Light-harvesting chlorophyll *a*/*b*-protein complexes of photosystem II from the green alga, *Bryopsis maxima*. Pigment composition and excitation energy transfer

緑藻オオハネモの系II集光性クロロフィル *a*/*b* 蛋白質複合体の色素組成と励起エネルギー移動

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Light-harvesting chlorophyll *a/b*-protein complexes of photosystem II from the green alga, *Bryopsis maxima*. Pigment composition and excitation energy transfer

by

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Abbreviations

Chl, chlorophyll
CP24, a minor antenna chlorophyll *a*/*b* protein complex of PS II containing an apoprotein of 21 kDa
CP26, a minor antenna chlorophyll *a*/*b* protein complex of PS II containing an apoproteins of 28.8 and 29 kDa
CP29, a minor antenna chlorophyll *a*/*b* protein complex of PS II containing an apoprotein of 29-31 kDa
CP I, chlorophyll protein complex of reaction center I
CPa, chlorophyll protein complex of reaction center II
DCIP, 2,6-dichlorophenolindophenol
DEAE, diethylaminoethyl
DNA, deoxyribonucleic acid
DPC, diphenylcarbazide
EDTA, ethylenediaminetetracetic acid
FCPA, fucoxanthin-Chl *a*/*c* protein assembly
FP, free pigments
LHC, light-harvesting Chl *a*/*b*-protein complexes including LHC I and LHC II
LHC I, light-harvesting Chl *a*/*b*-protein complex of PS I
LHC II, major light-harvesting Chl *a*/*b*-protein complex of PS II
LHC II-1, a form of LHC II with slowest electrophoretic mobility
LHC II-2, a form of LHC II with intermediate electrophoretic mobility
LHC II-3, a form of LHC II with fastest electrophoretic mobility
LHC II', minor antenna chlorophyll *a*/*b* protein complex of PS II containing an apoprotein of 35 kDa
HPLC, high performance liquid chromatography.
Nd-YAG, neodymium-yttrium aluminium garnet
PAGE, polyacrylamide gel electrophoresis
PMSF, phenylmethyl sulfonyl fluoride
P.I., polarity index
PS, photosystem
PS I, photosystem I
PS II, photosystem II
SDS, sodium dodecylsulfate
Tris, tris(hydroxymethyl)aminomethane

Contents

Chapter I.

General introduction

Chlorophylls and carotenoids, which are collectively called photosynthetic pigments, function to absorb solar energy and transfer it to the reaction centers (Thornber 1986). Photosynthetic pigments are present in several distinct Chl-protein complexes, which are functionally divided into two groups, the reaction center complexes and light-harvesting or antenna Chl-protein complexes. In higher plants and green algae, the reaction center complex of photosystem II is associated with at least four Chl-protein complexes, *i. e.* the major light-harvesting Chl *a* / *b*-protein complex called LHC II and three minor antenna complexes, CP29 (Camm and Green 1980), CP26 (Bassi et al. 1987, Dunahay et al. 1987) and CP24 (Dunahay and Staehelin 1986). The reaction center complex of photosystem I (P700-Chl *a* protein complex) is attached with antenna Chl *a* / *b*-protein called LHC I (Ish-Shalom and Ohad 1983, Dunahay and Staehelin 1985) (Fig. I–1).

LHC II is the most abundant light-harvesting Chl-protein complex present in the thylakoid membranes. This Chl-protein accounts for approximately one third of the total membrane proteins and binds nearly half of the total Chl and most of Chl *b* in higher plants (Thornber 1975). LHC II is mostly present in the stacked region of the grana thylakoids and regulates the distribution of excitation energy between the two photosystems by reversibly moving to stroma thylakoid membranes (Haworth et al. 1982).

LHC II was first isolated as a single green band by SDS-PAGE and named CP II (Ogawa et al. 1966, Thornber and Highkin 1974).

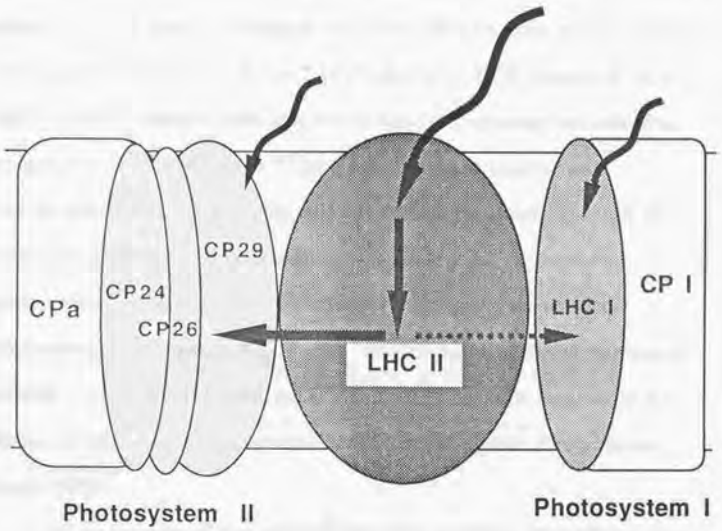


Fig. I-1 Model of the supermolecular organization of reaction centers and light-harvesting complexes in thylakoid membrane. CP1, reaction center of PS I; LHC I, light-harvesting Chl *a*/*b*-protein complex of PS I, CPa, reaction center of PS II; LHC II, major light-harvesting Chl *a*/*b*-protein complex of PS II. CP 29, CP26 and CP24, minor light-harvesting Chl *a*/*b* -protein complex of PS II.

Subsequently, the Chl *a*/*b*-protein has been isolated from higher plants and algae with various nonionic detergent such as digitonin, octyl β-D-glucopyranoside or Triton X-100, and extensively characterized in terms of polypeptide and pigment compositions. The Chl *a*/*b* ratio of LHC II from higher plants ranges from 1.1 to 1.47 (Burke et al. 1978, Hemelrijk et al. 1992). LHC II isolated from pea with *n*-octyl β-D-glucopyranoside was estimated to bind 8 Chl *a* and 7 Chl *b* per apoprotein (Butler and Kühlbrandt 1988). In addition, LHC II carries xanthophylls, such as lutein, violaxanthin and neoxanthin, at a molar ratio of about one carotenoid to five Chl (Ryrie et al. 1980, Siefermann-Harms and Ninnemann 1982, Eskins et al. 1983). However, the accurate number of individual carotenoids bound to LHC II is still unknown because of the release of pigments during preparation with detergents (Siefermann-Harms 1985).

LHC II consists of two apoproteins with apparent molecular masses of 24 to 27 kDa, which are encoded by a multi-gene family (*cab* genes) in the nucleus DNA (Thornber et al. 1991). The deduced amino acid sequences of the LHC II apoproteins from different higher plant species are highly homologous to each other (Jansson and Gustafsson 1990). Recently, two-dimensional crystals of pea LHC II were analyzed by electron diffraction, electron microscopy and image processing (Kühlbrandt and Wang 1991). The three-dimensional structure deduced shows that LHC II is a trimer composed of three subunit apoproteins, each of which has three membrane-spanning α-helices and binds 15 Chl

molecules. The porphyrin rings of the 15 Chl were distributed in the distances from 9 to 14 Å between nearest neighbors. These pigments are assumed to be noncovalently coordinated to histidine, glutamine or asparagine residues of the apoprotein (LaRoche et al. 1990).

Improved SDS-PAGE resolves three bands of LHC II with apparent molecular masses of 68-72, 48 and 26-27 kDa (Anderson 1980, Thornber 1986). They are considered to be two oligomer and one monomer forms of LHC II.

The absorption spectrum of spinach LHC II shows two maxima at 652 and 673 nm, which correspond to the red bands of Chl *b* and Chl *a*, respectively, at room temperature (Satoh 1979). Deconvolution of the absorption spectrum of pea LHC II resolved three different Chl *a* forms (Ca660, Ca670 and Ca678) and a single Chl *b* form (Cb649) at -184°C (Brown and Schoch 1981). However, the *in situ* state of Chl *b* appears heterogeneous because two components of Chl *b* (Cb640 and Cb648) were detected in circular dichroic and linear dichroic spectra at -196°C (van Metter 1977, Hemelrijk et al. 1992).

Fluorescence emission spectrum of spinach LHC II shows a single band of Chl *a* at 681 nm (Satoh 1979). Fluorescence emission from Chl *b* has never been observed in LHC II from higher plants, indicating a very high efficiency of energy transfer from Chl *b* to Chl *a* (van Metter 1977). The efficiency of energy transfer from carotenoids to Chl *a* has also been determined to be 100% in *Lactuca sativa* LHC II (Siefermann-Harms and Ninnemann 1982).

Picosecond time-resolved spectroscopy showed that the rate of energy

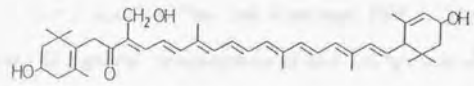
transfer from Chl *b* to Chl *a* was 6 ± 4 ps in isolated spinach LHC II (Gillbro et al. 1985). During the lifetime of excited Chl *b*, no energy transfer to differently oriented Chl *b* occurred. A faster energy transfer (0.5 ± 0.2 ps) from Chl *b* to Chl *a* was reported in thylakoid membranes of a mutant of *Chlamydomonas reinhardtii*, which contained LHCs but no reaction center complexes (Eads et al. 1989).

Three minor antenna complexes of photosystem II have been reported in higher plants. CP29 has a Chl *a*/*b* ratio of 1.8-4.0 (Henrysson et al. 1989) or no Chl *b* (Ghanotakis 1987), and consists of a single apoprotein of 29-31 kDa (Machold and Meister 1980, Camm and Green 1989). CP26 has two apoproteins of 28.5 and 29 kDa and shows a Chl *a*/*b* ratio of 1.8- 2.0 (Bassi et al. 1987, Dunahay et al. 1987). CP24 which consists of an apoprotein of 21 kDa has a Chl *a*/*b* ratio of 0.8-1.0 (Dunahay and Staehelin 1986). These minor components are considered to mediate energy transfer from LHC II to the reaction center complex of photosystem II.

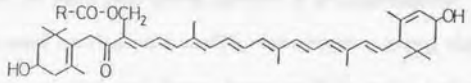
In contrast to higher plants which invariably show Chl *a*/*b* ratios of about 3.0, the abundance of Chl *b* relative to Chl *a* widely varies among green algae. This diversity of pigment composition in algae may be related to their habitats in water where light environment varies to great extents both in intensity and spectral quality. LHC II has been isolated from various green algae (Kan and Thornber 1976, Apel 1977, Bar-Nun et al. 1977, Anderson et al. 1980, Green et al. 1982). The Chl *a*/*b* ratio of LHC II is 2.6 in *Euglena gracilis* (Cunningham and Schiff 1986) and 5.4

in *Dunaliella tertiolecta* (Sukenik et al. 1988). On the other hand, the amino acid sequences of LHC IIs from *Chlamydomonas reinhardtii* and *Dunaliella tertiolecta* are 80% homologous to each other and, when the membrane spanning regions are compared, 70% – 74% homologous to those from higher plants (Jansson and Gustafsson 1990, LaRoche et al. 1990). Thus, in spite of a large diversity in the Chl *a*/*b* ratio, the primary structures of the LHC II apoproteins appear to be similar among Chl *b*-containing organisms.

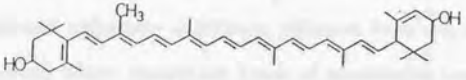
Generally, Chl *a*/*b* ratios of marine green algae are lower than those of terrestrial plants (Nakamura et al. 1976, Kunifuji et al. 1977). They also have a wider variety of carotenoids. Siphonous marine green algae contain unique xanthophylls, siphonaxanthin and siphonein, of which structures are shown in Fig. I-2. The two pigments, which show absorption bands at long wavelengths (above 500 nm) serve as important light-capturing pigments for the algae which inhabit in blue-green environment of the shallow sea bottom (Kageyama et al. 1977). Chl-protein complexes of a siphonous alga, *Codium* sp., have been partially characterized (Anderson 1983, 1985, Benson and Cobb 1983). The algal LHC II had a Chl *a*/*b* ratio of 0.67 and contained significant amounts of siphonaxanthin and siphonein. Fluorescence analysis showed a high efficiency of energy transfer from siphonaxanthin to Chl *a*. However, the LHC II preparation appears to be still impure because five polypeptides of 35.5, 34, 29.1, 28.5 and 27 kDa were resolved from the complexes (Anderson 1985). Spectral properties and pigment compositions of photosystem I and II reaction center complexes isolated from the alga were similar to the



Siphonaxanthin



Siphonein R=$(CH_2)_{10}$



Lutein

Fig. I-2 Structures of siphonaxanthin, siphonein and lutein

corresponding complexes of higher plants. LHC I, which consisted of several polypeptides of 20-25 kDa, had Chl *a*/*b* ratio of 1.73 and contained siphonaxanthin and siphonein (Chu and Anderson 1985). However, detailed analyses of pigment compositions of and energy transfer between pigments in these Chl-protein complexes have not yet been reported. The occurrence of minor antenna Chl *a*/*b*-proteins of photosystem II such as CP29, CP26 and CP24 in the siphonous algae is not known either.

The aim of the present study is elucidation of function of Chls and carotenoids in the light-harvesting system of photosynthesis. Experiments were carried with a siphonous marine green alga, *Bryopsis maxima*. The material was chosen because of its unique pigment composition: Because Chl *b* is present more abundantly than Chl *a* in LHC II, the algal preparation is expected to serve as an excellent material for study of excitation energy transfer involving Chl *b*. The absorption spectrum of the algal thylakoid membranes shows a distinct band(s) of siphonaxanthin and siphonein at 530 nm, different from the membranes from higher plants, where absorption bands of carotenoids (mainly lutein) mostly overlap the Soret bands of Chl *a* and Chl *b* (Fig. I-3). This is also a unique advantage of the alga for investigation of energy transfer from carotenoids to Chl.

In the first half of the present study, Chl-protein complexes were isolated in highly purified states from *B. maxima* and the pigment and polypeptide compositions and spectral properties of the complexes were analyzed (Chapter II). In particular, attempts were made to isolate

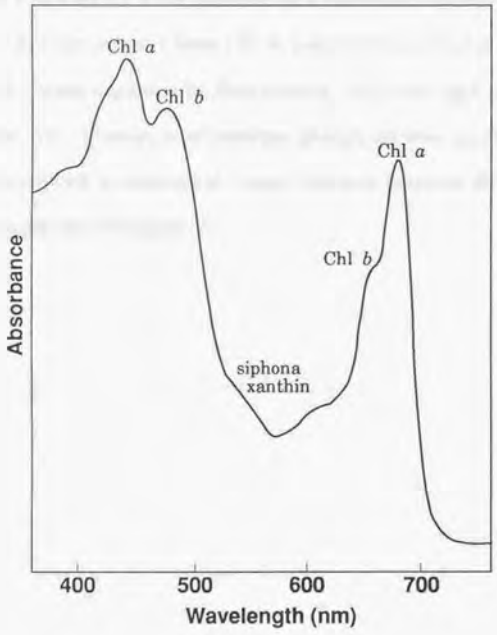


Fig. I-3 Absorption spectrum of chloroplasts of *Bryopsis maxima*

purified LHC II without significant release of pigments and detailed quantitative analyses of pigments and constituent polypeptides of the Chl-protein complex were carried out (Chapter III).

The second half of the present study focused on optical properties and energy transfer between pigments associated with LHC II. Different Chl *a* forms and Chl *b* forms were resolved by a computer-assisted deconvolution. Energy transfer from Chl *b*, siphonaxanthin and siphonein to Chl *a* were analyzed by fluorescence excitation and emission spectra (Chapter IV). Finally, time-resolved photolysis with picosecond laser pulses was applied to analysis of energy transfer between different photosynthetic pigments (Chapter V).

Chapter II

Chl-protein complexes of photosystem I and II from the siphonous green alga, *Bryopsis maxima*.

Summary

Seven chlorophyll (Chl) protein complexes were resolved by sodium dodecylsulfate-polyacrylamide gel electrophoresis from thylakoid membranes of *Bryopsis maxima*. Two Chl-proteins were identified as CP I and CPa, Chl-carrying proteins of the reaction center complexes of photosystem I and II, respectively. Light-harvesting chlorophyll *a*/*b*-protein complexes were divided into three categories; (1) three major light-harvesting Chl *a*/*b*-protein complexes associated with photosystem II, LHC II-1, LHC II-2 and LHC II-3, (2) a minor antenna Chl-protein of photosystem II, LHC II', and (3) a Chl *a*/*b* protein of photosystem I, LHC I. LHC II-1, an oligomeric form of LHC II, carries more then a half of the total Chl and major fractions of siphonaxanthin and siphonein. LHC II' was identified as a new antenna Chl-protein of photosystem II. LHC I also contained siphonaxanthin, siphonein and β-carotene. LHC I was resolved into to six bands with apparent molecular masses of 136-430 kDa, indicating different degree of aggregation.

Introduction

The reaction center complexes of Photosystem I and II are associated with specific light-harvesting complexes called LHC I and LHC II, respectively, in green algae and higher plants (Anderson et al. 1981, Wollman and Bennoun 1982). In addition to the major LHC II, there are several antenna Chl *a*/*b*-proteins of PS II called CP29 (Camm and Green 1980), CP26 (Dunahay et al. 1987) and CP24 (Dunahay and Staehelin 1986). The occurrence of minor antenna Chl-protein of CP I has not yet been reported. These light-harvesting Chl-proteins of the two photosystems bind all Chl *b* and most, if not all, of xanthophylls present in chloroplasts, whereas the reaction center complexes carry only Chl a and β-carotene (Siefermann-Harms 1985).

LHC II and LHC I isolated from higher plants have been extensively characterized in terms of composition and structure (Thornber et al. 1991). Relatively less is known about the light-harvesting Chl *a*/*b*-proteins of algae. In contrast to higher plants, which have uniform composition of photosynthetic pigments, a wider variety of pigments is found in fresh-water and marine algae. Siphonous green algae have unique xanthophylls called siphonaxanthin and siphonein and contain larger amounts of Chl *b* relative to Chl *a* (Anderson 1983). The distribution of pigments among several Chl-protein complexes from the siphonous alga, *Codium fragile* has been reported (Benson and Cobb

1983). However, detailed pigment compositions and optical properties of light-harvesting Chl *a*/*b*-proteins are not known yet.

In this chapter, Chl-protein complexes of a siphonous alga, *Bryopsis maxima*, were analyzed by SDS-PAGE. Compositions of pigments and polypeptides of isolated Chl-protein complexes were determined. Spectral properties of the Chl-protein complexes were also investigated.

Materials and Methods

Preparation of thylakoid membranes ----- *B. maxima* which has 10 to 30 cm long unicellular thalli with pinnate (Fig II-1) was collected from intertidal rock pool in Choshi, Chiba. Thalli were cut with scissors in 50 mM Tris-HCl buffer (pH 7.5) and squeezed through four layers of gauze (Nakayama et al. 1974). The green juice was centrifuged at 1,000 x *g* for 10 min and the thylakoids precipitated were washed once with the same buffer, suspended in 1 mM Na-EDTA (pH 8.0), left for 10 min at 20°C and then collected by centrifugation at 30,000 x *g* for 30 min. The EDTA-wash was further repeated 3 times and the thylakoid membranes were finally suspended in 50 mM Tris-HCl buffer (pH 7.5).

Isolation of Chl-proteins by SDS-PAGE ----- The thylakoid membranes were solubilized with 1% SDS (a SDS/Chl weight ratio of 10) for 1 min at 0°C and centrifuged at 1,000 x *g* for 10 min to remove insoluble matters. The supernatant was applied to disk SDS-PAGE (Laemmli 1970). Stacking gel (pH 6.8) and separation gel (pH 8.8) contained 5% and 6% or 10% acrylamide, respectively, together with 50 mM Tris-HCl and 0.1% SDS. The reservoir buffer contained 50 mM Tris, 380 mM glycine and 0.1% SDS (pH 8.3). Electrophoresis was carried out at 5°C in the dark and at 100 V until free pigments reached an end of the separation gel.

Determination of polypeptides ----- Chl-protein complexes were

Fig. II-1 The thalli of siphonous green alga, *Bryopsis maxima*

extracted from homogenized gels with a medium containing 0.1% SDS and 1 mM Tris-glycine buffer (pH 8.3), treated with 2% SDS and applied to slab gels which consisted of 5% acrylamide stacking gel (pH 6.8) and 10% acrylamide separation gel (pH 8.8). Electrophoresis was performed at 100 V at 5°C.

Spectrophotometric measurements ----- The absorption spectra of Chl-protein complexes in gels were measured with a Hitachi 356 spectrophotometer connected to a computer system (DEC PDP-11). Spectra were stored in digital form, normalized and recorded on an X-Y plotter (Miplot, Watanabe). Fluorescence spectra at -196°C were measured with a Hitachi 835 fluorescence spectrophotometer.

Analysis of pigments ----- Pigments were extracted from lyophilized Chl-protein complexes with 100% methanol and the extract was filtered through a cellulose membrane (pore size, 0.2 μm, Bioanalytical Systems). Extracted pigments were analyzed by high performance liquid chromatography (Twincle, Nihonbunko) with an octadecyl silica column (Finepak SIL C_{18}, 4.6 x 250 mm). Developing solvent was 97% methanol and a flow rate 0.5 ml per min. The concentration of each pigment was determined from the peak area of the elution curve which had been determined at 440 nm with an UV-visible detector (UV10EC-100-II, Nihonbunko). Absorption coefficients of Chl *a* and Chl *b* at this wavelength were estimated from the absorption coefficients of the Chls determined in hexane (Svec, 1978) by comparing absorption spectra of a known amount of Chl *a* or Chl *b* dissolved in

ether and 97% methanol. Absorption coefficients of carotenoids were similarly determined based upon the values in ethanol reported by Davies (1976). The absorption coefficients (εmM) thus determined were 67.1 for Chl *a*, 147 for Chl *b*, 152 for siphonaxanthin, 161 for siphonein, 135 for neoxanthin, and 145 for β-carotene. Chl was also determined in 80% acetone according to the method described by Arnon (1949).

