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Chlorophyll forms and excitation energy transfer pathways in light-harvesting chlorophyll *a* / *b*-protein complexes isolated from the siphonous green alga, *Bryopsis maxima*

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In this study, examination was made of chlorophyll (Chl) forms and energy transfer pathways in light-harvesting Chl *a* / *b*-protein complex (LHC II) isolated from the siphonous green alga, *Bryopsis maxima*. Three major Chl *a* forms (Ca664, Ca672 and Ca679) and one minor form (Ca688) were resolved at 15°C. Two Chl *b* forms were resolved at 648 and 653 nm. Based on the number of Chl bound to an apoprotein, two Chls *a* were assigned to each of the three major Chl *a* forms, and three and five Chls *b*, to Cb648 and Cb653, respectively. At 15°C, fluorescence spectra were identical, irrespective of the excitation conditions of Chl *a*, Chl *b* and siphonaxanthin. Fluorescence from Chl *b* was detected in addition to that from all Chl *a* forms. Very efficient energy transfer from siphonaxanthin or Chl *b* to Chl *a* and even uphill transfer from Chl *a* to Chl *b*, were noted by measurement of the excitation spectra. At 15°C, the equilibrium of energy distribution was established among pigments. However, Chl *b* was found not to mediate energy transfer from siphonaxanthin to Chl *a*. The partial amino acid sequence of *Bryopsis* LHC II was similar to those of green algae and higher plants. The energy transfer pathway between pigments and molecular organization of *Bryopsis* LHC II were compared with LHC II isolated from spinach.

Introduction

Light-harvesting pigments in photosynthesis in higher plants and green algae are Chl *a*, Chl *b* and carotenoids [1] and are confined to several types of pigment-protein complex. Among them, the light-harvesting Chl *a* / *b*-protein complex of Photosystem II (LHC II) is the most abundant [2]. In higher plants, pigment composition is 8 Chl *a*, 7 Chl *b* and several (in a range of 3.5 to 5.4) molecules of carotenoid per unit peptide [1,3]. The three-dimensional structure of pea LHC II was analyzed by electron diffraction on two-dimensional crystals [4]. LHC II has a C_3-symmetry (trimeric) structure. A polypeptide has three membrane-spanning α-helix regions, which are highly conserved in many organisms [5]. Chl molecules are localized in two layers parallel to the membrane surface. 8 Chl molecules are present on the stroma side and 7, on the lumen side, even though actual molecular species have get to be distinguished. Such an arrangement of Chl molecules is considered favorable for the absorption and transmission of light energy.

There are a few significant differences in the pigment composition of LHC II in marine green algae [6]; they contain more Chl *b* relative to Chl *a* than higher plants [7]. Siphonous green algae contain unique xanthophylls, siphonaxanthin and siphonein in place of lutein in higher plants. These carotenoids show prominent absorption around 500 to 540 nm and are essential to the efficient capture of green light for photosynthesis in deep-water type green algae [8]. These xanthophylls in LHC II exhibit light-induced absorbance shift in response to an electric field generated across thylakoid membranes when irradiated [9].

We purified LHC II from a siphonous green alga, *Bryopsis maxima* without significant loss of pigments [10]. The fraction of LHC II was obtained by DEAE-Toyopearl column-chromatography. The fraction, P5, with apparent molecular mass of 295 kDa, yield a single Chl-protein band at 72 kDa when subjected to mild SDS-PAGE. It consists of one 28 kDa and two 32

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Abbreviations: Chl, chlorophyll; LHC II, light-harvesting chlorophyll *a* / *b*-protein complex of Photosystem II.

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kDa polypeptides [10], like 27 and 28 kDa polypeptides in spinach LHC II [11]. The pigment composition of P5 is 6 Chl *a*, 8 Chl *b*, 3 siphonaxanthin and one each of siphonein and neoxanthin per one polypeptide. The total number of pigments and amino acid compositions in this LHC II were essentially the same as those in LHC II from higher plants [10]. In siphonous green algae, minor Chl *a*/*b*-protein complexes, corresponding to CP29, CP 26 and CP24 reported in other plants [2], were not detected [12]. Instead, one minor Chl *a*/*b*-protein complex (CP35), consisted of 35 kDa polypeptide was observed in P2 fraction isolated from *Bryopsis* by DEAE-Toyopearl column chromatography [10].

Since the total number of Chl per unit peptide of *Bryopsis* LHC II is the same as that in higher plants and its amino acid composition is also similar to those of higher plants, it is reasonably assumed that the basic structure of LHC II is conserved in *Bryopsis*. On the other hand, the difference in pigment composition will give rise to the discrimination of conserved and modified optical properties of LHC II. Since there are no comparable preparations of LHC II, *Bryopsis* LHC II can be regarded as a modification, or perturbation to the system, through which we can deduce the characterization of LHC II. Thus, a partial amino acid sequence, composition of Chl forms and energy transfer processes in *Bryopsis* LHC II were examined and compared with those in spinach LHC II.

