Chlorophyll *b* can serve as the major pigment in functional photosystem II complexes of cyanobacteria

Hong Xu, Dmitrii Vavilin, and Wim Vermaas†

Department of Plant Biology and Center of the Study of Early Events in Photosynthesis, Arizona State University, Box 871601, Tempe, AZ 85287-1601

Communicated by Elisabeth Gantt, University of Maryland, College Park, MD, October 5, 2001 (received for review March 2, 2001)

An *Arabidopsis thaliana* chlorophyll(ide) *a* oxygenase gene (*cao*), which is responsible for chlorophyll *b* synthesis from chlorophyll *a*, was introduced and expressed in a photosystem I-less strain of the cyanobacterium *Synechocystis* sp. PCC 6803. In this strain, most chlorophyll is associated with the photosystem II complex. In line with observations by Satoh *et al.* [Satoh, S., Ikeuchi, M., Mimuro, M. & Tanaka, A. (2001) *J. Biol. Chem*. 276, 4293–4297], chlorophyll *b* was made but accounted for less than 10% of total chlorophyll. However, when *lhcb* encoding light-harvesting complex (LHC)II from pea was present in the same strain (*lhcb*$^+$/*cao*$^+$), chlorophyll *b* accumulated in the cell to levels exceeding those of chlorophyll *a*, although LHCII did not accumulate. In the *lhcb*$^+$/*cao*$^+$ strain, the total amount of chlorophyll, the number of chlorophylls per photosystem II center, and the oxygen-evolving activity on a per-chlorophyll basis were similar to those in the photosystem I-less strain. Furthermore, the chlorophyll *a*/*b* ratio of photosystem II core particles (retaining CP47 and CP43) and of whole cells of the *lhcb*$^+$/*cao*$^+$ strain was essentially identical, and PS II activity could be obtained efficiently by chlorophyll *b* excitation. These data indicate that chlorophyll *b* functionally substitutes for chlorophyll *a* in photosystem II. Therefore, the availability of chlorophylls, rather than their binding specificity, may determine which chlorophyll is incorporated at many positions of photosystem II. We propose that the transient presence of a LHCII/chlorophyll(ide) *a* oxygenase complex in the *lhcb*$^+$/*cao*$^+$ strain leads to a high abundance of available chlorophyll *b* that is subsequently incorporated into photosystem II complexes. The apparent LHCII requirement for high chlorophyll(ide) *a* oxygenase activity may be instrumental to limit the occurrence of chlorophyll *b* in plants to LHC proteins.

Oxygenic photosynthetic organisms contain chlorophyll *a* and other (accessory) pigments to harvest light energy. Higher plants, many algae, and prochlorophytes contain chlorophyll *b* as one of the accessory pigments in light-harvesting chlorophyll complexes (LHCs), and in these organisms, the chlorophyll *a*/*b* ratio generally is 2–4. The two chlorophylls differ only at position 3 (ring B): chlorophyll *a* contains a methyl group at this position, whereas chlorophyll *b* contains an aldehyde group (1). The gene encoding chlorophyll(ide) *a* oxygenase (CAO) that catalyzes conversion of the methyl to the aldehyde group has been cloned (2–4); the natural substrate of CAO is as yet unknown, as recombinant CAO produced in *Escherichia coli* catalyzed the formation of chlorophyllide *b* from chlorophyllide *a* (5) but did so at a very low rate. Overexpressed CAO *in vitro* did not catalyze a clear conversion of chlorophyll *a* to *b* (5), but this may be related to very limited chlorophyll *a* solubility in aqueous media. No CAO is found in cyanobacteria, and therefore they are unable to synthesize chlorophyll *b*. Cyanobacteria do not possess LHC and instead contain phycobilisomes as the peripheral light-harvesting apparatus.

In plants, LHC stability and chlorophyll *b* synthesis appear to be mutually correlated. Chlorophyll *b* synthesis requires the presence of LHC apoproteins in the thylakoid (6), and chlorophyll *b*-less strains of barley (7), rice (8), and *Arabidopsis* (9) have very low levels of LHCII [LHC associated with photosystem (PS) II]. Even though it is clear that chlorophyll *b* binding is required for LHCII stability (10, 11), the mechanism for the dependence of chlorophyll *b* synthesis on the presence of LHC has remained unexplained.

The wild type of the cyanobacterium *Synechocystis* sp. PCC 6803 lacks the capability to synthesize either LHC or chlorophyll *b*. However, the corresponding higher-plant genes can be introduced into this cyanobacterium. On introduction of the *cao* gene, a *Synechocystis* strain has been generated that converted a small percentage of chlorophyll *a* to chlorophyll *b* (the chlorophyll *a*/*b* ratio was about 15, depending on the growth stage) (12). Another strain was created into which a pea *lhcb* gene (coding for a LHCII polypeptide) was introduced, and even though LHCII was synthesized, it was not stable in the membrane (13). This strain lacked PS I because of a *psa*AB deletion and made chlorophyll only in light because of a lack of *chl*L (13). This strain, as well as the PS I-less/*chl*L$^-$ parental strain (14) contained little chlorophyll, as most chlorophyll in cyanobacteria is associated with PS I (15).