Results

Separation of pigment-protein complexes ----- Fig. II-2 shows Chl-protein complexes resolved by SDS-PAGE from the thylakoid membranes of *B. maxima* solubilized with 1% SDS. The apparent molecular masses of green bands are also shown. Six Chl bands were identified as LHC I, CP I, LHC II-1, LHC II-2, CPa and LHC II-3 from their electrophoretic mobilities, polypeptide compositions and optical properties (see below). Because resolution pattern of Chl-protein complexes resembled that reported by Ryrie et al. (1980) and Anderson et al. (1981) for spinach preparations, the Chl-protein bands were labelled according to their terminology. CP I is the PS I reaction center complex that carries P-700 (Thornber and Highkin 1974), whereas CPa is Chl-bind protein of the

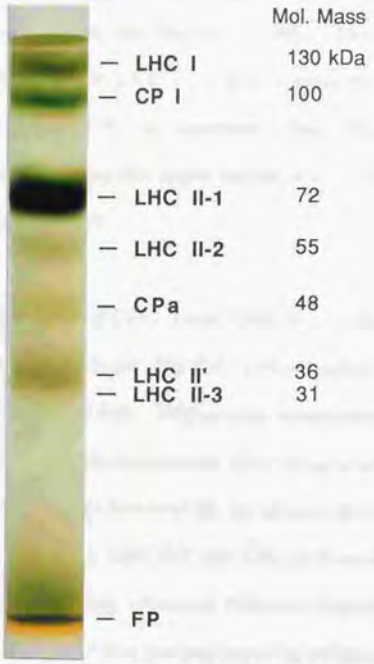


Fig. II-2 Pigment-protein complexes resolved by SDS-polyacrylamide gel electrophoresis from thylakoid membranes of *B. maxima*. The membranes were solubilized with 1% SDS and applied to 10% polyacrylamide gel.

reaction center complex of PS II (Waldron and Anderson 1979). LHC I is an antenna Chl *a*/*b*-protein complex of PS I which migrates in a high molecular mass region (Dunahay and Staehelin 1985). Three different forms of the major light-harvesting Chl *a*/*b* protein complex of PS II were resolved, LHC II-1 being the most prominent band among them. A band which migrated overlapping the upper region of the LHC II-3 band was labelled LHC II' (see below)

Polypeptide compositions of LHC I and LHC II ---- LHC I and II bands were excised from the disk gel (Fig II-3) and extracted with 0.1% SDS and 1 mM Tris-glycine (pH 6.8). Polypeptide composition of each LHC II band was analyzed by electrophoresis after denaturation with 2% SDS (Fig. II-3). Four polypeptide bands of 22, 24, 25 and 26 kDa were resolved from LHC I. LHC II-1, LHC II-2 and LHC II-3 contained a 28 kDa polypeptide. Thus, they may represent different oligomeric states of the same Chl-protein. LHC II-2 was contaminated by polypeptides of 47 to 55 kDa, which may be subunits of CF1 (Süss 1980). Note that LHC II' had no 28 kDa protein and a major component resolved from this Chl-protein was a 35 kDa polypeptide. This suggests that LHC II' is a minor antenna Chl-protein of PS II, distinct from LHC II.

Absorption spectra ----- The absorption spectra of LHC II-1, LHC II-2, LHC II-3 and LHC II' showed maxima of the red band of Chl *a* at 673, 670, 672 and 673 nm, respectively, and the Soret band at 437 nm

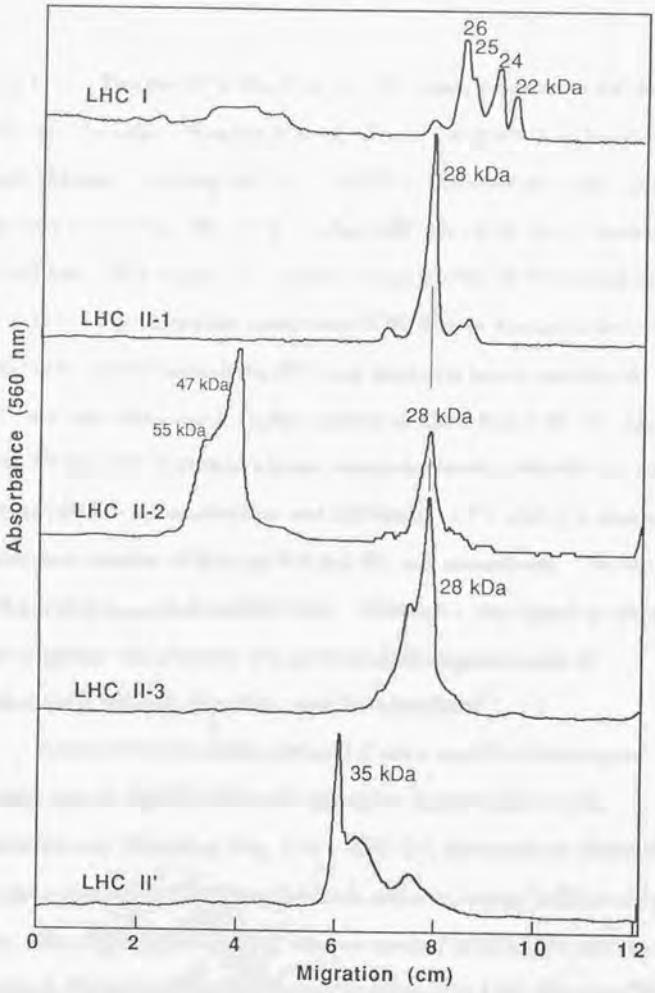


Fig. II-3 Polypeptide compositions of LHC I, LHC II-1, LHC II-2, LHC II-3 and LHC II'. Chl-protein complexes were extracted from gels, treated with 2% SDS and run on 10% polyacrylamide gel.

(Fig. II-4). Two peaks (or shoulder) of Chl *b* appeared at about 650 and 470 nm. As judged from the spectra, LHC II-1 appears to contain Chl *b* most abundantly among LHC IIs. LHC II-1 also showed an absorption maxima at 474 nm different from other LHC IIs which had a maximum at 467 nm. This suggests binding of a large amount of carotenoids to LHC II-1. The absorption spectrum of LHC II' was similar to that of LHC II-3. LHC I showed the Chl *a* red band at a longer wavelength (677 nm) and contained a smaller amount of Chl *b* than LHC II. All LHC IIs and LHC I showed a broad absorption band at 500-550 nm which are ascribed to siphonaxanthin and siphonein. CP I and CPa showed absorption maxima of Chl *a* at 676 and 671 nm, respectively. The two Chl-proteins apparently lacked Chl *b*. Although a shoulder(s) at 450-500 nm suggested the presence of a carotenoid, absorption bands of siphonaxanthin and siphonein were not significant.

Spectral features of the red band of Chl *a* and Chl *b* were more clearly seen at -196°C where each absorption band is known to be narrowed and intensified (Fig. II-5). LHC II-1 showed three absorption maxima of Chl *a* at 658, 669 and 672 nm and a maximum of Chl *b* at 649 nm. The maximum at 672 nm was less marked in LHC II-2 and the peaks at 649 and 658 nm were much diminished in LHC II-3 and LHC II'. LHC I showed a broad band of Chl *a* with a maximum at 674 nm.

Pigment compositions ---- Pigment compositions of the Chl-protein complexes and thylakoid membranes were analyzed by HPLC

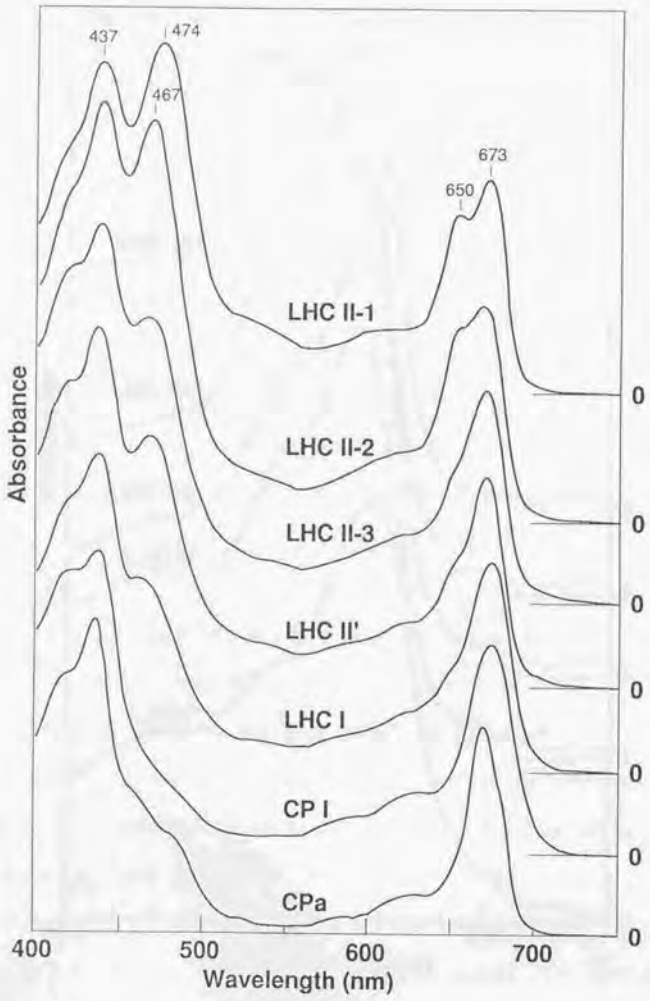


Fig. II-4 Absorption spectra of seven pigment-protein complexes at room temperature. Each spectrum was measured in the gel and normalized at the red absorption maxima.

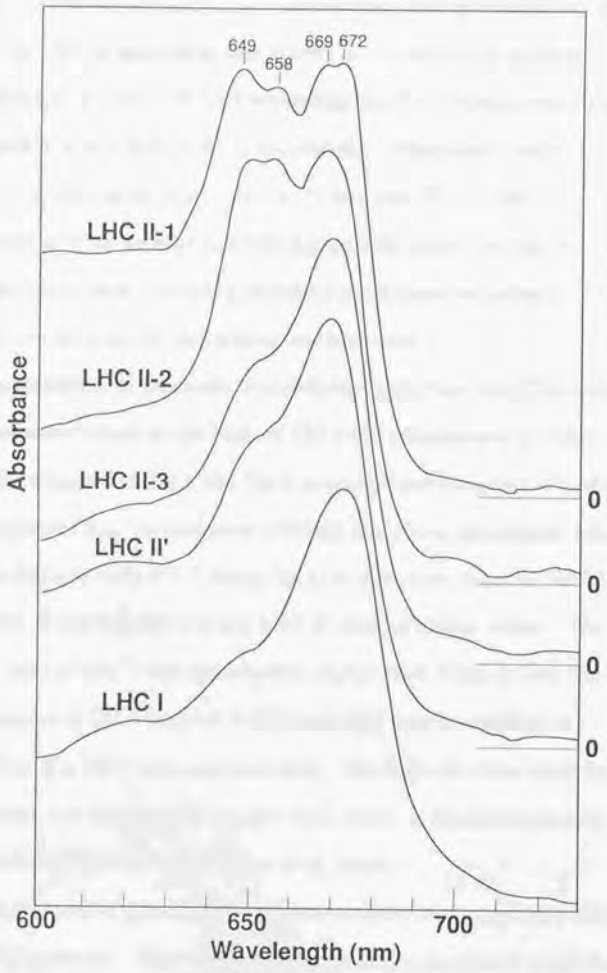


Fig. II-5 Absorption spectra of five Chl *a* / *b* protein complexes at -196°C. Each spectrum was measured in the gel and normalized at the red absorption maxima.

(Fig. II-6). The major peaks resolved were identified by comparison with the retention times and absorption spectra of the standard samples. Peaks, labelled 4' and 5', showed absorption spectra identical with those Chl *b* (peak 4) and Chl *a* (peak 5), respectively. These peaks were, therefore, considered to be derivatives of Chl *a* and Chl *b*. The degradation of Chls appears to occur during electrophoresis but not during SDS treatment, since the thylakoid membranes solubilized by SDS showed none of these Chl derivatives (see top trace).

Compositions of pigments of thylakoid membranes and Chl-protein complexes determined on the basis of Chl *a* are summarized in Table II–1. The amounts of Chl *a* and Chl *b* presented are the sums of native and denatured Chl. As compared with the thylakoid membranes which showed a Chl *a*/*b* ratio of 1.7, lower Chl *a*/*b* ratios were found in LHC II-1 and LHC II–2 but LHC II-3 and LHC II' showed higher ratios. The Chl *a*/*b* ratio of LHC I was considerably higher than those of LHC IIs. Small amount of Chl *b* detected in CP I and CPa may be ascribed to comigration of a Chl *b*-carrying protein(s). The Chl *a*/*b* ratios of all the Chl-proteins are significantly smaller than those of the corresponding Chl-proteins of higher plants (Eskins et al. 1983).

Abundances of xanthophylls relative to Chl *a* were markedly different among Chl-proteins. Siphonaxanthin is the major carotenoid present in the thylakoid membranes, followed by neoxanthin and siphonein. The molar ratio of siphonaxanthin, siphonein and neoxanthin in LHC II-1 was similar to that in the thylakoid membranes. However, the total xanthophyll to

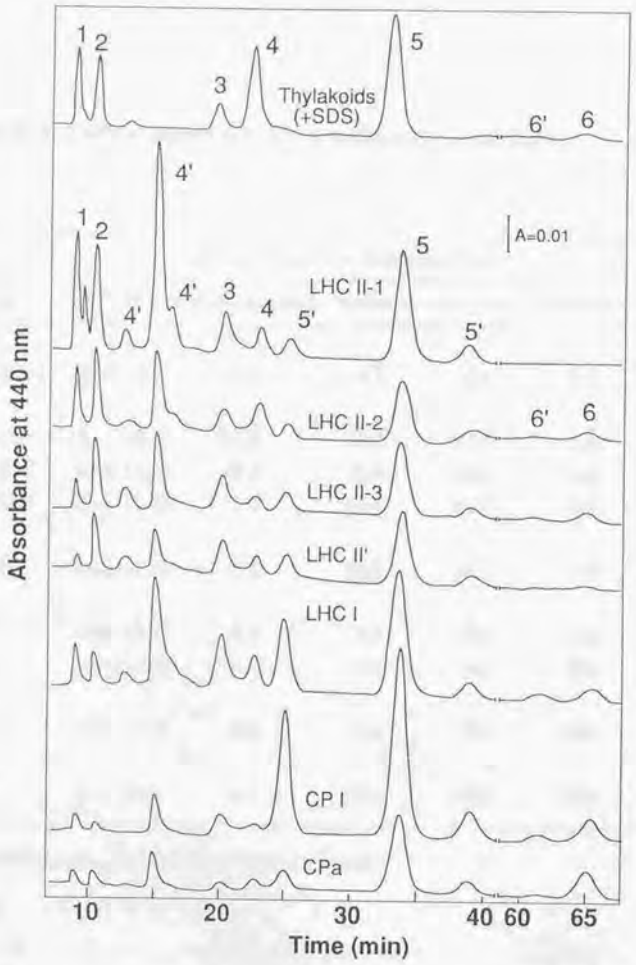


Fig. II-6 Elution profiles of pigments of Chl-protein complexes by high performance liquid chromatography. The pigments were developed with 97% methanol at flow rate of 0.5 ml/min and detected at 440 nm. Peak 1, siphonaxanthin; 2, neoxanthin; 3, siphonein; 4, Chl *b* ; 4', Chl *b* derivatives; 5, Chl *a*; 5', Chl *a* derivatives; 6 and 6', β– carotene.

Table II-1 Ratio of pigments to Chl *a* in Chl-protein complexes.

Chl-protein complex	pigment content (n mol/100 n mol Chl *a*)						
	Chl *b*	(Chl *a*/*b*)	Siphonaxanthin	Siphonein	Neoxanthin	β-carotene	(xan/Chl *a*)[a]
Thylakoid	59.0	(1.7)	25.6	9.7	12.4	5.1	(0.48)
LHC II-1	116	(0.9)	51.3	15.2	27.8	n.d.[b]	(0.94)
LHC II-2	91.2	(1.1)	24.6	18.9	19.2	8.4	(0.63)
LHC II-3	65.8	(1.5)	9.8	19.0	14.7	5.7	(0.44)
LHC II'	56.2	(1.8)	3.6	18.5	10.2	1.6	(0.32)
LHC I	41.3	(2.4)	6.3	4.7	2.4	4.6	(0.13)
CP I	9.1	(11.0)	1.7	3.4	0.4	5.2	(0.06)
CPa	21.3	(4.7)	3.2	3.4	2.8	13.5	(0.09)
FP	28.7	(3.5)	50.7	21.0	16.6	16.4	(0.88)

[a] total xanthophyll/ Chl *a*, [b] not detected,

Chl *a* ratio of the antenna Chl-protein was twice as large that of the membranes. The relative abundance of siphonaxanthin markedly decreased in the order of LHC II-1, LHC II-2 and LHC II-3, whereas variations in the content of siphonein and neoxanthin among LHC IIs were less. LHC II-2 and LHC II-3 had significant amount of β-carotene but no β-carotene was detected in LHC II-1. LHC II' had a lower xanthophyll / Chl *a* ratio (0.3) than LHC IIs. LHC I contained smaller amounts of siphonaxanthin and siphonein. Low concentrations of the xanthophylls were present in CP I and CPa but this may be ascribed to contamination of antenna Chl-proteins because the corresponding reaction center Chl-proteins from higher plants totally lack xanthophylls (Siefermann-Harms 1985).

Distribution of the pigments among isolated Chl-proteins was calculated based upon the amounts of Chl *a* present in the Chl-protein complexes resolved by SDS-PAGE (Table II-2). About 75% of Chl *b*, 66% of siphonaxanthin and 47% of siphonein were associated with LHC II-1. By contrast, both LHC II-2 and LHC II-3 carry only a few percentage of each pigment. CP I is the second in the abundance of pigment, followed by LHC II'. Note that about 18% of Chl *a*, 8% of Chl *b*, 30% of both siphonaxanthin and siphonein and 59% of β-carotene were solubilized with SDS and migrated at the free pigment zone. Thus, the amounts of pigments bound to the Chl-proteins estimated here should be regarded as the lower limits.

Table II-2 Percent distribution of Chls and carotenoids among the eight Chl-protein complexes

Chl-protein complexes	pigment distribution (%)						
	Total Chl	Chl *a*	Chl *b*	Siphonaxanthin	Siphonein	Neoxanthin	β-carotene
LHC II-1	58.4	41.0	75.1	66.4	47.1	71.2	n.d.[a]
LHC II-2	1.3	1.2	1.7	0.9	1.7	1.4	2.0
LHC II-3	0.9	0.9	0.9	0.3	1.3	0.8	1.0
LHC II'	8.1	9.4	8.3	1.1	13.1	6.0	2.9
LHC I	1.1	1.3	0.8	0.3	0.5	0.2	1.2
CP I	14.6	24.0	3.4	1.3	6.2	0.6	24.3
CPa	2.8	3.8	1.3	0.4	1.0	0.7	10.0
FP	12.8	18.4	8.3	29.4	29.2	19.1	58.7

[a] not detected

Separation of LHC I ---- LHC I migrated as a single band in 10% acrylamide gel (Fig. II-2). When thylakoid membranes solubilized with SDS were applied to a 6% acrylamide gel, six green bands were resolved above CP I (Fig. II-7). These bands were named as LHC I-1, LHC I-2, LHC I-3, LHC I-4, LHC I-5, LHC I-6, in the order of increasing mobility. The apparent molecular masses of these Chl-proteins were 430 (LHC I-1), 240 (LHC I-2), 185 (LHC I-3), 170 (LHC I-4), 150 (LHC I-5), 136 (LHC I-6) and 96 kDa (CP I). CPa and LHC IIs comigrated in the 6% acrylamide gel.

Absorption spectra of LHC Is ---- The absorption spectra of six LHC Is resembled each other and had an absorption peak at 677 nm and a shoulder at 650 nm, due to Chl *a* and Chl *b*, respectively (Fig. II-8). This contrasts to *Codium* LHC I which shows a maxima at 671 nm (Chu and Anderson 1985). All LHC Is had an absorption band around 530 nm due to siphonaxanthin and siphonein.

Fluorescence Spectra ---- The fluorescence emission spectra of LHC I-1, LHC I-2, LHC I-4, LHC I-6 and CP I at -196°C are shown in Fig. II-9. The spectra of four LHC Is resembled each other, showing a maximum at 695 nm. CP I had two maxima at 721 and 680 nm, different from the corresponding Chl-protein of *Codium* (Anderson 1983), which had peaks at 712 and 677 nm. The fluorescence excitation spectra for the emission at 695 nm were essentially the same among the LHC Is,

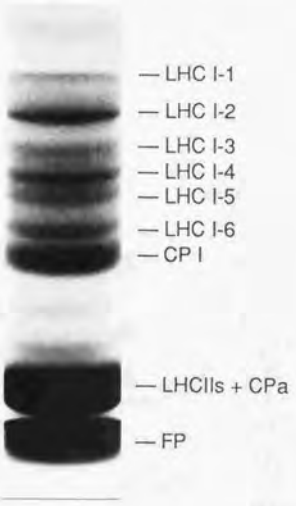


Fig. II-7 Pigment-protein complexes resolved by SDS-polyacrylamide gel electrophoresis from thylakoid membranes of *B. maxima* . The membranes were solubilized with 1% SDS and applied to a 6% polyacrylamide gel.

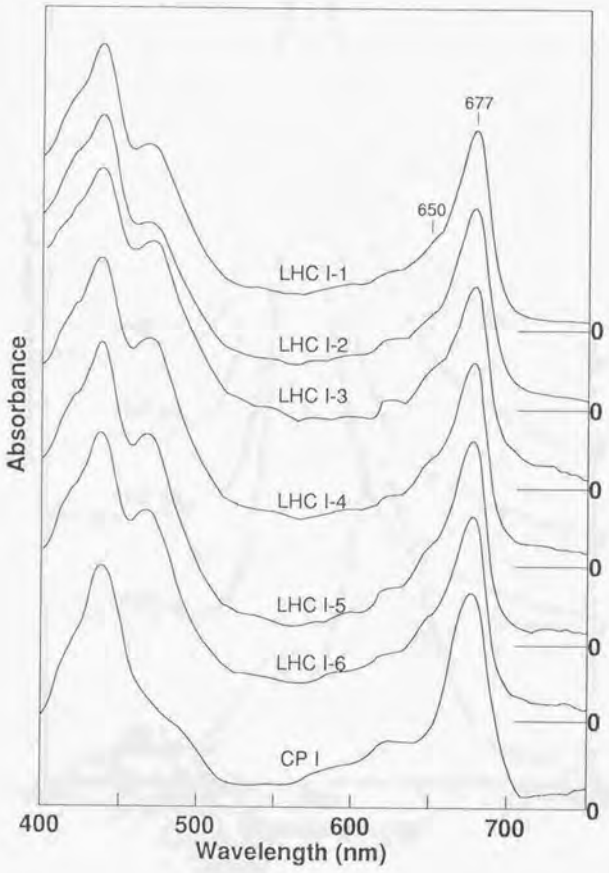


Fig. II-8 Absorption spectra of LHC Is measured at room temperature.

