced lines show HPLC elution profiles monitored by measuring A_{663}. Chl, Chlorophyll.

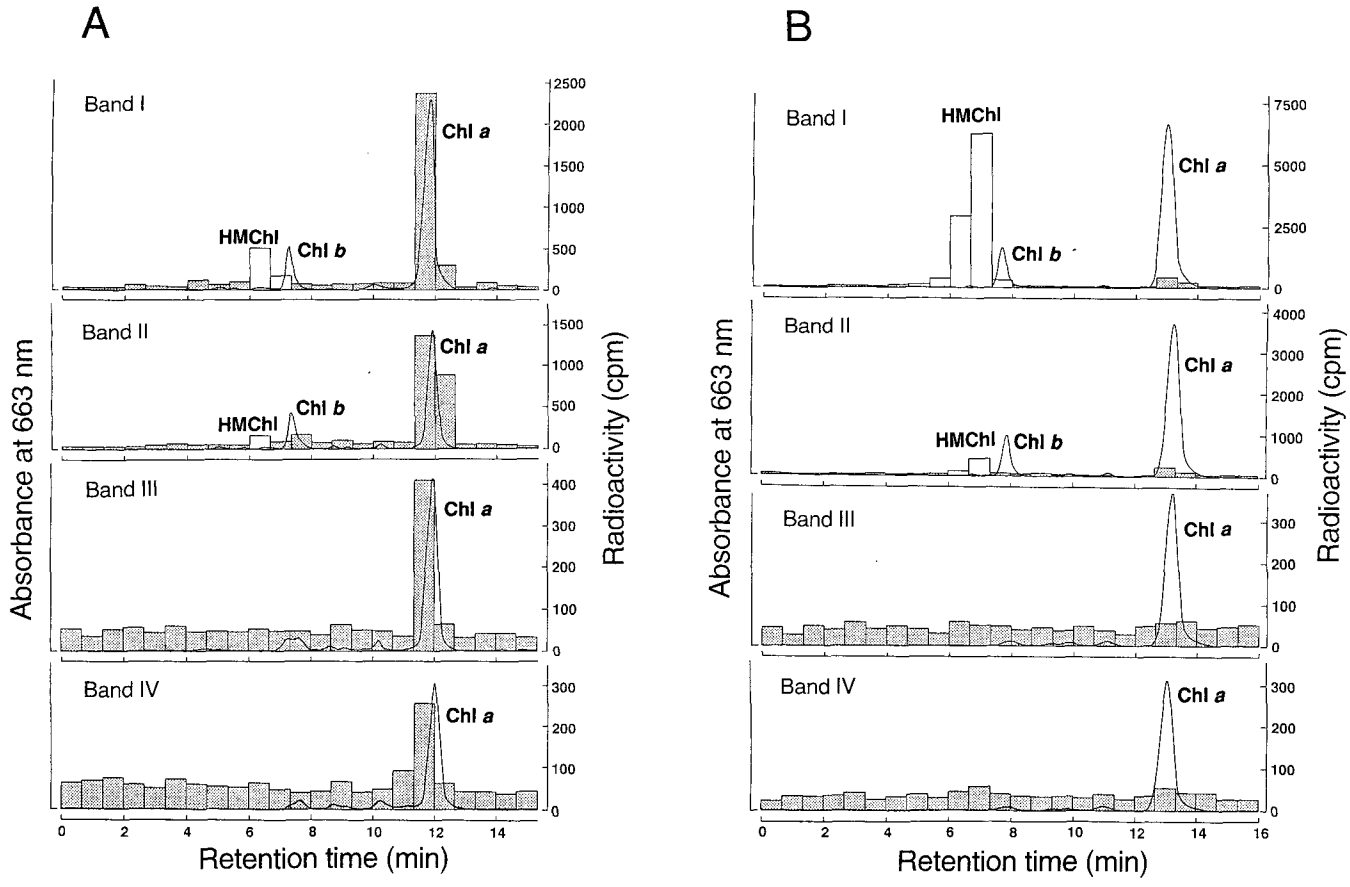

Figure 8. Synthesis of 7-hydroxymethyl chlorophyll and chlorophyll *a* from 7-hydroxymethyl chlorophyllide and their incorporation into photosynthetic particles. After the incubation of [^{14}C]7-hydroxymethyl chlorophyllide with intact chloroplasts (A, 0.25 mg of chlorophyll) or thylakoid membrane fraction (B, 0.25 mg of chlorophyll) isolated from 12-h illuminated cotyledons, thylakoid membranes were fractionated by Triton X-100 Suc density gradient centrifugation, as described in Figure 1. Chlorophyll samples extracted from bands I to IV were subjected to HPLC using an octadecyl silica column. Eluates were collected every 40 s and the radioactivity of each fraction was measured by liquid scintillation counting (shown by columns; open columns indicate the fractions of 7-hydroxymethyl chlorophyll). Traced lines show HPLC elution profiles monitored by measuring A_{663}. For band I, 40% of the chlorophyll collected from the Suc gradient was analyzed here. Chl, Chlorophyll; HMChl, 7-hydroxymethyl chlorophyll.

[^{14}C]chlorophyll *a* were smaller than in the experiments with intact chloroplasts (Fig. 8). Despite the increased accumulation of [^{14}C]7-hydroxymethyl chlorophyll, almost all of the [^{14}C]7-hydroxymethyl chlorophyll was found in band I, with very small amounts in band II (Fig. 8B, open columns); 98 and 2% of the [^{14}C]7-hydroxymethyl chlorophyll were distributed in bands I and II, respectively (Fig. 9B). If we assume that 7-hydroxymethyl