Chlorophyll *b* inhibits the formation of photosystem I trimer in *Synechocystis* sp. PCC6803

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Abstract **Chlorophyllide *a* oxygenase (CAO) catalyzes two-step oxygenation reactions and converts chlorophyllide *a* to chlorophyllide *b*. When CAO was introduced into the *Synechocystis* sp. PCC6803 genome, chlorophyll *b* was synthesized and incorporated into P700–chlorophyll *a*–protein complexes. Curve analysis of photosystem I particles showed that Ca687 was decreased with a concomitant increase in Cb652 suggesting that chlorophyll *b* was incorporated into Ca687-binding sites. When the level of chlorophyll *b* was high, Ca704, which is known as red chlorophyll and photosystem I trimers were decreased. Formation of photosystem I trimers is discussed in relation to red chlorophyll and chlorophyll *b* accumulation.**

Key words: Chlorophyll *b*; Chlorophyllide *a* oxygenase; Photosystem I; *Synechocystis* sp. PCC6803

1. Introduction

Photosynthetic organisms have diverse light-harvesting pigments such as chlorophyll *a*, chlorophyll *b*, chlorophyll *c*, phycobilins and carotenoids [1]. Each of these pigments has distinct spectral properties and can harvest light energy of a wide-range spectrum. The diversity of pigments contributes not only to photosynthesis under various light environments but also to the establishment of photosynthetic groups such as green algae, brown algae and red algae.

Light-harvesting systems of photosynthesis consist of core and peripheral antenna complexes [2]. P700–chlorophyll *a*–protein complex (CP1), CP43 and CP47 are the core antenna complexes of photosystems (PSs) that contain chlorophyll *a* as a light-harvesting pigment. Light-harvesting chlorophyll *a*/*b*–protein complex (LHC), peridinin–chlorophyll *a*–protein complexes and phycobilisomes are known as peripheral light-harvesting complexes. The X-ray crystallographic analysis of these pigment–protein complexes have shown that the arrangements of pigments in the complexes are strictly determined [3–5]. This strict arrangement of pigments in proteins was clearly demonstrated with light-harvesting complexes of photosynthetic bacteria [6]. These findings suggest that the molecular species and localization of the pigments in pigment–protein complexes are strictly determined. This strict arrangement is thought to be important for the efficient energy transfer among pigments. However, in vitro reconstitution experiments have shown that certain chlorophyll-binding sites in higher plant LHCII are able to bind either chlorophyll *a* or chlorophyll *b* interchangeably [7]. Red algae use chlorophyll *a* and phycobilins as photosynthetic pigments, and their LHCI bind chlorophyll *a* in chloroplasts. However, the LHCI apoproteins have been shown to functionally bind the major types of chlorophylls (chlorophyll *b* and chlorophyll *c*) and carotenoids (lutein, fucoxanthin and peridinin) that are known as accessory pigments of other photosynthetic organisms [8]. In vivo studies also suggested that LHCs have flexibility to bind various chlorophylls [9]. LHC was stable in thylakoid membranes of a chlorophyll *b*-less mutant of *Chlamydomonas reinhardtii*. Moreover, the chlorophyll antenna size of PSI in the mutant was the same with that in the wild type, suggesting that chlorophyll *a* molecules were incorporated into chlorophyll *b*-binding sites of LHCI. These reports indicated that LHCs have flexibility to bind various chlorophyll and carotenoid molecules.

As well as the peripheral antenna complexes, core antennae of oxygenic photosynthetic organisms have flexibility for pigments. Cyanobacteria use chlorophyll *a* and phycobilins as photosynthetic pigments, but they accumulated chlorophyll *b* when chlorophyllide *a* oxygenase (CAO) was introduced into the genomes [10]. Chlorophyll *b* synthesized in cyanobacteria was incorporated into the core antenna complex of PSI. The chlorophyll *a*–protein complexes of PSI were functionally transformed to the chlorophyll *a*/*b*–protein complexes. Furthermore, when CAO was introduced into a PSI-less mutant of cyanobacteria, chlorophyll *b* replaced part of chlorophyll *a* in the PSII core, and the energy absorbed by chlorophyll *b* was efficiently transferred to the PSII reaction centers [11]. These results strongly indicate that both core and peripheral antenna complexes have a flexibility to bind various photosynthetic pigments.