Here we show that in the transient presence of LHCII (but not in its absence), introduction of *cao* into *Synechocystis* sp. PCC 6803 causes chlorophyll *b* to become the major pigment that functionally replaces chlorophyll *a* from many binding sites in the PS II complex. This result indicates a lack of specificity of pigment binding in PS II complexes and highlights the important role LHC appears to play in CAO activity *in vivo*.

Materials and Methods

Strains and Growth Conditions. PS I-less *Synechocystis* sp. PCC 6803 strains were cultivated at 30°C in BG-11 medium (16) buffered with 5 mM *N*-tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid-NaOH (pH 8.2) and supplemented with 5 mM glucose. The light intensity was 0.5 μmol of photons $m^{-2}{\cdot}s^{-1}$.

Introduction of the *cao* Gene into the *Synechocystis* sp. PCC 6803 Genome. The *cao* gene (starting from codon 57, essentially corresponding to the coding region for the mature protein) was PCR-amplified from the *Arabidopsis thaliana* cDNA clone 103D24T7 [GenBank accession no. T22255 (17)] with primers creating a *Bsp*HI site and an AUG codon at the 5′ end of the region of the gene corresponding to the mature protein, and a *Sal*I site directly downstream of the stop codon. Moreover, a 0.75-kb region directly upstream of and including the *psa*A translation start site was PCR-amplified by using genomic *Synechocystis* sp. PCC 6803 DNA, introducing restriction sites for *Eco*RI (0.75 kb upstream of *psa*A) and *Nco*I (at the *psa*A

Abbreviations: CAO, chlorophyll(ide) *a* oxygenase; LHC, light-harvesting complex; PS, photosystem.

†To whom reprint requests should be addressed. E-mail: wim@asu.edu.

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www.pnas.org/cgi/doi/10.1073/pnas.251530298

translation start site). These PCR fragments were cloned together in pUC18, yielding a construct with the upstream *psa*A region and the part of the *cao* gene coding for the mature protein, linked together in-frame at the *psa*A translation start site. At the 3′ end of *cao*, a *Pst*I/*Pst*I fragment from pUC4K carrying the kanamycin-resistance cassette was inserted, and downstream of the cassette, a 0.6-kb sequence identical to that immediately downstream of *psa*B, but with introduced *Sph*I and *Nar*I sites, was inserted. The resulting plasmid was used to transform the PS I-less/*chl*L$^-$/*lhcb*$^+$ (13) and PS I-less/*chl*L$^-$ (14) strains of *Synechocystis* sp. PCC 6803, placing *cao* under control of the *psa*AB promoter.

Pigment Analysis. Pigments were extracted from *Synechocystis* cells with methanol, and the methanol extract was subjected to HPLC analysis. A 15-min gradient of ethyl acetate (0–100%) in acetonitrile–water–triethylamine (9:1:0.01, vol/vol/vol) at a flow rate of 1.5 ml/min was used to elute the HPLC column. For a preliminary estimation of the chlorophyll *a*/*b* ratio, concentrations of chlorophyll *a* and *b* in the methanol extract were determined spectrophotometrically according to ref. 18.

Mass Spectroscopy. Chlorophyll *b* was collected after HPLC analysis. Solvents were evaporated under nitrogen, and dry chlorophyll *b* was stored at −20°C in the dark. Mass spectra were obtained by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Before analysis, 10 μg of chlorophyll *b* was mixed with terthiophene (used as a matrix) dissolved in acetone.

Isolation of PS II Core Particles. Thylakoid membranes (13) were washed with 20 mM sodium pyrophosphate dissolved in 50 mM Mes–NaOH (pH 6.4) buffer. The pellet of washed membranes was resuspended in thylakoid buffer [20 mM Mes–NaOH, pH 6.4/5 mM $MgCl_2$/5 mM $CaCl_2$/20% glycerol (vol/vol)/1 mM benzamidine] to a final chlorophyll concentration of ≈0.1 mg/ml. Dodecyl maltoside was added to a concentration of 0.4% (wt/vol), and the mixture was incubated in the dark for 40 min at 4°C. The sample was centrifuged in the microfuge for 3 min, and the solubilized material was loaded on a sucrose gradient [50 mM Mes–NaOH, pH 6.4/10–30% (wt/vol) sucrose/5 mM $MgCl_2$/5 mM $CaCl_2$/10 mM NaCl/0.04% (wt/vol) dodecyl maltoside] and centrifuged overnight at 35,000 rpm and 4°C in a Beckman SW41 rotor. The most intense green band was recovered and subjected to anion-exchange chromatography (19). The resulting PS II core particles retained the PS II core antenna proteins CP43 and CP47.

SDS-Urea/PAGE. SDS-urea/PAGE was performed by using a continuous 16–22% (wt/vol) polyacrylamide gradient gel containing 6.5 M urea, as described (20).

[^{35}S] Protein Labeling and Chase. For pulse–chase experiments, 50 ml of *Synechocystis* cells was incubated in [^{35}S] protein-labeling mix (EXPRE^{35}S ^{35}S, containing 73% L-[^{35}S]methionine and 22% L-[^{35}S]cysteine, 11 mCi/ml) (DuPont/NEN) at a final concentration of 1 μCi/ml for 10 min. The radioactivity was chased by addition of 100 μM unlabeled methionine and cysteine. Cells were harvested 0, 10, and 30 min after the start of the chase, rapidly chilled, and thylakoids were prepared (13). Thylakoid proteins were separated by SDS-urea/PAGE. Labeled protein bands were detected by autoradiography by using a Storm PhosphorImager (Molecular Dynamics), and the integrated intensity of radiolabeled LHCII was determined by IMAGEQUANT software (Molecular Dynamics).