chlorophyll was distributed to LHCII monomer and oligomer in the same proportion as chlorophyll *a* (67% to LHCII monomer and 33% to LHCII oligomer), then 94% of the [^{14}C]7-hydroxymethyl chlorophyll is estimated to have existed as free chlorophylls. In contrast to the distribution of [^{14}C]7-hydroxymethyl chlorophyll, [^{14}C]chlorophyll *a* was distributed to all of the bands when [^{14}C]chlorophyllide *a* was incubated with the thylakoid membrane fraction (Fig. 2); 78.7, 16.4, 3.7, and 1.2% of the [^{14}C]chlorophyll *a* were found in bands I to IV, respectively (Fig. 9A), whereas 98, 1.9 to 2.0, <0.01, and <0.01% of the [^{14}C]7-hydroxymethyl chlorophyll were found in bands I to IV, respectively, after incubation with [^{14}C]7-hydroxymethyl chlorophyllide (Fig. 9B). These results indicate that 7-hydroxymethyl chlorophyll is not associated with apoproteins of chlorophyll-protein complexes but are localized in lipid bilayers of the thylakoid membrane during chlorophyll *b* to chlorophyll *a* conversion.

DISCUSSION

Labeled apoproteins have been used for studies of the assembly of chlorophyll with the apoproteins by isolated chloroplasts or thylakoids and the process has been clarified to some extent (Yalovsky et al., 1990; Adam and Hoffman, 1993; Nechushtai et al., 1995). However, these experiments used preexisting chlorophyll that had been bound by other apoproteins. To know how the newly synthesized chlorophyll is distributed among apoproteins, experiments with labeled chlorophyll are required. In this study we tried to investigate the assembly of chlorophyll with apo-

