

# The Subcomplex Organization of the Major Chlorophyll *a/b*-Protein Light-Harvesting Complex of Photosystem II (LHCII) in Barley Thylakoid Membrane

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Z. Naturforsch. **51c**, 454–463 (1996); received November 23, 1995/March 4, 1996

Barley, Isoelectric Focusing, Light Harvesting Complex, Polypeptide, Subcomplex, Trimer

The major chlorophyll *a/b*-protein light-harvesting complex of photosystem II (LHCII) isolated from barley photosynthetic membrane was shown to contain five major polypeptides only two of which (26.7 and 25.6 kDa) were found to be its true constituents as judged by the ability to migrate as oligomers in various analytical systems. When analyzed by a vertical-bed non-denaturing isoelectric focusing the LHCII was resolved into five trimeric subcomplexes (designated 1–5 in order of decreasing pI) containing either only 26.7 kDa polypeptide (subcomplexes 1 and 2) or 26.7 and 25.6 kDa ones associated at 2:1 ratio (subcomplexes 3–5). The polypeptide of 26.7 kDa could be split by denaturing isoelectric focusing into fifteen molecular forms while nine molecular species were found to be constituents of 25.6 kDa polypeptide. The subcomplexes 1–5 contained molecular forms of one or both polypeptides associated in sets of 7–9.

Our findings favour the view that the apoproteins of LHCII are much more heterogeneous than thought before.

## Introduction

Light interception by photosystem II (PSII) is accomplished by a large set of antenna pigments coordinated by specific polypeptides in the form of a family of at least four pigment-proteins. The main constituent of the antenna apparatus of PSII is the light-harvesting chlorophyll *a/b*-protein complex, designated LHCII by the majority of research groups, binding more than 50% of chlorophyll (Chl) in the thylakoid membrane (Chitnis and Thornber, 1988). Structural studies performed on highly-ordered two dimensional crystals of LHCII or LHCII solubilized with a nonionic detergent revealed that the holocomplex is a trimer with 3-fold symmetry (Kuhlbrandt 1984, 1987; Butler and Kuhlbrandt, 1988). It is most likely,

therefore, that the holocomplex forms trimers also inside the photosynthetic membrane. In the last few years the heterogeneity of LHCII at the level of trimeric pigment-proteins was recognized as it was shown that holocomplex could be resolved by non-denaturing isoelectric focusing (IEF) into some subcomplexes differing with respect to polypeptide composition (Bassi *et al.*, 1988, Spangfort and Andersson, 1989) and  $Mg^{2+}$ -dependent aggregation in vitro (Spangfort and Andersson, 1989). It was suggested that the subcomplexes may correspond to peripheral and inner subpopulations i.e. LHCII building blocs anticipated earlier to be responsible for the heterogeneity exhibited by LHCII during phosphorylation-mediated state transitions and thought to have different arrangement around PSII center complex (Staehelin and Arntzen, 1983; Larsson *et al.*, 1987).

It is generally accepted that LHCII is a flexible system able to rearrange structurally and functionally in response to environmental stimuli (Melis 1991) and changing developmental context (Dreyfuss and Thornber, 1994). The elucidation of the molecular events leading to these acclimations depends on better understanding of the subcomplex organization of intact LHCII holocomplex itself. In the current study we addressed the subcomplex organization of LHCII isolated from photosyn-

**Abbreviations:** CBB G-250, Coomassie Brilliant Blue; chl, chlorophyll; DM, *n*-dodecyl- $\beta$ -D-maltoside; IEF, isoelectric focusing; LDS, lithium dodecyl sulphate; LHCII, the major light-harvesting chlorophyll *a/b*-protein complex of photosystem II; LHCPII, apoprotein of LHCII; OG, octyl-  $\beta$ -D-glucopyranoside; PSII, photosystem II; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; TCA, trichloroacetic acid.

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thetic membranes of barley which is widely used in research concerning development – related rearrangements of PSII antenna apparatus (Dreyfuss and Thornber, 1994; Harrison and Melis, 1992; Harrison *et al.*, 1993; Sigrist and Staehelin, 1994). Our data indicate that barley LHCII exists inside the thylakoid membrane as a set of five trimeric subcomplexes with each subcomplex comprising a unique assembly of pI isoforms of two polypeptides (26.7 and 25.6 kDa).

## Material and Methods

### *Plant material*

Barley (*Hordeum vulgare* L.) seeds were germinated in moist lignin in complete darkness for 3 days followed by 7 days of illumination with a continuous white light of  $75 \mu\text{E m}^{-2} \text{s}^{-1}$ . For all experiments 10-days-old primary leaves were used.

### *Isolation of PSII and LHCII*

PSII particles were isolated from the primary barley leaves according to the method of Berthold *et al.*, (1981) with the modifications described by Dunahay *et al.*, (1984). The Triton X-100:Chl ratio of 4.5:1 was used to solubilize thylakoid membranes. LHCII was isolated from PSII particles essentially as described earlier (Jackowski and Kluck, 1994). PSII and LHCII samples were stored in 10% glycerol in dry ice.

### *Isolation of LHCII subcomplexes*

LHCII samples were solubilized, focused and eluted from non-denaturing, isoelectric focusing (IEF) gels as described by us previously (Jackowski and Przymusiński, 1995) except that focusing was performed for 7–8 h at voltage increasing in 50–480 V range.

### *Sucrose-gradient ultracentrifugation*

LHCII was pelleted by centrifugation at  $40\,000\times g$  for 4 min. The pellet was resuspended at 1.2 mg Chl/ml in 2 mM Tris/maleate, pH 7.0 and LDS, OG and DM stock solutions were added to yield final concentrations of 1.2%, 2.4% and 1.2% respectively. The samples were incubated on ice for 30 min, spun at  $12\,300\times g$  for 5 min, loaded on the top of 0.2–0.8 M sucrose gradient containing 2 mM Tris/maleate, pH 7.0 and run at  $300\,000\times g$  in

Beckman SW 50.1 rotor for 6 h at 4°C. Green bands of monomeric and trimeric forms of LHCII were harvested with an automatic micropipette.

