Chlorophyll \(b\) Expressed in Cyanobacteria Functions as a Light-harvesting Antenna in Photosystem I through Flexibility of the Proteins*

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Photosynthetic pigments bind to their specific proteins to form pigment-protein complexes. To investigate the pigment-binding activities of the proteins, chlorophyll \(b\) was introduced for the first time to a cyanobacterium that did not synthesize that pigment, and expression of its function in the native pigment-protein complex of cyanobacterium was confirmed by energy transfer. Arabidopsis CAO (chlorophyll \(a\) oxygenase) cDNA was introduced into the genome of Synechocystis sp. PCC6803. The transformant cells accumulated chlorophyll \(b\), with the chlorophyll \(b\) content being in the range of 1.4 to 10.6% of the total chlorophyll depending on the growth phase. Polyacrylamide gel electrophoresis analysis of the chlorophyll-protein complexes of transformant cells showed that chlorophyll \(b\) was incorporated preferentially into the P700-chlorophyll \(a\)-protein complex (CP1). Furthermore, chlorophyll \(b\) in CP1 transferred light energy to chlorophyll \(a\), indicating a functional transformation. We also found that CP1 of Chlamydomonas reinhardtii, believed to be a chlorophyll \(a\) protein, bound chlorophyll \(b\) with a chlorophyll \(b\) content of \(-4.4\%\). On the basis of these results, the evolution of pigment systems in an early stage of cyanobacterial development is discussed in this paper.

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† The abbreviations used are: PSI/II, photosystems I and II; LHC, light-harvesting complex; CP1, P700-chlorophyll \(a\)-protein complex; HPLC, high-performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis.

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by biochemical, physiological, and biophysical methods. However, we considered that the introduction of a new pigment into cells by a molecular genetics method would be a useful means of investigating the distribution of a new pigment among light-harvesting complexes to understand their pigment-binding activity. We therefore introduced the chlorophyll b synthesis gene, i.e. chlorophyll a oxygenase (CAO) (13), into a cyanobacterium that does not synthesize chlorophyll b. This is the first report on the introduction of a new pigment into a photosynthetic organism. Chlorophyll b was synthesized in transformant cyanobacteria cells and incorporated into the P700-chlorophyll a-protein complex (CP1). The chlorophyll a-protein was then functionally transformed to the chlorophyll a/b protein. It was found that CP1 of C. reinhardtii, believed to be a chlorophyll a protein, bound chlorophyll b. We propose herein a hypothesis for the evolution of light-harvesting systems on the basis of flexibility of antenna proteins.

**EXPERIMENTAL PROCEDURES**

**Introduction of the CAO Gene into the Synechocystis sp. PCC6803 Genome**—We predicted the cleavage site after the 50th amino acid residue of the CAO cDNA cloned from C. reinhardtii—less mutant (cbs3) of Synechocystis sp. PCC6803 together with a chloramphenicol-resistant cartridge (cb1–3). The control mutant was generated with DNA lacking the CAO gene but having the psbAII promoter and a kanamycin-resistant cartridge. It is very likely that the insertion of the initiation codon for translation (15). Spontaneous deletion of a 154-base pair segment including a putative reading frame had no effect on the cells, because slr2031 is not functional in this strain due to spontaneous deletion of a 154-base pair segment including a putative initiation codon for translation (15).

**Culture Conditions—**Synechocystis cells were grown at 22 °C in BG11 medium (16) under continuous illumination (30 micro-einsteins/m²/s) with 20 μg/ml of kanamycin (control) or 20 μg/ml of chloramphenicol (cb1–3). Cell density was monitored using a Shimadzu UV-160A to measure the absorbance of the culture medium at 677 nm.

**Preparation of Thylakoid Membranes from Synechocystis sp.**—Synechocystis sp. PCC6803 and C. reinhardtii—Cells of Synechocystis sp. PCC6803 or C. reinhardtii were harvested by centrifugation, suspended in 50 mM HEPES (pH 8.0), and broken with a Vibogen with glass beads at 0 °C. After removal of the beads by centrifugation, homogenates were centrifuged at 10,000 × g for 20 min. The green pellets were suspended in 50 mM HEPES (pH 8.0) containing 5 mM EDTA and centrifuged at 10,000 × g for 20 min. The pellets were resuspended in 50 mM HEPES (pH 8.0) and sonicated. After centrifugation at 2,500 × g for 5 min, the supernatants were used as thylakoid membranes.

**Optical Measurements—**Fluorescence spectra of cells were measured using a Hitachi F4500 spectrophotometer. For the excitation spectra at 77 K, a Dewar bottle and a home-made holder were used (17).