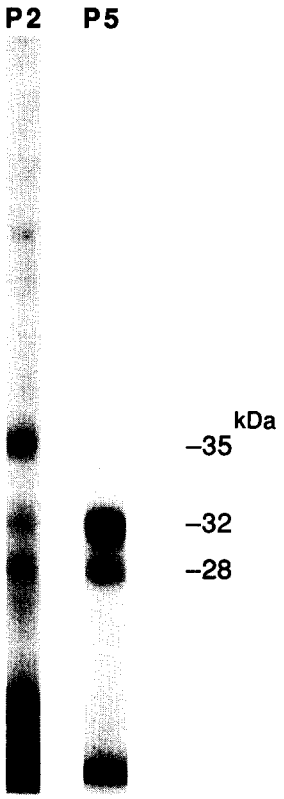

Fig. 1. Polypeptide compositions of two fractions, P2 and P5 (LHC II), separated from LHC II enriched fraction by DEAE-Toyopearl column chromatography. Fractions were treated with 2% SDS. LHC II is free from CP35 observed in P2 fraction.

Materials and Methods

Isolation of LHC II. LHC II of *B. maxima* was purified as described previously [10]. Thylakoid membranes were washed with 1 mM EDTA (pH 8.0) and treated with 0.8% digitonin (a weight ratio of detergent to Chl of 20) in a medium containing 50 mM Tris-HCl (pH 7.5), 2 mM PMSF and 40 mM ϵ-aminocaproic acid at 4°C for 8 h. LHC II was purified by DEAE-Toyopearl column chromatography (2.2 × 15 cm) and gel-permeation chromatography on a column of Superose 6 prep grade (1.6 × 50 cm). The purified main LHC II fraction (P5 fraction in Fig. 1) was concentrated by dialysis in a cellulose tube against poly(ethylene glycol) 20000. Thylakoid membranes of spinach were prepared in the above medium and treated with 0.8% digitonin (a ratio of detergent to Chl of 15) in the same medium at 4°C for 8 h. After centrifugation at 30000 × *g* for 1 h, the supernatant was applied onto a DEAE-Toyopearl column (2.2 × 20 cm) equilibrated with 20 mM Tris-HCl (pH 7.5), 20 mM NaCl and 0.15% digitonin. LHC II was eluted with a linear gradient of NaCl from 20 to 125 mM. The purified LHC II showed the spectral properties same as those reported before [11].

SDS-PAGE was carried out by the method of Laemmli (1970) [13]. For analysis of polypeptides, samples were treated with 2% SDS at 20°C for 30 min and run on a 12% polyacrylamide gel that contained 4 M urea and 0.1% SDS. Gels were stained with silver by the method of Morrissey [14].

Spectroscopic analysis. Absorption spectra were measured with a Hitachi 330 spectrophotometer. For measurement at −196°C, the samples were suspended in 50% potassium glycerophosphate and 25% glycerol. Fluorescence emission and excitation spectra and polarized fluorescence spectrum were measured with a Hitachi 850 spectrofluorometer equipped with glass plate polarizers. The band widths of excitation and emission light were 2 nm. For measurement at −196°C, the samples were each mixed with an equal volume of 30% poly(ethylene glycol) 4000. Absorption and fluorescence spectra were stored in a computer at 0.2 nm intervals, subjected to smoothing and drawing of the second derivative spectra according to the method of Savitzky and Golay [15] and resolved into their components by the least-square procedure described by Mimuro et al. [16]. The spectral sensitivity of fluorometer was numerically corrected using a microcomputer [16].

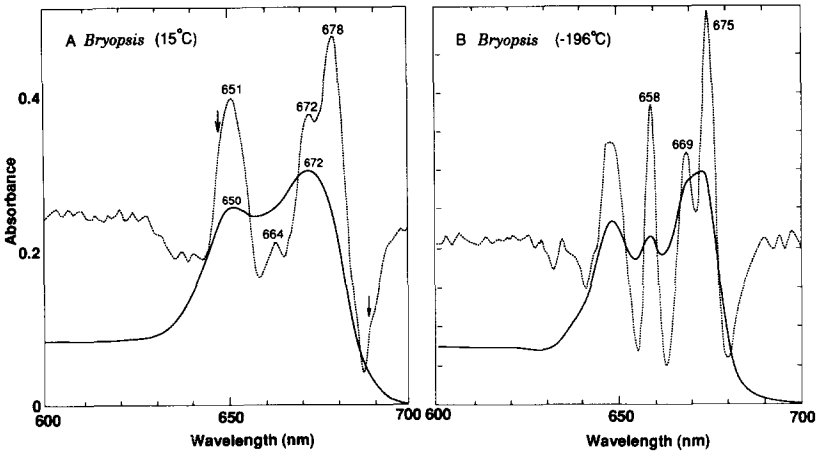

Fig. 2. Absorption and second derivative spectra of LHC II from *B. maxima* at 15°C (A) and −196°C (B). Solid lines, absorption spectra; Dotted lines, second derivatives of the spectra, following inversion.