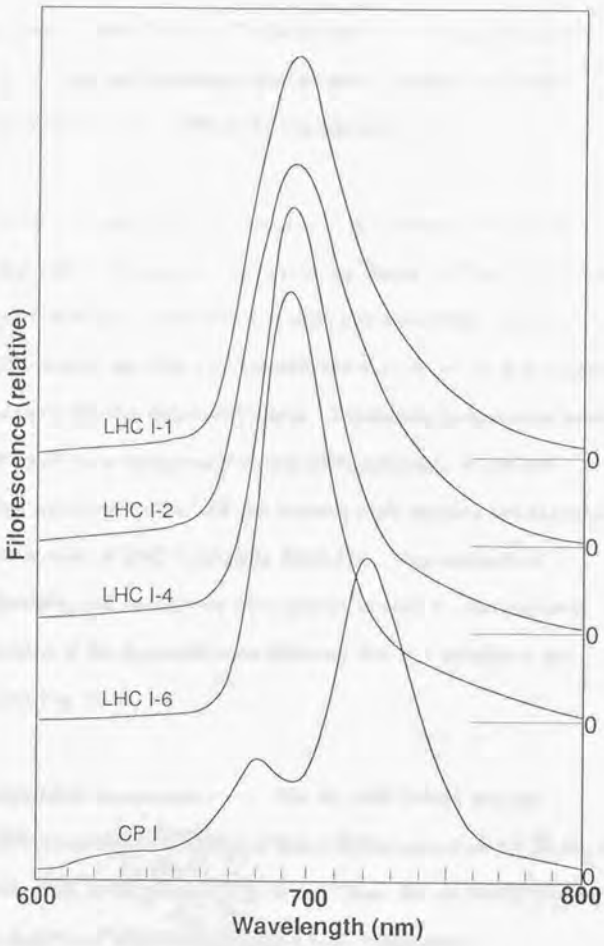


Fig. II-9 Fluorescence emission spectra of LHC I-1, LHC I-2, LHC I-4, LHC I-6 and CP I at the temperature of liquid nitrogen. The excitation wavelength was 430 nm.

showing peaks at 438 (Chl *a*), 470 (Chl *b*) and 540 nm (siphonaxanthin) (Fig. II-10). The spectra indicate that excitation energy absorbed by Chl *b* and carotenoids are efficiently transferred to Chl *a*.

Pigment compositions ----- Generally, all the six LHC Is had similar pigment compositions. However, as shown in Table II-3, there were small differences in the Chl *a*/*b* ratio and carotenoid contents. Neoxanthin was detected but its concentration could not be determined due to comigration of a denatured Chl *b*. Xanthophyll appears to have been extracted more extensively during electrophoresis in the 6% acrylamide gels than in the 10% gel because their relative abundances are less than that of LHC I shown in Table II-1. The contents of siphonaxanthin and β–carotene were lowest in LHC I-1 but accurate determination of the pigments were different due to a paucity of the sample (see Fig. II-7).

Polypeptide compositions ----- The six LHC Is had similar polypeptide composition consisting of four polypeptides of about 22, 24, 25 and 26 kDa (Fig. II-11, also see Fig. II-2). Thus, the six bands represent different degrees of oligomerization of a LHC I monomer.

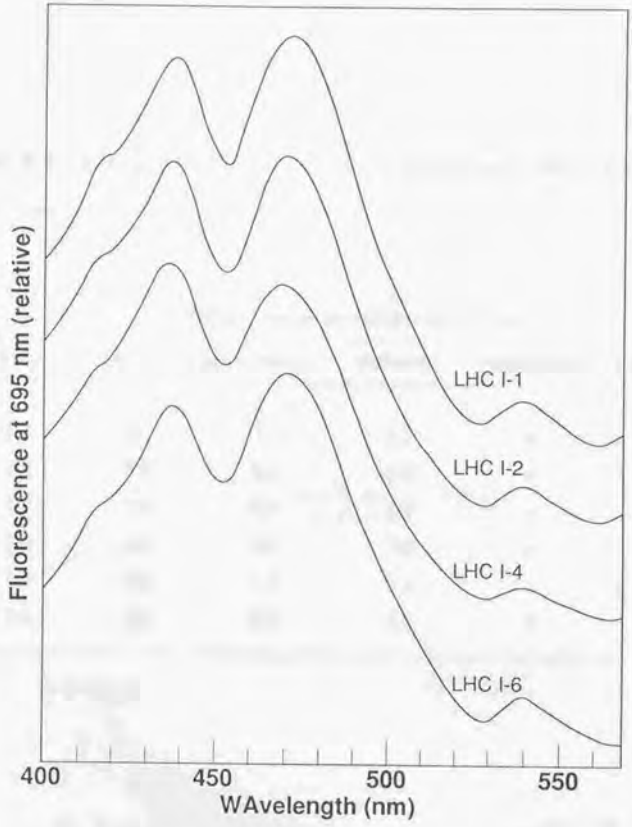


Fig. II-10 Fluorescence excitation spectra of LHC I-1, LHC I-2, LHC I-4, LHC I-6 at -196°C. The fluorescence emission at 695 nm were monitored.

Table II-3 Relative abundances of Chl b and carotenoids in PS I Chl-protein complexes.

Chl-protein complex	Pigment content (n mol/100 n mol Chl *a*)				
	Chl *a*/*b*	Siphonaxanthin	Siphonein	Neoxanthin	β-carotene
LHC I-1	2.1	+ [a]	1.7	+	0.9
LHC I-2	3.5	3.1	1.8	+	7.0
LHC I-3	2.0	3.8	2.4	+	6.8
LHC I-4	2.0	3.9	2.9	+	6.6
LHC I-5	2.8	4.4	3.1	+	6.2
LHC I-6	2.5	4.2	2.6	+	6.8

[a], detected

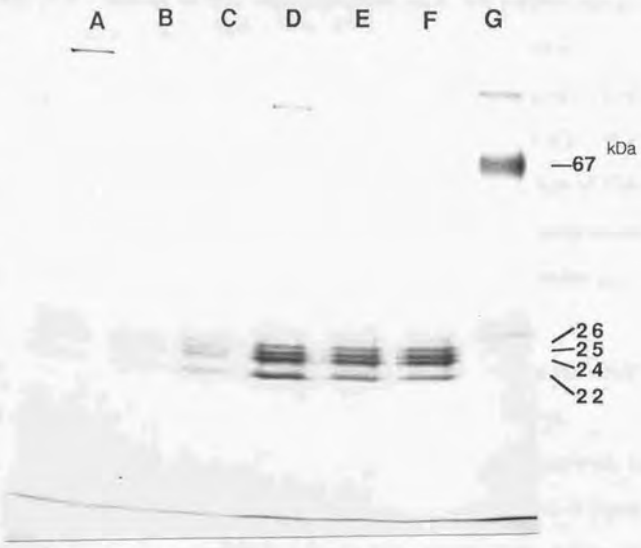


Fig. II-11 Polypeptides resolved from LHC I. (A) LHC I-1, (B) LHC I -2, (C) LHC I-3, (D) LHC I-4, (E) LHC I-5, (F) LHC I-6, (G) CP I

Discussion

Seven Chl-protein complexes were resolved from the thylakoid membranes of *B. maxima* by SDS-electrophoresis on a 10% acrylamide gel. They were identified as Chl-carrying proteins of the reaction center complexes of PS I (CP I) and PS II (CPa), and antenna Chl *a*/*b*-proteins of PS I (LHC I) and PS II (LHC II-1, LHC II-2, LHC II-3 and LHC II'). The order of migration of the Chl-protein complexes was similar to that of Chl-proteins from higher plants (Anderson et al. 1981). Thus, the composition and organization of Chl-protein assemblies of *B. maxima* are similar to those of other Chl *b*-containing organisms

Three different forms of LHC IIs were resolved as reported for other plant materials (Ryrie et al. 1980, Anderson 1981). In *B. maxima*, however, an oligomeric form, LHC II-1, was the major LHC II resolved, in contrast to *Codium* and higher plants, where a predominant LHC II band is a monomeric form corresponding to LHC II-3 (Anderson 1983, Eskins et al. 1983). Thus, *Bryopsis* LHC II appears to be more stable than LHC II of other organisms. All the three LHC IIs showed a single polypeptide of 28 kDa when electrophoresed in gel which contained no urea (see Chapter III for electrophoresis in the presence of urea), however, pigment compositions of the three LHC IIs differed from each other. The Chl *a*/*b* ratio increased and xanthophyll/Chl *a* ratio decreased in the order of LHC II-1, LHC II-2 and LHC II-3. LHC II-1 lacked β-carotene, whereas small amounts of β-carotene were detected in other LHC IIs. We interpret that

the different pigment compositions of the three LHC II forms may derive from extraction of pigments during the detergent-treatments and electrophoresis and comigration of other Chl-proteins.

LHC II' contained a 35 kDa protein but no 28 kDa protein. Anderson (1985) assigned a 35.5 kDa protein, which had been resolved from LHC II of *Codium*, to an apoprotein of LHC II. Our results do not support this assignment. It is suggested the 35 kDa protein is an apoprotein of a new minor antenna Chl *a*/*b*-protein of PS II (Camm and Green 1980, Machold and Meister 1980). Although, its Chl *a*/*b* ratio of 1.8 is smaller than that of CP29 from higher plants which ranges from 2.20 to 2.25 (Thornber et al. 1991).

The LHC I was separated into six bands by SDS-PAGE in 6% acrylamide gel. All these LHC Is had four polypeptides of 22 24, 25 and 26 kDa. The occurrence of four polypeptides was reported for LHC I from *Chlamydomonas* (Wollman and Bennoun 1982). The Chl *a*/*b* ratios of the LHC Is were 2.0 to 3.5, which were considerably lower than the ratio of 6.3 determined for LHC I in *Chlamydomonas* (Wollman and Bennoun 1982) but higher than that of *Codium* (1.73) (Chu and Anderson 1985). Thus, all the antenna Chl-proteins of PS I and PS II contain larger amounts of Chl *b* relative to Chl *a* in *B. maxima*. Because pigment compositions and absorption and fluorescence spectra of the six LHC Is are similar to each other, they are considered to represent different oligomeric forms. Comparison of the absorption spectra with the fluorescence excitation spectra of LHC Is (Fig. II-7 and Fig. II-8) indicates that Chl *b*, siphonaxanthin and siphonein transfer excitation energy efficiently to

Chl *a*. CP I showed an emission maximum at 720 nm similar to *Chlamydomonas* (720 nm) but different from that of higher plants (730 nm).

Distribution of photosynthetic pigments among Chl-protein complexes of *B. maxima* is illustrated as Fig II-12. Pigments which were extracted during preparation are neglected. The ratio of antenna size of PS I to that of PS II was estimated to about 0.21 by taking all Chls and carotenoids into account. Thus, there is a large imbalance in the light-harvesting capacity between the two photosystems. The imbalance may be compensated by spill-over of light-energy absorbed by LHC II to PS I (Howerth et al. 1982).

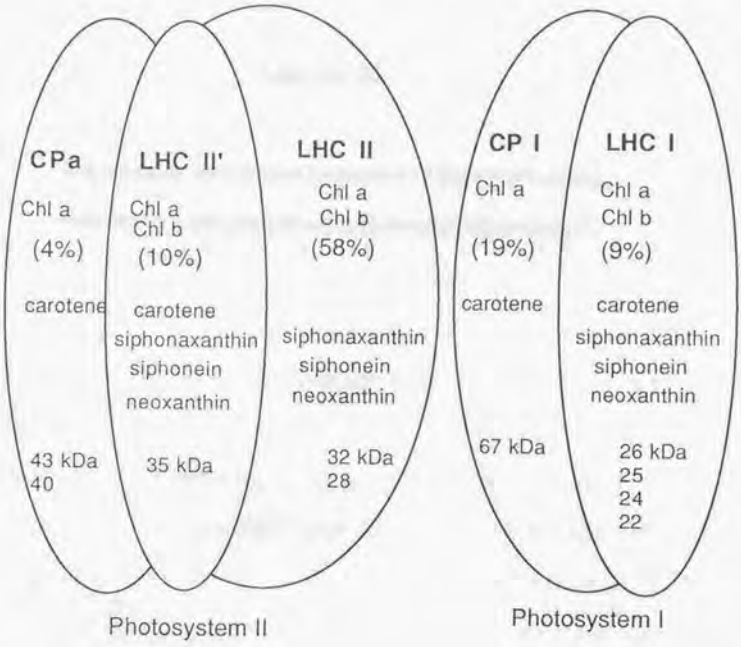


Fig. II-11 The distribution of pigments and polypeptides among the Chl-protein complexes separated by SDS-PAGE

Chapter III

Purification and characterization of light-harvesting chlorophyll *a/b*-protein complexes of photosystem II.

Summary

Light-harvesting chlorophyll *a*/*b*-proteins of photosystem II (LHC II) were isolated and highly purified from thylakoid membranes of the green alga, *Bryopsis maxima*, with digitonin without solubilization of significant amounts of chlorophylls (Chl) and carotenoids. Two purified preparations of LHC II with apparent particle sizes of 280 and 295 kDa, which are called P4 and P5, respectively, were obtained by sucrose density gradient centrifugation and chromatography on a DEAE-toyopearl column. P4 and P5 showed similar absorption spectra with Chl *a* maxima at 674, 658 and 438 nm and Chl *b* maxima at 649 and 476 nm at -196°C. Fluorescence excitation spectra demonstrated that Chl *b*, siphonaxanthin and siphonein could efficiently transfer absorbed light energy to Chl *a*. The two LHC II complexes contained two apoproteins of 28 and 32 kDa, which had similar but not identical amino acid compositions. N-terminal amino acid sequence of the 32 kDa polypeptide was homologous to those of LHC II from *Chlamydomonas reinhardtii* and *Dunaliella tertiolecta*. The stoichiometry of pigments bound to P5 was determined: A single polypeptide carries 6 Chl *a*, 8 Chl *b* and 5 xanthophyll molecules (3 siphonaxanthin and 1 each of siphonein and neoxanthin).

Introduction

LHC II is the major light-harvesting Chl *a*/*b* protein of PS II, which carries nearly half of the total Chl *a* in higher plants and green algae (Thornber 1986). LHC II also contains a large amount of Chl *b*. Higher plant LHC II shows Chl *a* to Chl *b* ratios of 1.1 and 1.47, compared to the ratio of about 3 for thylakoid membranes. In addition, LHC II contains significant quantities of xanthophylls, while carotene is only a minor component (Siefermann-Harms and Ninnemann 1982). LHC II isolated from different green algae considerably differ in their compositions of Chl and carotenoids, reflecting a wide diversity of pigment composition in green algae (Kan and Thornber 1976, Apel 1977, Bar-Nun et al. 1977, Anderson et al. 1980, Green et al. 1982, Cunningham and Schiff 1986). However, different pigment compositions may also derive from release of pigments during preparation with detergents.

Chl-proteins of the siphonous algae, *Codium* sp., have been isolated and partially characterized (Anderson 1983, 1985, Benson and Cobb 1983, Chu and Anderson 1985). SDS-PAGE of the algal thylakoid membranes resolved eight Chl-proteins. LHC II-1 and LHC II-3 were shown to contain siphonaxanthin and siphonein and have similar absorption and fluorescence spectra (Anderson 1983). However, details of pigment compositions of these antenna complexes are not known.

In Chapter II, three forms of the major Chl *a*/*b*-protein (LHC II-1,

LHC II-2 and LHC II-3) and a minor Chl-protein (LHC II') were separated by SDS-PAGE from the thylakoid membranes of the siphonous alga, *Bryopsis maxima*, and characterized in terms of absorption spectra and compositions of pigments and polypeptides. Relative to Chl *a*, Chl *b* and siphonaxanthin were most abundant in LHC II-1 (72 kDa) and decreased in amount with increasing electrophoretic mobility of the LHC IIs. LHC II' had a high ratio of Chl *a* to Chl *b* and a low level of siphonaxanthin. However, significant amounts of Chl and carotenoids were solubilized during SDS-PAGE and, in particular, about half of β–carotene and about 30% of siphonaxanthin and siphonein migrated in a free-pigment zone of the gels. Therefore, the amounts of Chl and carotenoids intrinsically associated with LHC II remain to be determined.

In this chapter, attempts were made to isolate LHC II from *B. maxima* without loss of pigments. Use of a mild detergent, digitonin, for solubilization of thylakoid membranes and purification of Chl-proteins by column chromatography enabled us to isolate two forms of highly purified LHC II with negligible solubilization of the pigments. Spectral and some chemical properties of the Chl-proteins were analyzed and the stoichiometry of Chls and carotenoids bound to an apoprotein of LHC II was determined.

Materials and Methods

Thylakoid membranes were prepared from fresh thalli of *B. maxima* by the method described in the preceding chapter. The thylakoid membranes were treated with 0.8% digitonin (a ratio of detergent to Chl of 1:20, w/w) in 50 mM Tris-HCl buffer (pH 7.5), 2 mM PMSF and 40 mM ε-aminocaproic acid at 4°C for 8 h with stirring. After removal of membrane fragments enriched in PS I by centrifugation at 30,000 x *g* for 1 h, the supernatant was layered on a sucrose density gradient (0.4 - 0.7 M sucrose in 50 mM Tris-HCl, pH. 7.5) in a vertical rotor (Hitachi, RPRV-18) and centrifuged at 27,000 x *g* for 17 h at 4°C. A green fraction was collected with a syringe and dialyzed against 2 liters of 20 mM Tris-HCl (pH 7.5) at 4°C for 12 h and subsequently applied to a column of DEAE-toyopearl (2.2 x 15 cm) equilibrated with 20 mM Tris-HCl (pH 7.5), 50 mM NaCl and 0.15% digitonin. The column was developed with a linear gradient of NaCl from 50 to 250 mM. Eluates containing LHC II were pooled, concentrated with polyethylene glycol 20,000 and subjected to gel-permeation chromatography on a column of Superose 6 (prep grade, 1.6 x 50 cm). The flow rate of the running buffer, which contained 50 mM Tris-HCl (pH 7.5) and 0.02% digitonin, was 20 ml/h. Particle sizes of the Chl-protein complexes were estimated with a calibration standards (Pharmacia Fine Chemicals).

SDS-PAGE was carried out by the method of Laemmli (1970). 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 (1981). Electrophoresis of the Chl-protein complexes was carried out by the method described in the preceding chapter. LHC II fractions separated by chromatography with a DEAE-toyopearl column were run on a 10% polyacrylamide gel that contained 0.1% sodium deoxycholate and 0.1% SDS, according to the method of Waldron and Anderson (1979). The two polypeptides with pigments were resolved by treating LHC II with 1% SDS in the presence of 20 mM $MgCl_2$, followed by electrophoresis in a 12% polyacrylamide gel that contained 4 M urea, 20 mM $MgCl_2$, 10% glycerol and 0.1% SDS.

Fluorescence excitation and emission spectra at -196°C were recorded with a Hitachi 850 fluorescence spectrophotometer. The band passes of excitation and emission were 5 and 2 nm, respectively.

DCIP photoreduction in the presence or absence of DPC as electron donor was determined by measuring absorption changes at 580 nm with a Hitachi 356 spectrophotometer. The actinic light from a 100 w halogen lamp passed through a 5 cm water layer and a VR 64 cut-off filter (Toshiba) (2.1×10^5 ergs $cm^{-2} \cdot sec^{-1}$). The reaction mixture contained 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 40 μM DCIP and, if present, 1 mM DPC.

Chls and carotenoids were extracted from lyophilized Chl-proteins with 100% methanol and analyzed with a Waters HPLC system

(ALC/GPC 208) with an octadecyl silica column (Unisil C_{18}, 0.6 x 10 cm). The developing solvent was 98% methanol (flow rate, 1.0 ml per min). The concentration of Chl was determined in 80% acetone (Arnon 1949).

Amino acid compositions were determined with a Hitachi 835 amino acid analyzer after samples had been hydrolyzed in 6 M HCl and 1% thioglycolic acid at 110°C for 24 or 64 h. For analysis of N-terminal amino acid sequence, polypeptides were separated by SDS-PAGE, transferred onto polyvinilidene difluoride membranes (Applied Biosystems) and applied to a ABI 473A peptide sequencer (Applied Biosystems).

Results

Purification of two LHC II complexes ----- For extraction of LHC II, thylakoid membranes were solubilized with 0.8% digitonin and subjected to sucrose density gradient centrifugation in a vertical rotor. A single green band containing large amounts of Chl *b* and xanthophylls appeared separately from heavier Chl-containing materials near the bottom of the rotor (Fig. III-1). Chls and carotenoids were not detected in the topmost layer of the density column, where detergent micelles

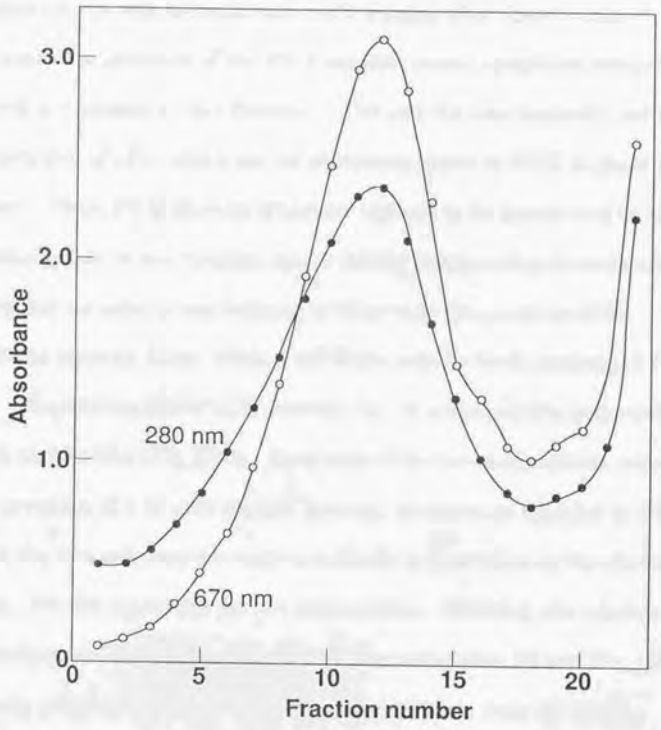


Fig. III-1 Sucrose density gradient centrifugation of digitonin-treated thylakoid membranes The volume of each fraction was 1.0 ml.

containing free pigments were expected to occur.

When the LHC II enriched fraction was subjected to chromatography on a DEAE-toyopearl column, five green bands labelled P1 to P5 were resolved (Fig. III-2). DCIP photoreduction with H_2O as electron donor was detected only in P3 fraction (Fig. III-3). This indicates the presence of the PS II reaction center complexes competent in water-oxidation in this fraction. The activity was markedly enhanced by addition of DPC, which served as electron donor to PS II in place of water. Thus, PS II electron transport appears to be inactivated on the oxidizing side of the reaction center during fractionation procedures. Note that no activity was detected in P5 even in the presence of the artificial electron donor, while a tail of the activity band overlapped P4.