The molecular structure of chlorophyll *b* is different from that of chlorophyll *a*, because chlorophyll *b* has a formyl group on its B ring instead of a methyl group. Considering the difference in molecular structures, it is unlikely that all of the chlorophyll-binding sites can accept chlorophyll *b*. However, it has not been determined whether chlorophyll *b* is incorporated into all of the chlorophyll-binding sites or into specific sites. If some chlorophyll *a*-binding sites accept chlo-

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Abbreviations: CAO, chlorophyllide *a* oxygenase; CP1, P700–chlorophyll *a*–protein complex; PS, photosystem; LHC, light-harvesting chlorophyll *a*/*b*–protein complex; PAGE, polyacrylamide gel electrophoresis

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rophyll *b* instead of chlorophyll *a*, the difference in molecular structure would induce structural changes in chlorophyll-binding proteins or in super complexes of PSs. However, there are no reports of structural changes in pigment–protein complexes when other pigments are incorporated.

In this study, we found that chlorophyll *b* was incorporated into some specific chlorophyll *a*-binding sites of PSI in CAO-expressed cyanobacteria. Chlorophyll *b* in PSI also induced spectral changes in chlorophyll *a* and inhibited the formation of PSI trimers.

2. Materials and methods

2.1. Mutant strains and culture condition

The control mutant of Cyanobacteria *Synechocystis* sp. PCC6803 and cb1-3 having CAO gene were grown as previously reported [10].

2.2. Isolation of thylakoid membranes

Thylakoid membranes were prepared as described by Chitnis and Chitnis [12] with some modifications. Cells were harvested by centrifugation at various growth phases and suspended in SMN buffer (0.4 M sucrose, 10 mM NaCl, 50 mM MOPS, pH 7.0) containing 200 μM phenylmethylsulfonyl fluoride and 5 mM benzamidine. The cells were broken with glass beads (100 μm in diameter) at 4°C using a Vibrogen for sucrose-gradient centrifugation or a Mini-Bead Beater for sodium dodecyl sulfate (SDS)–dodecyl β-D-maltoside polyacrylamide gel electrophoresis (PAGE). After removal of the beads and unbroken cells by centrifugation at 2500×*g* for 10 min at 4°C, the supernatants were centrifuged at 50 000×*g* for 1 h at 4°C. The green pellets were resuspended in SMN buffer and used as thylakoid membranes.

2.3. Preparation of PSI trimers by sucrose-gradient centrifugation

Thylakoid membranes were incubated for 30 min at room temperature with 1 mM $CaCl_2$ in SMN and solubilized by the addition of dodecyl β-D-maltoside and incubation for 15 min on ice. The ratio of chlorophyll to detergent was 1:15 (w/w). After centrifugation at 10 000×*g* for 10 min at 4°C, the supernatants were layered on a 10–30% linear gradient of sucrose in 0.05% dodecyl β-D-maltoside. The samples were centrifuged at 160 000×*g* for 14 h at 4°C, and the green bands corresponding to PSI trimer bands were collected with a syringe [12].

2.4. Separation of PSIs by SDS–dodecyl β-D-maltoside PAGE

Thylakoid membranes were solubilized with dodecyl β-D-maltoside for 15 min on ice and centrifuged at 10 000×*g* for 10 min at 4°C. The ratio of chlorophyll to detergent was 1:15 (w/w). The supernatants were applied onto a 4% polyacrylamide disk gel (5 mm in diameter) containing 0.05% dodecyl β-D-maltoside. Electrophoresis was carried out at 4°C for about 1.5 h at 0.5 mA/tube according to the method of Anderson [13], provided that the upper reservoir buffer contained 0.02% SDS and the lower reservoir buffer contained no SDS. After electrophoresis, the gels were scanned at 675 nm using a spectrophotometer (model 556, Hitachi Co., Tokyo, Japan).