Results

Chlorophyll forms in Bryopsis LHC II

The absorption spectrum of *Bryopsis* LHC II at 15°C showed a broad band with two maxima at 650 and 672 nm. The second derivative spectrum indicated the presence of four maxima at 651, 664, 672 and 678 nm and shoulders at 645 and 688 nm as indicated by arrows (Fig. 2A). These components were quite clearly resolved at −196°C; the component at 658 nm was observed as a peak in the absorption spectrum (Fig. 2B). The second derivative of the low temperature spectrum indicated an absorption maximum of Chl *b* at 646 nm by a plateau. The maxima shifted by 1 to 6 nm to a shorter wavelength at −196°C.

Chl forms were resolved into components assuming Gaussian band shape; a component was shown present by the second derivative spectrum. The absorption spectrum at 15°C was resolved into four Chl *a* forms (Ca664, Ca672, Ca679 and Ca688) and two Chl *b* forms (Cb648 and Cb653) (Fig. 3A). The peak positions of Chl *a* forms differed only by less than 1 nm from the maxima as estimated by the second derivative spectrum. Relative peak areas of the resolved components, estimated by integration of resolved bands in wavenumber unit, were 0.36, 0.28, 0.29 and 0.07 for Ca664, Ca672, Ca679 and Ca688, respectively, and 0.36 and 0.64 for Cb648 and Cb653, respectively.

At −196°C, four Chl *a* forms were resolved into those with maxima at 659, 668, 675 and 682 nm and

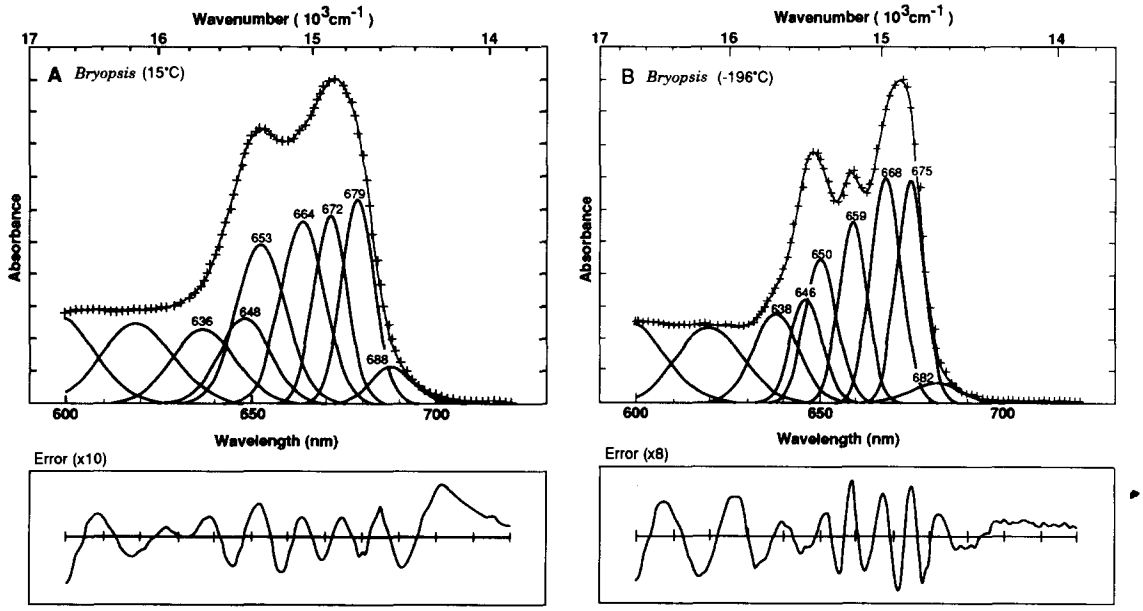

Fig. 3. Deconvolution patterns of absorption spectra of LHC II from *B. maxima* at 15°C (A) and −196°C (B). Plus signs indicate real data points. Thick solid lines show deconvolution curves.