Denaturing SDS-PAGE showed that P5 contained two polypeptides of 28 and 32 kDa (Fig. III-4). Resolution of the two polypeptides required the presence of 4 M urea in gels, because, as shown in Chapter II (Fig. II-2), the two polypeptides migrated closely to each other in the absence of urea. P4 also contained the two polypeptides. However, the relative abundances of the polypeptides were different between P5 and P4. The 32 kDa polypeptide was present in greater amounts than the 28 kDa polypeptide in P5, whereas the reverse was the case with P4. The two polypeptides were present in both P2 and P3 but not in P1. P3 additionally contained polypeptides that migrated in the molecular-mass region of 41 to 72 kDa, some of which may be ascribed to the PS II reaction center complexes. Two polypeptides of 35 and 22 kDa were also resolved from

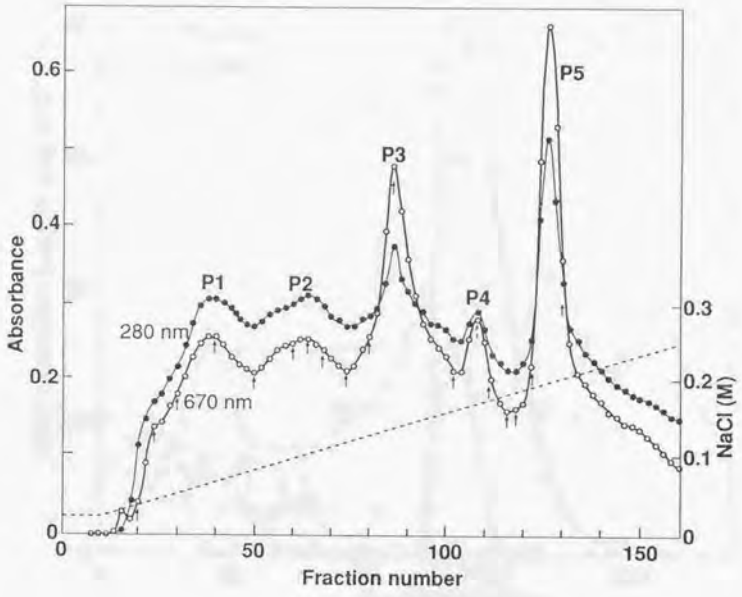


Fig. III-2 Elution profile of DEAE-toyopearl column chromatography of LHC II enriched fractions obtained by sucrose density gradient centrifugation. The concentration of NaCl increased as indicated by a broken line. Arrows, see Fig. III-4

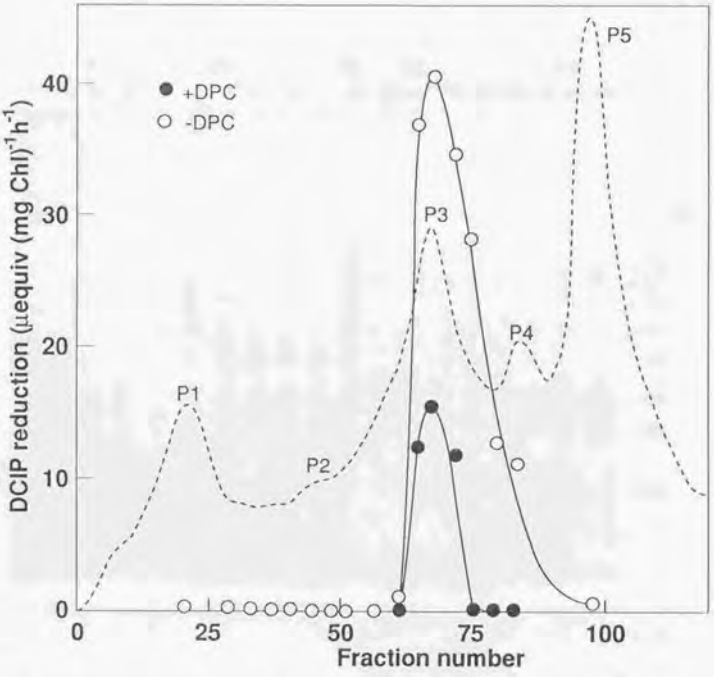


Fig. III-3 DCIP photoreduction in fractions obtained by column chromatography. Broken line, absorbance at 280 nm.

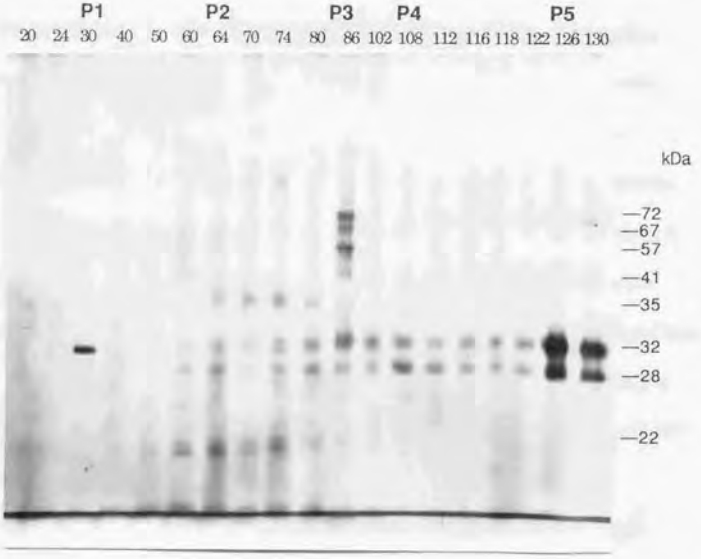


Fig. III-4 Polypeptide compositions of fractions separated from LHC II enriched preparation by the column chromatography. The fractions indicated by arrows in Fig. III-2 were treated with 2% SDS and analyzed by electrophoresis in gels that contained 4 M urea. Numbers above the gel are fraction numbers. The gel was stained with silver.

P2. This suggests the presence of LHC II' in this fraction. Elution profile of column chromatography showed a very small peak of Chl immediately after NaCl concentration was raised (Fig. III-2 fraction 15-18). Because no polypeptide was detected, Chl present in this fraction may be ascribed to free Chl (Kühlbrandt and Barber 1988). Thus, that amounts of Chl released by the procedures used was negligibly small.

When P4 and P5 were chromatographed on a gel-permeation column, P5 was eluted slightly faster than P4 (Fig. III-5). The particle sizes of P4 and P5 were estimated to be 280 and 295 kDa, respectively. A Chl-containing band of 400 kDa (P5') which come down before P5 also contained the 28 kDa and 32 kDa polypeptides. Re-chromatography of the main P5 fractions (indicated by a bracket in the figure) produced only a single peak with the same retention time as that of P5. Properties of P5' was not further studied.

SDS-PAGE of P4 and P5 under mild conditions yielded a single green band at the same positions (Fig. III-6A). Their molecular mass of 72 kDa corresponds to that of LHC II-1 described in chapter II. The result indicates that P4 and P5 are aggregates of LHC II-1.

P5 was found to be resolved into two Chl-proteins, P5a and P5b, by SDS-PAGE in the presence of 0.1% sodium deoxycholate, 4 M urea, 20 mM $MgCl_2$ and 10% glycerol (Fig. III-5B). Figure III-5C shows that P5a contained the 32 kDa polypeptide, whereas the 28 kDa polypeptide was resolved from P5b, although there were some cross contaminations of the polypeptides. The result indicates that the 32 kDa and 28 kDa

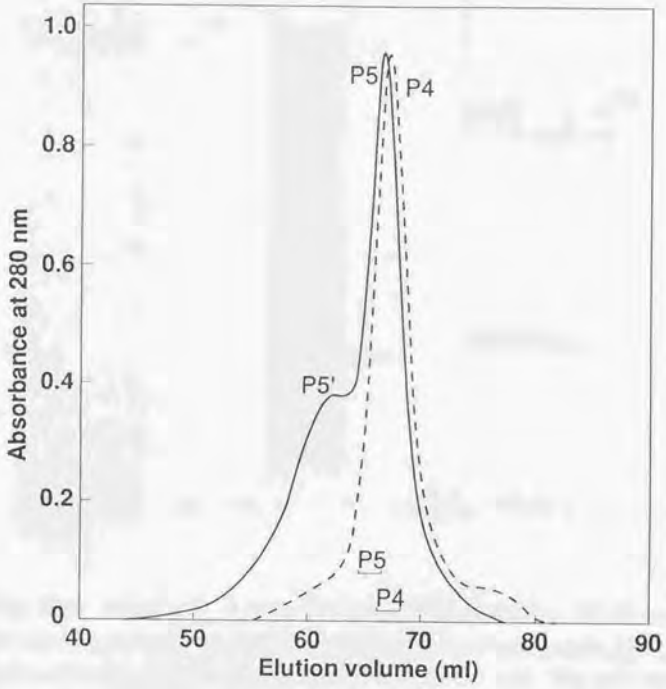


Fig. III-5 Elution profiles of P4 and P5. The running buffer contained 50 mM Tris-HCl (pH 7.5) and 0.02% digitonin. Particle size was estimated with thyroglobulin (669 kDa), ferritin (440 kDa), aldolase (158 kDa) and bovine serum albumin (67 kDa) as molecular standards.

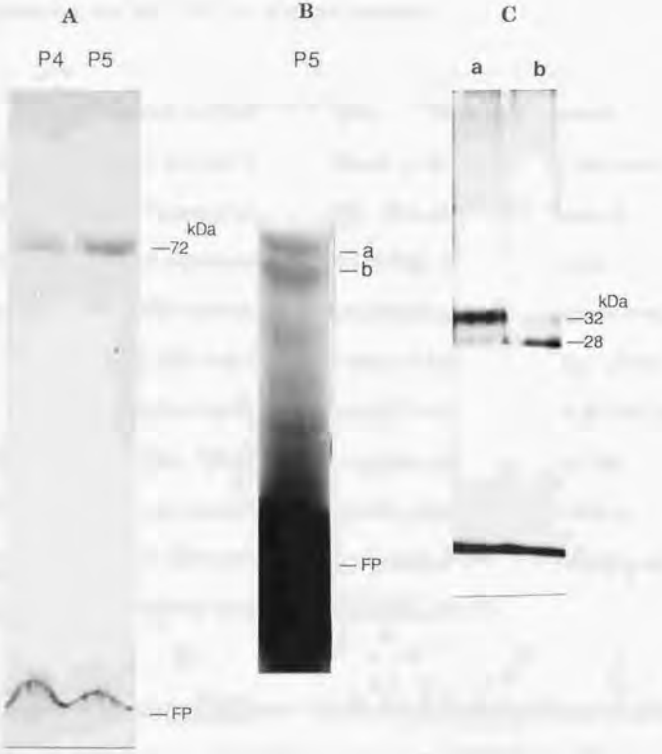


Fig. III-6 SDS-PAGE of P4 and P5 under mild conditions. (A) P4 and P5 were treated with 1% SDS and subjected to electrophoresis in 10% polyacrylamide gel that contained 0.1% deoxycholic acid. The gels were not stained. (B) Resolution of two Chl proteins from P5. P5 was treated with 1% SDS and 20 mM $MgCl_2$ and subjected to electrophoresis in a 12% polyacrylamide gel that contained 0.1% deoxycholic acid, 4 M urea, 20 mM $MgCl_2$, 10% glycerol and 0.1% SDS. The gel was not stained. (C) Polypeptide compositions of P5a and P5b. The bands a and b in (B) were excised from a disk gel, treated with 2% SDS at 100°C for 5 min and run on the gel that contained 4 M urea and 0.1% SDS. The gel was stained with silver.

polypeptides are both Chl-carrying polypeptides.

Absorption and fluorescence spectra ----- P4 and P5 showed identical absorption spectra with prominent peaks of Chl *b* at 650 and 474 nm at room temperature (Fig. III-7). The absorption bands of siphonaxanthin and siphonein appeared at 530 - 540 nm. At the temperature of liquid nitrogen, four maxima or shoulder were observed at 674, 669 (shoulder), 658 and 649 nm in the red band region (Fig. III-8).

P4 and P5 emitted an intense band of Chl *a* fluorescence at 680 nm at -196°C (Fig. III-9A). The excitation spectra determined for this emission resembles their absorption spectra, indicating that Chl *b*, siphonaxanthin and siphonein transfer excitation energy efficiently to Chl *a* in the two purified forms of LHC II (Fig. III-9B).

Amino acid composition ----- The amino acid compositions of the 28 kDa and 32 kDa polypeptides of P5 are shown in Table III-1. The two polypeptides had similar but not identical amino acid compositions. For the comparison, the amino acid compositions of analogous polypeptides from green algae and higher plants are presented. There are notable similarities among the compositions of amino acid of different LHC II. The polarity indexes of the 28 and 32 kDa polypeptides were 37-38, which were also comparable to those of the corresponding polypeptides from green algae and higher plants. By assuming that the 28 kDa and 32 kDa polypeptides contain three and two histidine residues, respectively, the

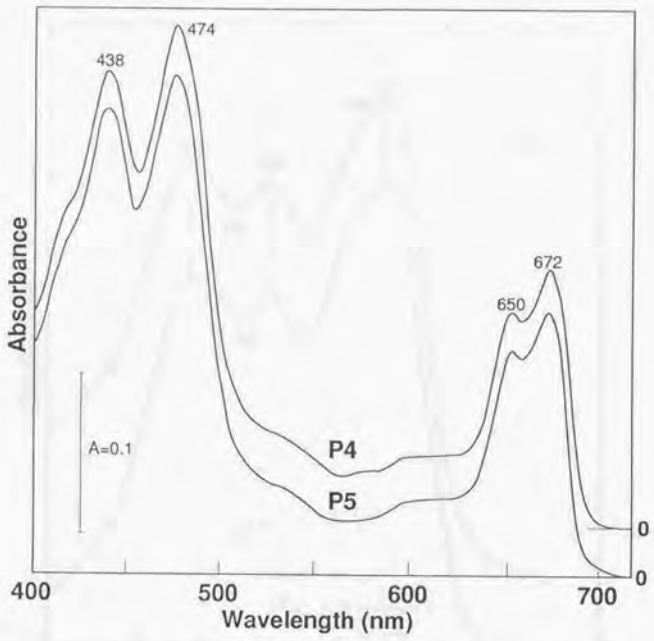


Fig. III-7 Absorption spectra of P4 and P5 at room temperature. Spectra were normalized at the absorption maxima of the red band but vertically displaced for the convenience of comparison.

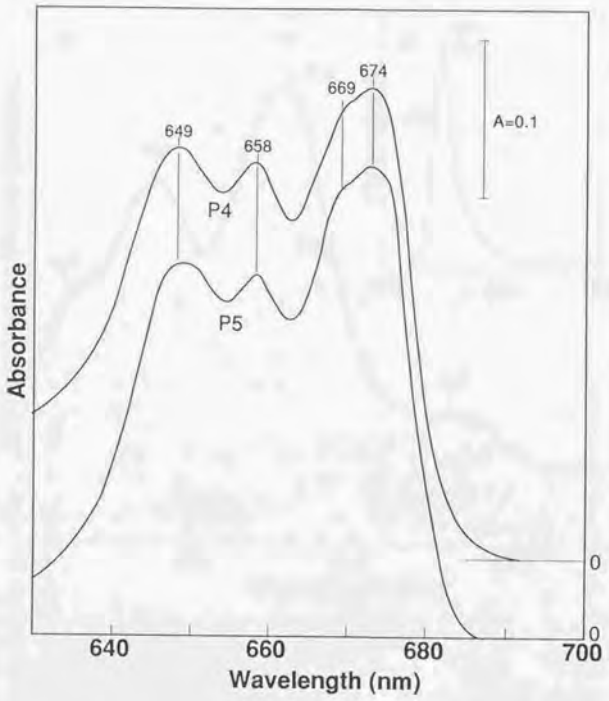


Fig. III-8 Absorption spectra of P4 and P5 at the temperature of liquid nitrogen. The two spectra were normalized at the absorption maximum of the red band but vertically displaced

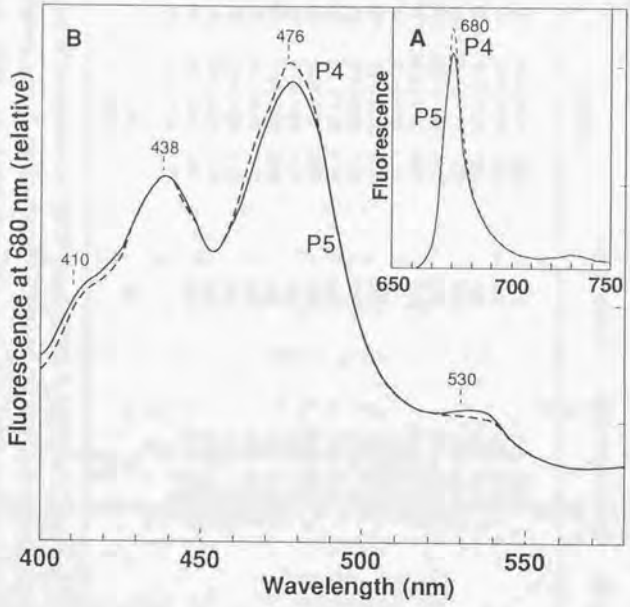


Fig. III-9 Fluorescence emission and excitation spectra of P4 and P5 at the temperature of liquid nitrogen. Solid line, P5; Broken line, P4. (A) Emission spectra excited at 476 nm. (B) Excitation spectra for the emission band at 680 nm. Samples purified by gel-permeation chromatography were used.

Table III-1 Amino acid compositions (mol %) of the polypeptides of LHC II (P5) from *Bryopsis maxima* and other plants and green algae

Amino acid	Polypeptides of P5		*Acetabularia*[a]	*Chlamydomonas*[b]	*Dunaliella*[c]	*Lemna*[d]	pea[e]
	28 kDa	32 kDa					
Asx	8.4 ± 0.11 (19)	8.4 ± 0.15 (20)	9.1	8.5 (24)	9.4	8.8 (20)	7.9 (18)
Thr	4.8 ± 0.13 (11)	4.6 ± 0.17 (11)	4.5	4.9 (14)	3.8	3.5 (8)	3.5 (8)
Ser	7.1 ± 0.15 (16)	6.3 ± 0.14 (15)	5.8	3.6 (10)	3.8	3.1 (8)	7.5 (17)
Glx	10.6 ± 0.41 (24)	11.2 ± 0.38 (27)	9.3	8.6 (25)	9.8	7.8 (21)	8.3 (19)
Gly	12.8 ± 0.51 (29)	12.6 ± 0.57 (30)	15.6	12.1 (34)	19.0	12.7 (29)	12.3 (28)
Ala	12.0 ± 0.56 (28)	12.2 ± 0.51 (29)	9.6	11.1 (31)	7.4	11.4 (26)	9.7 (22)
Cys	0.5 ± 0.11 (1)	0.4 ± 0.09 (1)	-	0.5 (1.5)	-	0.4 (1)	0.4 (1)
Val	5.4 ± 0.26 (12)	5.5 ± 0.21 (13)	5.3	4.3 (12)	8.7	6.6 (13)	7.5 (18)
Met	1.8 ± 0.24 (4)	2.0 ± 0.26 (5)	1.4	1.7 (5)	1.3	2.2 (5)	1.8 (4)
Ile	3.6 ± 0.17 (8)	3.7 ± 0.12 (9)	4.5	4.2 (12)	3.1	4.8 (11)	3.1 (7)
Leu	8.4 ± 0.43 (19)	9.2 ± 0.48 (22)	9.1	11.4 (32)	8.8	10.1 (23)	11.0 (24)
Tyr	3.6 ± 0.58 (8)	3.7 ± 0.63 (9)	4.2	3.2 (9)	2.3	2.6 (6)	3.4 (8)
Phe	5.7 ± 0.28 (13)	5.8 ± 0.23 (14)	5.7	6.7 (19)	5.8	5.7 (13)	6.4 (14)
Lys	3.6 ± 0.42 (8)	3.4 ± 0.45 (8)	4.6	7.1 (15)	4.7	4.4 (10)	3.5 (8)
His	1.4 ± 0.51 (3)	0.9 ± 0.60 (2)	1.1	1.4 (4)	0.5	1.3 (3)	1.8 (4)
Arg	2.7 ± 0.46 (6)	2.5 ± 0.17 (6)	3.2	3.2 (9)	3.5	3.1 (7)	2.6 (6)
Pro	6.3 ± 0.62 (14)	6.3 ± 0.43 (15)	7.3	7.1 (20)	8.2	7.0 (16)	8.0 (16)
Trp	1.4 ± 0.15 (3)	1.3 ± 0.12 (3)				2.6 (6)	2.7 (6)
P.I.	38	37	38	37	35	38	38
mol wt (x1000)	27.6	29.6		29.5		28.3	28.5

[a], the 23 kDa subunit of *Acetabularia* LHCP (Apel 1977); [b], LHCP of *C. reinhardtii* (Kan and Thornber 1976); [c], LHC II of *D. tertiolecta* (Sukenik et al. 1988); [d] and [e],compositions deduced from the nucleotide sequence of AB30 of *L. gibba* (Kohorn et al. 1986) and pAB96 of pea (Coruzzi et al. 1983), respectively. The number of residues per protein is shown in parentheses. P.I., polarity index calculated by the method of Capaldi and Vanderkooi (1972)

molecular masses are estimated as 27.6 kDa for the 28 kDa polypeptide and 29.4 kDa for the 32 kDa polypeptide. The total numbers of amino acid residues in the 28 and 32 kDa polypeptides were 226 and 239, respectively. They agree with the residue numbers of higher plant preparations, which range from 226 (Kohorn et al. 1986) to 233 (Leutwiler et al. 1986). The amino acid composition and the polarity index of P4 resemble those of the P5 polypeptides (Table III-II).

Amino Acid sequence of the 32 kDa polypeptide. N-terminal amino acid sequence of the 32 kDa polypeptide of P5 was determined as far as the 38th residue, a region which is considered to extrude to the stroma (Kohorn et al. 1986) (Fig. III-10). The sequence is very homologous to those of LHC II apoproteins from *Chlamydomonas reinhardtii* and *Dunaliella tertiolecta*. A high homology also exists between *B. maxima* and higher plant proteins, in particular, in the region from the 22nd to 38th residues.