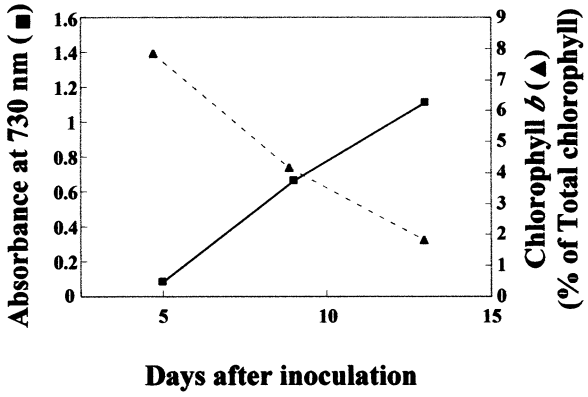

Fig. 1. Growth curve and chlorophyll *b* content in cb1-3. cb1-3 and control cells were grown at 22°C in BG11 medium under continuous illumination. Cell densities were monitored by measuring absorbance at 730 nm of culture.

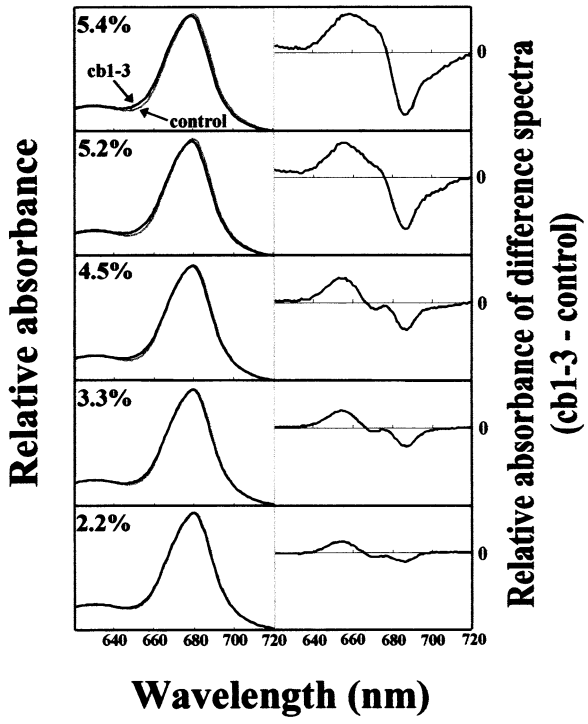

Fig. 2. Absorption spectra of PSI. Absorption spectra and difference absorption spectra measured at various phases of growth. The percentages indicate the chlorophyll *b* content in PSI particles isolated by sucrose-gradient centrifugation. Left, absorption spectra of cb1-3 (black) and control (gray). Right, cb1-3 minus control difference absorption spectra.

2.5. Separation of PSI proteins by SDS–PAGE

The green bands of PAGE corresponding to PSI were cut and immersed in solubilizing buffer containing 8% SDS for 30 min. Gels were loaded on a gel. The proteins were separated by Tricine–urea–SDS–PAGE according to the method of Schagger and von Jagow [14] on slab gels containing 14% polyacrylamide and 6 M urea. After electrophoresis, the gels were stained with silver.

2.6. Chlorophyll determination

Chlorophyll was extracted with 80% acetone and subjected to high-performance liquid chromatography. The chlorophyll was quantified from the chromatographic peak areas after calibration of the chromatographic response with known quantities of the relevant pigments [15].

2.7. Optical measurement

Absorption spectra of isolated PSI trimers were measured using a Hitachi U-3310 spectrophotometer. Curve analysis was carried out as described previously [16].