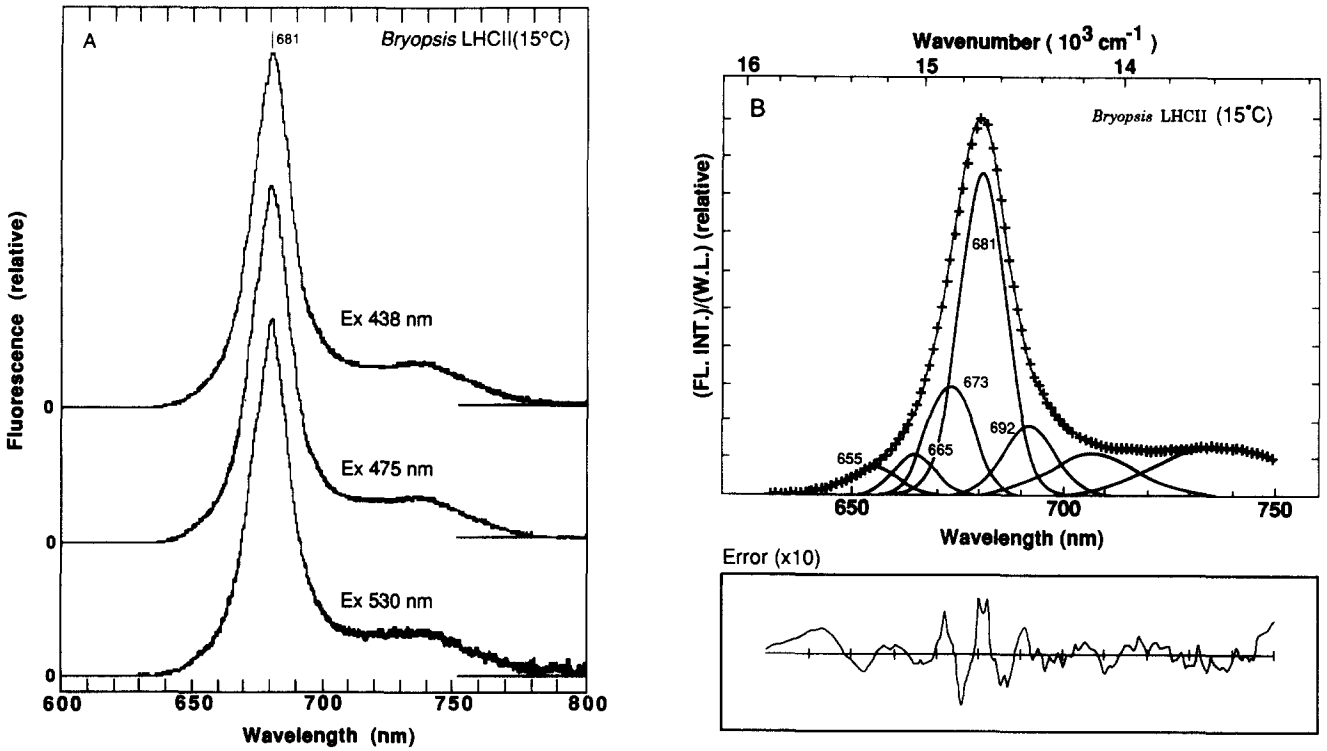

Fig. 4. Fluorescence emission spectra of LHC II from *B. maxima* at 15°C. (A) Excitation wavelengths were 438, 475 and 530 nm. (B) Deconvolution of the fluorescence emission spectrum at 15°C. Excitation wavelength was 475 nm. Plus signs indicate real data points. Thick solid lines show deconvolution curves.

two Chl *b* forms, with maxima at 646 and 650 nm (Fig. 3B). The stoichiometry of individual components was the same as at 15°C. The relative amount of Ca688 became smaller. The 638 nm component at −196°C was not assigned to Chl *b* [17] but to a vibrational structure of a certain band(s) of Chl *a* [18]. The occurrence of the two Chl *b* forms is known in spinach LHC II at 644–645 and 652 nm [19,20]. These two bands were clearly observed in the absorption spectrum of *Bryopsis* LHC II, at almost the same sites.

Fluorescence spectra

Fig. 4A shows fluorescence emission spectra of LHC II at 15°C sensitized by Chl *a* (438 nm), Chl *b* (475 nm) or siphonaxanthin (530 nm). Irrespective of the pigments excited, in each case, the spectra showed a single major emission at 681 nm with a small but significant shoulder or tailing around 655 nm. Gaussian deconvolution of the fluorescence spectrum, based on the second derivative spectrum, gave rise to five emission components, at 655, 665, 673, 681 and 692 nm (Fig. 4B). The number and locations of the emission components match those of Chl *a* forms; the major 681 nm fluorescence band was ascribed to emission from Ca679, and minor components at 665, 673 and 692 nm were from Ca664, Ca672 and Ca688, respectively. A small but significant emission band was noted at 655 nm and ascribed to Chl *b* (most likely Cb653).