**Separation of Chlorophyll-Protein Complexes—**Thylakoid membranes from Synechocystis sp. PCC6803 (0.5 mg chlorophyll/ml) were dissolved in 0.5% SDS and electrophoresed with 8% polyacrylamide disc gels at 4 °C in the dark for 1 h (12).

**Isolation of the P700-Chlorophyll a-Protein Complex from C. reinhardtii—**Thylakoid membranes prepared from wild type (c-e) and a chlorophyll b-less mutant (cbs3) of C. reinhardtii (13) were solubilized with 2% SDS at room temperature. Solubilized membranes were loaded on a 12% polyacrylamide gel and electrophoresed for 14 h in the dark. Green bands corresponding to CP1 were cut and immersed in solubilizing buffer containing 2% SDS. After heat treatment at 90 °C for 2 min, gels were loaded on a 12% polyacrylamide gel, electrophoresed, and stained with silver.

**Northern Blot Analysis—**Total RNA was extracted from Synechocystis cells. Two μg of total RNA was electrophoresed on a 1% agarose/formaldehyde gel. The RNA was blotted onto a Hybond-N nylon membrane and hybridized with a probe for CAO.

**Isolation of Chlorophyll from the Green Bands—**The green bands on the gel were cut and homogenized with 0.1% SDS. Chlorophyll was extracted from the gel slices with 0.1% SDS. After centrifugation of the homogenate, 100% acetone was added to the green supernatant to a final concentration of 8% and centrifuged. Chlorophyll Determination—Chlorophyll was extracted with 80% acetone and subjected to high-performance liquid chromatography (HPLC). The chlorophyll was quantified from the chromatographic peak areas after calibration of the chromatographic response with known quantities of the relevant pigments (18).

**RESULTS**

**Expression of the CAO Gene in Synechocystis—**Enzymatic studies have shown that CAO catalyzes out two-step oxygenation and converts chlorophyllide a to chlorophyllide b by itself (14), suggesting that photosynthetic organisms can synthesize chlorophyll b when they acquire the CAO gene. We introduced Arabidopsis CAO cDNA into the genome of Synechocystis sp. PCC6803 (cb1–3) under the control of a PsbAII promoter. Northern blot analysis showed that the introduced CAO gene was expressed in a cb1–3 transformant (Fig. 1). We then analyzed the pigment composition by HPLC. Besides chlorophyll a, we observed a new peak (X) (Fig. 2) for which the absorption maximum was located at 650 nm in acetone. The results of coinjection experiments on HPLC (data not shown) and fluorescence spectra (Fig. 3) showed that peak X corresponded to chlorophyll b. The chlorophyll b content varied with the growth phase. During the early phase, chlorophyll b was actively synthesized, and the chlorophyll b content was in the range of 7.0 to 10.6% (Fig. 4). However, the chlorophyll b content decreased as the culture period was prolonged. A possible explanation for this decrease is that chlorophyll b synthesis was halted by the nature of the promoter used for expression of CAO or that chlorophyll b was cleared out because of a greater turnover of chlorophyll-binding proteins that contain chlorophyll b. At present, we have no direct evidence, and further studies are required. There was no significant difference between cell growth in the control group and that in the cb1–3 transformant group during the whole growth phase (Fig. 4), indicating that chlorophyll b did not induce photodamage and was not toxic for this species.

**Function of Chlorophyll b in PSI—**The localization of chlorophyll b in pigment-protein complexes was investigated. Thylakoid membranes were prepared from Synechocystis cells, and chlorophyll-protein complexes were resolved by green gel (15) (Fig. 5). It was found that 57.5 and 34.9% of chlorophyll a was confined to the P700-chlorophyll a-protein complex (CP1) and the chlorophyll a-protein complexes of PSII (CP43/47), respectively (Table 1). Less than 7% of chlorophyll a was of a free form, indicating the reliability of separation. Most of the chlorophyll b (76.5%), on the other hand, was associated with CP1 with a small amount (15.0%) in CP43/47 and free chlorophyll b (8.5%). The chlorophyll b content of CP1 to total chlorophyll was 8%, higher than that of CP43/47 (2.7%) and of free chloro-
corresponding to chlorophyll b shows a newly synthesized pigment. Chlorophylls extracted with 80% acetone from C. reinhardtii transformants were separated by reversed-phase HPLC. The peak X spectra were measured in methanol by excitation at 470 nm.

Chlorophyll content varied with the growth phase. Cells grown under continuous light were harvested at various times. Chlorophyll was extracted and determined by HPLC. Cell densities were monitored by measuring absorbance at 677 nm of culture.