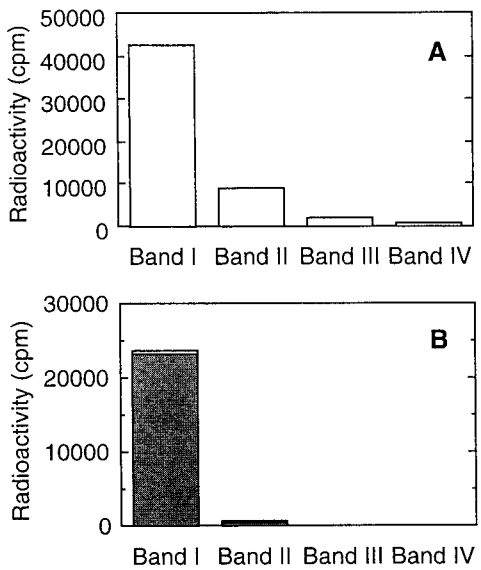

Figure 9. Distribution of 7-hydroxymethyl chlorophyll and chlorophyll *a* synthesized from 7-hydroxymethyl chlorophyllide or chlorophyllide *a* among photosynthetic particles. [^{14}C]Chlorophyllide *a* (A) or [^{14}C]7-hydroxymethyl chlorophyllide (B) was incubated with thylakoid membrane fractions. Radioactivities and chlorophyll were calculated from those in Figure 8 as for Figure 4. Open and closed columns indicate the fractions of chlorophyll *a* and 7-hydroxymethyl chlorophyll, respectively.

proteins by using chlorophyllide, which is rapidly converted to chlorophyll only by esterification with phytol. If we use phytyl PPi as the substrate, esterification occurs by the thylakoid membranes without additional substrates. This enables us to study the process of the assembly of newly synthesized chlorophyll with apoproteins by chloroplasts and thylakoids. We can also use many other chemically modified chlorophyll derivatives as substrates. Experiments with the chlorophyll derivatives would show what moieties of the chlorophyll molecule are important for the binding with apoproteins.

When [^{14}C]chlorophyllide *a* was incubated with chloroplasts, chlorophyllide *a* was esterified with phytyl PPi and the newly synthesized chlorophyll *a* was distributed among various apoproteins. When [^{14}C]chlorophyllide *b* was used instead of chlorophyllide *a*, chlorophyllide *b* was esterified and a part of the chlorophyll *b* was converted to chlorophyll *a*, followed by incorporation into chlorophyll-protein complexes. This indicates that chlorophyll *b* to chlorophyll *a* conversion is coupled with the formation of the chlorophyll-protein complex.

Proper assembly of chlorophyll with apoproteins in these experiments was assessed by the ratio of [^{14}C]chlorophyll *a* to [^{14}C]chlorophyll *b* of individual chlorophyll-protein complexes. LHCII had a lower ratio and CP1 had a higher ratio compared with that for the total thylakoid membranes, indicating the specific binding of the pigments with the apoproteins. Additional indication of the proper assembly of chlorophyll with apoproteins comes from the fact that 7-hydroxymethyl chlorophyll did not assemble with any apoproteins. The apoproteins of various chlorophyll-protein complexes bind chlorophyll *a* and chlorophyll *b* at specific sites, which distinguish chlorophyll *b* from chlorophyll *a* by the structure of 7C of chlorophyll. If the binding of chlorophyll with apoproteins is specific in our system, it is reasonable that all of the apoproteins did not bind 7-hydroxymethyl chlorophyll. This specificity is in contrast to the observation that chlorophyll with various prenyl chains can become assembled with apoproteins to form functional chlorophyll-protein complexes that have photosynthetic functions (Bollivar et al., 1994).

Because apoproteins were not synthesized during incubation in our system, chlorophyll would be incorporated into chlorophyll-protein complexes, which preexisted in the thylakoid membrane in our experiments. Bhaya and Castelfranco (1985) also showed that chlorophyll was incorporated into chlorophyll-protein complexes even when plastid protein synthesis was strongly inhibited by chloramphenicol and spectinomycin. These results from in vivo experiments indicate that the formation of the chlorophyll-protein complex does not require strong co-regulation between the syntheses of chlorophyll and apoproteins in the short term.