### *Sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) and denaturing IEF*

LHCII was pelleted by centrifugation at  $12\,300\times g$  for 8 min. The pellet was resuspended at 1.2 mg Chl/ml in 0.3 M Tris/HCl, pH 8.8 containing 10% glycerol and 20% SDS was added to yield final concentration of 0.9%. Monomeric and trimeric forms of LHCII were resolved under mildly denaturing SDS-PAGE conditions using the buffer system of Tanaka *et al.*, (1987). The green bands of monomeric and trimeric forms of LHCII were electroeluted from gels using Model 422 Electroeluter (BioRad, Italy) filled with the electrode buffer of Laemmli (1970).

The polypeptides of PSII, LHCII and its oligomeric forms and subcomplexes were precipitated by adding 5 volumes of 80% acetone. The pellets, recovered by centrifugation at  $12\,300\times g$  for 10 min, were dissolved and the polypeptides were fractionated by denaturing SDS-PAGE using a separating gel with 8–22% linear acrylamide gradient and the buffer system of Fling and Gregerson (1986). The electrophoreses were run at 25 mA/gel for 2–3 h. The gels were fixed for 25 min at 12% TCA, stained overnight with 0.08% CBB G-250 by an improved, highly sensitive method (Neuhoff *et al.*, 1988) and destained in 25% methanol.

For denaturing IEF green LHCII subcomplexes were electroeluted from the non-denaturing IEF gel using Model 422 Electroeluter filled with 20 mM Tris/maleate pH 7.0. The apoproteins of the subcomplexes were precipitated with 80% acetone and the pellets, recovered by centrifugation, were applied to the wells of a preparative, 1.5 mm thick, polyacrylamide gel. Preparative SDS-PAGE was performed using gradient gels, the buffer system and staining protocol as described above. Gel pieces containing individual, stained polypeptide bands were excised, the polypeptides electroeluted from the gel using 25 mM Tris pH 8.3 containing 192 mM glycine as an elution buffer, and precipitated by adding 80% acetone. The pellets were washed extensively with 80% acetone and 50% isopropanol to remove residual stain and SDS and

the samples underwent denaturing IEF and staining procedure as described by us previously (Jackowski and Przymusiński, 1995) except that only 3.5/10 pH carrier ampholites were used.

To perform a relative quantitation of CBB-stained bands the gels were scanned at 600 nm in a zig-zag mode applying Shimadzu CS-9000 Flying Spot Dual-Wavelength Scanner with on-board integration system. To determine a relative content of non-stained LHCII subcomplexes within LHCII holocomplex non-denaturing IEF gels were scanned at 650 and 675 nm and the relative areas under the bands of individual subcomplexes were calculated as described by Genge *et al.*, (1974).

### Immunoblotting

Polypeptides belonging to LHCII subcomplexes were resolved by SDS-PAGE and blotted onto nitrocellulose membranes (Serva, FRG) using transfer apparatus filled with buffer of Towbin *et al.*, (1979) containing 0.1% SDS. The immunoblot assays were then carried out according to the protocol of Legocka *et al.*, (1990) using biotinylated secondary antibodies in conjunction with streptavidine-peroxidase complex.

For this study antibodies obtained from Dr Staehelin's laboratory were applied. Anti-CP26 was raised against synthetic oligopeptide derived from amino acids 56–69 in the tomato "CP29 type 1" sequence (Falbel and Staehelin, 1992).

### Other methods

Chl concentration was determined according to Arnon (1949).

## Results

LHCII was isolated from barley thylakoid membranes by  $K^+$ -induced aggregation of *n*-heptylthioglucoside-treated PSII particles and centrifugation (Jackowski and Kluck, 1994). When analyzed by denaturing SDS-PAGE the LHCII samples yielded five predominant polypeptides of 29.5, 26.7, 25.6, 24 and 22.5 kDa (Fig. 1) associated at 1.6 : 5.1 : 1 : 0.25 : 0.17 stoichiometry as judged by an integrating densitometry of CBB-stained bands. LHCII samples contained also variable amounts of colourless 33 kDa protein of the water splitting

system (oxygen evolution enhancer). As LHCII preparations obtained by methods based on cation-induced precipitation are often contaminated by polypeptides belonging to other Chl *a/b* pigment-proteins (Dainese *et al.*, 1990; Harrison and Melis, 1992; Jackowski and Kluck, 1994) we performed an identification of true LHCII constituents based on the ability to migrate as oligomers in various electrophoretical and sucrose gradient ultracentrifugation systems. For this purpose an LHCII preparation was separated into two green bands corresponding to trimeric and monomeric aggregation states using ultracentrifugation in 0.2–0.8 M sucrose gradient (Fig. 2A) or mildly denaturing SDS-PAGE (Fig. 2B). The monomeric form of LHCII contained all the polypeptides present in initial LHCII while the trimeric form was found to contain exclusively 26.7 and 25.6 kDa ones (Fig. 3) thus it appears that these polypeptides are the only authentic apoproteins of LHCII defined as PSII pigment-protein able to form stable oligomers.