Herbicide Binding. ^{14}C-3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU)-binding analysis was carried out to quantify the amount of PS II in the cells, as described (15).

Table 1. Chlorophyll *b* amount as a percentage of total chlorophyll (chlorophyll *a* + chlorophyll *b*) in the *Synechocystis* sp. PCC 6803 PS I-less/*chl*L$^-$ (parental) strain and mutants derived from this strain carrying either *cao* (*cao*$^+$), *lhcb* (*lhcb*$^+$), or both (*cao*$^+$/*lhcb*$^+$)

Strain	Parental	*cao*$^+$	*lhcb*$^+$	*cao*$^+$/*lhcb*$^+$	*cao*$^+$*
Chlorophyll *b* (%)	0	6 ± 1	0	59 ± 6	3 ± 2

Chlorophyll *a* and *b* contents were determined by comparing the respective peak areas in the HPLC profile with chlorophyll standards, monitoring absorbance at 440 nm.

*This strain initially carried both *cao* and *lhcb*, but *psb*A3 was reintroduced in lieu of *lhcb*.

Fluorescence Induction. Fluorescence induction was measured in the presence of 5 μM DCMU by using a FluoroLog (Spex Industries, Metuchen, NJ) spectrophotometer equipped with a manually triggered UNIBLITZ-26L2A0T5 electronic shutter (3-ms opening time). The emission wavelength was set at 680 nm (the bandwidth at half maximum was 8 nm). To correct for the difference in the intensity of the excitation beam at the two wavelengths, the excitation bandwidths at half maximum were 1.00 and 0.85 nm at 436 and 462 nm, respectively.

Low-Temperature Fluorescence Emission Measurements. Fluorescence emission spectra (77 and 15 K) were measured by using a Spex FluoroLog 2 instrument. Intact cells or isolated PS II particles (5 μg of chlorophyll/ml) were placed between two fused glass plates about 1.2 mm apart and cooled in a temperature-controlled cryostat (Air Products and Chemicals, Allentown, PA). The excitation and emission bandwidths were 4 and 1 nm, respectively.

Results

Introduction of the *cao* Gene. An *A. thaliana cao* gene was introduced under the *Synechocystis psa*AB promoter and with the native *psa*A translation start site in two strains of *Synechocystis* sp. PCC 6803. One was the PS I-less/*chl*L$^-$ strain lacking *psa*AB (coding for the PS I reaction center proteins) and *chl*L (coding for a subunit of the light-independent protochlorophyllide reductase); this strain is referred to as the "parental strain" in this study. The other strain was the PS I-less/*chl*L$^-$/*lhcb*$^+$ strain (referred to as *lhcb*$^+$), which carries the pea gene for the major LHCII subunit under the *psb*A3 promoter (13). The *psb*A3 gene is one of the genes coding for the D1 protein of PS II and is dispensable for PS II activity when *psb*A2 is present (21).

Chlorophyll *a* and *b* Levels. When introducing *cao* into the parental (PS I-less/*chl*L$^-$) strain, the resulting transformant, referred to as *cao*$^+$, contained very little chlorophyll *b* relative to *a* (Table 1), in line with the findings of Satoh *et al.* (12). However, when *cao* was introduced together with *lhcb*, the chlorophyll *b* amount was increased by an order of magnitude at the expense of chlorophyll *a*, and the resulting strain (named *cao*$^+$/*lhcb*$^+$) contained more chlorophyll *b* than *a* (Table 1 and Fig. 1). To verify that the major increase in the chlorophyll *b* content in the *cao*$^+$/*lhcb*$^+$ strain vs. the *cao*$^+$ stain indeed is caused by the presence of *lhcb*, the *lhcb* gene in the *cao*$^+$/*lhcb*$^+$ strain was replaced by the native *psb*A3 gene resulting in the *cao*$^+$* strain. In this strain, the chlorophyll *b* content dropped by an order of magnitude to levels comparable to that in the *cao*$^+$ strain (Table 1), confirming that the presence of *lhcb* was required to generate high chlorophyll *b* levels in the strain.

We verified that the major compound appearing in the *cao*$^+$/*lhcb*$^+$ strain was indeed chlorophyll *b*. As shown in Fig. 1*B*, the absorption spectrum corresponds to that of chlorophyll *b*. Furthermore, laser desorption mass spectrometry was carried