Next, we carried out spectral analysis to determine whether chlorophyll b in Synechocystis cells could function as photosynthetic pigment. Upon excitation of chlorophyll a at 440 nm, a typical PSIII fluorescence was observed at 685 nm both at room temperature and at 77 K, and an additional two fluorescence components were detected at 695 and 722 nm, as in the case of the control cells (data not shown). Upon excitation of chlorophyll b at 465 nm, no fluorescence from chlorophyll b was observed on intact cells either at room temperature or at 77 K (data not shown), indicating that free chlorophyll b did not exist in cells. This finding is consistent with the results that most of the chlorophyll b was bound to some chlorophyll proteins as shown by the green gel. When we measured the excitation spectrum of the PSI fluorescence (725 nm) at 77 K (Fig. 6, top), a significant difference was observed at around 470 nm between the control and the cb1–3 transformant; the difference spectrum corresponded to the chlorophyll b Soret band. These results, together with those of the green gel, clearly showed that chlorophyll b was incorporated into CP1 and transferred light energy to PSI chlorophyll a. On the other hand, the excitation spectrum of PSIII chlorophyll a fluorescence at 685 nm showed that the contribution of chlorophyll b to sensitization of chlorophyll a was very small (Fig. 6, bottom), consistent with the low chlorophyll b content of CP43/47 purified by the green gels. These results indicate that the P700-chlorophyll a-protein complex was functionally transformed to a P700-chlorophyll a/b-protein complex when this cyanobacterium acquired chlorophyll b.

Existence of Chlorophyll b in the PSI Core Complex in C. reinhardtii—Because the amino acid sequences of CP1 apoproteins in cyanobacteria and green plants are very similar, binding of chlorophyll b to CP1 in Synechocystis cells would indicate the possibility that CP1 of green plants also binds chlorophyll b. We therefore re-examined whether CP1 of green plants binds chlorophyll b. Thylakoid membranes were prepared from Chlamydomonas and Synechocystis cells and subjected to PAGE of the Laemmli system (19) without heat treatment. This PAGE system was so harsh that all of the chlorophyll was released from CP43/47, and a large amount of chlorophyll was also released from LHII. However, CP1 still bound a considerable amount of chlorophyll b (6.8%), indicating that chlorophyll b was associated preferentially with CP1. Considering that CP1 has 100 chlorophyll molecules/P700, 8 chlorophyll b molecules were bound to CP1 in the cb1–3 transformant. On the other hand, CP43/47 bound at most 1 chlorophyll b molecule on the basis of 40 chlorophyll a molecules in CP43/47.

Chlorophyll b Expressed in Cyanobacteria

![Figure 2](image2)

**Fig. 2.** HPLC elution profiles of chlorophyll. Chlorophylls extracted with 80% acetone from C. reinhardtii, a control, and the cb1–3 transformant were separated by reversed-phase HPLC. The peak X shows a newly synthesized pigment.

![Figure 3](image3)

**Fig. 3.** Fluorescence spectra of chlorophylls. Fractions of HPLC corresponding to chlorophyll b from *Chlamydomonas* and the peak X from the cb1–3 transformant were collected, and their fluorescence spectra were measured in methanol by excitation at 470 nm.

![Figure 4](image4)

**Fig. 4.** Chlorophyll b content varied with the growth phase. Cells grown under continuous light were harvested at various times. Chlorophyll was extracted and determined by HPLC. Cell densities were monitored by measuring absorbance at 677 nm of culture.

![Figure 5](image5)

**Fig. 5.** Separation of chlorophyll-protein complexes. Thylakoid membranes were isolated from *Synechocystis* cells, and chlorophyll-protein complexes were separated by nondenaturing SDS-PAGE (12).

| Chlorophyll a content in each fraction | Chlorophyll b content in each fraction | Chlorophyll a | Chlorophyll b |
|--------------------------------------|---------------------------------------|---------------|---------------|
| %                                    | %                                     | %             | %             |
| CP1                                  | 57.5                                  | 76.5          | 8.0           |
| CP43/47                              | 34.9                                  | 15.0          | 2.7           |
| Free chlorophyll                      | 7.6                                   | 8.5           | 6.8           |

**TABLE I**

Distribution of chlorophyll-protein complexes

After nondenatured SDS-PAGE, the green bands of CP1, CP43/47, and free pigments were excised, and chlorophyll was extracted.
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FIG. 6. Excitation spectra of individual photosystems at liquid nitrogen temperatures. The excitation spectra of chlorophyll of intact cells were measured by monitoring fluorescence at 685 nm (PSII) and 725 nm (PSI). The difference spectrum of PSI excitation spectra between the control and the cb1-3 transformant exhibited a peak at 465 nm, indicating an energy transfer from chlorophyll b to chlorophyll a in PSI.