Newly synthesized chlorophyll from exogenous chlorophyllide would be bound to unoccupied chlorophyll-binding sites on chlorophyll-protein complexes. Some reports suggested the existence of unoccupied binding sites for chlorophyll on LHCII. Sukenik et al. (1987) reported that the chlorophyll *a* to chlorophyll *b* ratio of purified LHCII from *Dunaliella tertiolecta* grown under high light was twice that grown under low light. This suggests that LHCII apoproteins are fairly stable even if not fully pigmented. The accumulation of LHCII apoproteins in a chlorophyll *b*-less barley mutant indicated that the apoproteins may be partially stabilized by a supply of chlorophyll *a* alone (Apel and Kloppstech, 1980). The amount of LHCII apoproteins increased with additional levels of chlorophyll *b* but somewhat more rapidly during the early phase of greening of cucumber cotyledons. This observation also supports the idea that LHCII has an unoccupied site for chlorophyll in vivo. Although LHCII apoproteins would be degraded when the unoccupied chlorophyll-binding sites are below a certain threshold level (Bennett 1981; Herrin et al., 1992; Tanaka et al., 1992, 1993, 1994), newly synthesized chlorophyll from exogenous chlorophyllide would be incorporated into unoccupied sites. However, exchange mechanisms cannot be excluded at present. We showed that newly synthesized chlorophyll was incorporated into preexisting apoproteins, but that apoproteins are supplied continuously and chlorophylls are distributed to the newly synthesized apoproteins during greening under continuous illumination. These newly synthesized apoproteins become pigmented gradually and assemble with photosynthetic particles before becoming fully pigmented because PSI and LHCII of greening chloroplasts has unoccupied sites for chlorophyll.

We investigated the distribution of 7-hydroxymethyl chlorophyll, an intermediate molecule for chlorophyll *b* to chlorophyll *a* conversion, in the thylakoid membranes.

When 7-hydroxymethyl chlorophyllide was incubated with chloroplasts, 7-hydroxymethyl chlorophyll and chlorophyll *a* accumulated. Chlorophyll *a* was incorporated into chlorophyll-protein complexes, but 7-hydroxymethyl chlorophyll was not. When we use thylakoid membranes instead of intact chloroplasts, a large amount of 7-hydroxymethyl chlorophyll accumulates with very little chlorophyll *a*, because conversion of 7-hydroxymethyl chlorophyll to chlorophyll *a* requires stromal components. In this case, 7-hydroxymethyl chlorophyll was not incorporated into apoproteins. These results indicate that 7-hydroxymethyl chlorophyll is not associated with chlorophyll-protein complexes but is localized in lipid bilayers of the thylakoid membrane.

At early stages of greening, a drastic increase in the chlorophyll *a* to *b* ratio is observed during dark incubation after a short period of illumination (Argyroudi-Akoyunoglou et al., 1982; Tanaka and Tsuji, 1983). These changes in chlorophyll contents are associated with the disorganization of LHCII and the formation of CP1 and CPa. Based on these observations, we suggest that chlorophyll *a* of LHCII is used for the formation of CPa and CP1. During this reorganization of the photosynthetic apparatus in the dark, chlorophyll *b* is released from the apoproteins and exists as free chlorophyll in the thylakoid membranes because LHCII apoproteins are digested by some proteases.

In the present study we showed by using [^{14}C]chlorophyllide *b* that free chlorophyll *b* in the thylakoid membranes is rapidly converted to chlorophyll *a*. Incorporation of the chlorophyll *a* into CP1 was confirmed by the observation that purified CP1 had [^{14}C]chlorophyll *a*, which was synthesized from [^{14}C]chlorophyllide *b*. These in vivo and in vitro experiments suggest that chlorophyll *b* is released from LHCII apoproteins, converted to chlorophyll *a* via 7-hydroxymethyl chlorophyll, and then incorporated into CP1, the core complex of PSI, during reorganization of the photosynthetic apparatus. Chlorophyll *b* of LHCII would also be used for the formation of CPa, because CPa increased during the reorganization. Previously, we also showed the opposite movement of chlorophyll from CPa to LHCII with the calcium-treated cucumber cotyledons (Tanaka and Tsuji, 1982; Tanaka et al., 1991, 1995). During these movements, some chlorophyll *a* was converted to chlorophyll *b* and assembled with LHCII. Thus, chlorophyll *a* and chlorophyll *b* are interconverted by the chlorophyll cycle (Ito et al., 1996) and redistributed among various apoproteins (Fig. 10). The formation of chlorophyll-protein complexes by the chlorophyll cycle and chlorophyll redistribution would be important for the fine regulation of the organization of photosystems.