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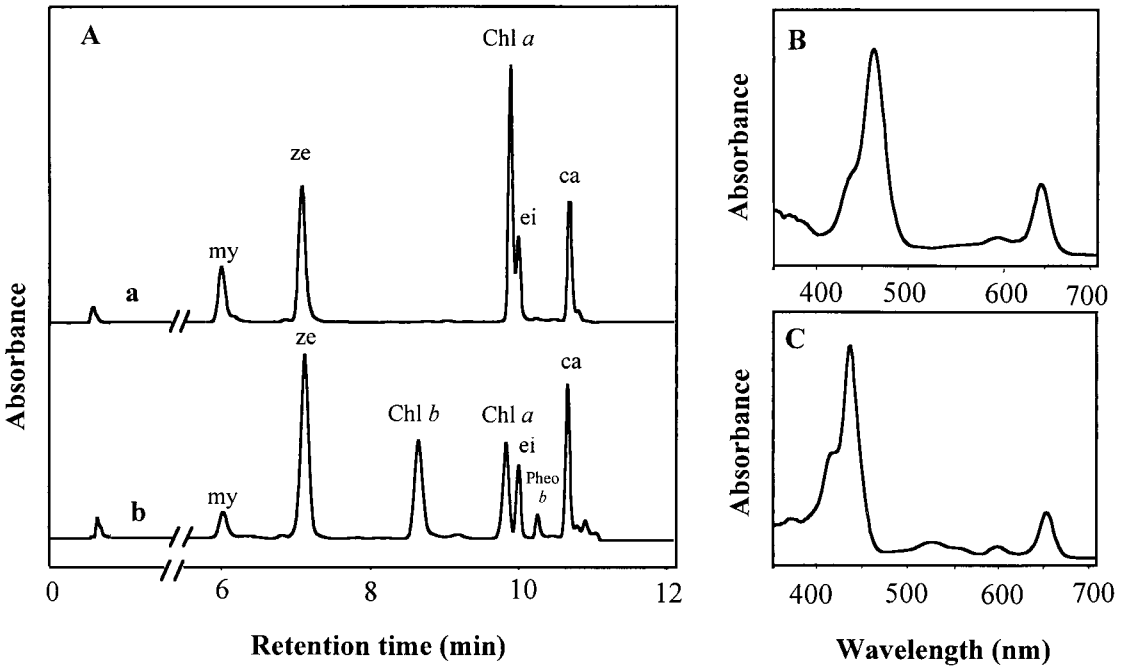

Fig. 1. HPLC analysis of cyanobacterial pigments. (*A*) HPLC profiles of pigments from methanol extracts of whole cells from the *lhcb*$^+$ (a) and *cao*$^+$/*lhcb*$^+$ (b) strains. my, myxoxanthophyll; ze, zeaxanthin; Chl *a*, chlorophyll *a*; Chl *b*, chlorophyll *b*, ei, echinenone; ca, β-carotene. Pigment assignments are based on absorption spectra and HPLC retention time. Detection was at 440 nm, at which wavelength the molar extinction coefficients of chlorophylls *a* and *b* are similar. Absorption spectra of the chlorophyll *b* (*B*) and pheophytin *b* (*C*) peaks are shown as well. No peaks were visible at retention times between 2 and 6 min and after 12 min.

out on HPLC-purified chlorophyll *b* from *A. thaliana* leaves and from the *Synechocystis* sp. PCC 6803 *cao*$^+$/*lhcb*$^+$ strain (Fig. 2). The mass spectrum of the two isolates was essentially identical and showed two peaks at *m*/*z* 907.7 and 630.7, corresponding to chlorophyll *b* itself and to chlorophyll *b* that lost the phytyl chain during mass spectrometry (22).

A minor new peak occurring in the *cao*$^+$/*lhcb*$^+$ strain was assigned to pheophytin *b* on the basis of its mobility (Fig. 1*A*) and optical spectrum (Fig. 1*C*); its abundance was about 4% of that of total chlorophyll in the strain.

Chlorophyll *b* Replaces Chlorophyll *a* in PS II. The next question to be addressed is: With which protein complex is chlorophyll *b* in the *cao*$^+$/*lhcb*$^+$ strain (which lacks PS I) associated? In the *cao*$^+$/*lhcb*$^+$ strain, the total amount of chlorophyll, the number of chlorophylls per PS II center, and the oxygen evolution rate of PS II were essentially indistinguishable from that in the parental strain (Table 2). These data suggest that chlorophyll *b* replaces most of the chlorophyll *a*. To verify that chlorophyll *b* indeed is part of the PS II core complex, PS II core particles were isolated from the parental and *cao*$^+$/*lhcb*$^+$ strains. The protein composition and absorption spectra of PS II core particles (i.e., PS II particles retaining the core antenna proteins CP43 and CP47) from the two strains are shown in Fig. 3. The SDS/PAGE protein patterns of PS II core particle preparations from the parental and *cao*$^+$/*lhcb*$^+$ strains were similar, whereas in the PS II core particle, preparation from the *cao*$^+$/*lhcb*$^+$ strain chlorophyll *b* made up about 60% of the total chlorophyll. This percentage is similar to that of intact cells from which the PS II core particles were isolated.

Fluorescence excitation and emission spectra (77 K) of the PS II core particles from the parental and *cao*$^+$/*lhcb*$^+$ strains are shown in Fig. 4. Excitation spectra show a significant contribution of chlorophyll *b* to 684-nm emission. The emission spectrum is essentially identical for the two strains, regardless of whether, in PS II core particles from the *cao*$^+$/*lhcb*$^+$ strain, chlorophyll *a* (at 436 nm) or chlorophyll *b* (at 462 nm) was excited preferen-