3. Results

Cyanobacteria accumulated chlorophyll *b* when they acquired the CAO gene, but chlorophyll *a*/*b* ratios drastically changed during growth (Fig. 1). Chlorophyll *b* was actively synthesized and the chlorophyll *a*/*b* ratio was low during the early phase of growth, but the ratios became high as the culture periods were prolonged. In spite of the presence of chlorophyll *b*, there was no significant difference between

the growth of control cells and that of cb1-3 cells [10]. We harvested cells at various growth phases and chlorophyll *b* contents of these cells were between 1.8% and 7.8% of the total chlorophyll.

We previously reported that chlorophyll *b* was preferentially incorporated into PSI in cb1-3 cells [10]. There are three possible explanations for the manner of chlorophyll *b* binding to PSI proteins. One possible explanation is that chlorophyll *b* binds to PSI proteins non-specifically. This hypothesis is very unlikely, however, because the efficiency of energy transfer from chlorophyll *b* to chlorophyll *a* is almost 100% in PSI [10]. The second possible explanation is that chlorophyll *b* is randomly incorporated into chlorophyll *a*-binding sites in PSI, meaning that all chlorophyll *a*-binding sites can accept chlorophyll *b*. The last possible explanation is that chlorophyll *b* is incorporated into some specific chlorophyll *a*-binding sites in PSI.

Chlorophyll *a* and *b* have distinct spectral characteristics in apoproteins as well as in an organic solvent. Chlorophyll *a* molecules exhibit various spectral properties in PSs due to their different localizations in apoproteins. In order to know the binding manner of chlorophyll *b*, we performed spectrum analysis of PSI particles that contained various amount of chlorophyll *b* (Fig. 2).

In order to determine the spectral changes in PSIs induced by chlorophyll *b* incorporation, we isolated trimeric PSI particles by sucrose-gradient centrifugation and measured the difference spectrum of PSI particles between control cells and cb1-3 cells (Fig. 2). As the chlorophyll *b* content increased, absorbance at 650 nm increased with a concomitant decrease at 685 nm. Increase at 670 nm and decrease at 700 nm were observed when the level of chlorophyll *b* was high. The increase at 650 nm was due to the incorporation of chlorophyll *b*, and other spectral changes were attributed to chlorophyll *a*.

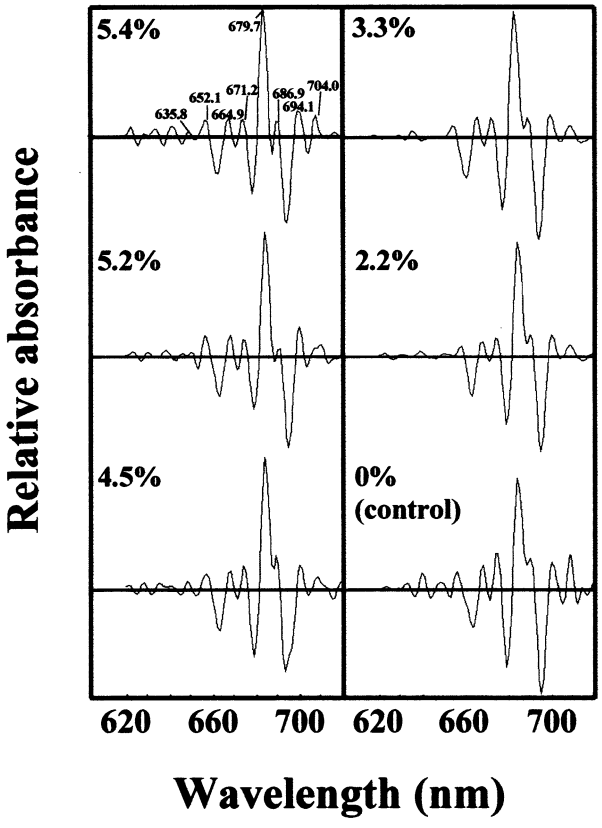

Fig. 3. Fourth-derivative spectra of PSI. The percentages indicate the chlorophyll *b* content in PSI isolated by sucrose-gradient centrifugation. Numerals given on the peaks represent the wavelength of absorption maximum.