Lindahl et al. (1995) reported that the amount of LHCII decreased with a concomitant increase in CPa and the chlorophyll *a* to *b* ratio during acclimation of a full-grown spinach plant from low light intensity to high light intensity. They also showed that LHCII was degraded by the protease extrinsically bound to the outer surface of the thylakoid membranes. These findings, together with our present results, suggest that chlorophyll *b* is released from LHCII and converted to chlorophyll *a*, which is used for the formation of core complexes of photosystems during adaptation. If chlorophylls of LHCII could not be reused, free chlorophyll would accumulate during reorganization of the photosystems. It is well known that chlorophyll and its precursors are toxic molecules that generate active oxygen under illumination. Diphenylether-type herbicides inhibit protoporphyrinogen oxidase activity and this leads to the accumulation of protoporphyrin IX (Matringe et al., 1992), which is toxic for plants. Feeding of ALA, a precursor of tetrapyrroles, also induces the accumulation of free chlorophyll and its precursors, resulting in inhibition of the greening of intact leaves (Tanaka et al., 1992). Chlorophyll metabolism must be regulated so that it does not cause accumulation of free chlorophyll and its precursors. The chlorophyll cycle and chlorophyll redistribution would reduce the free chlorophyll and support the immediate reorganization of photosystems during acclimation.

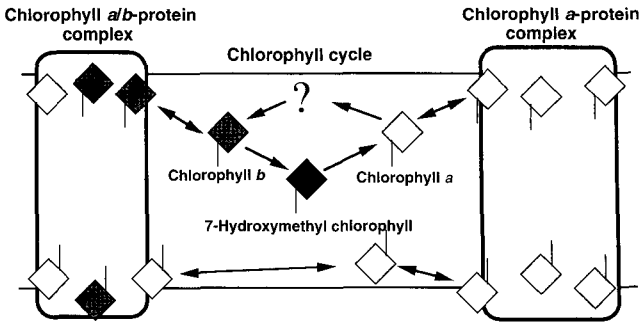

Figure 10. Chlorophyll cycle and chlorophyll redistribution among chlorophyll-protein complexes. Chlorophyll *a* and chlorophyll *b* are interconverted by the chlorophyll cycle and redistributed among chlorophyll-protein complexes. See text for explanation.

Received July 15, 1996; accepted September 9, 1996.
Copyright Clearance Center: 0032–0889/97/113/0137/11.

LITERATURE CITED

Adam Z, Hoffman N (1993) Biogenesis of a photosystem I light-harvesting complex. Evidence for a membrane intermediate. Plant Physiol **102:** 35–43

Anderson JM (1980) P-700 content and polypeptide profile of chlorophyll-protein complexes of spinach and barley thylakoids. Biochim Biophys Acta **591:** 113–126

Apel K, Kloppstech K (1980) The effect of light on the biosynthesis of the light-harvesting chlorophyll *a/b* protein. Evidence for the requirement of chlorophyll *a* for the stabilization of the apoproteins. Planta **150:** 426–430

Argyroudi-Akoyunoglou JH, Akoyunoglou A, Kalosakas K, Akoyunoglou G (1982) Reorganization of 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

Arnon D (1949) Copper enzyme in isolated chloroplasts. Plant Physiol **24:** 1–14

Bennett J (1981) Biosynthesis of the light-harvesting chlorophyll *a/b*-protein. Polypeptide turnover in darkness. Eur J Biochem **118:** 61–70

Benz J, Rüdiger W (1981) Chlorophyll biosynthesis: various chlorophyllides as exogenous substrates for chlorophyll synthetase. Z Naturforsch **36c:** 51–58