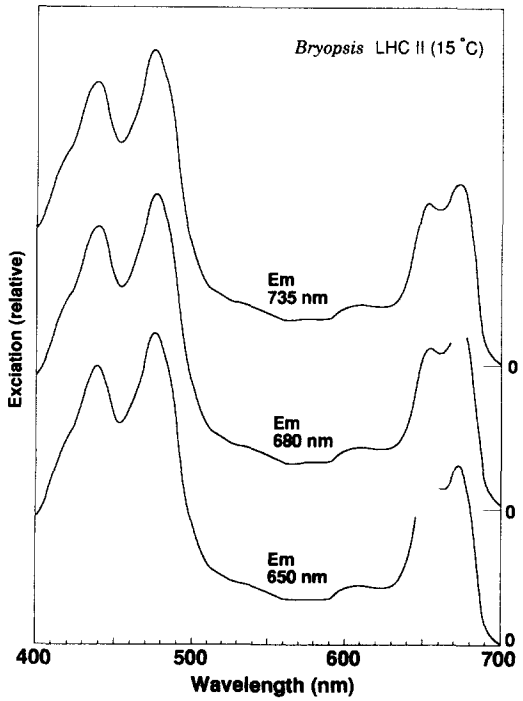

Fig. 5. Excitation spectra of LHC II at 15°C monitored at 735, 680 and 650 nm, respectively. Spectra were normalized at their maxima. Gaps appeared due to the scattering of excitation light.

The excitation spectra of Chl *a* fluorescence were monitored at 680 and 735 nm at 15°C (Fig. 5). The two spectra were almost the same and quite similar to the

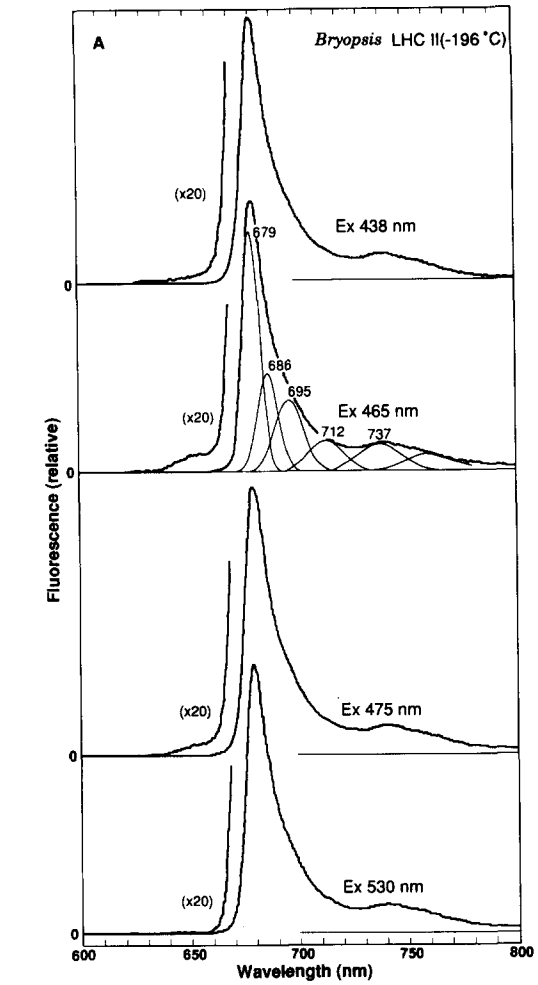

Fig. 6. Fluorescence emission and excitation spectra of *Bryopsis* LHC II at −196°C. (A) Emission spectra excited at 438, 465, 475 and 530 nm, respectively. Component bands resolved are also shown for the spectrum excited at 645 nm. (B) Excitation spectra monitored at 645 and 655 nm, respectively.

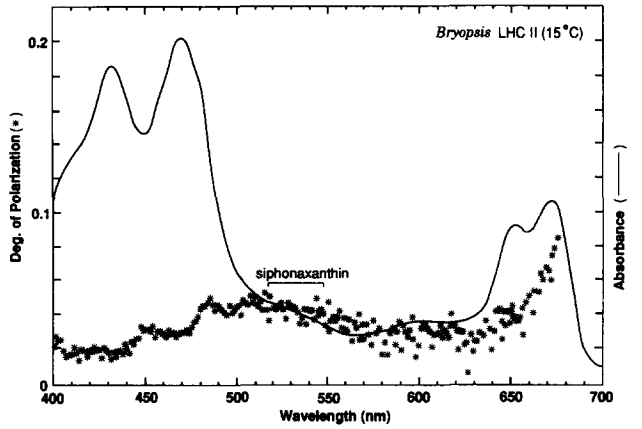

Fig. 7. Degree of polarization of Chl *a* fluorescence as a function of excitation wavelength. Fluorescence was monitored at 680 nm. Degrees of polarization and absorption spectrum are indicated by asterisks and the full line, respectively.

absorption spectrum (see Fig. 7), thus showing the efficiency of energy transfer from Chl *b* and siphonaxanthin to Chl *a* to be very high. The excitation spectrum for emission from Chl *b* at 650 nm was also determined and found quite similar to those for the Chl *a* emission. It is significant that the excitation spectrum of Chl *b* fluorescence showed a peak at 670–680 nm, indicating light energy absorbed by the red band of Chl *a* to be transferred to Chl *b*, that is, an uphill energy transfer with high efficiency. At 15°C, equilibration of the energy distribution among antenna components was established.