Pigment compositions ---- The pigment compositions of P4 and P5 were analyzed by HPLC (Table III-3). Chl *b* was about 30% more abundant than Chl *a* in both P4 and P5. Denatured Chl was not detected in the two Chl-protein complexes, in contrast to LHC IIs prepared by SDS gel electrophoresis, which showed significant amounts of several degradation products of both Chl *a* and Chl *b* (Fig II-5). The amounts of siphonaxanthin in P4 and P5 were about half that of Chl *a* on the

Table III-2 Amino acid composition (mol %) of the polypeptides of P4

Amino Acid	P4
Asx	7.8 ± 0.31
Thr	4.9 ± 0.73
Ser	3.9 ± 0.80
Glx	10.0 ± 0.46
Gly	13.5 ± 0.64
Ala	12.3 ± 0.35
Cys	0.6 ± 0.11
Val	6.5 ± 0.14
Met	1.3 ± 0.59
Ile	4.8 ± 0.40
Leu	9.9 ± 0.21
Tyr	3.2 ± 0.47
Phe	6.6 ± 0.25
Lys	4.5 ± 0.26
His	1.4 ± 0.35
Arg	3.1 ± 0.46
Pro	5.7 ± 0.38
Trp	-a
P.I.	38

[a] not determined

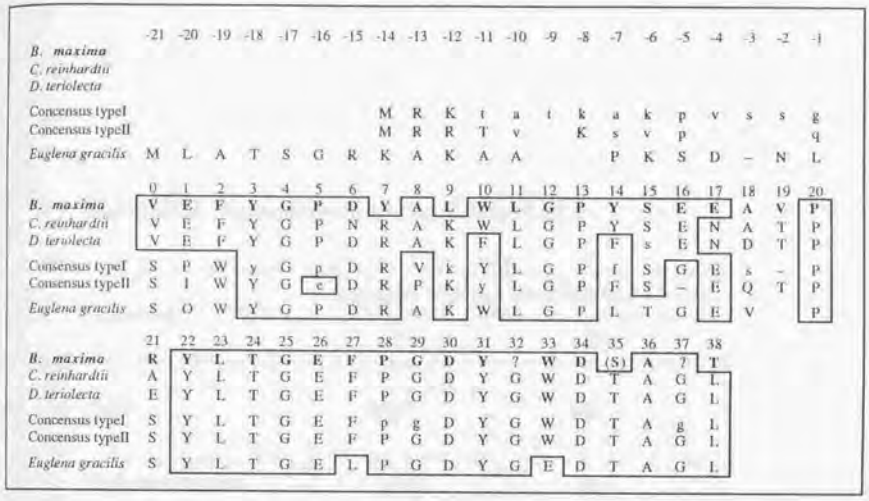


Fig III-10 N-terminal amino acid sequence of the 32 kDa polypeptide from *B. maxima* LHC II (P5). For the comparison, amino acid sequences of LHC II apoproteins from *Dunaliella tertiolecta* (LaRoche et al. 1990), *Chlamydomonas reinhardtii* (Imbault et al. 1988), *Euglena gracilis* (Muchhal and Schwartzbach 1992) and consensus sequence of two types of LHC II from higher plants LHC II, which were deduced from the sequences of all the published cab genes (Jansson and Gustafsson, 1990) are shown. The upper and lower case letters in the consensus sequences indicate the conserved and most frequent amino acid residues, respectively. A dash represents a gap which is introduced to maximize homology.

Table III-3 Pigment compositions of P4 and P5

Pigments	Pigment content (mol/100 mol Chl *a*)	
	P4	P5
Chl b	129	135
Siphonaxanthin	48.9	50.4
Siphonein	18.0	16.8
Neoxanthin	13.1	19.2
Total xanthophylls	80.0	86.4
β-carotene	n.d[a].	n.d.

[a], not detected.

molar basis. Siphonein was more abundant than neoxanthin in P4, while the reverse was the case in P5. Thus, P4 and P5 have similar but not identical pigment compositions. Molar ratios of the total xanthophylls to Chl *a* of the two complexes were significantly larger than those of LHC II isolated from spinach (0.38, Ryrie et al. 1980) and *Dunaliella* (0.43, Sukenik et al. 1988). No β-carotene was detected in the two complexes. The pigment compositions of P4 and P5 are similar to that of LHC II-1 (chapter II). This supports the notion that P4 and P5 are aggregates of LHC II-1.

Stoichiometry of pigments bound to an apoprotein --- Molar ratios of Chl *b*, siphonaxanthin, siphonein and neoxanthin to Chl *a* are 1.4, 0.5, 0.17 and 0.9, respectively, in P5 (Table III-4). Quantitative analyses of amino acids and pigments of P5 indicated that there are 187 ng Chl *a* per mg of protein. LHC II occurs usually in a trimeric form (Butler and Kühlbrandt, 1988). Judged from the intensities of the Coomassie stained bands, a molecular ratio of the 28 kDa and 32 kDa polypeptides appeared to be 1 : 2 in P5. When the two assumptions were made that a LHC II unit contained one copy of the 28 kDa polypeptide and two copies of the 32 kDa polypeptide and that the pigments were evenly distributed among the three polypeptides, a stoichiometry of 6 Chl *a* and 8 Chl *b*, 3 siphonaxanthin and 1 each of siphonein and neoxanthin per polypeptide is obtained (Table III-3). The amounts of each pigment bound to a trimeric LHC II unit are shown in parentheses.

Table III-4 Numbers of Chls and carotenoids associated with one apoprotein of P4 and P5.

Pigments	Pigment content			
	B. maxima		spinach[a]	*Dunaliella*[b]
	P4	P5	LHC II	LHC II
Chl *a*	5.4 (16)	6.1 (18)	8 (24)	4.67
Chl *b*	7.0 (21)	7.9 (24)	7 (21)	1.92
Siphonaxanthin	2.6 (8)	3.1 (9)	0	0
Siphonein	1.0 (3)	1.0 (3)	0	0
Neoxanthin	0.7 (2)	1.1 (3)	1.2 (4)	-
Lutein	0	0	2 (6)	1.96
Violaxanthin	0	0	0.6 (2)	-
Total xanthophyll	4.3 (13)	5.2 (15)	3.8 (12)	2
β-carotene	n.d.	n.d.	0.1	-

[a] Ryrie et al. (1980) and Butler and Kühlbrandt (1988), and [b] Sukenik et al. (1987). Numbers in parentheses are numbers of pigments per trimer of polypeptides.

P4 contained 170 ng Chl *a* per mg of protein and had an amino acid composition similar to that of P5. Based upon an assumption that P4 is composed of two copies of the 28 kDa polypeptide and one copy of the 32 kDa polypeptide, molar ratio of Chl a, Chl b, siphonaxanthin, siphonein and neoxanthin is estimated as 5 : 7 : 3 : 1 : 1.

Purification and polypeptide composition of LHC II' ----- P2 fraction separated by DEAE-toyopearl column chromatography (Fig. III-2) contained a 35 kDa polypeptide. This polypeptide was assigned to the apoprotein of a new minor LHC II (LHC II') resolved by SDS-PAGE (Fig II-3). Attempts were, therefore, made to purify this new Chl-protein from P2. When this fraction was subjected to gel permeation chromatography, two major peaks of 430 and 270 kDa were resolved (Fig. III–12). As shown in Fig.III-13, the 290-270 kDa fractions contain LHC II consisting of the 28 and 32 kDa polypeptides. The 430 kDa fraction contained a 35 kDa polypeptide. Two other polypeptides of 21 and 17 kDa were also resolved from this fraction. Re-chromatography of the 430 kDa fraction removed residual amounts of the 28 and 32 kDa polypeptides of LHC II but not the 21 and 17 kDa polypeptides. However, LHC II' which was isolated by SDS-PAGE lacked the two polypeptides (see Fig II-3). A possibility remains that the 21 and 17 kDa polypeptides present in P2 are contamination.

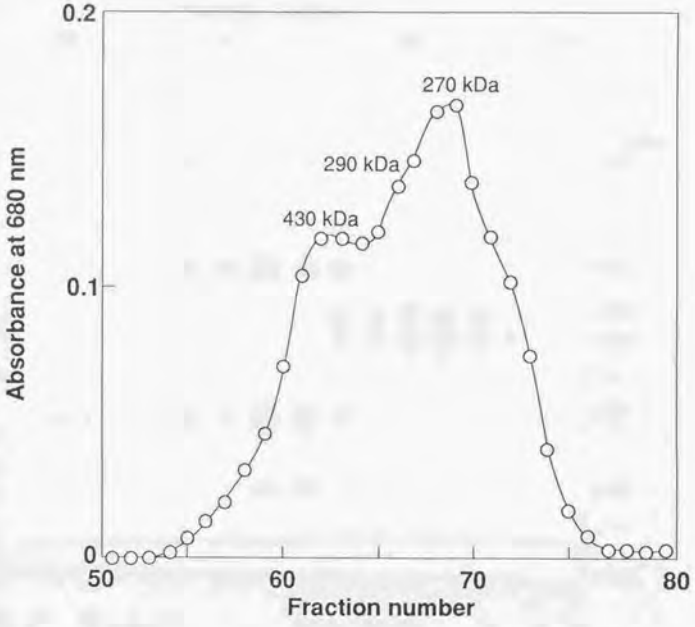


Fig. III-12 Elution profile of P2 fraction from a column of superose 6.

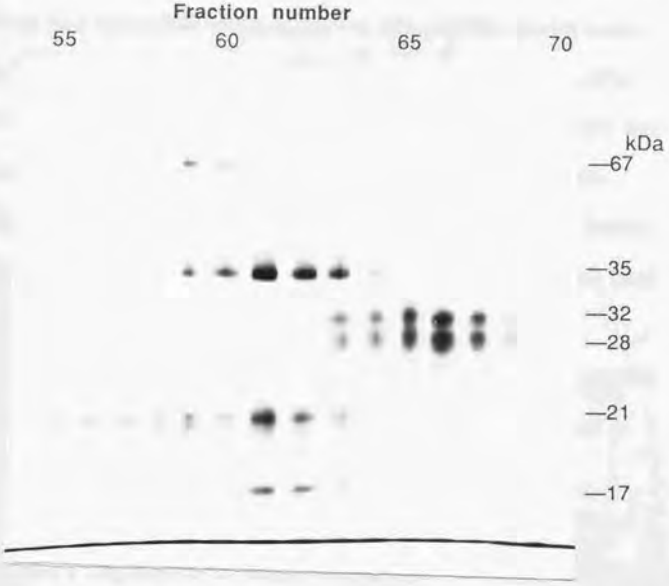


Fig. III-13 Polypeptide compositions of fractions in Fig. III-12. Fractions were treated with 2% SDS and analyzed by electrophoresis in 12% polyacrylamide gel that contained 4 M urea. The gel was stained with silver.

Absorption and fluorescence spectra of LHC II' ----- The absorption spectrum of the peak fraction of the 430 kDa Chl-protein complex is shown in Fig. III-14. The spectrum differs from that of P5 in that absorption bands of Chl *b* at 474 and 650 nm are much less significant and there is no appreciable band at 530 nm (Fig. III-14). The spectrum suggests that the Chl-protein complex has only small amounts of Chl *b*, siphonaxanthin and siphonein. Analysis of the pigment composition showed that this was indeed the case (Table III-5). The molar ratios of Chl a to Chl b and total xanthophylls to Chl were 8.4 and 0.1, respectively. Larger abundances of Chl *b* and xanthophylls present in LHC II' isolated by gel electrophoresis (see Table II-1) are, therefore, ascribed to contamination of some LHC II.

The red band of Chl *a* determined at -169°C was broad and showed a long tail on the longer wavelength side of the peak (Fig. III-15). The fluorescence emission spectrum of the purified LHC II' had a sharp peak at 680 nm (Fig. III-16).

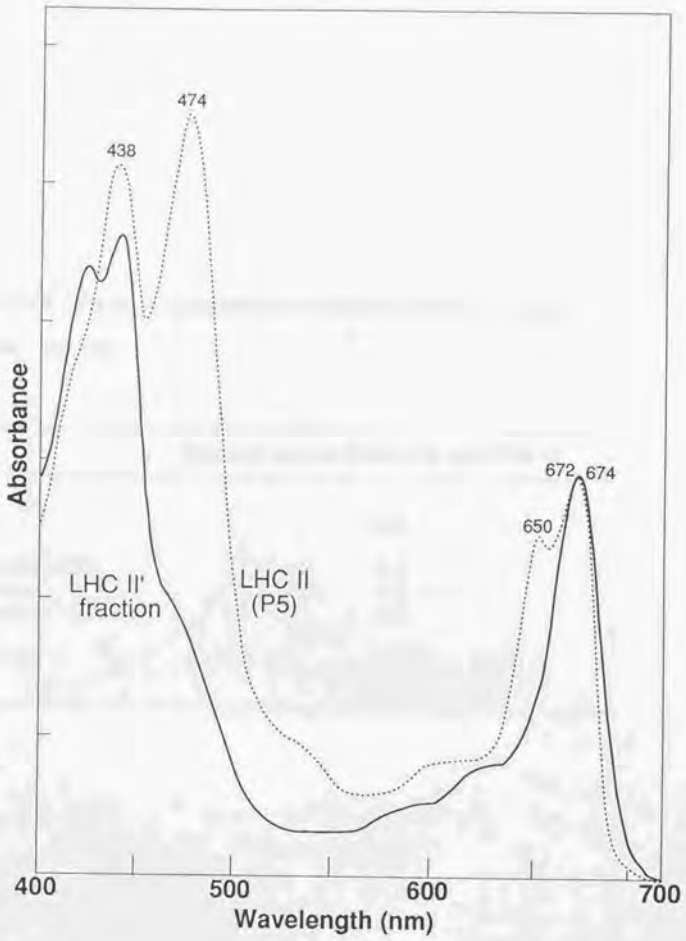


Fig. III-14 Absorption spectrum of LHC II' at room temperature. For comparison, absorption spectrum of LHC II (P5) is also shown. The two spectra were normalized at the absortion maxima of the red bands.

Table III-5 Pigment compositions of fraction of LHC II' from *Bryopsis maxima*

	Pigment content (nmol/100 nmol Chl *a*)
Chl *b*	11.9
Siphonaxanthin	3.2
Siphonein	6.2
Neoxanthin	0.8
β-carotene	6.3

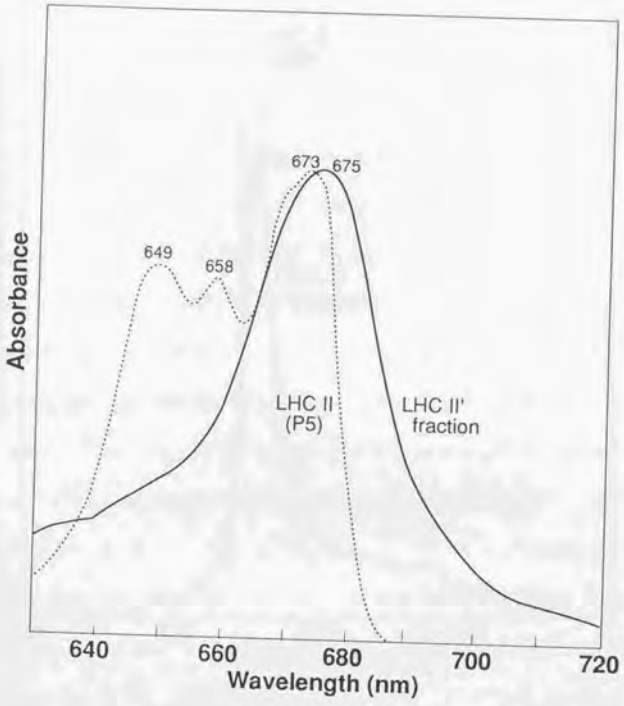


Fig. III-15 Absorption spectra of LHC II' and LHC II (P5) at liquid nitrogen temperatute.

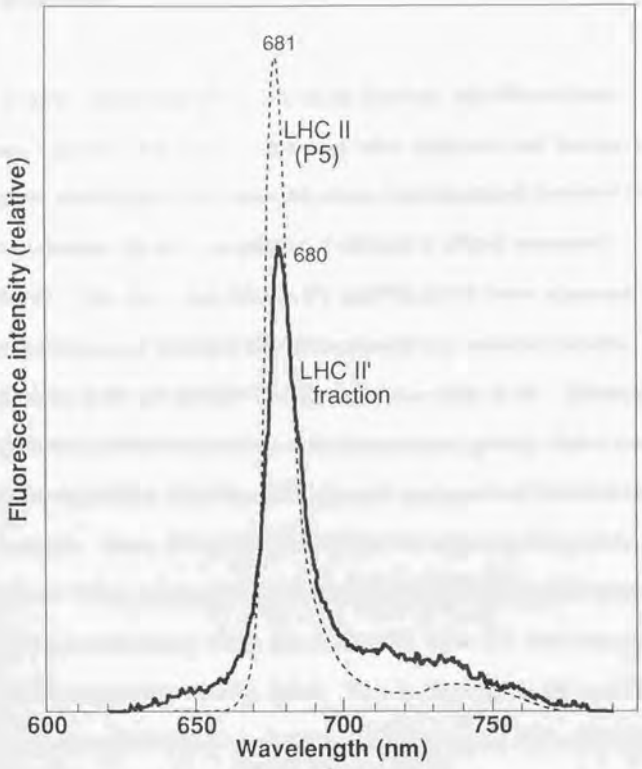


Fig. III-16 Fluorescence emission spectra of LHC II' and LHC II (P5) at the liquid nitrogen temperqture. Excitaion wavelength was 438 nm.

Discussion

A light-harvesting Chl *a*/*b*-protein fraction, solubilized from *Bryopsis maxima* thylakoid membranes with digitonin and fractionated by sucrose density gradient centrifugation, was separated into five bands by ion-exchange chromatography on a column of DEAE-toyopearl (Fig. III-2). The two major bands, P4 and P5, which have apparent molecular masses of 280 and 295 kDa, respectively, contain the two polypeptides of 28 and 32 kDa in different ratios (Fig III-4). Although they showed identical absorption and fluorescence spectra, there were slight but significant differences in pigment compositions between the two preparations. Thus, P4 and P5 are similar but distinct Chl-protein complexes. When subjected to mild SDS-PAGE, both P4 and P5 yielded a single Chl-protein band, which corresponds to LHC II-1 with an apparent molecular mass of 72 kDa (Fig. III-6). This indicates that P4 and P5 are oligomeric forms of LHC II-1, which possibly represent the more native state of LHC II.

The occurrence of two types of LHC IIs, which have two apoproteins in different ratios, has been reported in higher plants (Staehelin and Arntzen 1983). The major population of LHC II which binds tightly to PS II reaction center complexes in the appressed grana region of the thylakoids, while another loosely bound population migrates laterally in the thylakoid membranes when phosphorylated. The phosphorylated

LHC II has a higher content of a low molecular mass polypeptide than the bulk of LHC II (Larsson and Andersson 1985). P4 and P5 may correspond to the loosely and tightly bound forms of LHC II, respectively, since the former contains the 28 kDa polypeptides more abundantly than the 32 kDa polypeptide and the reverse is the case with the later.

It is to be stressed that P4 and P5 were highly purified without significant loss of pigments. This enabled us to determine accurate and native pigment composition of the Chl-protein complexes. Assuming even distribution of the pigment among the three polypeptides, one polypeptide of P5 is estimated to carry 6 Chl *a*, 8 Chl *b* and 5 xanthophylls. The stoichiometry of 5 Chl *a*, 7 Chl *b* and 5 xanthophylls per polypeptides was obtained with P4. When amounts of the bound pigments are taken into calculation, the total molecular mass of LHC II-1, which consists of one 28 kDa polypeptide and two 32 kDa polypeptides, is 130 kDa. The complex would also carry the detergent and lipids. We suggest, therefore, that P5 (and P4), which has an apparent molecular mass of 295 kDa (and 280 kDa), contains two LHC II-1 trimers. On the other hand, the apparent molecular mass of LHC II-1 determined by gel electrophoresis is 72 kDa. This may be ascribed to an undenatured conformation of the complexes in gel. Anomalous behaviors of Chl-proteins in gel have been well documented (Thornber 1986). A model of molecular organization of P5 is shown in Fig. III-11.

Bryopsis LHC II resemble the corresponding Chl *a*/*b* protein of higher plants in several respects. The amino acid compositions of the

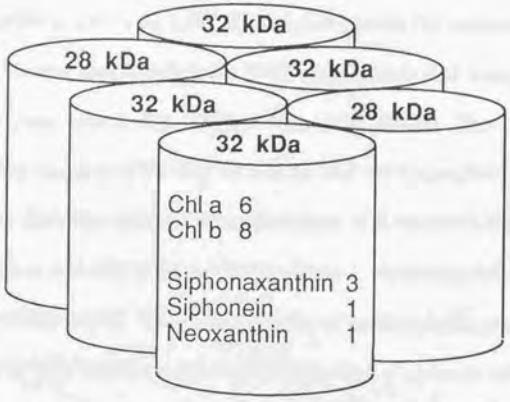


Fig. III-11 The model of molecular organization of P5

28 kDa and 32 kDa polypeptides were similar to those of LHC II from green algae and higher plants (Coruzzi et al. 1983, Kohorn et al. 1986). The N-terminal amino acid sequence of the 32 kDa polypeptide is highly homologous to the corresponding parts of LHC II polypeptides of other green algae and higher plants. The total number of Chl molecules bound to a polypeptide is 14-15 for LHC II of higher plants (14 molecules) (Burke et al. 1978, Butler and Kuhlbrandt 1988 Kuhlbrandt and wang 1992) and *Chlamydomonas reinhardtii* y-1 (Bar-Nun et al. 1977). The corresponding number of P5 and P4 are 14 and 12, respectively. These data suggest that the molecular organization of *Bryopsis* LHC II is similar to those of LHC II from higher plants. However, two Chl *a* are replaced by Chl *b* in P5. The total number of xanthophylls per a P5 polypeptide is also significantly larger than that of spinach LHC II polypeptide which contains 4 xanthophylls per polypeptide (Ryrie at al. 1980).

Histidine is considered to be involved in the binding of Chl. Sukenik et al. (1988) suggested binding of Chl *b* to histidine residues of LHC II proteins from *Dunaliella*. However, the number of histidine residues in LHC II apoproteins of *B. maxima* was significantly smaller than that of Chl *b*. This suggests that Chl *b* (and Chl *a*) binds to other amino acid residues such as glutamine and asparagine residues (LaRoche et al. 1990)

LHC II' was also highly purified in the present study. This Chl-protein contains the 35 kDa polypeptide but lacks the 28 and 32 kDa

polypeptides. The two other polypeptides of 17 and 22 kDa were present in this preparation but not in LHC II' isolated by gel electrophoresis (Fig. II-2). Thus, the 35 kDa polypeptide appears to be the sole apoprotein of LHC II'. The results conclusively rule out a possibility that the 35 kDa polypeptide is an apoprotein of LHC II (Anderson 1985). The present study also shows that the abundances of Chl *b* and xanthophylls in LHC II' determined in Chapter II are overestimation due to comigration of LHC II with the Chl-protein in gel. The purified LHC II' contains only small amount of Chl *b* and xanthophylls. Because the Chl-protein was isolated together with LHC II and PS II reaction center complexes, LHC II' is considered to be a minor antenna Chl *a*/*b* protein which, like CP29 or CP26, functions in excitation energy transfer from LHC II to the PS II reaction center complex.

Chapter IV

Chlorophyll forms and excitation energy transfer between pigments of LHC II.

Summary

Chlorophyll (Chl) forms in light harvesting Chl *a*/*b* protein complex (LHC II) from the siphonous green alga, *Bryopsis maxima*, were analyzed by means of the Gaussian deconvolution. Three major Chl *a* forms (Ca664, Ca672 and Ca679) and one minor form (Ca688) were resolved. In addition, two Chl *b* forms were resolved at 648 and 653 nm. Taking the number of Chl *a* and Chl *b* bound to an apoprotein into consideration, two Chl *a* each are assigned to the three major Chl *a* forms, and three and five Chl *b* to Cb648 and Cb653, respectively. Ca688 was suggested to be a Chl *a* form which occurred only a trimeric form of LHC II. Gaussian deconvolution of the fluorescence emission spectra showed that all the Chl *a* forms emitted fluorescence, Ca679 being the strongest emitter. A fluorescence emission from Chl *b* was also detected for the first time. The occurrence of very efficient energy transfer from siphonaxanthin or Chl *b* to Chl *a*, and even an uphill transfer from Chl *a* to Chl *b*, were demonstrated by measurement of the excitation spectra at 15°C. Efficient energy transfer from siphonaxanthin or Chl *b* to Chl *a* was also observed at the liquid nitrogen temperature. However, Chl *b* was found not to mediate energy transfer from siphonaxanthin to Chl *a*. Measurement of polarization of the Chl fluorescence also suggested that siphonaxanthin transferred its excitation energy directly to Chl *a*.