Bhaya D, Castelfranco PA (1985) Chlorophyll biosynthesis and assembly into chlorophyll-protein complexes in isolated developing chloroplasts. Proc Natl Acad Sci USA **82:** 3570–3574

Bollivar DW, Wang S, Allen JP, Bauer CE (1994) Molecular genetic analysis of terminal steps in bacteriochlorophyll *a* biosynthesis: characterization of a *Rhodobacter capsulatus* strain that synthesizes geranylgeraniol-esterified bacteriochlorophyll *a*. Biochemistry **33:** 12763–12768

Burke JJ, Ditto CL, Arnzen CJ (1978) Involvement of the light-harvesting complex in cation regulation of excitation energy distribution in chloroplasts. Arch Biochem Biophys **187:** 252–263

Chow WS, Melis A, Anderson JM (1990) Adjustments of photosystem stoichiometry in chloroplasts improve the quantum efficiency of photosynthesis. Proc Natl Acad Sci USA **87:** 7502–7506

Dainese P, Bassi R (1991) Subunit stoichiometry of the chloroplast photosystem II antenna system and aggregation state of the component chlorophyll *a*/*b* binding proteins. J Biol Chem **266:** 8136–8142

Delepelaire P, Chua N-H (1979) Lithium dodecyl sulfate/polyacrylamide gel electrophoresis of thylakoid membranes at 40°C: characterization of two additional chlorophyll *a*-protein complexes. Proc Natl Acad Sci USA **76:** 111–115

Eichacker LA, Soll J, Lauterbach P, Rüdiger W, Klein RR, Mullet JE (1990) *In vitro* synthesis of chlorophyll *a* in the dark triggers accumulation of chlorophyll *a* apoproteins in barley etioplasts. J Biol Chem **264:** 13566–13571

Golbeck JH (1992) Structure and function of photosystem I. Annu Rev Plant Physiol Plant Mol Biol **43:** 293–324

Herrin DL, Battey JF, Greer K, Schmidt GW (1992) Regulation of chlorophyll apoprotein expression and accumulation. J Biol Chem **268:** 8260–8269

Holden M (1960) The breakdown of chlorophyll by chlorophyllase. Biochem J **78:** 359–364

Holt AS (1959) Reduction of chlorophyllides, chlorophylls and chlorophyll derivatives by sodium borohydride. Plant Physiol **34:** 310–314

Ito H, Ohtsuka T, Tanaka A (1996) Conversion of chlorophyll *b* to chlorophyll *a* via 7-hydroxymethyl chlorophyll. J Biol Chem **271:** 1475–1479

Ito H, Takaichi S, Tsuji H, Tanaka A (1994) Properties of synthesis of chlorophyll *a* from chlorophyll *b* in cucumber etioplasts. J Biol Chem **269:** 22034–22038

Ito H, Tanaka Y, Tsuji H, Tanaka A (1993) Conversion of chlorophyll *b* to chlorophyll *a* in isolated cucumber etioplasts. Arch Biochem Biophys **306:** 148–151

Kühlbrandt W, Wang DA (1991) Three-dimensional structure of plant light-harvesting complex determined by electron crystallography. Nature **350:** 130–134

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:** 680–685

Lindahl M, Yang D-H, Anderson B (1995) Regulatory proteolysis of the major light-harvesting chlorophyll *a*/*b* protein of photosystem II by a light-induced membrane-associated enzymic system. Eur J Biochem **231:** 503–509

Maloney MA, Hoober JK, Marks DB (1989) Kinetics of chlorophyll accumulation and formation of chlorophyll-protein complexes during greening of *Chlamydomonas reinhardtii* y-1 at 38°C. Plant Physiol **91:** 1100–1106

Markwell JP, Thornber JP, Boggs RT (1979) Higher plant chloroplast: evidence that all the chlorophyll exists as chlorophyll-protein complex. Proc Natl Acad Sci USA **76:** 1233–1235