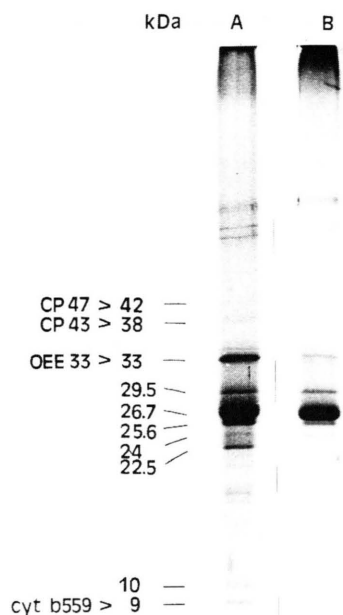


Fig. 1. Polypeptide composition of LHCII and PSII particles. 2  $\mu$ g of Chl of PSII (A) and LHCII (B) was separated by denaturing SDS-PAGE and the gels were stained with CBB G-250. Apparent molecular weights of the polypeptides were determined relative to those of protein standards. The identity of some PSII polypeptides is shown on the left. CP47, CP43 – Chl/*a* – proteins of photosystem II; OEE33 – oxygen evolving enhancer 33 kDa; Cyt – cytochrome.

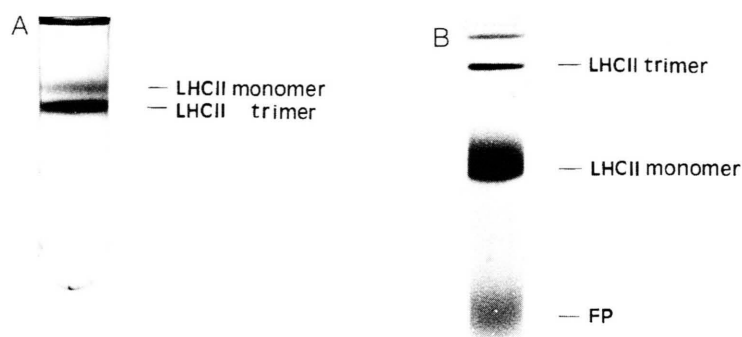


Fig. 2. Resolution of trimeric and monomeric forms of LHCII. (A) Sucrose gradient ultracentrifugation of LHCII. (B) Mildly-denaturing, "green" SDS-PAGE of LHCII.

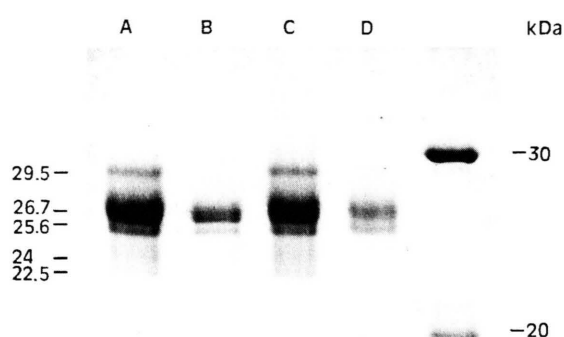


Fig. 3. Polypeptide composition of monomeric and trimeric forms of LHCII. 2  $\mu$ g Chl of the apoproteins of pigment-proteins harvested from sucrose gradient (A,B) or eluted from the "green" gel (C,D) were separated by denaturing SDS-PAGE and stained with CBB G-250. The bands represented monomeric (A,C) and trimeric forms (B,D) of LHCII. FP – free pigments.

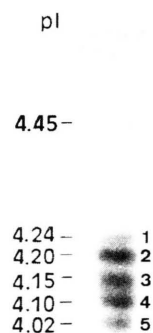


Fig. 4. Resolution of subcomplexes of LHCII by non-denaturing IEF. 16  $\mu$ g Chl of LHCII was fractionated in a 0.50 mm-thick analytical, vertical, 7% polyacrylamide gel containing 0.375% DM (pH 3.5–5.0). The symbols and pI of the subcomplexes are indicated.

When the LHCII sample was treated with 1% DM and the solubilization mixture fractionated by vertical-bed, non-denaturing IEF in 7% acrylamide containing 0.375% DM (Jackowski and Przymsiński, 1995), five prominent green bands at pH range of 4.02–4.24 and a diffused, faint fraction around pH 4.45 were resolved with no signs of the disruption of pigment-protein interaction (Fig. 4). The prominent bands, labelled 1–5 in order of decreasing pI value, focused at 4.24, 4.20, 4.15, 4.10 and 4.02 respectively. They were associated at a proportion of 1.00 : 1.55 : 1.01 : 1.13 : 1.12 as determined by an integrating densitometry of non-stained gels at 650 and 675 nm. The fractions exhibited both spectroscopic and polypeptide composition peculiarities indicating unequivocally that they were LHCII subcomplexes. Namely, the fractions displayed absorption spectra (almost identical) in 400–700 nm range with dominating maxima at 672, 651, 469 and 439 nm, representative of LHCII (data not shown) and very similar chl a/chl b ratios of 1.03 : 1(1), 1.00 : 1(2), 1.13 : 1(3), 1.09 : 1(4) and 0.92 : 1(5). The polypeptide composition of individual subcomplexes was analyzed by denaturing SDS-PAGE followed by an integrating densitometry of CBB-stained bands (Fig. 5A). The fractions yielded apoproteins of LHCII, i.e. 26.7 and 25.6 kDa ones, in different relative amounts – the subcomplexes 1 and 2 contained single 26.7 kDa polypeptide while the subcomplexes 3–5 could be resolved into 26.7 and 25.6 kDa polypeptides associated at ratios of 2.34 : 1, 2.20 : 1 and 2.16 : 1, respectively.