(Fig. 7b), indicating that LHCl was released completely from CP1 in the green gel. However, the HPLC analysis showed that the chlorophyll b content of purified CP1 was 4.4%, indicating that CP1 of green algae bound chlorophyll b. Namely, the core complex of PSI in green algae was not a P700-chlorophyll a-protein complex but a P700-chlorophyll a/b-protein complex in situ.

DISCUSSION

In a previous paper, we described a possible reaction mechanism of CAO based on the results of in vitro experiments with CAO gene products (14). CAO catalyzed two-step oxygenation reactions and converted chlorophyllide a to chlorophyllide b without any other enzymes. The results of those in vitro experiments are supported by the results of present experiments showing that cyanobacteria accumulated chlorophyll b by acquiring only the CAO gene. An in vitro study (14) also showed that reduced ferredoxin was required for the oxygenation reactions by CAO. The cb1–3 transformant cells probably utilized endogenous ferredoxin for chlorophyll b synthesis.

Chlorophyll b synthesized in the cb1–3 transformant cells was incorporated preferentially into CP1 apoproteins, and chlorophyll a transferred light energy to chlorophyll a in PSI. Fluorescence from free chlorophyll b was not observed on the transformant cells. These results indicate that chlorophyll b was incorporated into the chlorophyll a binding sites of CP1 instead of chlorophyll a, because it is generally observed that nonspecifically bound chlorophyll fluoresces even in a low yield (21). The notion of specific binding of chlorophyll b was also supported by the observation that chlorophyll b was found only in CP1. CP43/47 and free chlorophyll bands on the green gels and that of other colorless proteins never bound chlorophyll b. The chlorophyll b content of 8% in CP1 indicated that at least eight chlorophyll binding sites could be replaced with chlorophyll b. These results are consistent with recent reports that some chlorophyll binding sites in LHC are replaceable by either chlorophyll a or chlorophyll b and that the chlorophyll b content of LHClI could change (10, 17).

Native SDS-PAGE is a powerful tool for isolating chlorophyll-protein complexes without contamination. This method has been used in many studies to determine the chlorophyll contents of chlorophyll-protein complexes. Most of these studies showed that CP1 has no chlorophyll b (12), although some studies suggested the presence of chlorophyll b in CP1 (22). Ikegami and Re (23), using the ether extraction method, reported the existence of chlorophyll b in PSI reaction center particles in which the chlorophyll/P700 ratio was 13. These discrepant results concerning the chlorophyll a/b ratio of CP1 may be due in part to the method used for chlorophyll determination, because high chlorophyll a/b ratios cannot be determined spectrophotometrically. Although CP1 is believed to be a chlorophyll a-protein complex, there have been no results providing clear evidence of this idea. In the present study, we found by HPLC that CP1, which was completely free from LHClI, bound a considerable amount of chlorophyll b. Our results suggested that CP1 is not a chlorophyll-a protein complex but a chlorophyll a/b-protein complex in C. reinhardtii. Our observation was also supported by the results of experiments showing that cyanobacterial CP1 apoproteins, which have amino acid se-
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...full length homology to that of green plants, bound chlorophyll $b$. Further studies are needed to determine whether core antenna complexes of oxygen-evolving photosynthetic organisms bind exclusively chlorophyll $a$.

On the basis of the above results, we propose the progression of an antenna system in which a new pigment is acquired without the presence of a corresponding new protein. Our results demonstrated that the PSI core complex in a prokaryote has the capacity to incorporate chlorophyll $b$ flexibly to its functional sites and that the complex in green algae indeed binds chlorophyll $b$. These findings led us to hypothesize as to how chlorophyll $b$, a new pigment, is incorporated into an antenna system of a prototype of cyanobacteria. When photosynthetic organisms acquired a CAO gene during an early evolutionary phase (24), chlorophyll $b$ began to be synthesized and bound to the core antenna of PSI through its flexibility. Chlorophyll $b$ in CP1 immediately began to function as a photosynthetic pigment, and the organisms became able to use light energy at around 470 and 650 nm, which would be favorable for competition for light energy capturing. It would also have been important for the organisms that a new pigment did not induce photodamage. Our experiments reproduced this process. Cyanobacteria do not contain chlorophyll $b$, and it is therefore probable that they lost the CAO gene. Prochlorophytes bind chlorophyll $b$ to prochloroocyte chlorophyll $b$-binding protein (25) by acquiring a new protein. In the evolutionary progression to eukaryotes, CP1 in green plants has retained an ancestral character to bind chlorophyll $b$ by keeping a CAO gene in the common ancestor. On the other hand, chlorophyll $b$ in peripheral antenna systems has changed its locations from prochlorophyte chlorophyll $b$-binding protein to LHC, which appears after an endosymbiotic event by the duplication of high light-inducible protein (26).

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