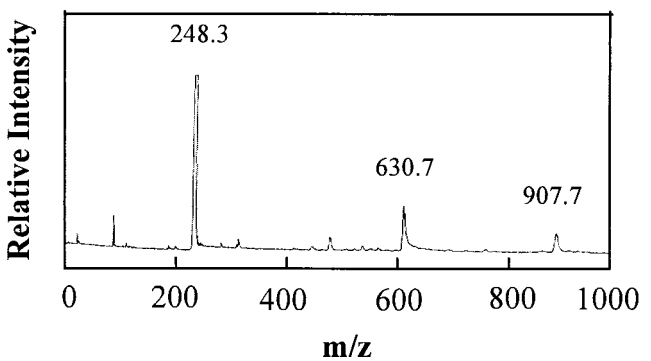

Fig. 2. Matrix-assisted laser desorption/ionization (MALDI) mass spectra of chlorophyll *b* isolated from the *cao*$^+$/*lhcb*$^+$ strain. Molecular masses are indicated. The matrix used was terthiophene (molecular weight 248.4). The molecular mass of chlorophyll *b* is 907.5. The peak around 630 is because of chlorophyll *b* that has lost the phytyl chain during the MALDI experiment.

Table 2. Properties of *Synechocystis* sp. PCC 6803 strains

Strain	Chlorophyll, μg/ml/OD_{730}	Chlorophyll/PS II ratio	O_2 evolution rate (μmol O_2/mg chlorophyll·h)
Parental	0.81 ± 0.06	75	2,360 ± 100
cao$^+$/*lhcb*$^+$	0.90 ± 0.08	75	1,950 ± 150

Cells were grown photoheterotrophically to $OD_{730} \sim 0.5$ for all assays. Chlorophyll is the sum of chlorophylls *a* and *b*. Chlorophyll/PS II ratios were determined by ^{14}C-DCMU binding. The chlorophyll *a*/*b* ratio in cells of the *cao*$^+$/*lhcb*$^+$ strain used for these assays was 0.7 ± 0.2.

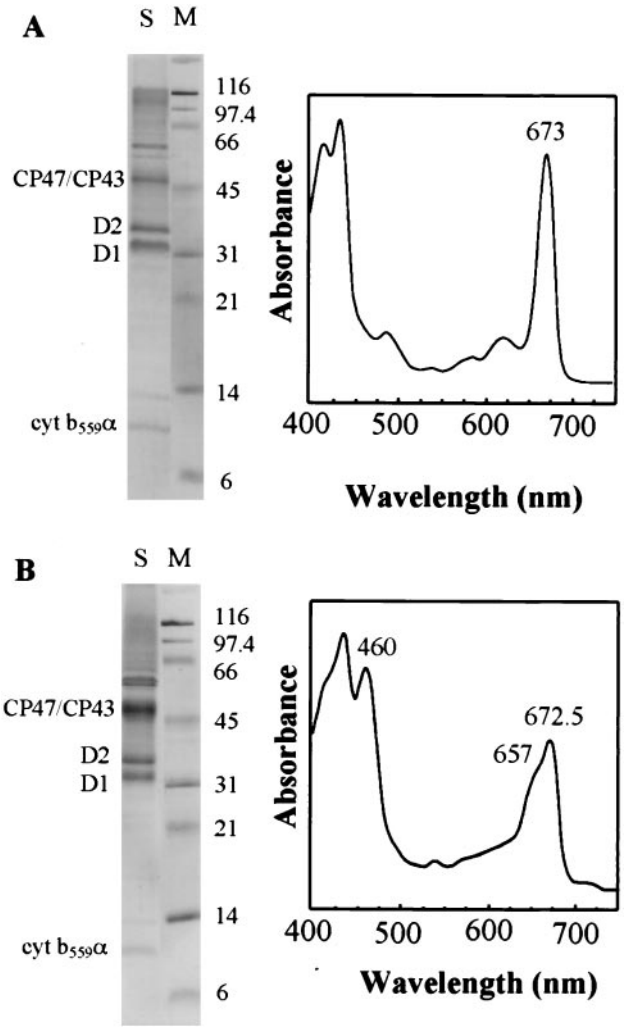

Fig. 3. Protein composition and absorption characteristics of PS II core particles isolated from the parental (*A*) and *cao*$^+$/*lhcb*$^+$ (*B*) strains. Proteins from PS II core particles were separated by denaturing SDS-urea/PAGE and visualized by Coomassie brilliant blue staining (lane S). Lane M contains marker proteins (Bio-Rad). The molecular masses of standard marker proteins are indicated in kilodaltons. Bands in the 55-kDa region are ATPase contaminations. Protein band assignments were made after blotting and immunodetection or N-terminal sequencing. Total chlorophyll (0.1 μg) (chlorophylls *a* plus *b*) was loaded per sample lane. *Right* represents room-temperature absorbance spectra of the respective PS II core particles.