The absorption spectrum of the faint fraction focusing around pH 4.45 showed features characteristic for CP29 and CP26 including chl b red maxi-

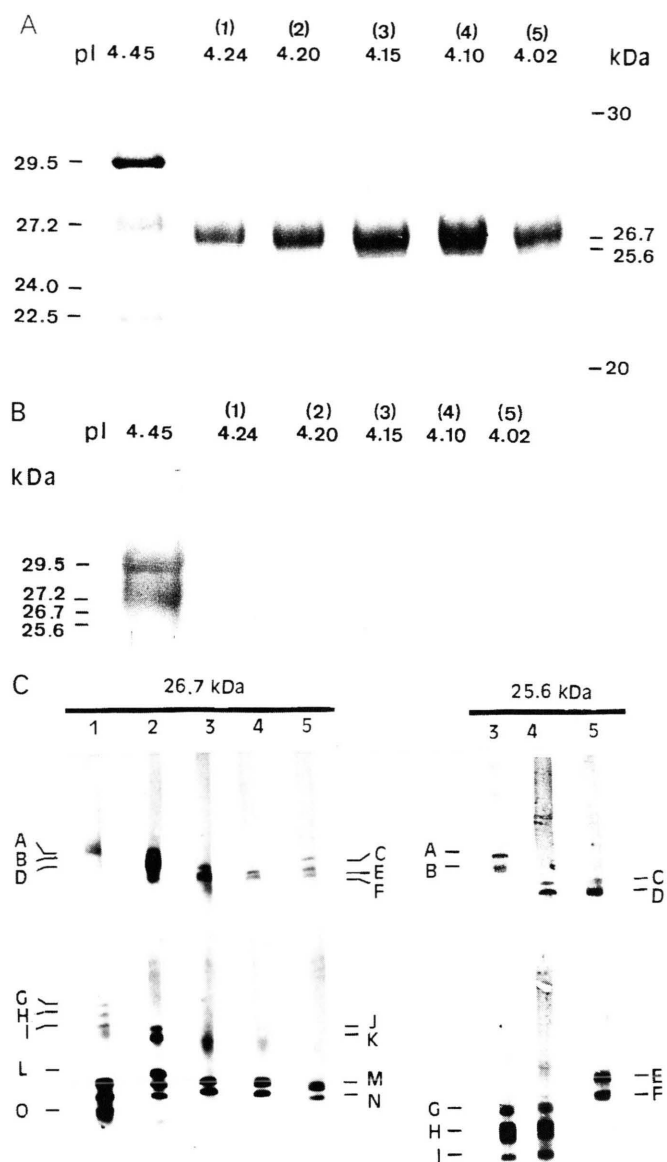


Fig. 5. Polypeptide composition of subcomplexes of LHCII. (A) 2  $\mu$ g Chl of the subcomplexes were separated by denaturing SDS-PAGE and stained with CBB G-250 (A) or electrotransferred on nitrocellulose and immunostained with anti-CP26 antibodies (B). 2  $\mu$ g of polypeptides of subcomplexes 1–5 were resolved by denaturing IEF and stained with CBB G-250 (C).

mum at 641 nm (data not shown). As seen in Fig. 5A the faint fraction contained all the polypeptides, apart from authentic LHCII apoproteins, identifiable in initial LHCII samples i.e. 29.5, 24 and 22.5 kDa ones along with a band migrating as 27.2 kDa which in initial LHCII was not noticeable due to comigration with 26.7 kDa LHCII apoprotein. To establish if the polypeptides yielded by a green fraction focusing at pH 4.45 contaminated LHCII subcomplexes 1–5, the apoproteins of all

six green fractions found in LHCII preparation were resolved electrophoretically, transferred onto nitrocellulose membranes and immunostained with anti-CP26 antibodies. The results are shown in Fig. 5B. Interestingly, the antibodies showed a remarkable cross-reactivity toward LHCII apoproteins but definitively no traces of polypeptides found in pI 4.45 fraction were detected as constituents of LHCII subcomplexes 1–5. Anti-CP26 that we obtained from Dr Staehelin's laboratory was

shown to bind to apo CP29 (Falbel and Staehelin, 1992). On our gels anti-CP26 recognized both 29.5 and 27.2 kDa polypeptides thus establishing their assignment as apo CP29 and apo CP26, respectively.

In spite of the differences in pI the subcomplexes 1 and 2 as well as 3–5 appear to have identical polypeptide composition. It may imply that these groups of subcomplexes represent various oligomeric forms of LHCII, it was shown, however, using mildly denaturing SDS-PAGE that the prevailing majority of the material of all subcomplexes was recovered as trimers (data not shown). The discrepancy between the differences in pI and the apparent identity of polypeptide composition of LHCII subcomplexes 1–2 and 3–5 could be alternatively explained by assuming that the trimers were composed of distinct subsets of pI isoforms of 26.7 and 25.6 kDa polypeptides. In order to verify this prediction all the subcomplexes were electroeluted from non-denaturing IEF gels and their polypeptides further fractionated by denaturing IEF. Figure 5C presents the pattern of pI isoforms of the 26.7 and 25.6 kDa polypeptides originating from five LHCII subcomplexes. The 26.7 kDa polypeptide was found to comprise a total of 15 pI isoforms covering pH range of 3.9–6.4 designated 26.7/A – O. As seen in Fig. 5C there were substantial qualitative and quantitative differences among the patterns of pI isoforms of 26.7 kDa polypeptide recovered from defined LHCII subcomplexes – ten molecular species were found exclusively in single subcomplexes whereas only two isoforms (M,N) were common to all the subcomplexes. In accordance with the expectations pI isoforms were distributed in LHCII subcomplexes (in sets of 4–8 species) in such a manner that, in general, more alkaline isoforms prevailed in more alkaline trimers while more acidic ones were enriched in acidic trimers. The polypeptide 25.6 kDa found in the LHCII subcomplexes 3–5 was resolved into a total of nine pI isoforms, designated 25.6/A–I, distributed in the subcomplexes in distinct sets of 4–6 species. The majority of the isoforms were detectable in two subcomplexes but no one isoform appeared to be common to all three subcomplexes. The isoforms covered pI range of 3.7–6.4. A summary of distribution of molecular species of both barley LHCII apoproteins within the subcomplexes is shown in Table I.