The fluorescence emission spectra at −196°C differed from those at 15°C (Fig. 6A). Irrespective of the pigments excited, the main emission at 681 nm was prominent. A clear shoulder was observed around 690 nm. Tailing or a shoulder at the shorter wavelength side of the main peak was much less significant at low temperature. The fluorescence spectrum excited at 465 nm was resolved into six emission components, at 679, 686, 695, 712, 737 and 760 nm. Fluorescence bands at 679 and 686 nm were ascribed to emission from Ca679 and Ca688 (675 and 682 nm at −196°C), respectively. A Chl form corresponding to emission at 695 nm could still not at this time be identified in the absorption spectrum.

When the scale of the ordinate was expanded 20-times, an emission from Chl *b* was detected around 650 nm. Emission around 650 nm was ascribed to two Chl *b* forms. The magnitude of the 650 nm band varied depending upon the pigment excited. When the Soret band of Chl *b* was preferentially excited (465 or 475 nm), there was a notable emission at 650 nm. The intensity of emission was much less with excitation of the Soret band of Chl *a* at 438 nm. A weak emission was ascribed to a small absorption fraction of Chl *b* at this wavelength. It may thus be concluded that excita-

tion energy transfer from Chl *a* to Chl *b* is negligible or inefficient at −196°C. The 530-nm excitation did not sensitize any emission from Chl *b*, indicating light energy absorbed by siphonaxanthin and/or siphonein not to be transferred to Chl *b*. Excitation spectra for emission at 645 and 655 nm showed the spectrum for the 655 nm emission to slightly but significantly red-shift compared with that for 645 nm emission (Fig. 6B). This is evidence for independent fluorescence emission from the two Chl *b* forms (Cb648 and Cb653). No distinct band could be seen in the 530 nm region in the two excitation spectra, thus showing siphonaxanthin and siphonein not to transfer excitation energy to Chl *b*. Energy transfer from Chl *a* to Chl *b* was substantially prohibited at −196°C, as evident from the diminished blue bands of Chl *a* at 430 nm in the excitation spectrum.

Fluorescence polarization spectrum

Fig. 7 shows excitation wavelength dependence of fluorescence polarization monitored at 680 nm at 15°C. The magnitude of decrease in fluorescence polarization was indication of the number of energy transfer steps prior to the fluorescing component [21]. The degree of polarization was highest in the wavelength region of 510 to 530 nm, where light was mainly absorbed by siphonaxanthin and siphonein, except for the red band of Chl *a*, fluorescing component. Lesser polarization was noted when Chl *b* was excited at about 650 nm. This is consistent with the notion that siphonaxanthin and siphonein transfer their excitation energy directly, not through Chl *b*, to Chl *a*.

Fluorescence emission spectra of LHC II from spinach

Emission from Chl *b* in LHC II of *B. maxima* is an exceptional case. This fluorescence was compared with that in LHC II of spinach in which no emission from Chl *b* has been detected [19]. The fluorescence emission spectra of spinach LHC II showed the main band at 681 nm at −196°C (Fig. 8). No emission band could be seen in the 650 nm region even when excited at 465 or 475 nm mainly absorbed by Chl *b*. Emission from the Chl *a* form whose maximum was less than Ca679 was insignificant, as also noted for *Bryopsis* LHC II. When excited at 510 nm, mainly absorbed by lutein [22], fluorescence from Chl *a* was observed.

Amino acid sequence of the 32 kDa polypeptide

The N-terminal amino acid sequence of the 32 kDa polypeptide of *Bryopsis* LHC II was determined up to the 38th residue (Fig. 9), a region which is considered to extrude to the stroma [23]. The sequence was very homologous to those of LHC II apoproteins from *Chlamydomonas reinhardtii* [24] and *Dunaliella tertiolecta* [25]. High homology was also observed between *B. maxima* and higher plant proteins, particularly in the region from the 22nd to 38th residues.