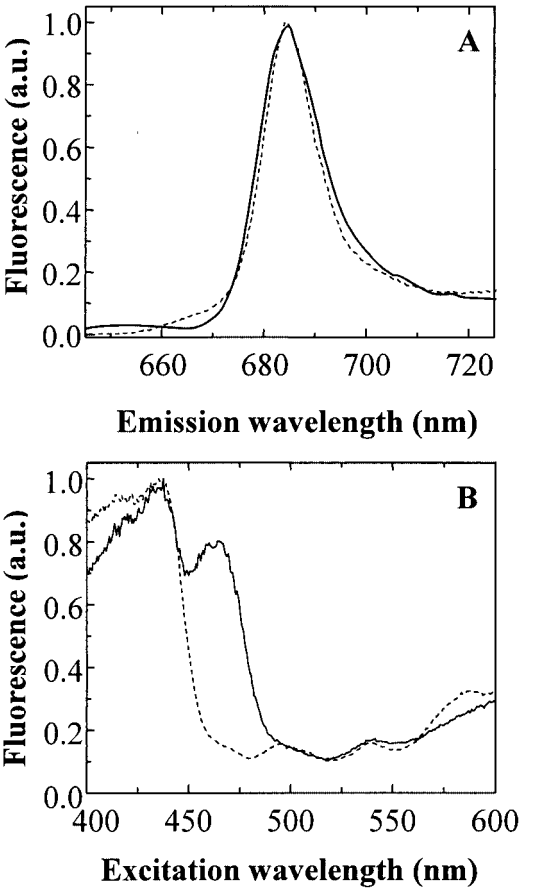

Fig. 4. Fluorescence spectra (77 K) of PS II core particles isolated from the parental and *cao*$^+$/*lhcb*$^+$ strains. (*A*) Emission spectra of PS II core particles on excitation at 436 nm (chlorophyll *a*; parental strain, dashed line) or at 462 nm (chlorophyll *b*; *cao*$^+$/*lhcb*$^+$ strain, solid line). On excitation at 436 nm, the emission spectrum of the *cao*$^+$/*lhcb*$^+$ strain was essentially indistinguishable from that obtained on 462 nm excitation. (*B*) Excitation spectra of the parental (dashed line) and *cao*$^+$/*lhcb*$^+$ (solid line) PS II core particles on monitoring 684-nm emission. Spectra were normalized to 1.0 at the fluorescence maximum.

tially, indicating efficient energy transfer from chlorophyll *b* to chlorophyll *a*. However, a small 650- to 660-nm fluorescence emission shoulder is present in particles from the *cao*$^+$/*lhcb*$^+$ strain; this shoulder may originate from chlorophyll *b*.

Presence of LHCII. Interestingly, LHCII does not seem to contribute to chlorophyll *b* binding in the *cao*$^+$/*lhcb*$^+$ strain. According to Western blots, LHCII did not accumulate to significant levels in the *cao*$^+$/*lhcb*$^+$ strain (data not shown). Moreover, in chlorophyll *b*-rich PS II core particle preparations, no band corresponding to LHCII was observed (Fig. 3). On pulse labeling, the thylakoid protein pattern of *cao*$^+$/*lhcb*$^+$ cells (Fig. 5) was similar to that of the *lhcb*$^+$ strain (12). However, the presence of *cao* and thereby the presence of chlorophyll *b* stabilized LHCII to some degree: the half-life of LHCII increased from about 10 min in the *lhcb*$^+$ strain to $\approx$30 min in the *cao*$^+$/*lhcb*$^+$ strain; however, LHCII accumulation remained insufficient for immunodetection.

Functionality of Chlorophyll *b* in PS II. To test whether light absorbed by chlorophyll *b* in PS II complexes is capable of driving PS II photochemistry, fluorescence induction curves were recorded by using intact cells of the parental and *cao*$^+$/*lhcb*$^+$ strains in the presence of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (Fig. 6). In the *cao*$^+$/*lhcb*$^+$ strain, fluorescence induction, which reflects the rate of Q_A reduction in PS II, occurred at a similar rate on excitation at 462 nm (absorbed mostly by chlorophyll *b*) versus when excited at 436 nm (absorbed mostly by chlorophyll *a*). However, in the control strain, 436 nm light was much more effective in exciting PS II than 462 nm light. These results indicate a large contribution of chlorophyll *b* to light harvesting for PS II in the *cao*$^+$/*lhcb*$^+$ strain.

As chlorophyll *b* appears to be part of the PS II antenna in the *cao*$^+$/*lhcb*$^+$ strain, 77 K fluorescence excitation and emission spectra were determined in intact cells. As shown in Fig. 7*A*, the parental strain contained characteristic 685- and 695-nm peaks representing antenna/PS II chlorophyll and a "low-energy" chlorophyll *a* presumably associated with His-114 of CP47 (23, 24), respectively, in intact cells. However, in the *cao*$^+$/*lhcb*$^+$ strain, the two peaks were merged to one with a maximum around 691 nm. Cooling of the sample to 15 K, which intensifies emission from the low-energy chlorophyll (25), did not lead to