Table I. Distribution of pI isoforms of LHCII apoproteins in LHCII subcomplexes. The isoforms were separated by denaturing IEF and stained with CBB G–250. No quantitative evaluation of the band intensity is given.

pI isoform	LHCII subcomplex				
	1	2	3	4	5
26.7/A	+	–	–	–	–
25.6/A	–	–	+	–	–
26.7/B	–	+	–	–	–
26.7/C	–	+	–	–	+
25.6/B	–	–	+	–	–
26.7/D	–	–	+	–	–
26.7/E	–	+	+	+	+
26.7/F	–	–	–	+	+
25.6/C	–	–	–	+	+
25.6/D	–	–	–	+	+
26.7/G	+	–	–	–	–
26.7/H	+	–	–	–	–
26.7/I	+	–	–	–	–
26.7/J	–	+	–	–	–
26.7/K	–	+	–	–	–
26.7/L	–	+	–	–	–
25.6/E	–	–	–	–	+
26.7/M	+	+	+	+	+
25.6/F	–	–	–	–	+
26.7/N	+	+	+	+	+
25.6/G	–	–	+	+	–
26.7/O	+	–	–	–	–
25.6/H	–	–	+	+	–
25.6/I	–	–	+	+	–

## Discussion

In spite of a wide usage of barley photosynthetic membrane in research in the area of structural organization of PSII antenna apparatus (Dreyfuss and Thornber, 1994; Harrison and Melis, 1992; Harrison *et al.*, 1993; Król *et al.*, 1995; Peter and Thornber, 1991) up to now the trimeric subcomplexes (subpopulations) of barley LHCII were not identified directly as biochemically resolvable entities and the existing models describing LHCII organization are of indirect origin. Upon examination of the composition of a series of PSII large building blocs and multimetric LHCII complexes isolated by Deriphat-PAGE of various surfactants' extracts of barley PSII holocomplex Thornber *et al.*, (1992) have suggested that PSII reaction center complex is served by three distinct kinds of LHCII subcomplexes containing 28, 27 and 25 kDa apoproteins in the form of 28<sub>3</sub>, 28<sub>2</sub>25 and 27<sub>2</sub>28 trimeric assemblies. On the other hand quantitative SDS-PAGE and immunoblot analysis

of the light-harvesting proteins in the thylakoid membranes of barley wild-type and chlorina f2 mutant let Harrison and Melis (1992) propose a model in which PSII reaction center complex binds two kinds of LHCII subcomplexes, organized as 28<sub>3</sub> and 28<sub>2</sub>27 assemblies while 25 kDa apoprotein is linked in a monomeric form. The results produced in the present study permit unequivocal conclusions about the structural organization of barley LHCII as the utilization of a recently described, non-denaturing IEF system (Jackowski and Przymusiński, 1995) allowed the resolution of LHCII into its trimeric subcomplexes which could undergo direct biochemical characterization. Treatment of LHCII sample with DM followed by a fractionation on this system let us purify five trimeric subcomplexes, designated 1–5. In accordance with the observations made earlier for spinach (Spangfort and Andersson, 1989) and carnation (Jackowski and Przymusiński, 1995) we could not find any significant differences among barley LHCII subcomplexes with respect to their chl content and organization. The subcomplexes contained two polypeptides which in our gel system migrated with apparent molecular weight of 26.7 and 25.6 kDa. They most probably correspond to LHCII apoproteins described as 28 and 27 kDa or 27.5 and 26.9 kDa in various laboratories (Peter and Thornber, 1991; Harrison and Melis, 1992; Sigrist and Staehelin, 1992) – depending on the gel system used the estimations of apparent molecular weight of Chl *a/b*-binding proteins may vary by as much as 9% even in a single laboratory (Henrysson *et al.*, 1989). It is not possible to decide at present which of the two apoproteins is coded for by *Lhcb1* and which by *Lhcb2* sequences. In almost all species investigated to date (petunia, spinach and tomato) LHCII apoproteins coded for by *Lhcb1* genes are heavier than those coded for by *Lhcb2* sequences (Allen and Staehelin, 1992; Green *et al.*, 1992; Sigrist and Staehelin, 1992). In contrast, Sigrist and Staehelin have reported that barley LHCII apoproteins exhibited *Lhcb1/Lhcb2* dependence reversal i.e. have identified 27.5 kDa LHCPII as containing *Lhcb2* apoproteins and 26.9 kDa band as containing *Lhcb1* apoproteins (Sigrist and Staehelin, 1994). However, these findings were not confirmed by other study on barley LHCII apoproteins (Król *et al.*, 1995) demonstrating that in

the case of barley LHCII the identification of the subtypes of individual apoproteins is still not conclusive.

The subcomplexes 1–5 did not contain any other of polypeptides found in an initial LHCII sample, including 24 kDa one, which most probably constitutes *Lhcb 3* gene product reported as migrating to 24–26 kDa by other groups (Peter and Thornber, 1991; Harrison and Melis, 1992; Sigrist and Staehelin, 1992; Harrison *et al.*, 1993). This observations together with the absence of 24 kDa polypeptide in the bulk LHCII trimeric form isolated by mildly denaturing SDS-PAGE or ultracentrifugation in sucrose gradient (Fig. 2 and 3) favour the view that this polypeptide is not a constituent of LHCII defined as PSII pigment-protein able to form stable oligomers (Harrison and Melis, 1992).