Introduction

In the preceding chapter, the two forms of purified LHC II from *B. maxima* were prepared and analyzed in terms of polypeptide and pigment compositions. The stoichiometry of photosynthetic pigments per a unit polypeptide was determined to be six Chl *a*, eight Chl *b*, three siphonaxanthin and one each of siphonein and neoxanthin. Although the total number of Chl per polypeptide is close to that of pea LHC II (Butler and Kühlbrandt 1988), the abundance of Chl *b* relative to Chl *a* is the reverse to that of LHC II in higher plants.

It is well known that there are several distinct groups of Chl *a* molecule, which show different absorption maxima in the red band region *in situ* (Brown et al. 1974). They are called Chl forms and spectrophotometrically resolved by means of a computer simulation by assuming a Gaussian for the absorption band of each component. In the first half of this chapter, Chl *a* and Chl *b* forms of *Bryopsis* LHC II were analyzed. The Gaussian deconvolution of the fluorescence emission spectrum was also carried out to relate the fluorescence components to the Chl forms. *Bryopsis* LHC II was found to show a fluorescence emission from Chl *b* at 655 nm at 15°C. This allowed us to investigate the function of Chl *b* in excitation energy transfer from xanthophylls to Chl for the first time. Excitation energy transfer between different pigments was investigated in detail by spectrofluorometry techniques in the second half of this chapter.

Materials and Methods

Isolation of LHC II ----- LHC II (P5) of *Bryopsis maxima* was prepared as described in chapter III. LHC II from spinach were purified by the method adopted for preparation of *Bryopsis* LHC II. Thylakoid membranes were washed three times with 1 mM EDTA (pH 8.0) and treated with 0.8 % digitonin (detergent to Chl ratio of 15) at 4°C for 8 h. After centrifugation at 30,000 x g for 1 h, the supernatant was applied onto a DEAE-toyopearl column (2.2 x 20 cm) equilibrated with 2 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.

Spectroscopic analyses ----- Absorption spectra were measured with a Hitachi 330 spectrophotometer. For measurements at -196°C, samples were suspended in 50% potassium glycerophosphate and 25% glycerol. Emission and excitation spectra of fluorescence were measured with a Hitachi 850 spectrofluorometer. For the measurement of polarized excitation spectrum, fluorescence emissions parallel and perpendicular to the direction of polarized excitation light were detected through glass plate polarizers. The degree of polarization, p, is defined as

$$p = \frac{I_{/\!/} - I_{\perp}}{I_{/\!/} + I_{\perp}}$$

where $I_{/\!/}$ and $I_{\perp}$ are the intensities of the emissions, which are oriented parallel and perpendicular to the polarization of exciting light. The band widths of excitation and emission light were 2 nm. Low temperature

fluorescence was determined with samples which had been mixed with an equal volume of 30% polyethyleneglycol 4000. For the deconvolution of absorption and fluorescence spectra, spectral data were stored in a computer at 0.2 nm intervals and analyzed by the curve-fitting procedure described by Mimuro et al. (1982). The second derivative of the spectra were obtained according to the method of Savitzky and Golay (1964).

Results

Deconvolution of absorption spectra ----- As described in chapter III (Fig. III-8), the absorption spectrum of *B. maxima* LHC II showed a broad band with two maxima at 650 and 672 nm in the red region at 15°C. The second derivative of the spectrum showed four maxima at 651, 664, 672 and 678 nm. In addition, two shoulder appeared at 645 and 688 nm as indicated by arrows (Fig. IV-1A). The maxima at 664, 672, 678 nm are assigned to Chl *a* and those at 645 and 651 nm to Chl *b*. Additional peak of Chl *a* was clearly resolved at 658 nm in the spectrum determined at -196°C (Fig. IV-1B). The second derivative of the low temperature spectrum more clearly showed an absorption maximum of Chl *b* at 646 nm. The maxima were shifted by 1 to 6 nm to shorter wavelength at -196°C.

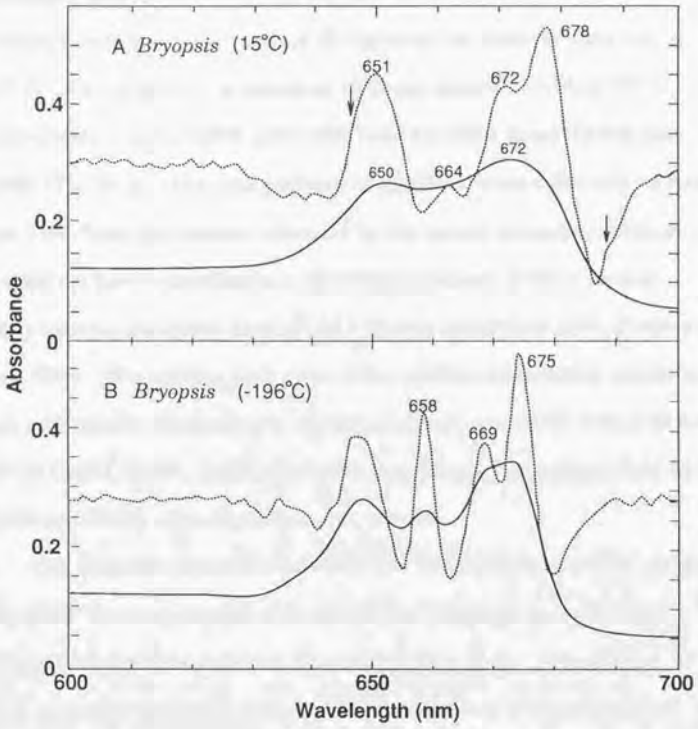


Fig. IV-1 Absorption and second derivative spectra of LHC II from *B. maxima*. (A) 15°C, (B) -196 C. Solid lines, absorption spectra; Dotted line, the second derivatives of the spectra.

Gaussian deconvolution of the red Chl band was carried out by a program constructed by Mimuro (1982). The relative amplitude, center wavelength and band width of the Gaussian were varied to obtain the best fit of the sum of spectra of resolved Chl forms to the observed spectrum of LHC II. 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. IV-2). The peak positions of the Chl *a* forms differ only by less than 1 nm from the maxima estimated by the second derivative spectrum. The 636 nm band is ascribed to a vibrational structure of Chl *a* form(s) rather than an absorption band of Chl *b* (Brown and Schoch 1981, Fragata et al. 1988). The relative peak areas of the resolved components, which had been estimated by integration 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.

The Gaussian deconvolution of the low temperature spectrum yielded essentially the same results, although all the components resolved had narrower band widths and were blue-shifted (Fig. IV-3). Two Chl *b* forms were also resolved at 646 and 650 nm. Ratios of the peak areas of individual components were also similar to those estimated at 15°C.

Deconvolution of fluorescence spectra ----- Fig. IV-4A shows fluorescence spectra of LHC II at 15°C excited by light which is predominantly absorbed by Chl *a* (438 nm), Chl *b* (475 nm) or

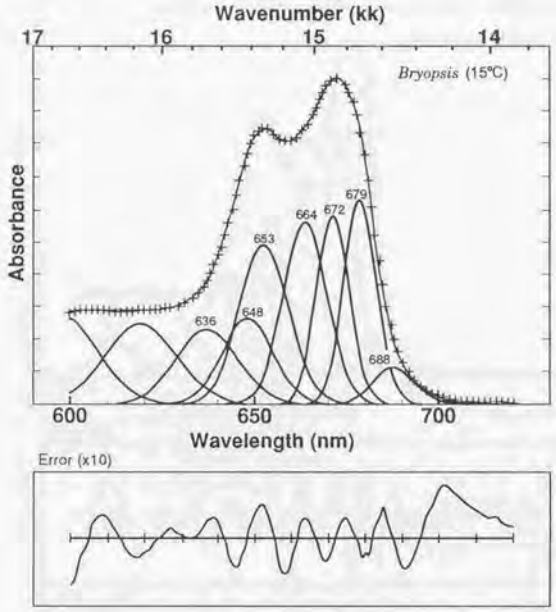


Fig. IV-2 Deconvolution of absorption spectra of LHC II at 15°C. Plus sign indicate real data points. Thick solid lines show deconvoluted components. Thin solid line is the spectrum constructed by summation of all the component bands resolved. The difference between the observed and estimated absorption spectra is shown in the lower part of the figure.

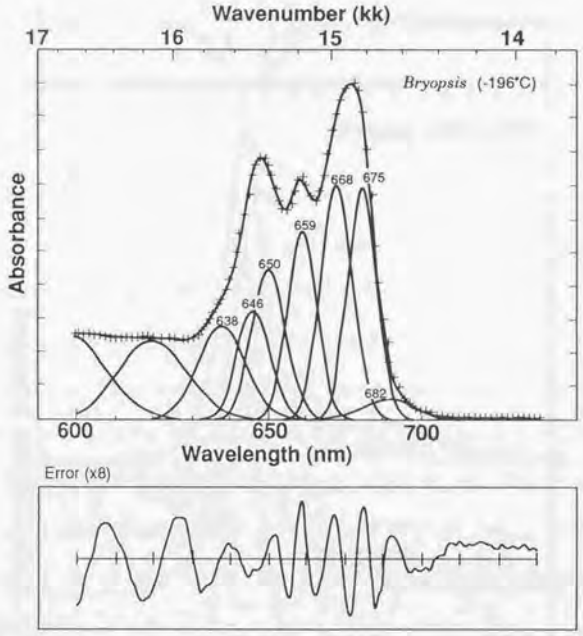


Fig. IV-3 Deconvolution patterns of absorption spectra of LHC II at -196°C. Plus sign indicate real data points. Thick solid lines showed deconvolution curves.

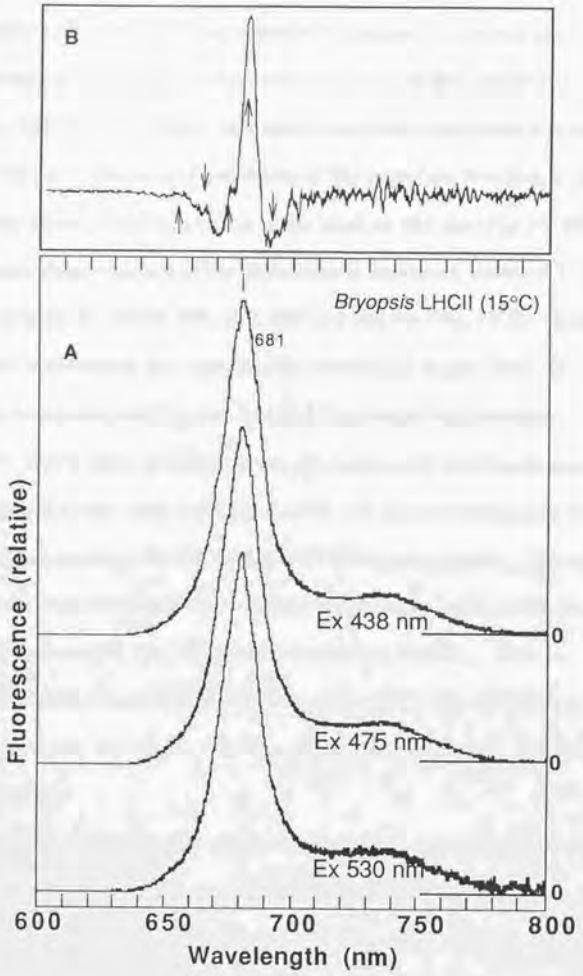


Fig. IV-4 Fluorescence emission spectra of LHCII at 15°C. (A) Excitation wavelengths were 438, 475 and 530 nm. (B) Second derivative of the fluorescence emission spectrum. Excitation wavelength was 475 nm. Arrows in (b) indicate the locations of components adopted for the deconvolution in Fig. IV-5.

siphonaxanthin (530 nm). Irrespective of the pigments excited, the spectra showed a single major emission from Chl *a* at 681 nm with a shoulder at 720-740 nm. There was also a small but significant shoulder or tailing at 650 nm. The second derivative of the spectrum revealed several peaks or shoulders in addition to the major peak at 681 nm (Fig. IV-4B). The Gaussian deconvolution of the fluorescence spectrum resolved 5 emission components at 655, 665, 673, 681 and 692 nm (Fig. IV-5). Because Chl *a* emits fluorescence at a wavelength several nm longer than its absorption maximum and the number and location of the emission components match those of Chl *a* forms, the major 681 nm fluorescence band is ascribed to the emission from Ca679, and minor components at 665, 673 and 692 nm are from Ca664, Ca672 and Ca688, respectively. It is to be noted that there is also a weak but significant emission band at 655 nm, which can be ascribable to a Chl *b* (most likely from Cb653). This is remarkable because fluorescence emission from Chl *b* has never been reported previously in cells, thylakoids or LHC II from higher plants and other green algae.

In order to determine the efficiency of excitation energy transfer from Chl *b* and carotenoids to Chl *a*, the excitation spectra of Chl *a* fluorescence were determined at 680 and 735 nm at 15°C (Fig. IV-6). The two spectra obtained were similar to each other and also to the absorption spectrum (see Fig. IV-9). This indicates that efficiency of energy transfer from Chl *b* and siphonaxanthin to Chl *a* is very high. The excitation spectrum for the emission from Chl *b* at 650 nm was also determined.

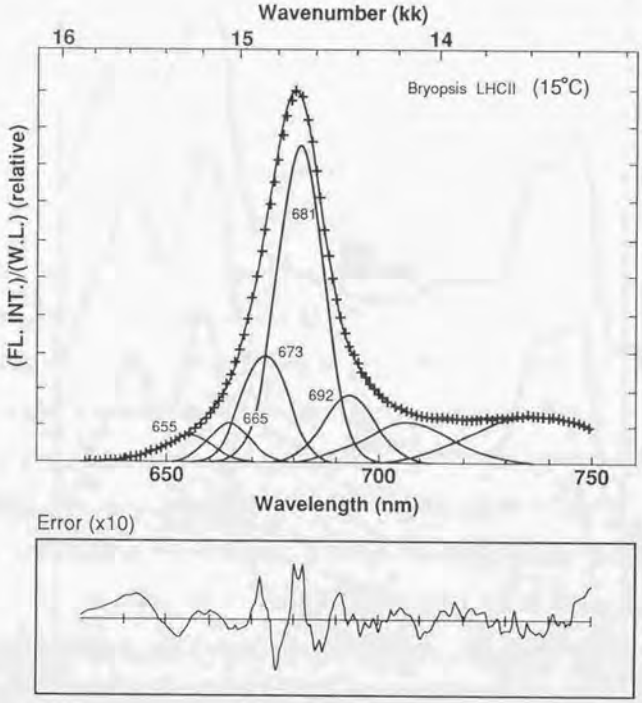


Fig. IV-5 Deconvolution of the fluorescence emission spectrum at 15°C. Excitation wavelength was 475 nm. Plus signs indicate real data points.

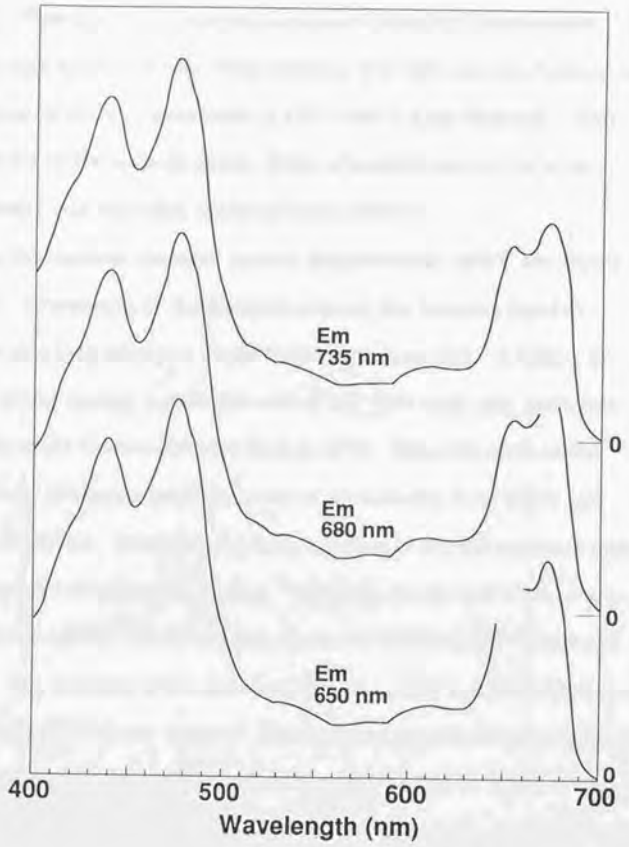


Fig. IV-6 Excitation spectra of LHC II at 15 C monitored at 735, 680 and 650 nm, respectively. Spectra were normalized at their maxima. Gaps appeared around the measuring wavelength.

The spectrum obtained was similar to those determined for the Chl *a* emission. Note that the excitation spectrum of the Chl *b* fluorescence showed a peak at 670-680 nm. This indicates that light energy absorbed by the red band of Chl *a* is transferred to Chl *b* with a high efficiency. This is an uphill-transfer because energy levels of excited state of Chl *a* are considerably lower than that of excited state of Chl *b*.

The fluorescence emission spectra determined at -196°C are shown in Fig. IV-7. Irrespective of the pigments excited, the emission band at 681 nm with a long tailing at longer wavelength appeared. A tailing or shoulder at the shorter wavelength side of the main peak was much less significant at the low temperature than at 15°C. When the scale of the ordinate was 10 times expanded, however, an emission from Chl *b* was detected at 650 nm. Interestingly, the magnitude of the 650 nm band varied depending upon the pigments excited. When the Soret band of Chl *b* was preferentially excited (465 or 475 nm), a notable emission was observed at 650 nm. The intensity of the emission band was much less on excitation of the Soret band of Chl *a* at 438 nm. The weak emission can be ascribed to a small fraction of incident light which is absorbed by Chl *b*. Thus, excitation energy transfer from Chl *a* to Chl *b* is inefficient or negligible at -196°C. Note that the 530-nm excitation did not induce any emission from Chl *b*. This result indicates that light energy absorbed by siphonaxanthin and siphonein is not transferred to Chl *b* at the low temperature.

The emission spectrum of Chl *b* fluorescence determined with the 465-nm excitation was different from that determined with the 475-nm

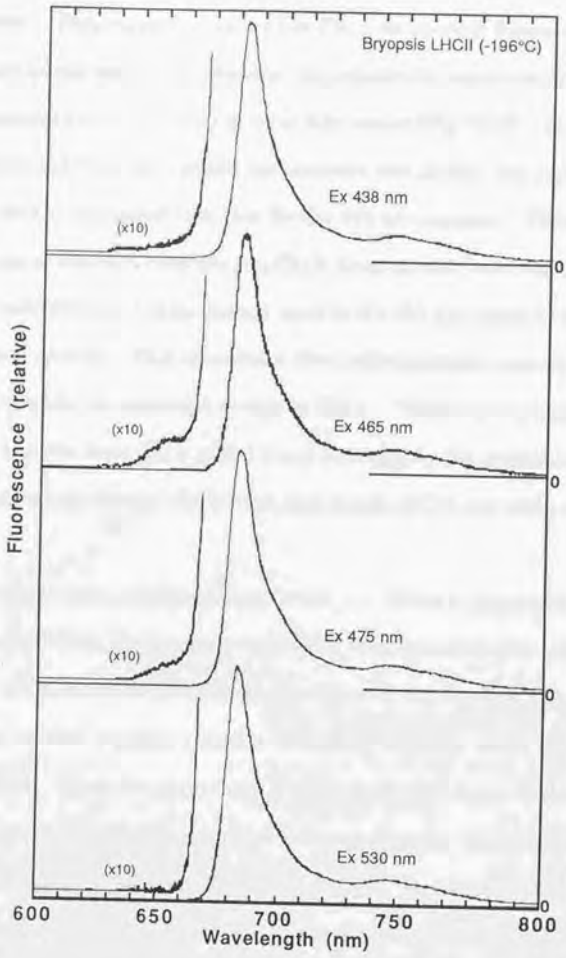


Fig. IV- 7 Fluorescence emission spectra of LHC II at –196°C.

excitation. This suggests that the two Chl *b* forms emit fluorescence at different wavelengths. To examine this possibility, excitation spectra for the emission at 645 and 655 nm were determined (Fig. IV-8). The excitation spectrum for the 655 nm emission was slightly but significantly red-shifted as compared with that for the 645 nm emission. This supports fluorescence emission from the two Chl b forms (Cb648 and Cb653). It is also of note that there is no distinct band in the 530 nm region in the two excitation spectra. This is evidence that siphonaxanthin and siphonein cannot transfer its excitation energy to Chl *b*. Furthermore, inefficient energy transfer from Chl *a* to Chl *b* was indicated by the excitation spectrum which showed diminished blue bands of Chl *a* at 430 nm.

Fluorescence polarization spectrum ----- When a pigment is excited by polarized light, fluorescence emits from the pigment is also polarized. Degree of fluorescence polarization decreases as light energy is transferred from the excited pigment molecule to another molecule which has different orientation. Thus, the magnitude of decrease in the degree of fluorescence polarization is a measure of number of energy transfer steps involved prior to the fluorescence emission (Fragata et al. 1988, van Metter 1977, Whitmarsh and Levine 1974). Fig IV-9 shows the data obtained from experiments, in which different pigments were excited by polarized light and the degrees of polarization of fluorescence from Chl *a* were monitored at 680 nm. Experiments were carried out at 15°C. It is seen that the degree of polarization is highest in the wavelength region of 510 to 530 nm,

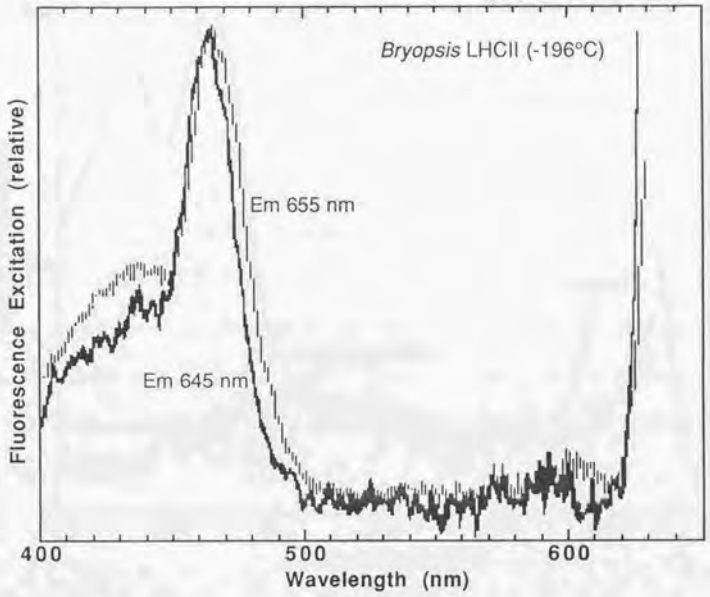


Fig. IV-8 Fluorescence excitation spectra of LHC II for the emission monitored at 645 and 655 nm at –196°C.