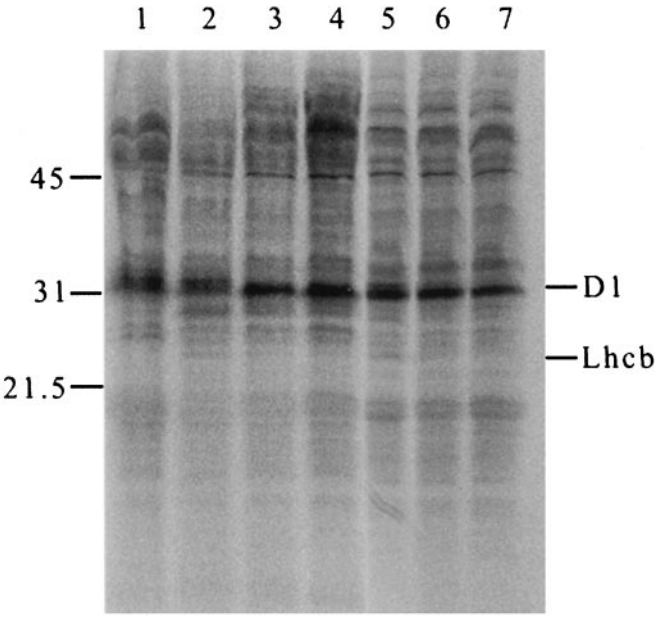

Fig. 5. Pulse–chase labeling of thylakoid membrane proteins. Cells of the PS I-less/*chl*L$^-$ parental strain (lane 1) or of the *lhcb*$^+$ (lanes 2–4) or *cao*$^+$/*lhcb*$^+$ (lanes 5–7) strains were pulse-labeled with [^{35}S]methionine and [^{35}S]cysteine for 10 min. Subsequently, radioactivity was chased by addition of a 15,000-fold excess of unlabeled methionine and cysteine for 0 min (lanes 1, 2, and 5), 10 min (lanes 3 and 6), and 30 min (lanes 4 and 7). Thylakoids were then isolated, and thylakoid proteins were separated by SDS-urea/PAGE. The D1 and Lhcb proteins are indicated. Numbers (*Left*) indicate the position of marker proteins (molecular mass, kDa).

spectral shifts in the *cao*$^+$/*lhcb*$^+$ strain (Fig. 7*B*), indicating that in this strain, the 695-nm fluorescence emission band has shifted to the blue, and the low-energy chlorophyll *a* associated with His-114 of CP47 has disappeared and is now perhaps chlorophyll

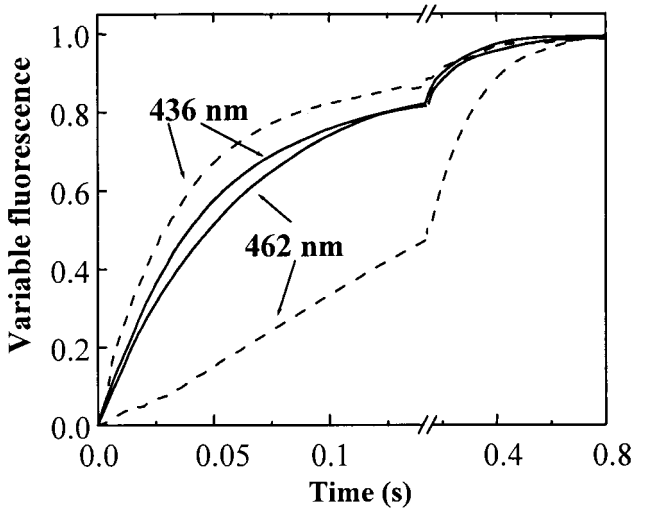

Fig. 6. Variable fluorescence induction kinetics in *Synechocystis* sp. PCC 6803 strains. Cells from the parental (dashed lines) and *cao*$^+$/*lhcb*$^+$ (solid lines) strains were excited at 436 or 462 nm, the *in vivo* chlorophylls *a* and *b* absorption maxima, respectively. The fluorescence signal (F_o) detected immediately on turning on the excitation light was subtracted from the induction curve. The variable fluorescence was normalized to the maximum fluorescence value reached after 2 s of illumination. The F_o intensity was comparable to that of maximal variable fluorescence in the *cao*$^+$/*lhcb*$^+$ strain, and about half the maximal variable fluorescence intensity in the parental strain.

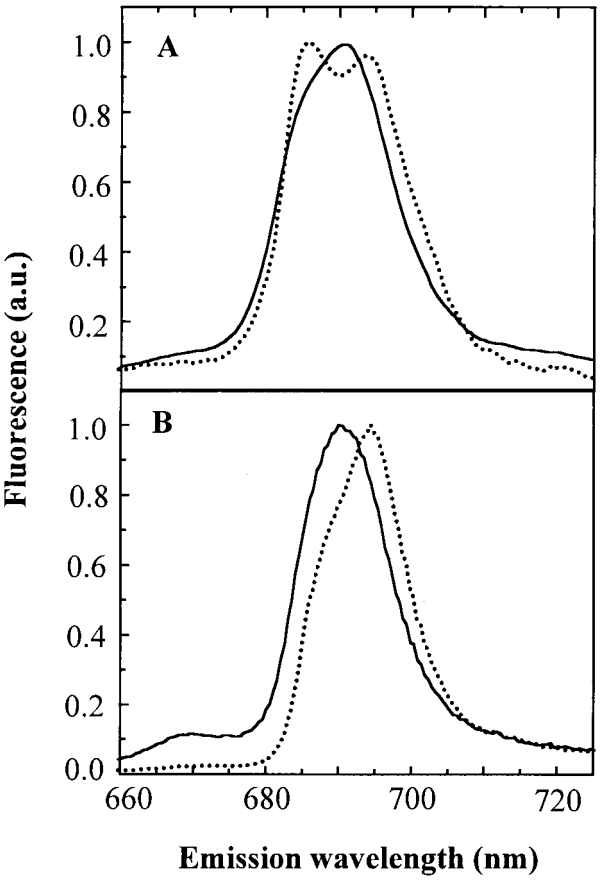

Fig. 7. Low-temperature fluorescence emission spectra of intact cells of the parental and *cao*$^+$/*lhcb*$^+$ strains. Emission spectra from the parental (dotted lines) and the *cao*$^+$/*lhcb*$^+$ (solid lines) strains were measured at 77 K (*A*) and 15 K (*B*). Excitation was at 436 nm, and spectra were normalized to 1.0 at the fluorescence maximum. The emission spectra of the *cao*$^+$/*lhcb*$^+$ strain were qualitatively indistinguishable on excitation at 462 nm (data not shown), indicative of efficient energy transfer between the chlorophylls at both temperatures.