The subcomplexes 1 and 2 contained exclusively 26.7 kDa polypeptide while the subcomplexes 3–5 were composed of 26.7 kDa and 25.6 kDa ones associated at a ratio close to 2:1. The sole occurrence of two kinds of LHCII trimeric subcomplexes (26.7<sub>3</sub> and 26.7<sub>2</sub>/25.6), out of four theoretically predictable (26.7<sub>3</sub>, 26.7<sub>2</sub>/25.6, 25.6<sub>2</sub>/26.7, 25.6<sub>3</sub>) is fully consistent with the results of earlier studies in which spinach and carnation LHCII subcomplexes were resolved and characterized (Spangfort and Andersson, 1989; Jackowski and Przymusiński, 1995). However, the reasons why only two types of trimers are allowed to be assembled within LHCII holocomplex are not clear at present.

Our data concerning the polypeptide composition of LHCII subcomplexes are also consistent with the composition anticipated by Harrison and Melis (1992), however, the estimation of the stoichiometric ratios of the subcomplexes provided by these authors are different from the ratios measured by us. Taking into the account the 26.7/25.6 kDa ratios determined by us for 1–5 subcomplexes and the proportion for the subcomplexes (very close to 1: 1.5: 1: 1: 1 respectively), the 26.7/25.6 kDa ratio for the entire LHCII may be calculated yielding a value 4.4: 1, while the value measured for an initial LHCII was found to be 5.1: 1. The values seem to be compatible since the ratio measured for an initial LHCII was overestimated due to the comigration of apo CP26 with 26.7 kDa LHCII apoprotein. Considering the ex-

perimental errors the interpretation of both figures as 4.5 : 1 seems plausible. When we tried to use this estimate and a measured proportion of the subcomplexes to construct a model of the organization of LHCII subcomplexes within a single, dimeric PSII holocomplex we obtained fractional stoichiometry of LHCII subcomplexes per PSII center core and it may be inferred that our data can not arise from a sole occurrence of single type of PSII holocomplex in barley photosynthetic membrane. Instead, we suggest that there is a heterogeneity among barley PSII particles with respect to the arrangement of LHCII subcomplexes. This suggestion requires, however, more positive evidence and appropriate studies are in progress in our laboratory.

Using denaturing IEF we have found that 26.7 and 25.6 kDa polypeptides could be resolved into as many as 15 and 9 pI isoforms, respectively, associated in sets of 7–10 in LHCII subcomplexes. This finding demonstrates that LHCII contains considerably greater number of polypeptide species than thought before as the application of high-performance analytical systems for various plant species allowed the resolution of 5–11 LHCII apoproteins at maximum (Sigrist and Staehelin, 1992, 1994; Jackowski and Przymusiński 1995). For barley up to now only 2–4 LHCII apoproteins (plus *Lhcb 3* gene product) could be identified regardless of the running system applied (Darr and Arntzen, 1986; Jackowski *et al.*, 1991; Peter and Thornber, 1991; Sigrist and Staehelin, 1994). To side-step the possibility of artifactual generation of pI isoforms of LHCII apoproteins during the preparative procedure, we minimized the exposition of the apoproteins to SDS by electroeluting them from non-denaturing IEF and denaturing electrophoresis gels in the presence of detergent-free elution buffers. SDS which came into contact with the apoproteins during SDS-PAGE was washed out of the apoproteins pellet with 80% acetone and 50% isopropanol. Still, the other criticism can be raised against the results presented in this paper i.e. the possibility that some of pI isoforms interpreted as belonging either to 26.7 or 25.6 kDa polypeptide are cross-contaminants – a true number of isoforms would then be lower than 24. In fact a group of molecular species thought to represent 26.7 or 25.6 kDa apoproteins focus at a very similar pI (e.g. 26.7/A vs 25.6/A or 26.7/D

vs 25.6/B). There are, however, serious reasons why we regard a cross-contamination of 26.7 and 25.6 kDa pI isoforms as improbable – to analyze the pattern of their pI isoforms the apoproteins originating from defined LHCII subcomplexes were eluted from preparative gels electrophoresed long enough to maximize the expansion of LHCII apoproteins' region. 26.7 and 25.6 kDa polypeptides from the subcomplexes 3–5 (the only ones housing both apoproteins) migrated on such gels at a distance of at least 3 mm making cutting errors improbable, what more the electrophoretic purity of the apoproteins' samples was verified prior to denaturing IEF.

Thus, we believe that the pattern of molecular species of 26.7 and 25.6 kDa polypeptides does reflect a heterogeneity existing in thylakoid membrane. The complexity of this pattern stems most likely from yet undiscovered heterogeneity of barley *cab* genes as the results of other studies support the view that individual LHCII apoproteins may be distinct genes products (Jansson *et al.*, 1990; Sigrist and Staehelin, 1992). A second source of multiplicity of LHCII apoproteins may be differential N-terminal processing of precursor polypeptides (LHCP<sub>II</sub>). In vitro import assays have shown that single pLHCP<sub>II</sub> can be N-terminally converted (by proteolysis) into two or more polypeptides of various length (Kohorn and Tobin, 1986; Dietz and Bogorad, 1987). It is highly probable that such type of processing activity exists also in vivo as individual LHCII apoproteins of barley were immunostained differentially with antibodies raised against *Lhcb1* or *Lhcb2* unique sequence domains located at different distances from N-terminus (Sigrist and Staehelin, 1994). It is possible that posttranslational processing can generate a family of isopolypeptides differing in length only by 1–3 aminoacids. The isopolypeptides may have various pI but they yield only a single band in our electrophoretic system, working with a resolution of 0.3 kDa. A third source of multiplicity of LHCII apoproteins may be posttranslational acylation of residues. The mechanism, reported for LHCII apoproteins of *Spirodela* (Mattoo and Edelman, 1987) does not seem, however, to be in vivo a common phenomenon (Jansson *et al.*, 1990).