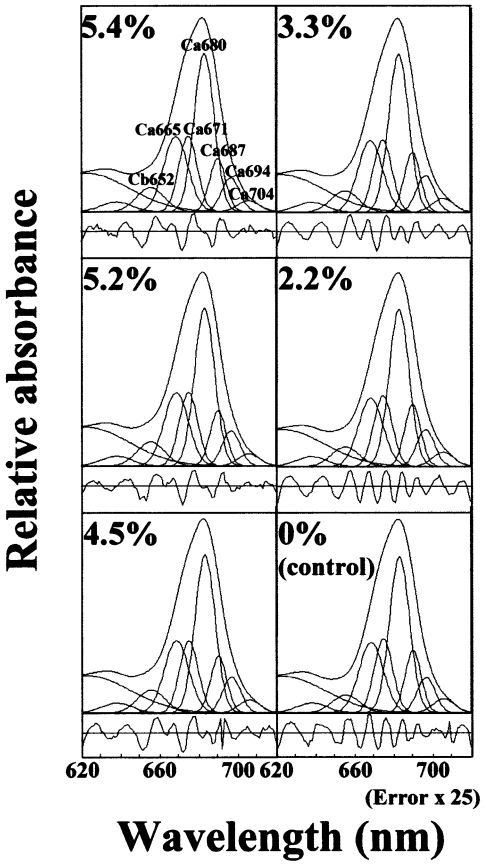

Fig. 4. Absorption spectra of PSI fitted with the component bands. The spectrum above all component bands is the sum of the component curves with parameters given in Table 1. The error of fit at each point is shown below the spectra on a scale with the designated magnification. Numerals given on the peaks represent the chlorophyll forms shown in Table 1.

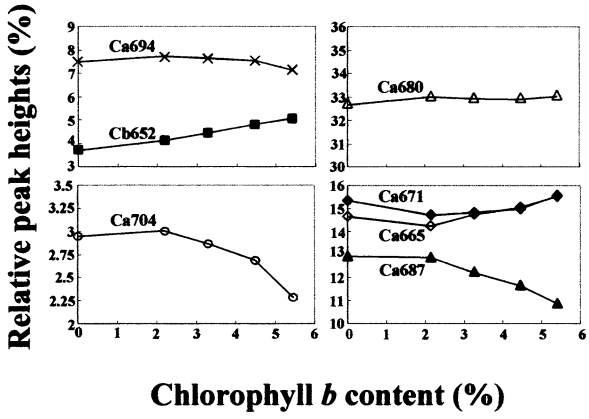

Fig. 5. Chlorophyll *b*-induced changes in spectral components. Areas of each component of the spectrum shown in Fig. 4 are expressed as percentages of the total areas of the components and plotted against the chlorophyll *b* content in PSI.

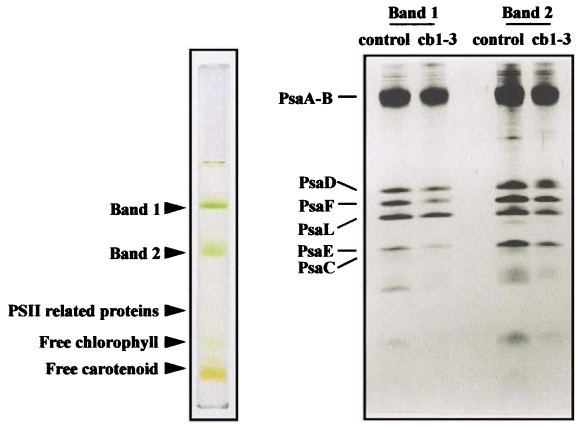

Fig. 6. Separation of PSs by SDS–dodecyl β-D-maltoside PAGE. Left: Electrophoresis of PSs by SDS–dodecyl β-D-maltoside PAGE. Right: The protein compositions of PSI particles.