b. This His-114-ligated chlorophyll *b* no longer is a long-wavelength emitter, and excitation energy is expected to be transferred efficiently to nearby chlorophyll *a* molecules. The fluorescence emission spectrum of the *cao*$^+$/*lhcb*$^+$ strain was essentially identical when excited at 436 vs. 462 nm (not shown), indicative of an efficient energy exchange between chlorophylls *b* and *a*, supporting the data shown in Fig. 4*B*. This conclusion was further confirmed by the fluorescence excitation spectrum of intact *cao*$^+$/*lhcb*$^+$ cells, where chlorophylls *a* and *b* both contributed to 690-nm fluorescence emission at 77 K (data not shown).

Discussion

The results obtained indicate the functional presence of chlorophyll *b* at the majority of chlorophyll-binding sites in the *cao*$^+$/*lhcb*$^+$ PS II core complex, apparently replacing chlorophyll *a*. Consequently, most of the chlorophyll-binding sites in the PS II core complex are not specific for chlorophyll *a* and can functionally accommodate chlorophyll *b* if offered. The chlorophyll *b* level increased about 10-fold when *lhcb* was present (Table 1), even though LHCII was not stable (Fig. 4), and did not accumulate. In agreement with the results of Satoh *et al.* (12), very little chlorophyll *b* accumulated in *Synechocystis* strains containing *cao* but lacking *lhcb*. Therefore, LHCII appears to be

needed for activation of CAO and/or for providing an initial binding niche for chlorophyll *b*. The requirement of LHCII for the high activity of CAD provides an explanation for the specific association of chlorophyll *b* with LHC in plants and for the requirement of LHC for chlorophyll *b* accumulation (6, 26). As LHCII is not stable in *Synechocystis* thylakoids, chlorophyll *b* may become available as LHCII degrades and may be incorporated into newly synthesized PS II core complexes. As indicated in Fig. 6 and Table 2, these PS II complexes are fully functional although the majority of chlorophyll *a* binding sites are occupied by chlorophyll *b*. The probability with which chlorophyll *b* is incorporated into these complexes may depend on the size of the pool of available chlorophyll *b* relative to that of chlorophyll *a*. This apparent lack of specificity of chlorophyll binding supports the notion that the chlorophyll complement of photosynthetic organisms depends on which enzymes for chlorophyll synthesis happen to be present (27).

In further support of this argument, in the oxygenic prokaryote *Acaryochloris marina*, chlorophyll *d* is the major pigment of the photosystems, whereas chlorophyll *a* is a minor component (28, 29). Unless this organism has adapted its photosystems to be able to bind and use a different chlorophyll at essentially all positions, the simplest explanation is that there is little specificity for exactly which chlorophyll is bound. A similar situation may occur in prochlorophytes, which have chlorophyll *a*/*b*-binding proteins that are closely related to the iron-stress-induced protein (Isi A) of cyanobacteria (30) and PS II core antenna proteins CP43 and CP47. The latter two bind only chlorophyll *a* in plants and cyanobacteria. Indeed, on *in vitro* reconstitution of LHCII with different ratios of chlorophyll *a* and *b*, one can obtain a situation where LHCII has bound much more chlorophyll *b* than *a* (31). Moreover, LHC from the red alga *Porphyridium cruentum*, which normally contains only chlorophyll *a*, under *in vitro* conditions can functionally bind chlorophylls *b* and *c* as well (32). Therefore, the pigment composition of an organism is not a reliable criterion for determining evolutionary relationships. Indeed, phylogenetically the chlorophyll *b*-containing prochlorophytes appear to be interspersed among cyanobacteria that lack this pigment (33).

Pigment analysis of isolated PS II complexes (Fig. 3) and fluorescence emission data (Figs. 4 and 7) demonstrate that chlorophyll *b* replaces part of chlorophyll *a* in the PS II core. Moreover, the energy absorbed by chlorophyll *b* can be used efficiently by the reaction centers and can cause Q_A reduction (Fig. 6). Because of the high amount of chlorophyll *b* in the *cao*$^+$/*lhcb*$^+$ cells and the limited specificity of the chlorophyll-binding sites, it is likely that chlorophyll *b* occupies chlorophyll *a*-binding sites even in the PS II reaction center itself and not only in the CP43 and CP47 core antenna proteins. Moreover, pheophytin *b*, which was detected in the pigment extracts from chlorophyll *b*-containing cells (Fig. 1), may also replace native pheophytin *a* in the PS II reaction centers.

We thank Dr. Judy Brusslan (California State University, Long Beach) for her generous gift of the *A. thaliana* expressed sequence tag cDNA clone containing the *cao* gene. We also thank Dr. Daniel Brune (Arizona State University) for his help in protein microsequencing. This work was funded by a grant from the Department of Energy (DE-FG03–95ER20180).

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