An interesting question arises concerning the accommodation of complexity of LHCII isopolypep-

tides within a PSII holocomplex particle. According to stoichiometric measurements performed in other laboratories (Morrissey *et al.*, 1989; Dainese and Bassi, 1991; Peter and Thornber, 1991) a single, dimeric PSII center core is served by 24 molecules of LHCII apoproteins, the value matching exactly a number of LHCII polypeptide isoforms found in our study (15+9). However, if all the isoforms were assembled in single barley PSII particles it should be inferred that the isoforms are associated at equimolar amounts and according to our data it is by far not the case (Fig. 5C). Thus we conclude that there should exist a number of barley PSII particles heterogenous with respect to the assembly of pI isoforms of LHCII apoproteins, in addition to the suggested heterogeneity of PSII particles at the level of the arrangement of LHCII subcomplexes.

The functional significance of the multiplicity of LHCII apoproteins is not yet known but it may be

suggested that differential expression of individual apoproteins reflects a fine tuning of PSII light-harvesting apparatus to changing environmental and developmental situation (Larsson *et al.*, 1987; Morrissey *et al.*, 1989). It is interesting in this context that Sheen and Bogorad (1986) were able to demonstrate the differences among maize LHCII mRNAs relative to their pattern of light inducibility and tissue-specific expression.

### Acknowledgements

The author gratefully acknowledges the skilled technical assistance of Mrs Julianna Kryzstofiak, the photographic work of Mr Zygmunt Bartkowiak and the help of Mrs Barbara Rochowiak in an initial phase of the experiments. The purchase of Shimadzu CS-9000 Scanning Densitometer was supported by Fundacja na Rzecz Nauki Polskiej.

- Allen K.D. and Staehelin L.A. (1992), Biochemical characterization of photosystem II antenna polypeptides in grana and stroma membranes of spinach. *Plant Physiol.* **100**, 1517–1526.
- Arnon D. (1949), Copper in isolated chloroplasts. Polyphe-noloxidase in *Beta vulgaris*. *Plant Physiol.* **24**, 1–15.
- Bassi R., Rigoni F., Barbato R. and Giacometti G.M. (1988), Light-harvesting chlorophyll *a/b* protein (LHCII) populations in phosphorylated membranes. *Biochim. Biophys. Acta* **936**, 29–38.
- Berthold D.A., Babcock G.T. and Yocum C.F. (1981), A highly resolved, oxygen-evolving photosystem II preparation from spinach thylakoid membranes. *FEBS Lett.* **34**, 231–234.
- Butler P.J.G. and Kuhlbrandt W. (1988), Determination of the aggregate size in detergent solution of the light-harvesting chlorophyll *a/b*-protein complex from chloroplast membranes. *Proc. Natl. Acad. Sci. U.S.A.* **85**, 3797–3801.
- Chitnis P.R. and Thornber J.P. (1988), The major light-harvesting complex of photosystem II: aspects of its molecular and cell biology. *Photosynth. Res.* **16**, 41–63.
- Dainese P., Hoyer-Hansen G. and Bassi R., (1990), The resolution of chlorophyll *a/b* binding proteins by a preparative method based on flat bed isoelectric focusing. *Photochem. Photobiol.* **51**, 693–703.
- Darr S.C. and Arntzen C.J. (1986), Reconstitution of the light-harvesting complex into developing chloroplasts membrane using a dialysable detergent. *Plant Physiol.* **80**, 931–937.
- Dietz K.J. and Bogorad L. (1987), Plastid development in *Pisum sativum* leaves during greening. *Plant Physiol.* **85**, 816–822.
- Dreyfuss B.W. and Thornber J.P. (1994), Assembly of the light-harvesting complexes (LHCs) of photosystem II. Monomeric LHC IIb complexes are intermediates in the formation of oligomeric LHC IIb complexes. *Plant Physiol.* **106**, 829–839.
- Dunahay T., Staehelin L.A., Seibert M., Ogilvie P.D. and Berg S.P. (1984), Structural, biochemical and biophysical characterization of four oxygen evolving photosystem II preparations from spinach. *Biochim. Biophys. Acta* **764**, 179–193.
- Falbel T.G. and Staehelin L.A. (1992), Species-related differences in the electrophoretic behaviour of CP29 and CP26: An Immunochemical analysis. *Photosynth. Res.* **34**, 249–262.
- Fling S.P. and Gregerson D.S. (1986), Peptide and protein molecular weight determination by electrophoresis using a high-molarity Tris buffer system without urea. *Anal. Biochem.* **157**, 83–88.
- Genge S., Pilger D. and Hiller R.G. (1974), The relationship between chlorophyll *b* and pigment-protein complex II. *Biochim. Biophys. Acta* **347**, 22–30.