Matringe M, Camadro J-M, Block MA, Joyard J, Rene S, Labbe P, Douce R (1992) Localization within chloroplasts of protoporphyrinogen oxidase, the target enzyme for diphenylether-like herbicides. J Biol Chem **267:** 4646–4651

Minami E, Shinohara K, Kuwabara T, Watanabe A (1986) In vitro synthesis and assembly of photosystem II proteins of spinach chloroplasts. Arch Biochem Biophys **244:** 517–527

Nechushtai R, Cohen Y, Chitnis PR (1995) Assembly of chlorophyll-protein complexes. Photosynth Res **44:** 165–181

Rüdiger W, Benz J, Guthoff C (1980) Detection and partial characterization of activity of chlorophyll synthetase in etioplast membranes. Eur J Biochem **109:** 193–200

Shimada S, Tanaka A, Takabe T, Takabe T, Tsuji H (1990) Formation of chlorophyll-protein complexes during greening. 1. Distribution of newly synthesized chlorophyll among apoproteins. Plant Cell Physiol **31:** 639–647

Shioi Y, Fukae R, Sasa T (1983) Chlorophyll analysis by high-performance liquid chromatography. Biochim Biophys Acta **722:** 72–79

Sukenik A, Wyman KD, Bennett J, Falkowski PG (1987) A novel mechanism for regulating the excitation of photosystem II in a green alga. Nature **327:** 704–707

Tanaka A, Tanaka Y, Takabe T, Tsuji H (1995) Calcium-induced accumulation of apoproteins of the light-harvesting chlorophyll *a*/*b*-protein complex in cucumber cotyledons in the dark. Plant Sci **105:** 189–194

Tanaka A, Tanaka Y, Tsuji H (1987) Resolution of chlorophyll *a*/*b*-protein complexes by polyacrylamide gel electrophoresis: evidence for the heterogeneity of light-harvesting chlorophyll *a*/*b*-protein complexes. Plant Cell Physiol **28:** 1537–1545

Tanaka A, Tanaka Y, Tsuji H (1994) Preferential accumulation of apoproteins of light-harvesting chlorophyll *a*/*b*-protein complex in greening barley leaves treated with 5-aminolevulinic acid. Planta **192:** 92–97

Tanaka A, 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, 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, Yamamoto Y, Tsuji H (1991) Formation of chlorophyll-protein complex during greening. 2. Redistribution of chlorophyll among apoproteins. Plant Cell Physiol **32:** 195–204

Tanaka K (1982) Purification and properties of chlorophyllase from greened rye seedlings. J Biochem **92:** 1763–1773

Tanaka Y, Tanaka A, Tsuji H (1992) Stabilization of apoproteins of light-harvesting chlorophyll *a*/*b*-protein complexes by feeding 5-aminolevulinic acid under intermittent illumination. Plant Physiol Biochem **30:** 365–370

Tanaka Y, Tanaka A, Tsuji H (1992) Effect of 5-aminolevulinic acid on the accumulation of chlorophyll *b* and apoproteins of light-harvesting chlorophyll *a*/*b*-protein complex of photosystem II. Plant Cell Physiol **34:** 465–472

Tang X-S, Satoh K (1984) Characterization of a 47-kilodalton chlorophyll-binding polypeptide (CP-47) isolated from a photosystem II core complex. Plant Cell Physiol **25:** 935–945

Thornber JP (1979) Chlorophyll-proteins: light-harvesting and reaction center components of plants. Annu Rev Plant Physiol **26:** 127–158

Vermaas W (1993) Molecular-biological approaches to analyze photosystem II structure and function. Annu Rev Plant Physiol Plant Mol Biol **44:** 457–481

Wettstein D, Gough S, Kannangara G (1995) Chlorophyll biosynthesis. Plant Cell **7:** 1039–1057

Yalovsky S, Schuster G, Nechushtai R (1990) The apoprotein precursor of the major light-harvesting complex of photosystem II (LHCIIb) is inserted primarily into stromal lamellae and subsequently migrates to the grana. Plant Mol Biol **14:** 753–764