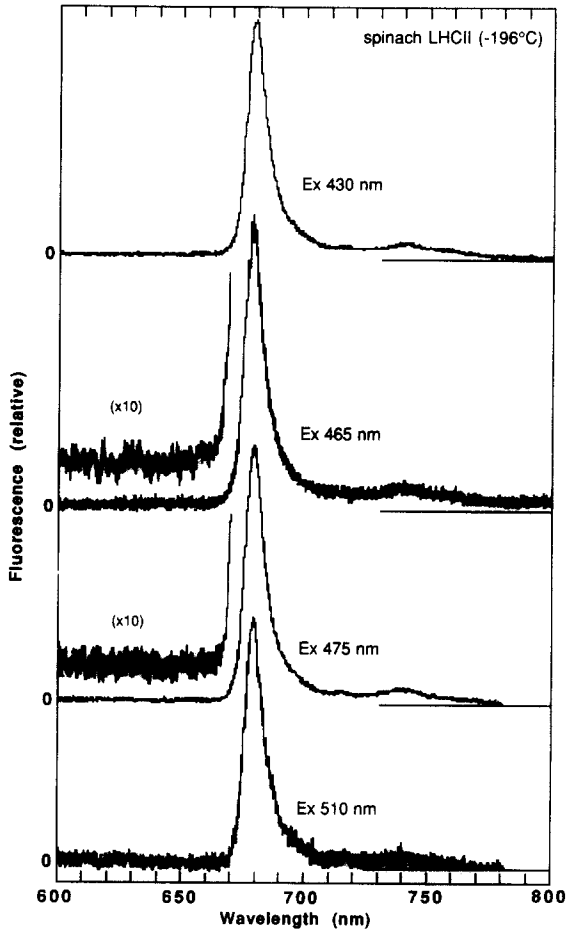

Fig. 8. Fluorescence emission spectra of LHC II from spinach at −196°C. Emission spectra excited at 430, 465, 475 and 510 nm, respectively.

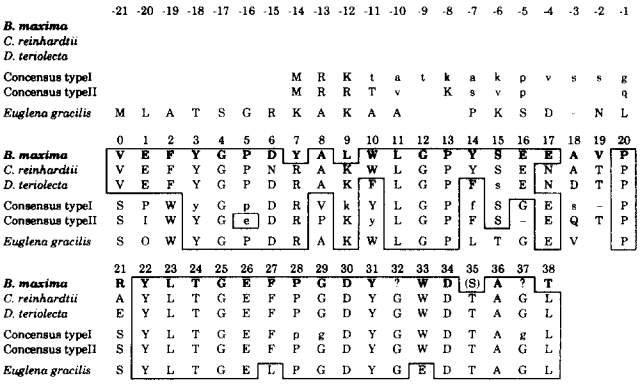

Fig. 9. N-terminal amino acid sequence of the 32 kDa polypeptide from *Bryopsis* LHC II. For comparison, amino acid sequences of LHC II apoproteins from *Dunaliella tertiolecta* [25], *Chlamydomonas reinhardtii* [24], *Euglena gracilis* [32] and consensus sequence of two types of LHC II from higher plants LHC II, deduced from the sequences of all published cab genes 5, are shown. Upper and lower case letters in consensus sequences indicate conserved and most frequent amino acid residues, respectively. Each dash represents a gap introduced to maximize homology.

Discussion

Polypeptide and pigment composition

Total numbers of Chl and xanthophyll molecules per polypeptide in *Bryopsis* LHC II were 14 and 5, respectively, these being virtually the same as those in LHC II of higher plants (14 and 3.5 to 5.4) [1,10]. Amino acid composition [10] and amino acid sequence in the N-terminus region of *Bryopsis* LHC II (Fig. 9) were much the same as those of higher plants [25]. The arrangement of Chls and xanthophylls in apoproteins would thus appear to be conserved from green algae to higher plants.

Chl forms and stoichiometry of pigments

Deconvolution of the absorption spectra of *Bryopsis* LHC II resolved four Chl *a* forms (Ca664, Ca672, Ca679 and Ca688) and two Chl *b* forms (Cb648 and Cb653). Based on pigment content and relative areas, the number of Chl molecules was estimated with reasonable accuracy. Two Chl *a* were assigned to each of Ca664, Ca672 and Ca679. Ca688 was estimated as less than one per unit polypeptide and thus assigned to a specific form appearing in trimeric form. For Chl *b*, three molecules were assigned to Cb648, and five to Cb653. Chl *a* forms resolved into *Bryopsis* LHC II resembled those of spinach LHC II (Ca660, Ca668 and Ca678) [26] in which Ca672 (or Ca668) is more abundant than in *Bryopsis* LHC II [17]. Different Chl *a* forms were ascribed to different molecular environments surrounding Chl *a* in an apoprotein. Thus, their being similar Chl *a* forms indicates the molecular organization of LHC II to be similar between *Bryopsis* and higher plants, such as spinach.

The Chl *b* form has not been definitely determined; only one form has been reported for LHC II from higher plants, *Dunaliella* and *Euglena* [27]. However, two Chl *b* components may possibly exist according to deconvolution of the absorption and circular dichroism spectra of spinach LHC II [19,20]. The two forms of Chl *b* could be more clearly resolved into *Bryopsis* LHC II. The larger amount of Chl *b* in this alga may be one reason for this.

Fluorescence properties and energy transfer in LHC II

A remarkable feature of fluorescence properties is the equilibrium of energy distribution among pigments at 15°C. Once energy is transferred to Chl *a* pools, it migrates within all components with thermal energy (ca. 200 cm^{-1}) (Fig. 10), thus clearly demonstrating the proximity of pigments to each other in LHC II.