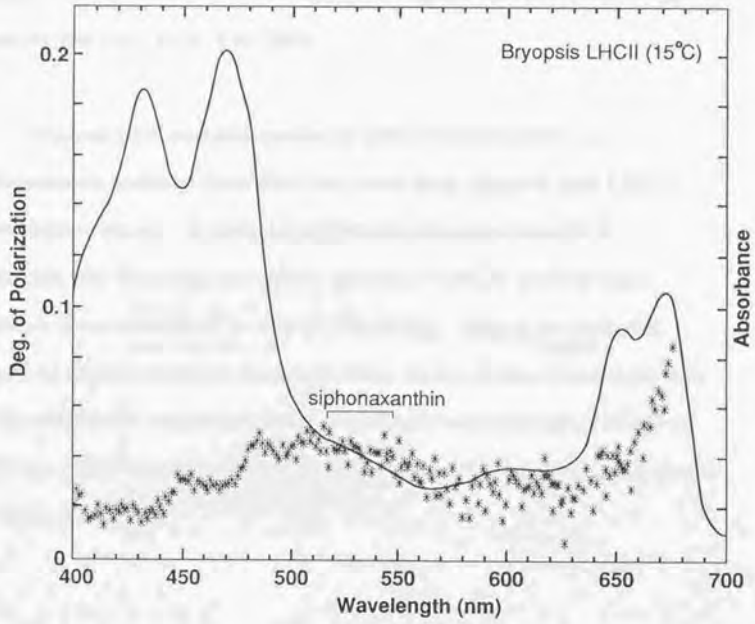


Fig. IV-9 Degree of polarization of Chl*a* fluorescence as a function of excitation wavelength. Solid line, absorption spectrum.

where light is mainly absorbed by siphonaxanthin and siphonein, except for the red band of Chl *a*. Lower degrees of the polarization were obtained when Chl *b* was excited at about 650 nm. This result is consistent with the notion that siphonaxanthin and siphonein transfer its excitation energy directly, but not via Chl *b*, to Chl *a*.

Fluorescence emission spectra of LHC II from spinach ----- Fluorescence emission from Chl *b* has never been reported with LHC II from higher plants. In order to confirm the absence of the Chl *b* emission, the fluorescence emission spectra of LHC II purified from spinach were determined at -196°C (Fig. IV-10). Even at an expanded scale, no distinct emission band from Chl *b* on the shorter wavelength side of the main band was detected even when Chl *b* had been excited at 465 or 475 nm. This confirms that the emission band of Chl *b* is a unique feature of *Bryopsis* LHC II.

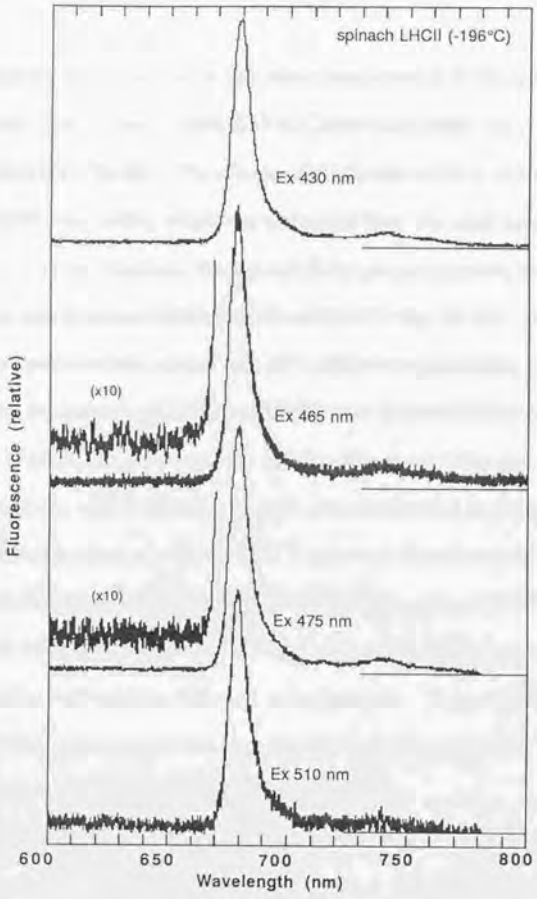


Fig. IV-10 Fluorescence emission spectra of LHCII from spinach at –196°C. Emission spectra excited at 430, 465, 475 and 510 nm, respectively.

Discussion

Gaussian 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). The relative abundances of Chl *a* in Ca664, Ca672, Ca679 and Ca688, which are estimated from the peak areas, are about 1 : 1 : 1 : 0.5. Because, there are 6 Chl *a* per polypeptide, two Chl *a* each can be attributed to Ca664, Ca672 and Ca679 (Fig. IV-11). Ca688 may be a Chl *a* species which occurs only in a trimeric organization of LHC II. The relative peak areas of Cb648 and Cb653 were 0.36 and 0.64 respectively. Thus, out of eight Chl *b* present in a polypeptide, three Chl *b* are assigned to Cb648 and the rest to Cb653. The Chl *a* forms resolved in *Bryopsis* LHC II resemble those of spinach LHC II reported (Satoh and Butler 1978), except that Ca672 is more abundant than others in spinach LHC II (Brown and Schoch 1981). Different Chl *a* forms are ascribed different molecular environments surrounding Chl *a* in an apoprotein. Thus, the occurrence of similar Chl a forms indicates that the molecular organization of LHC II is similar between the alga and higher plant.

Only one Chl *b* form has been reported for LHC II from higher plants (Satoh and Butler 1978, Brown and Schoch 1981), *Dunaliella* and *Euglena* (Brown et al. 1974). However, the occurrences of two Chl *b* components have been postulated by a deconvolution of the absorption and circular dichroism spectra of spinach LHC II (van Matter 1977). The two forms of Chl *b* were more clearly resolved in *Bryopsis* LHC II, which

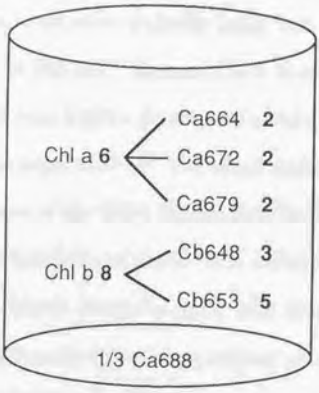


Fig. IV-11 A proposed composition of the Chl forms in an apoprotein of *Bryopsis* LHC II

contain larger amount of Chl *b*.

Energy transfer between different pigments in LHC II ----

A most interesting feature of the fluorescence spectrum of *Bryopsis* LHC II is the occurrence of an emission band from Chl *b* in the wavelength region at 655 nm. Because Chl *b* fluorescence has never been reported in LHC II from higher plants, this should be ascribed to the high Chl *b* content of the algal LHC II. The broad emission band pattern and the excitation spectra of the Chl *b* fluorescence indicate that fluorescence is emitted from the two forms of Chl *b*. It is evidence that Cb648 and Cb653 cannot transfer excitation energy to Chl *a* with 100% efficiency. However, this would not significantly affect the quantum efficiency of photosynthesis because the fluoresce is very weak.

The occurrence of the Chl *b* fluorescence provided novel and important information about energy transfer among different photosynthetic pigments. Generally, excitation energy is transferred according to the singlet excitation energy levels of pigments. The lowest excitation energy levels decrease in the order of carotenoid, Chl *b* and Chl *a*. It has long been postulated, therefore, that excitation energy is transferred in the following order.

carotenoids $\rightarrow$ Chl *b* $\rightarrow$ Chl *a*

The emission and excitation spectra determined in this study show that light energy absorbed by siphonaxanthin, siphonein and Chl *b* is transferred to Chl a with a very high efficiency. The most interesting

finding obtained in the present study is that Chl *b* does not mediate energy transfer from carotenoids to Chl *a*, contrary the long-held postulation. No emission from Chl *b* was detected at-196°C when siphonaxanthin and siphonein were excited, indicating the absence of energy transfer from the xanthophylls to Chl *b* (Fig. IV-7). The low temperature excitation spectrum clearly demonstrated that the Chl *b* fluorescence was sensitized only or predominantly by Chl *b* by itself and light absorbed by Chl *a* and carotenoids were ineffective in excitation of Chl *b*. Measurement of the polarization of Chl *a* fluorescence also supports direct energy transfer from siphonaxanthin to Chl *a*. These results altogether indicate the following order of energy transfer,

Chl *b* → Chl *a*

carotenoids → Chl *a*.

Another interesting finding is the occurrence of uphill-transfer from Chl *a* to Chl *b* at room temperature. An uphill-transfer, *i. e.*, energy transfer against a gradient of energy level is generally considered to occur with a low probability. In fact, no evidence was obtained for any uphill-transfer of excitation energy at -196°C. However, the excitation spectrum of the Chl *b* fluorescence revealed that light energy absorbed by the red band of Chl *a* (above 670 nm) was transferred to Chl *b* (650 nm) with a high efficiency at 15°C. At 15°C, the Chl *b* fluorescence at 655 nm was observed on excitation of siphonaxanthin and siphonein. This can be ascribed to the uphill-energy transfer from Chl *a* to Chl *b* because light energy absorbed by the carotenoids is efficiently transferred to Chl *a*.

Chapter V

Energy transfer from chlorophyll *b* and carotenoids to chlorophyll *a* studied by means of picosecond time-resolved fluorescence spectroscopy.

Summary

Energy transfer among different photosynthetic pigments of LHC II isolated from *Bryopsis maxima* was kinetically analyzed by picosecond time-resolved fluorescence spectroscopy. The emission spectra determined in the picosecond time region showed that Chl *b* is not involved in energy transfer between carotenoids (siphonaxanthin and siphonein) and Chl *a*. The carotenoids transferred excitation energy only and directly to one Chl *a* form, Ca672, at -196°C, indicating a tight coupling of siphonaxanthin or siphonein molecules with Chl *a* molecules of Ca672 in LHC II. At the physiological temperature, fluorescence emissions from the four Chl *a* forms were observed immediately after siphonaxanthin excitation and the relative intensities of the emission bands remained unchanged during measurement. This indicates that excitation energy is transferred from the carotenoids to Chl *a* and equilibrates among Chl *a* molecules within the limiting time of the equipment (< 3 ps). However, the energy equilibrium between Chl *b* and Chl *a* molecules was established at 25 ps when siphonaxanthin or Chl *b* was excited. The results obtained are discussed by comparison with the data obtained with spinach LHC II.

Introduction

LHC II is the most abundant Chl *a*/*b*-protein complex present in the thylakoid membranes of higher plants and green algae, which plays an important role in light-harvesting of photosynthesis (Thornber et al. 1991). Excitation energy absorbed by pigments of LHC II is rapidly and efficiently transferred to the reaction center of photosystem II. Efficient transfer of absorbed light energy is supported by the molecular organization of pigments in LHC II (Knox 1986). The arrangement of Chl molecules in LHC II and the three-dimensional structure of protein were investigated by the electron diffraction method (Kuhlbrandt and Wang 1991). In this model, 15 Chl molecules are distributed in an apoprotein with distances from 9 to 14 Å between nearest neighbors. However, the species and absorption forms of individual Chl molecules were not known. Recently, a model with 14 Chl molecules (8 Chl *a* and 6 Chl *b*) was proposed by the same investigators (Kuhlbrandt and Wang 1992).

The pigment composition of *Bryopsis* LHC II was determined to be 6 Chl *a*, 8 Chl *b*, 3 siphonaxanthin and one of each siphonein and neoxanthin per apoprotein (Chapter III). Each of Chl *a* forms (Ca664, Ca672, and Ca679) consists of two Chl *a* molecules, whereas two forms of Chl *b* (Cb648 and Cb653) contain 3 and 5 Chl *b* molecules, respectively.

Energy transfer from Chl *b* to Chl *a* is highly efficient in spinach LHC II (van Metter 1977) so that no fluorescence emission from Chl *b* is

detected in LHC II of higher plants even when Chl *b* is directly excited. The excitation polarization spectra of fluorescence monitored by Chl *a* emission showed a negative low degree of polarization in the wavelength region of Chl *b* (Hemelrijk et al. 1992, van Metter 1977), indicating a larger angle between their dipole moments. According to the model developed by van Metter and Knox, three Chl *b* molecules are strongly coupled (exciton) in a trimer of LHC II and Chl *a* molecules are located near the peripheral region of the LHC II molecule (Lin and Knox 1988). However, the strongly coupled Chl groups could not be detected in the three-dimensional structure proposed by Kuhlbrandt and Wang (1991). Significant interaction between Chl *b* and Chl *a* was observed in LHC II solubilized with *n*-octyl-β-D-glucopyranoside, but SDS disrupted the integration between pigments (Ide et al. 1987).

Carotenoids are also important light harvesting pigment of LHC II (Siefermann-Harms 1985). The efficiency of energy transfer from lutein to Chl *a* was reported to be 100% in LHC II of *Lactuca* (Siefermann-Harms and Ninnemann 1982). However, little is known about molecular arrangement of carotenoids and the mechanism of energy transfer from carotenoids to Chl *a*.

In the previous study, the steady state fluorescence spectra of LHC II from *B. maxima* showed an emission from Chl *b* when Chl *a*, Chl *b* or siphonaxanthin had been excited at 15°C (Chapter IV). This is a unique feature of *Bryopsis* LHC II because, as stated above, fluorescence emission from Chl *b* has never been observed in LHC II from higher

plants and other green algae. The excitation spectrum and excitation polarization spectrum also indicated that siphonaxanthin and siphonein directly transfered excitation energy to Chl *a*.

Picosecond time-resolved absorption or fluorescence spectroscopy provides important information about the mode and kinetics of energy transfer among different pigments (Breton and Geacintov 1980). A 6-ps component was assigned to the energy transfer from Chl *b* to Chl *a* (Gillbro et al. 1985). However, no energy transfer between Chl *b* molecules during the lifetime of the excited Chl *b* was detected. A faster transfer time of excitation energy from Chl *b* to Chl *a* was reported in the thylakoid membranes of a *Chlamydomonas reinhardtii* mutant which lacked the reaction centers of the two photosystems (Eads et al. 1989).

In this study, kinetics of energy transfer between pigment molecules in purified *Bryopsis* LHC II was studied with picosecond laser pulses. Changes in the fluorescence emission spectrum excited by laser pulses were measured at 15 and -196°C to determine energy transfer between different pigment species and different Chl forms.

Materials and Methods

Preparation of LHC II --- LHC IIs from *Bryopsis maxima* and spinach were purified by the method described in chapter III and IV.

Optical Measurements --- The time-resolved fluorescence spectra were determined with a single photon counting equipment assembled by Nishimura et al. (Yamazaki et al. 1984, Nishimura et al. 1991) (Fig V-1). The excitation pulse was obtained by a picosecond laser system composed of a synchronously-pumped, cavity-dumped dye laser and a mode-locked Nd-YAG laser connected with a pulse compressor (Spectra Physics 3800). The original pulse (1064 nm, 100 ps) was compressed to about 5 ps and converted to 532 nm. The repetition rate of the cavity-dumper was 4.0 MHz. For excitation of siphonaxanthin and siphonein, the 532-nm pulse was used. For Chl *b* excitation, the 645-nm pulse was obtained from the cavity-dumped dye laser. Fluorescence emission was detected by a microchannel plate photomultiplier (R1294U, Hamamatsu Photonics) after passing through a monochromator (Nikon P-250). Signals from the photomultiplier were used as stop pulses for a time-to-amplitude converter (TAC). Start pulses were obtained directly from a dye laser using a pin-photodiode. The data acquisition system including a multichannel pulse height analyzer (MCPHA) was operated under the microcomputer control system. Fluorescence decay curves were measured at 0.9994 nm steps. Time-resolved fluorescence spectra were compiled from the fluorescence decay curves measured at different wavelengtes.

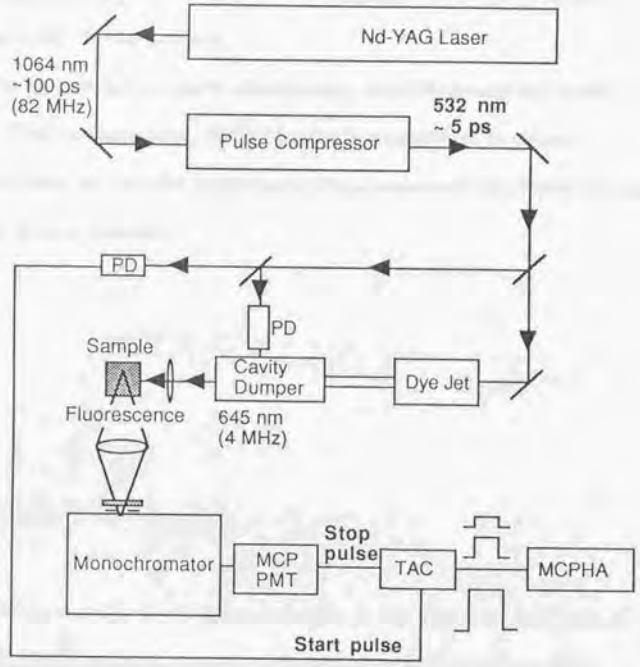


Fig. V-1 Diagram of the experimental apparatus for picosecond time-resolved fluorescence measurements. MCP-PMT, micor channel plate photomultiplier; PD, photodiode; TAC, time-to-amplitude converter; MCPHA, multichannel pulse hight analizer.

For measurements of fluorescence, the samples were suspended in 50 mM Tris-HCl (pH. 7.5) to give an absorbance of 0.1 at 672 nm. The experiments were performed at 15°C for *Bryopsis* LHC II and at 20°C for spinach LHC II, respectively.

For the low temperature spectroscopy, poly(ethylene)glycol 4000 was added (final concentration, 15 %) to sample suspensions to obtain homogeneous ice and the suspensions were immersed into liquid nitrogen during measurements.

Results

Energy transfer from siphonaxanthin to Chl a *in* Bryopsis *LHC II*

Fig. V-2 shows the time courses of fluorescence emission from Chl *a* (673 and 681 nm) and Chl *b* (655 nm), and the laser pulse profile at 532 nm, which was used for excitation of siphonaxanthin. The time zero corresponds to the time at which the excitation laser pulse reaches its maximum intensity. Due to a limited time response of the instrument, the laser pulse was spread in width. Time courses of fluorescence decay at 673 and 681 nm were similar to each other at 15°C (Fig. V-2A). However, the decay at 673 nm was faster than that at 681 nm at -196°C

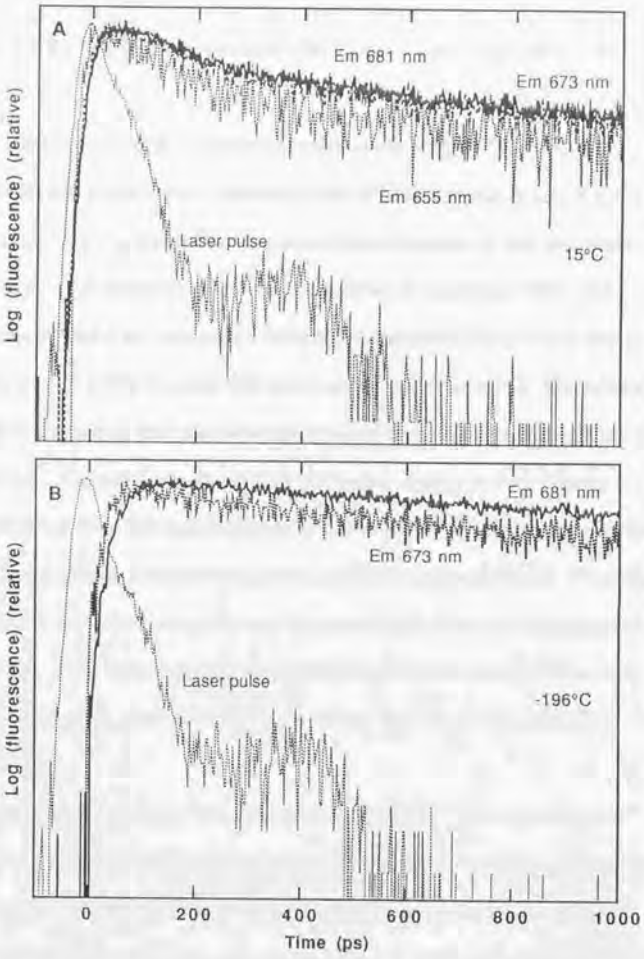


Fig. V-2 Time-resolved fluorescence from *Bryopsis* LHCII. Laser pulse of 532 nm was used to excite the sample. The decay of the pulse was apparently elongated by the time responce of the detecting system. Measurements were carried out at 15 (A) and -196°C (B).

(Fig. V-2B). No fluorescence from Chl *b* was observed at -196°C (see later).

Time-resolved fluorescence spectra were obtained from the fluorescence decay carves measured from 630 to 730 nm at about 1 nm intervals and shown in Fig. V-3 after normalization at the maximum intensity. Immediately after the excitation of siphonaxanthin and siphonein (-26.4 ps), emissions from plural forms of Chl *a* (from 660 to 710 nm) and Chl *b* (around 655 nm) were observed at 15°C. The emission peak remained at 680 nm throughout the course of measurement up to 2480 ps. The results indicate that excitation energy is very rapidly transferred from the xanthophylls to Chl *a* and equilibrates among three Chl forms within the resolution time of the instrument (3 ps). Note that the Chl *b* fluorescence at 650 nm decreased with time and disappeared at 29.1 ps. This indicates that the energy equilibrium among Chl *a* and Chl *b* molecules is established more slowly than that between Chl *a* molecules.