In order to determine the spectral changes in more detail, we carried out curve analysis using a program that we constructed previously [16]. Seven peaks were observed in the fourth-derivative spectrum between 640 nm and 720 nm. Peak numbers and wavelengths of the fourth-derivative spectrum of *Synechocystis* sp. PCC6803 (Fig. 3, control) were almost the same as previously reported with other cyanobacteria [17]. The profiles of fourth-derivative spectra were not affected by the presence of chlorophyll *b*, indicating that chlorophyll forms of control PSI were identical with those of chlorophyll *b*-containing PSI. We then carried out curve fitting using the parameters derived from fourth-derivative spectra (Fig. 4). The peak areas of each component are shown in Fig. 5. Among the many spectral components, Cb652 and Ca687 exhibited the most dynamic changes in response to chlorophyll *b* incorporation. Cb652 increased as the chlorophyll *b* content increased, whereas Ca687 decreased, suggesting that chlorophyll *b* was incorporated into Ca687-binding sites. Long-wavelength chlorophyll forms such as Ca694 and Ca704 decreased and Ca665 and Ca671 slightly increased when chlorophyll *b* content was high. The largest component of Ca680 was not influenced by the presence of chlorophyll *b*.

Curve analysis showed that chlorophyll *b* induced the spectral changes in chlorophyll *a* in PSI particles. Next, we investigated whether chlorophyll *b* induced the changes in trimeric structure and protein compositions of PSI. We developed methods for the quantification of trimeric and monomeric PSI particles by green gel systems using dodecyl β-D-maltoside and SDS. When thylakoid membranes were solubilized and subjected to electrophoresis, four green bands were resolved on a gel (Fig. 6). According to the molecular weights of the green bands, bands 1 and 2 were trimeric and monomeric PSI particles, respectively. Protein composition of band 3 indicated that this green band was PSII particles (data not shown). Band 4 was free chlorophylls released from proteins during electrophoresis.

Next, we analyzed the protein compositions of the PSI particles isolated by the green gel (Fig. 6). When the PSI particles were subjected to fully denatured SDS–PAGE, 10 proteins were resolved on a gel. This protein profile was very similar to that of PSI particles isolated by sucrose-gradient centrifugation, indicating that intact PSI particles were isolated by this method. There was no difference between protein compositions of monomeric and trimeric PSI particles. Furthermore,

Table 1
Parameters of component bands obtained from curve analysis of the absorption spectra of PSI

	Peak wavelength of component (nm)	Half width of component (nm)
Cb652	652.1	37.5
Ca665	664.9	32.9
Ca671	671.2	24.9
Ca680	679.7	27.2
Ca687	686.9	21.8
Ca694	694.1	24.2
Ca704	704.0	29.4

PSI was isolated by sucrose-gradient centrifugation. Ca and Cb indicate the spectral components of chlorophyll *a* and *b*, respectively.