At −196°C, equilibrium was not observed; the energy difference between pigments was larger than thermal energy at this temperature (ca. 50 cm^{-1}). The main emission came from Ca679, even though Ca688, with a maximum at longer wavelength than that of

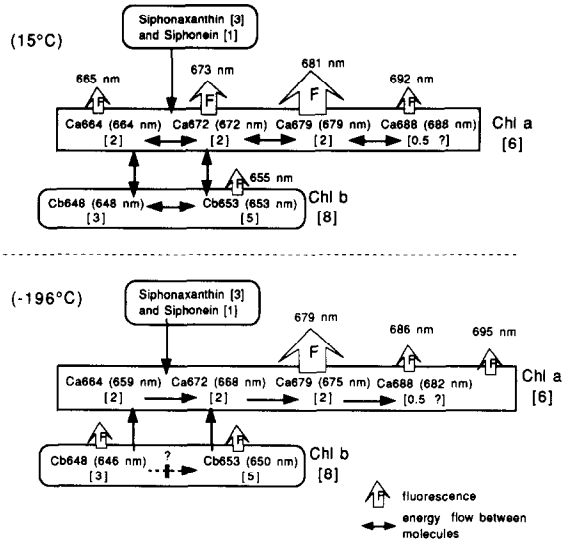

Fig. 10. Schematic presentation of Chl forms and energy flow in a unit polypeptide of *Bryopsis* LHC II at 15 and −196°C. Absorption maximum of each Chl form is shown in parentheses. Numbers in brackets indicate molecular numbers of carotenoids and Chl forms per polypeptides.

Ca679 was present (Fig. 10). If Ca688 is the energy sink of LHC II at −196°C, the main emission should originate therefrom. However, this was not the case, even when a weak emission from Ca688 was observed at −196°C (Fig. 6A). The energy difference between Ca679 and Ca688 was about 150 cm^{-1} and relative amount of Ca688 about 10 % of Ca679. Thus, more than 50 % of emission should come from Ca688 when both are equilibrated, but actual intensity was actually less than 30 %. The relatively weak emission from Ca688 thus cannot be explained by equilibrium and at present, there is no adequate explanation. This phenomenon has also been observed in spinach LHC II (Fig. 8), suggesting a general property of Chl forms in LHC II.

Another feature was fluorescence from two forms of Chl *b* (Fig. 6). Since Chl *b* fluorescence has rarely been found in LHC II from higher plants [28], this feature should be due to changes in pigment organization as well as a high Chl *b* content in *Bryopsis* LHC II. In spinach LHC II, no fluorescence from Chl *b* was observed at −196°C, even when Chl *b* was excited at 465 or 475 nm (Fig. 8). The molecular arrangement of pigments in LHC II of higher plants was essentially maintained at low temperature. The roles of individual molecules in energy transfer sequences in LHC II may be essentially the same irrespective of temperature and this would be applicable to the system in green algal LHC II. A decrease in the number of Chl *a* molecules of longer wavelength form in isolated *Bryopsis* LHC II may cause the fluorescence emission of shorter wavelength Chl *a* and Chl *b*.

Emission and excitation spectra showed light energy absorbed by siphonaxanthin, siphonein and Chl *b* to be transferred to Chl *a* with very high efficiency. Chl *b* does not mediate energy transfer from carotenoids to Chl *a*, contrary a long-held postulation. This is confirmed by fluorescence spectra sensitized by siphonaxanthin (Fig. 6A), excitation spectrum monitored by Chl *b* emission (Fig. 6B) and the fluorescence polarization spectrum (Fig. 7).

Siphonaxanthin transferred excitation energy directly to Chl *a* and indirectly to Chl *b* at 15°C. Fluorescence around 650 nm from Cb653 excited at 530 nm indicates the equilibration of excitation energy among the Chl *a* and Chl *b* molecules at 15°C (Fig. 5). Xanthophylls did not transfer excitation energy to either Chl *b* form at −196°C (Fig. 6B), as also noted for spinach (Fig. 8). Excitation energy transfer from xanthophylls to Chl *a* requires elaborate proximity between the acceptor and donor [29]. In this study, we could not determine the actual number of Chl *a* coupled with xanthophylls, as was done for brown alga [30]. The exact number of couples is not known at present. However, Ca672 appears to couple with xanthophylls, since rapid energy transfer from xanthophylls to this Chl forms was observed by the time-resolved fluorescence spectra (unpublished) (cf. Fig. 10).

Higher order structure of LHC II in B. maxima

Similarity in spectral properties, amino acid composition and partial sequence of N-terminus suggest the three-dimensional structure of *Bryopsis* LHC II to be quite similar to that of pea LHC II, resolved by a two-dimensional crystal structure [4]. Siphonaxanthin takes on a specific configuration; i.e., the s-*trans* configuration of a keto carbonyl group in relation to the conjugated double bond [31]. Siphonaxanthin may thus occupy a larger area in an apoprotein than lutein, thus possibly affecting the folding of the apoprotein. This matter will be studied in relation to evolution and habitats of these plants.

Acknowledgments

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