Time-resolved fluorescence spectra at -196°C were significantly different from those determined at 15°C (Fig. V-4). The fluorescence peak occurred initially at 675 nm. This indicates that excitation energy is initially transferred from siphonaxanthin and siphonein only to Ca672, which has an absorption maxima at 668 nm at -196°C. Then, the fluorescence maximum gradually shifted to 680 nm with time, indicating energy transferred from Ca672 to Ca679. Note that no emission band was present around 650 nm. This is an additional evidence that no energy

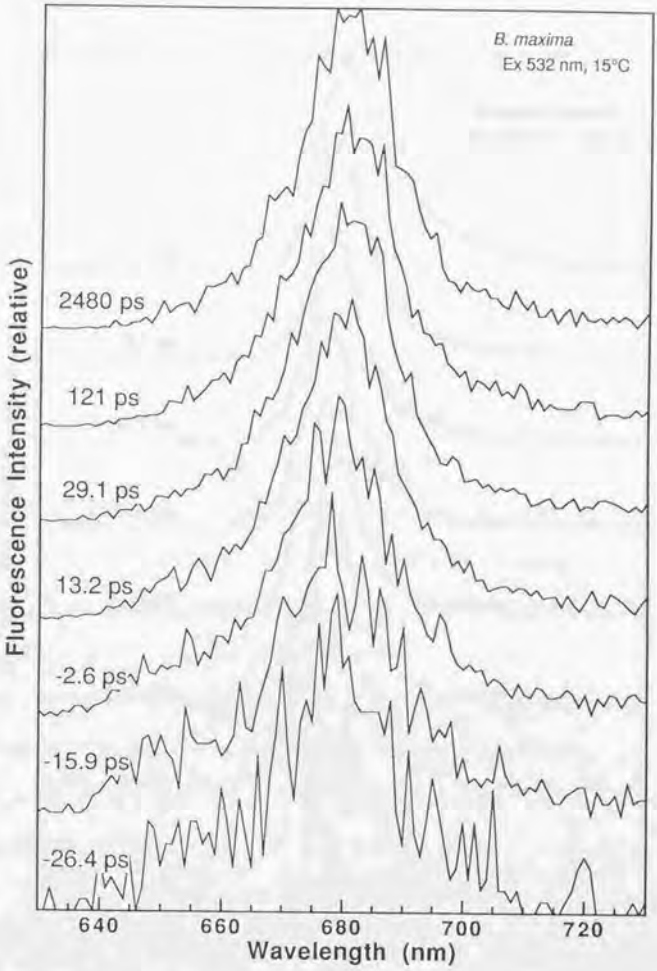


Fig. V-3 Time-resolved fluorescence spectra of *Bryopsis* LHC II at 15°C obtained by excitaion of siphonaxanthin at 532 nm. Each spectrum was normalized at the maximum fluorescence peak.

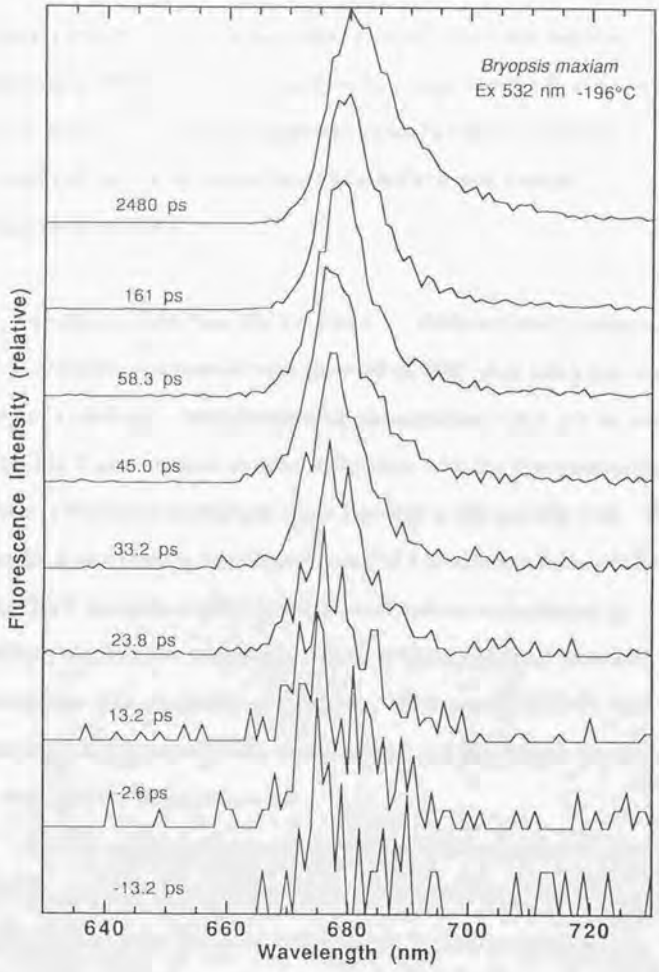


Fig. V-4 Time-resolved fluorescence spectra of *Bryopsis* LHC II obtained by excitaion of siphonaxanthin at -196°C.

transfer occurs from the carotenoids to Chl *b*. The Chl *b* emission observed at 15°C is, therefore, ascribed to energy transfer from Chl *a* to Chl *b*, rather than that from siphonaxanthin to Chl *b*. This uphill-transfer of excitation energy from Chl *a* to Chl *b* was strongly suppressed at -196°C.

Energy transfer from Chl b *to Chl* a --- Different kinetic behaviors of fluorescence components were observed at 15°C when Chl *b* had been excited at 645 nm. Immediately after the excitation (-23.8 ps), an emission from Chl *b* was observed at 655 nm, together with the fluorescence from Chl *a* in the broad wavelength range from 660 to 710 nm (Fig V-5). Thus, energy is very rapidly transferred from Chl *b* to different forms of Chl *a*. The Chl *b* emission rapidly decreased and became insignificant at 23.8 ps, whereas the emission spectrum of Chl *a* remained constant throughout the course of measurement. These results indicate that excitation energy equilibrates between Chl *b* and Chl *a* more slowly than it does between Chl *a* molecules.

For the comparison, time-resolved fluorescence spectra of spinach LHC II were determined at 20°C (Fig. V-6). When Chl *b* was excited, the emission maximum remained at 680 nm up to 2480 ps, whereas the emission from Chl *b* at 655 nm mostly disappeared at 23.8 ps. These features are essentially the same as those in *Bryopsis* LHC II.

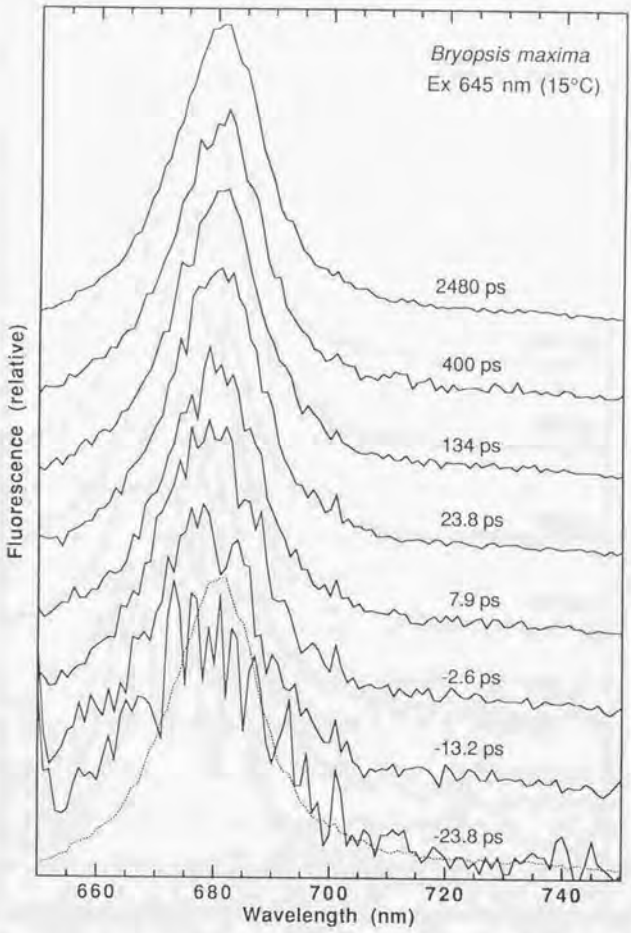


Fig. V-5 Time-resolved fluorescence spectra of *B. maxima* LHC II obtained by excitaion of Chl *b* at 645 nm. Each spectrum was normalized at the maximum fluorescence peak. For the comparison with the spectrum determined at -23.8 ps, the spectrum at 2480 ps is shown in dotted line.

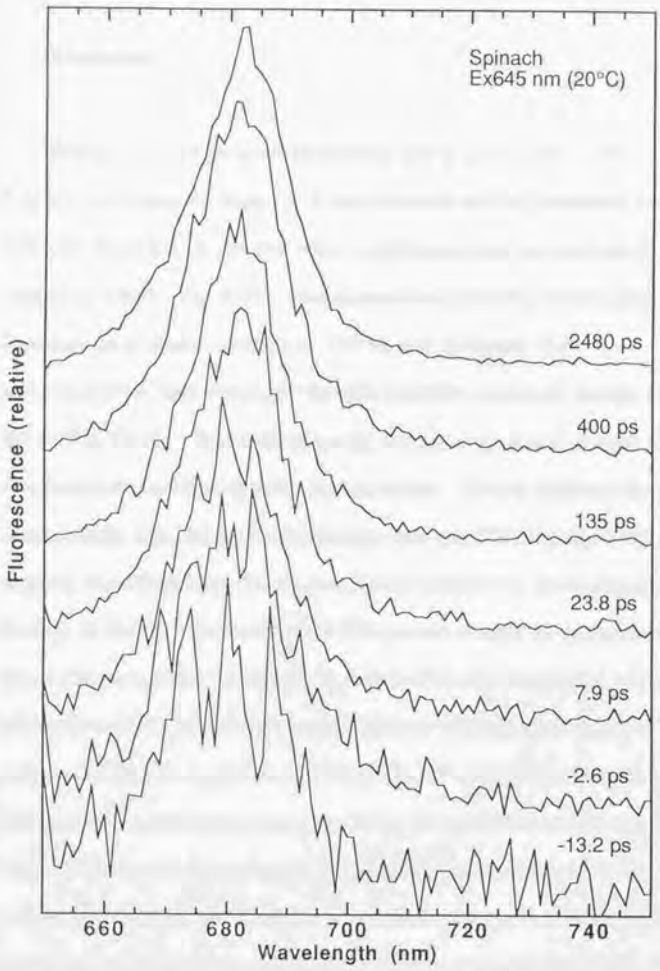


Fig. V-6 Time-resolved fluorescence spectra of spinach LHC II obtained by excitation of Chl *b* at 645 nm. Each spectrum was normalized at the maximum fluorescence peak.

Discussion

Energy transfer from carotenoids to Chl a *in LHC II* --- No fluorescence emission from Chl *b* was detected in the picosecond time-resolved fluorescence spectra when siphonaxanthin and siphonein were excited at -196°C (Fig. V-4). This is consistent with the steady-state fluorescence emission spectra at -196°C, and indicates that siphonaxanthin and siphonein directly transfer excitation energy to Chl *a* (Fig. IV-8). Interestingly, only fluorescence emission from Ca672 was detected immediately after the excitation. This is evidence that the xanthophylls transfer excitation energy only one Chl *a* form, Ca672. We suggest, therefore, that the xanthophyll molecules are located in close vicinity of Ca672. In fucoxanthin-Chl-protein complexes isolated from brown algae, a major xanthophyll, fucoxanthin was suggested to interact with one each of Chl a molecules of Ca669 or Ca673, based upon the stoichiometry of fucoxanthin and Chl *a* (Katoh et al. 1989, Mimuro et al. 1990). Such a one-to-one correspondence was not present in *Bryopsis* LHC II because three molecules of siphonaxanthin and one siphonein are present per two Chl a of Ca672 (Chapter IV). Spatial arrangement of siphonaxanthin, siphonein and Chl *a* molecules in *Bryopsis* LHC II remains to be studied.

Energy transfer from Chl b *to Chl* a --- Fluorescence emissions from all the Chl *a* forms were detected in *Bryopsis* LHC II immediately

after the excitation of Chl *b* at 15°C, while the emission from Chl *b* became negligible at 29.1 ps. Similar features were observed in the initial range of the time-resolved fluorescence spectra of spinach LHC II (Fig. V–5, 6). The results indicate that the excitation energy equilibrates between Chl *a* and Chl *b* molecules more slowly than between Chl *a* molecules both in *Bryopsis* and spinach LHC II. The degree of polarization for the excitation of the Chl *a* emission was very low (0.04) in the Chl *b* region in *Bryopsis* (Chapter IV) and in spinach (van Metter 1977). This indicates that orientation of Chl *b* to Chl *a* molecules is also similar in the two LHC IIs. However, the steady-state fluorescence spectra show the emission from Chl *b* in *Bryopsis* LHC II, but not in spinach LHC II. This is evidence that energetic coupling between Chl *b* and Chl *a* is different between the two organisms and a small population of chl *b* in *Bryopsis* LHC II cannot transfer excitation energy to Chl *a* with 100% efficiency. The emissions from two Chl *b* forms were clearly resolved at -196°C in *Bryopsis* LHC II when Chl *b* was excited. This indicates that efficiency of energy transfer from Cb648 to Cb653 or to Chl *a* is lower than 100% at the liquid nitrogen temperature.

An apoprotein of pea LHC II, of which the three dimensional structure has been analyzed recently, carries 8 Chl *a* and 6 Chl *b* (Kühlbrandt and Wang 1991, 1992). Steady-state and time-resolved fluorescence spectroscopy showed that optical properties of pigments bound to LHC II were similar between higher plants and *Bryopsis*. The primary structures of the LHC II apoproteins are also highly homologous

between higher plants and algae. It is plausible to assume that the molecular arrangement of pigments in *Bryopsis* Chl-protein which also contains 14 Chls in total is analogous to that in pea LHC II (Kühlbrandt and Wang, 1992). However, two Chl *a* molecules are replaced by Chl *b* in *Bryopsis* LHC II. The occurrence of Chl *b* emission in the algal LHC II can be related to the abundance of Chl *b*, combined with a smaller content of Ca672 in *Bryopsis* LHC II.

The results obtained in this and previous chapters may be summarized in a scheme shown in Fig. V-7, which illustrates the Chl forms and energy flow in a LHC II unit polypeptide of *B. maxima*.

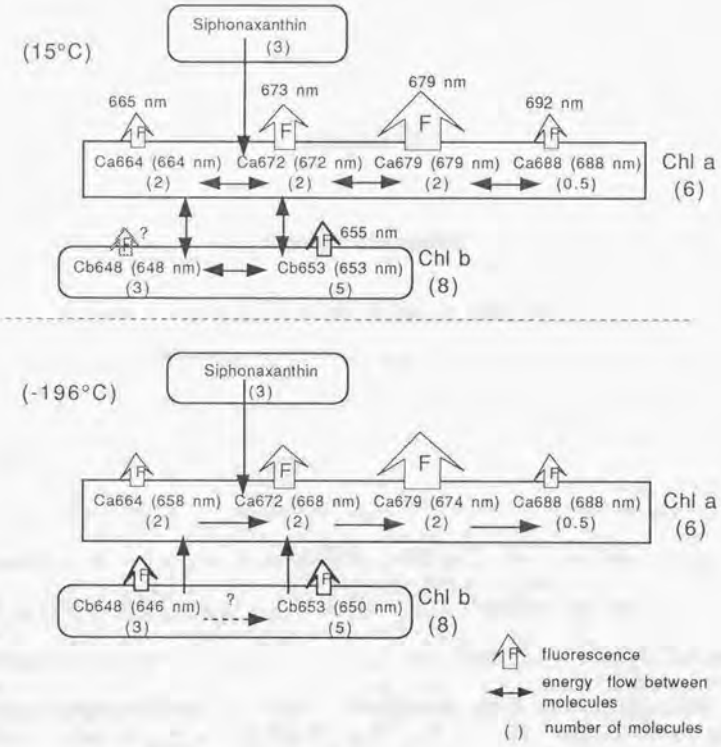


Fig. V-7 Schematic presentation of Chl forms and energy flow in a unit polypeptide of *B. maxima* LHC II at 15 and -196°C

Chapter VI

General discussion

The present study shows that composition of major Chl-proteins present in the marine siphonous green alga, *Bryopsis maxima*, is similar to those in higher plants. The two Chl-proteins, CP I and LHC I, which belong to PS I, and the two PS II components, CPa and LHC II, were resolved by SDS-PAGE from the algal thylakoids. The order of the electrophoretic migration of these Chl-proteins was identical to that of higher plant Chl-proteins (Anderson et al. 1981). LHC II was resolved into three bands as reported for LHC II from higher plants (Ryrie et al. 1980). These results indicate that the basic organization of the light-harvesting and light-energy converting systems of the alga is essentially the same as that of higher plants.

Besides the above-mentioned major Chl-proteins, the three minor antenna Chl *a*/*b* -proteins called CP29, CP26 and CP24 have been isolated from higher plants (Peter and Thornber 1991). They are considered to serve as a mediator of excitation energy from bulk LHC II to PS II reaction center complex (Bassi et al. 1987). The present study demonstrated the occurrence of a minor antenna Chl *a*/*b* -protein, LHC II', in green algae for the first time. This Chl-protein also appears to function in PS II because it was extracted together with the PS II reaction center complexes and LHC II. Whether or not green algae contain other minor Chl *a*/*b* -proteins of PS II remains to be studied.

LHC II from higher plants consists of the two major apoproteins of

24-28 kDa (Kühlbrandt and Barber 1988). *Bryopsis* LHC II contains the two apoproteins of 28 and 32 kDa. The amino acid composition and N–terminal sequence of the LHC II apoproteins are similar between the green alga and higher plants. The number of Chls bound to an apoprotein of *Bryopsis* LHC II is also comparable to those determined for higher plant preparations (Butler and Kühlbrandt 1988). There are two populations of LHC II present in the grana region of chloroplasts of higher plants, which are different in the composition of the two apoproteins. A small population of LHC II binds more loosely to the PS II reaction center complex than another and, when phosphorylated, moves to the stroma thylakoid and transfers excitation energy to PS I (Larsson and Andersson 1985). In this study, two purified forms of LHC II, P4 and P5, were successfully isolated without significant release of pigments. Interestingly, P4 and P5 are different in relative abundance of the 28 and 32 kDa polypeptides. Thus, they may correspond to the two populations of LHC II in higher plants, which bind differently to PS II reaction center complexes. *Bryopsis* chloroplasts have no grana. Thus, it is interesting to study whether P4 or P5 functions similar to higher plants LHC II. At any event, these data altogether indicate that the molecular organization of the algal LHC II is essentially the same as that of LHC II in higher plants.

However, there are notable differences in the pigment composition between *Bryopsis* and higher plants LHC II. An apoprotein of *Bryopsis* LHC II contains 6 Chl *a*, 8 Chl *b*. This Chl *a* to Chl *b* ratio is just the

reverse of that of an apoprotein of pea LHC II which carries 8 Chl *a* and 6 Chl *b* (Kühlbrandt and Wang 1991, 1992). As will be described blow, this replacement of two Chl *a* by two Chl *b* makes *Bryopsis* LHC II a unique and interesting material for investigation of excitation energy transfer involving Chl *b*.

The total numbers of carotenoids bound to one apoprotein of LHC II from several higher plants were reported to be 3.5 to 5.4 (Siefermann-Harms 1985). The number of carotenoid molecules associated with a *Bryopsis* apoprotein is 5, which corresponds to the largest number of the carotenoids bound to higher plant LHC II protein. The most abundant carotenoid present in higher plants is lutein (Eskins et al. 1983), whereas the major carotenoids present in the *Bryopsis* LHC II are siphonaxanthin and siphonein. Siphonaxanthin and siphonein have absorption bands at 530-540 nm, well separated from the Soret bands of Chl *a* and Chl *b*. Thus, *Bryopsis* LHC II has a unique advantage that the carotenoids can be excited without significant excitation of Chl *a* and Chl *b*.

In the second half of the present study, excitation energy transfer between different pigments of LHC II were investigated in detail. Interestingly, Chl *b* was found to emit weak fluorescence in *Bryopsis* LHC II at room and liquid nitrogen temperatures. Because emission from Chl *b* has never been observed in LHC II from higher plants, this should be ascribed to the presence of Chl *b* in excess over Chl *a*. The two new and important observations emerged from studies on the Chl *b*

fluorescence. First, the excitation energy transfer from long wavelength forms of Chl *a* to Chl *b* was demonstrated for the first time. This finding indicates that excitation energy rapidly equilibrates between Chl *a* and Chl *b* molecules at the physiological temperature, in spite of a large energy gap between the excited states of Chl *a* and Chl *b*. The absence of energy transfer from Chl *a* to Chl *b* at -196°C shows that the uphill-energy transfer requires thermal energy.

Second, it was clearly shown that Chl *b* is not involved in energy transfer from carotenoids to Chl *a*. No emission from Chl *b* was detected on excitation of siphonaxanthin and siphonein at liquid nitrogen temperature. Instead, siphonaxanthin and siphonein transfer excitation energy to only one Chl *a* form, Ca672. Thus, the present study provides convincing evidence against the long-held postulation that excitation energy absorbed by carotenoid is transferred to Chl *a* *via* Chl *b*. The results also suggest that siphonaxanthin and siphonein are not uniformly distributed in LHC II protein structure but located in close proximity of Ca672 in a molecular orientation suitable for energy transfer. It is expected, therefore, that *Bryopsis* LHC II serves as an interesting and promising materials for investigation of the molecular organization and function of photosynthetic pigments, in particular, Chl *b* and xanthophyll in the light-harvesting Chl *a*/*b*-protein complexes.

VII.

Acknowledgments

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VIII.

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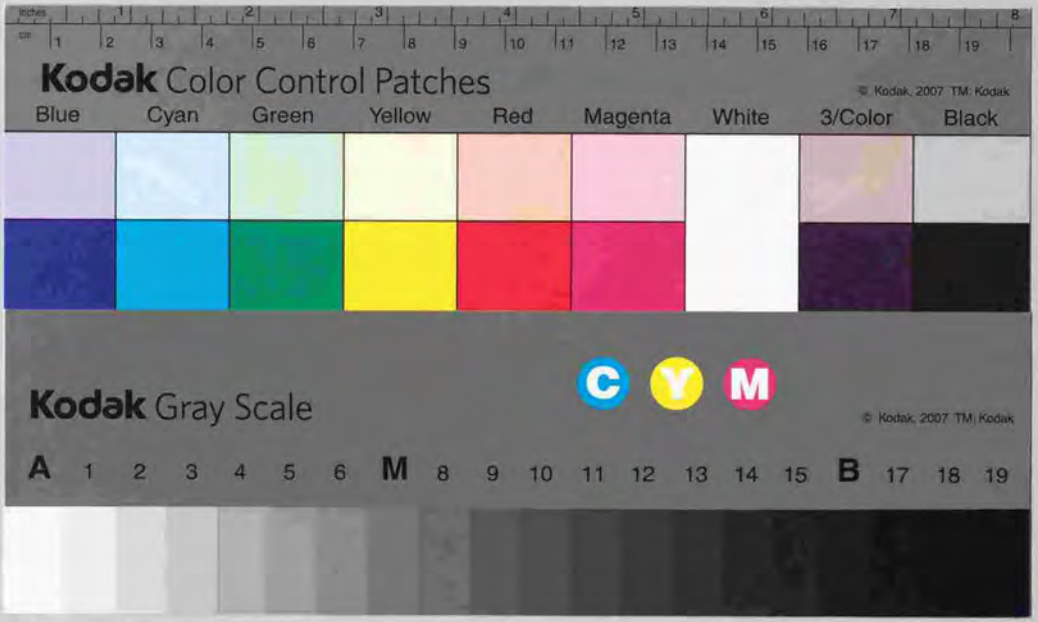
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