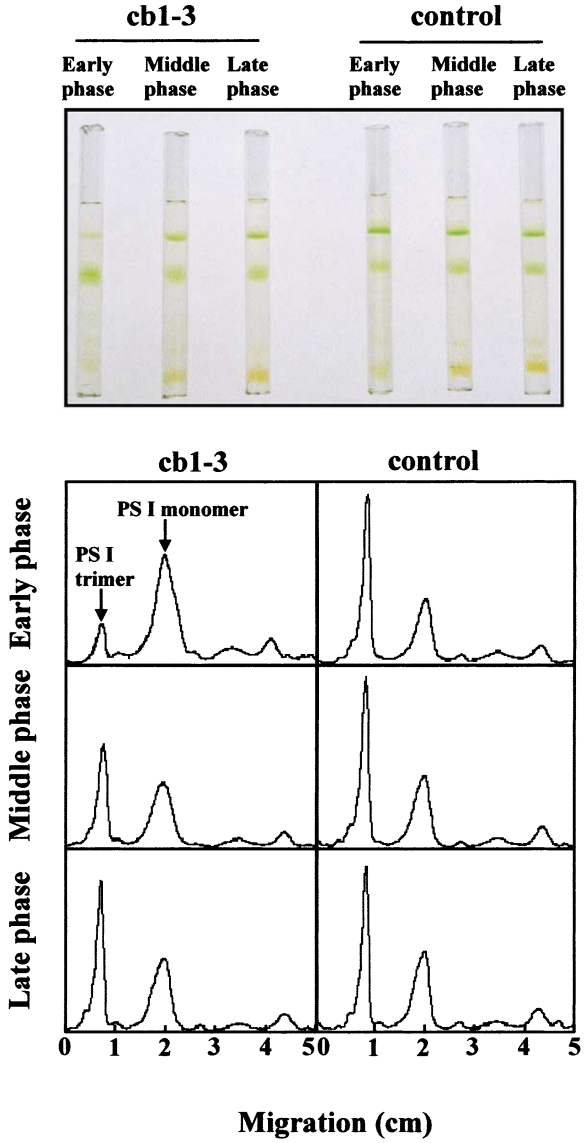

Fig. 7. Changes in the ratios of trimeric to monomeric PSI particles and chlorophyll *b* accumulation. Upper: Thylakoid membranes were isolated from early (5 days), middle (9 days) and late (13 days) phases of growth, and the PSs were separated by SDS–dodecyl β-D-maltoside PAGE. Lower: After electrophoresis, the gels were densitometrically scanned at 675 nm.

Table 2
Ratios of trimeric to monomeric PSI particles and chlorophyll *b* content

		Area		PSI trimer/PSI monomer	Chlorophyll *b* content of cb1-3 (%)
		PSI trimer	PSI monomer		
Control	Early phase	883	749	1.2	–
	Middle phase	787	767	1.0	–
	Late phase	687	769	0.9	–
cb1-3	Early phase	232	1344	0.2	7.8
	Middle phase	609	876	0.7	4.1
	Late phase	692	885	0.8	1.8

Areas corresponding to each PSI were calculated from Fig. 7.

chlorophyll *b* did not induce any changes in protein composition of PSI particles.

This PAGE system is very useful for determining the ratio of trimeric to monomeric PSI particles because, unlike in sucrose-gradient centrifugation, monomeric PSI particles were not contaminated by PSII particles. The trimeric form of PSI was very stable under these electrophoretic conditions, because re-electrophoresis of trimeric PSI purified by sucrose-gradient centrifugation did not give rise to monomeric PSI particles on the gel, indicating that trimeric PSI was not dissociated by the electrophoresis. We then determined the effect of chlorophyll *b* on the ratio of trimeric to monomeric PSI particles (Fig. 7, Table 2). The ratios were around 1 in control cyanobacteria and cb1-3, which had small amounts of chlorophyll *b*. The ratios were almost constant during growth of control cyanobacteria. However, when the chlorophyll *b* content increased to 7.8% of total chlorophyll, the ratio decreased to 0.2.

4. Discussion

Both curve analysis and differential spectra showed that the incorporation of chlorophyll *b* into PSI led not only to an increase in the absorbance corresponding to chlorophyll *b* but also to changes in the chlorophyll *a* spectrum. The most remarkable changes accompanying chlorophyll *b* incorporation were an increase in Cb652 and a decrease in Ca687. The decrease in Ca687 was almost two-times greater than the increase in Cb652. Considering the molecular coefficients of chlorophyll *a* and chlorophyll *b* [18], the reduced number of Ca687 molecules is almost the same as the increased number of Cb652 molecules, suggesting the replacement of chlorophyll *a* by chlorophyll *b* at the Ca687-binding sites. It is reasonable to speculate that some chlorophyll *a*-binding sites accept chlorophyll *b* with the same orientation as that of chlorophyll *a* in the pockets, because chlorophyll *a* and chlorophyll *b* molecules have almost the same structures. This would enable efficient energy transfer from chlorophyll *b* to chlorophyll *a*. If chlorophyll *b* associates with some moieties of PSI proteins that are not involved in pigment binding, energy transfer would not occur between chlorophyll *a* and chlorophyll *b*. However, we previously showed efficient energy transfer from chlorophyll *b* to chlorophyll *a* in PSI of transformant cyanobacteria [10]. These results indicate that some chlorophyll-binding sites of PSI core antenna could accept either chlorophyll *a* or chlorophyll *b*.