- Green B.R., Shen D., Aebersold R. and Pichersky E. (1992), Identification of the polypeptides of the major light-harvesting complex of photosystem II (LHCII) with their genes in tomato. *FEBS Lett.* **305**, 18–22.
- Harrison M.A. and Melis A. (1992), Organization and stability of polypeptides associated with the chlorophyll *a/b* light-harvesting complex of photosystem-II. *Plant Cell Physiol.* **33**, 627–637.
- Harrison M.A., Nemson J.A. and Melis A. (1993), Assembly and composition of the chlorophyll *a/b* light-harvesting complex of barley (*Hordeum vulgare* L.): immunochemical analysis of chlorophyll *b*-less and chlorophyll *b*-deficient mutant. *Photosynth. Res.* **38**, 141–151.
- Henrysson T., Schroder W.P., Spangfort M. and Akerlund H.-E. (1989), Isolation and characterization of the chlorophyll *a/b* protein complex CP29 from spinach. *Biochim. Biophys. Acta* **977**, 301–308.
- Jackowski G. and Kluck E. (1994), The oligomeric arrangement of the light-harvesting chlorophyll *a/b*-protein complex of photosystem II. *Z. Naturforsch.* **49c**, 337–342.
- Jackowski G. and Przymusiński R. (1995), The resolution and biochemical characterization of subcomplexes of the main light-harvesting chlorophyll *a/b*-protein complex of photosystem II (LHCII). *Photosynth. Res.* **43**, 41–48.
- Jackowski G., Su L.D. and Schneider J. (1991), Immunolocalization of the apoprotein of the light-harvesting chlorophyll *a/b*-protein complex of photosystem II in senescing leaves as influenced by cytokinin. *Acta Physiol. Plant.* **13**, 263–270.
- Jansson S., Selstam E. and Gustafsson P. (1990), The rapidly phosphorylated 25 kDa polypeptide of the light-harvesting complex of photosystem II is encoded by the type cab-II genes. *Biochim. Biophys. Acta* **1019**, 110–115.
- Kohorn B. and Tobin E.M. (1986), Chloroplast import of light-harvesting chlorophyll *a/b* proteins with different amino termini and transit peptides. *Plant Physiol.* **82**, 1172–1174.
- Król M., Spangfort D.M., Huner N.P.A., Öquist G., Gustafsson P. and Jansson S. (1995), Chlorophyll *a/b*-binding proteins, pigment conversions, and early light-induced proteins in a chlorophyll *b*-less barley mutant. *Plant Physiol.* **107**, 873–883.
- Kuhlbrandt W. (1984), Three-dimensional structure of the light-harvesting chlorophyll *a/b*-protein complex. *Nature* **307**, 478–480.
- Kuhlbrandt W. (1987), Three-dimensional structure of the light-harvesting chlorophyll *a/b*-protein complex from pea chloroplasts. *J. Mol. Biol.* **194**, 757–762.
- Laemmli U.K. (1970), Cleavage of structural proteins during the assembly of the hof bacteriophage T4. *Nature* **227**, 680–685.
- Larsson U.K., Anderson J.M. and Andersson B. (1987), Characterization of two different subpopulations of spinach light-harvesting chlorophyll *a/b*-protein complex (LHCII); polypeptide composition, phosphorylation pattern and association with photosystem II. *Biochim. Biophys. Acta* **894**, 59–68.
- Legocka J., Schneider J. and Jackowski G. (1990), Cytokinin-controlled expression of the apoprotein on the light-harvesting complex of photosystem II in the tissue culture of *Dianthus caryophyllus*. I. Protein identification. *Acta Physiol. Plant.* **12**, 303–309.
- Mattoo A.K. and Edelman M. (1987), Intramembrane translocation and posttranslational palmitoylation of the chloroplast 32-kDa herbicide-binding protein. *Proc. Natl Acad. Sci. U.S.A.* **84**, 1497–1501.
- Melis A. (1991), Dynamics of photosynthetic membrane composition and function. *Biochim. Biophys. Acta* **1058**, 87–106.
- Morrissey P.J., Glick R.E. and Melis A. (1989), Supramolecular assembly and function of subunits associated with the chlorophyll *a/b* light-harvesting complex II (LHCII) in soybean chloroplasts. *Plant Cell Physiol.* **30**, 335–344.
- Neuhoff V., Arold N., Taube D. and Erhardt W. (1988), Improved staining of proteins in polyacrylamide gels including isoelectric focusing gels with clear background at nanogram sensitivity using Coomassie Brilliant Blue G-250 and R-250. *Electrophoresis* **9**, 255–262.
- Peter G.F. and Thornber J.P. (1991), Biochemical composition and organization of higher plant photosystem II light-harvesting pigment-proteins. *J. Biol. Chem.* **266**, 6745–6754.
- Sheen J.Y. and Bogorad L. (1986), Differential expression of six light-harvesting chlorophyll *a/b* binding protein genes in maize leaf cell types. *Proc. Natl Acad. Sci. U.S.A.* **83**, 7811–7815.
- Sigrist M. and Staehelin L.A. (1992), Identification of type 1 and type 2 light-harvesting chlorophyll *a/b*-binding proteins using monospecific antibodies. *Biochim. Biophys. Acta* **1098**, 191–200.
- Sigrist M. and Staehelin L.A. (1994), Appearance of type 1,2 and 3 light-harvesting complex II and light-harvesting complex I proteins during light-induced greening of barley (*Hordeum vulgare*) etioplasts. *Plant Physiol.* **104**, 135–145.
- Spangfort M. and Andersson B. (1989), Subpopulations of the main chlorophyll *a/b* light-harvesting complex of photosystem II – isolation and biochemical characterization. *Biochim. Biophys. Acta* **977**, 163–170.
- Staehelin L.A. and Arntzen C.J. (1983), Regulation of chloroplast membrane function. Protein phosphorylation changes the spatial organization of membrane components. *J. Cell Biol.* **97**, 1329–1337.
- Tanaka A., Tanaka M. and Tsuji H. (1987), Resolution of chlorophyll *a/b* protein complexes by polyacrylamide gel electrophoresis: evidence for the heterogeneity of light-harvesting chlorophyll protein complexes. *Plant Cell Physiol.* **28**, 1537–1545.
- Thornber J.P., Peter G.F., Morishige D.T., Gomez S., Anandan S., Welthy B.A., Lee A., Kerfeld C., Takeuchi T. and Preiss S. (1992), Light harvesting in photosystem I and II. *Biochem. Soc. Transaction* **21**, 15–18.
- Towbin H., Staehelin T. and Gordon J. (1979), Electrophoretic transfer of protein from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. U.S.A.* **76**, 4350–4354.