Considering these results and previous reports [7,11], it could be concluded that almost all of the peripheral and core antenna complexes have flexibility to bind various pigment molecules. This gives rise to the question of how chlorophyll *a*-binding proteins of the core antenna system of green plants exclusively bind chlorophyll *a*, despite the fact that these apoproteins could potentially bind chlorophyll *b* and *a* and a large amount of chlorophyll *b* is supplied. There must be some mechanisms by which chlorophyll *b* is excluded during the formation of chlorophyll *a*–protein complexes.

We also observed other spectral changes in chlorophyll *a* when a large amount of chlorophyll *b* was accumulated. Long-wavelength components of Ca704 decreased with concomitant increases in Ca665 and Ca671. The changes in these components were small when the level of chlorophyll *b* was low. One possible explanation for this is the blue shift of red chlorophylls. Chlorophyll *b* in PSI induced structural changes in Ca704-binding moieties, and these structural changes induced a blue shift of Ca704 to Ca665 and/or Ca671. Other chlorophyll forms, including the largest component of Ca680, were not influenced by the presence of chlorophyll *b*, indicating that structural changes induced by chlorophyll *b* were restricted to small portion of PSI particles.

Analysis by sucrose-gradient centrifugation [12] and nondenaturing PAGE (Fig. 6) showed that PSI complexes exist as monomers or trimers in cyanobacterial cells. This trimeric form is a unique structure of cyanobacteria [19,20], because higher plants have only monomeric PSI [21]. However, the mechanisms for the formation or dissociation of trimeric PSI are still unknown. Formation of trimeric PSI was achieved in vitro under low salt conditions [22] and in proteoliposomes [23]. PsaL, a small polypeptide, was reported to participate in the formation of PSI trimers, because a cyanobacterial PsaL-less mutant contains only PSI monomers [12]. In this study, we found that most of the PSI existed as monomers when cyanobacteria accumulated a large amount of chlorophyll *b*. In this case, no differences between protein compositions of PSI particles in the control cells and the transformant cells containing chlorophyll *b* were found. These results indicate that not only the presence or absence of specific subunits is relevant but that subtle structural changes in them are also involved in the formation of PSI trimers.

Ca704 is the chlorophyll *a* form with the longest wavelength of a PSI trimer in *Synechocystis* sp. PCC6803. This component may correspond to Ca708 examined at 77 k [24]. According to the analysis of 77 k fluorescence spectra of PSI particles that are deficient in PSI peripheral subunits, Ca708 is not bound to peripheral subunits but to the core antenna of PSI [25]. Analysis of the three-dimensional structure of PSI trimer of *Synechococcus elongatus* revealed two chlorophyll *a* dimers that are the candidates for red chlorophylls [4]. One of these chlorophyll *a* dimers is located in the PSI core close to the PsaF subunit, and the other is located close to the PsaL subunit. PSI monomers isolated from a PsaL-deficient mutant

showed a decrease in Ca708, but PsaF-deleted PSI did not [24], indicating that Ca708 is the chlorophyll dimer close to the PsaL subunit that is located between PSI monomers in the trimeric structure. In this study, we showed that the decrease in Ca704 was closely related to the ratio of trimeric to monomeric PSI particles. The fact that Ca704 is located between monomer PSI particles in the trimeric structure together with our present results suggest that the red chlorophyll-binding moiety of PSI plays an important role in trimer formation.

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