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The resolution and biochemical characterization of subcomplexes of the main light-harvesting chlorophyll *a/b*-protein complex of Photosystem II (LHC II)

Grzegorz Jackowski[1] & Roman Przymusiński[2]
[1]*Adam Mickiewicz University, Department of Plant Physiology, Al. Niepodległości 14, 61–713 Poznań, Poland;*
[2]*Adam Mickiewicz University, Department of Plant Ecophysiology, Al. Niepodległości 14, 61–713 Poznań, Poland*

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Abstract

LHC II isolated from carnation leaves has been solubilized and resolved by a newly developed, vertical-bed non-denaturing isoelectric focusing in polyacrylamide slab gels to yield three trimeric subcomplexes focusing at pH 4.52, 4.42 and 4.37 (designated a, b and c, respectively), comprising approximately 38%, 24% and 38% of the chlorophyll. The spectroscopic data demonstrated a close similarity among LHC II subcomplexes concerning their chlorophyll content and organization. The most alkaline and the most acidic subcomplex contained the 27 kDa polypeptide of LHC II while the intermediate pI fraction contained both LHC II polypeptides, i.e. 27 kDa and 26 kDa ones associated at 2:1 stoichiometry. The 27 kDa polypeptide could be resolved by denaturing isoelectrofocusing into 10 pI molecular isoforms covering 5.90–4.20 pH range. Three of the isoforms were found in the subcomplexes a and b and eight in the subcomplex c. The 26 kDa polypeptide comprised the unique pI molecular isoform focusing at pH 5.61.

Abbreviations: CBB G-250 – Coomassie Brilliant Blue G-250; chl – chlorophyll; DM – *n*-dodecyl-β-D-maltoside; EDTA – ethylendiaminotetraacetic acid; IEF – isoelectric focusing; LHC II – the main light-harvesting chlorophyll *a/b*-protein complex of Photosystem II; LHCP II – apoprotein of the main light-harvesting chlorophyll *a/b*-protein complex of Photosystem II; NP-40 – polyethyleneglycol-*p*-isooctylphenyl ether; pI – isoelectric point; OG – octyl-β-D-glucopyranoside; PS II – Photosystem II; SDS-PAGE – sodium dodecylsulphate polyacrylamide gel electrophoresis; TCA – trichlorooacetic acid.

Introduction

The main light-harvesting chlorophyll *a/b*-protein complex of Photosystem II, designated LHC II by the majority of research groups, is an exceptionally complicated system with a structural heterogeneity recognized at the levels of DNA, protein and pigment-protein holocomplex. The apoproteins of LHC II (LHCP II) are coded for by numerous genes belonging to the CAB extended family (Dunsmuir et al. 1983; Green et al. 1991). The number of reported polypeptides in LHC II of a single species varies from 1 to 8 (most frequently 2–3) with molecular masses within 25–30 kDa range (Green 1988; Spangfort and Andersson 1989; Sigrist and Staehelin 1992). The polypeptides are synthesized in the cytoplasm as soluble precursors (pLHCP II) with cleavable N-terminal extensions called transit peptides. The pLHCP II is then translocated across both chloroplast envelopes, proteolytically processed to remove the transit peptide, complexed with chlorophyll and xantophyll molecules and assembled into thylakoid membrane (Schmidt et al. 1981; Cline 1988). In vitro import assays have shown that single pLHCP II can be proteolytically converted

into two or more polypeptides of different molecular weight (Kohorn and Tobin 1986; Dietz and Bogorad 1987). In spite of this the heterogeneity of LHCP II in vivo is thought to be dependent rather on the heterogeneity of coding genes than on different precursor processing (Jansson et al. 1990; Sigrist and Staehelin 1992). Structural in vitro studies performed on two-dimensional as well as three-dimensional crystals of LHC II revealed that the holocomplex had 3-fold symmetry (Kühlbrandt 1984, 1987) and analytical centrifugation of OG-solubilized LHC II finally established the aggregate size to be trimer (Butler and Kühlbrandt 1988). The trimer, therefore, is also believed to be the preferred form of LHC II inside the thylakoid membrane. Recently, it was shown that LHC II trimers are heterogenous themselves – in spinach two distinct trimers were isolated with polypeptide compositions corresponding to those predicted for peripheral and inner subpopulations, i.e. LHC II subcomplexes having different arrangement around PS II reaction center complex and exhibiting different behaviour during phosphorylation – mediated state transitions (Larrson et al. 1987; Spangfort and Andersson 1989). In corn at least seven LHC II trimers were resolved (Bassi et al. 1988; Bassi and Dainese 1992), three of which were suggested to build up the peripheral subpopulation the remaining trimers being the constituents of inner subpopulation (Bassi and Dainese 1992). For soybean it was suggested that LHC II-inner may contain two individual trimers while LHC II-peripheral is composed of one kind of trimer (Morrisey et al. 1989). Large complexes containing multiple LHC II trimers have been described recently (Peter and Thornber 1991a; Jackowski and Kluck 1994) demonstrating the non-uniformity of oligomeric status of LHC II in vivo. All these data feature LHC II as a supramolecular assembly of 2–7 distinct trimeric isoforms constituting inner and peripheral moieties which, at least partially, can be organized as higher order aggregates. However, to construct the detailed model of the structural organization of LHC II more exhaustive studies of biochemical composition of individual trimers are required. This study was therefore aimed at monitoring the organization of LHC II in terms of the quantity, polypeptide composition and pigment organization of its trimeric subcomplexes.

Material and methods

Plant material

The investigations were carried out using carnation (*Dianthus caryophyllus* L. cv. Rosalie) leaves obtained from a local supplier.

Isolation of LHC II and its subcomplexes

LHC II was isolated and stored essentially as described previously (Jackowski and Kluck 1994). For separation of LHC II subcomplexes the LHC II sample was pelleted by centrifugation at 40 000 *g* for 5 min, washed twice with 2 mM EDTA, pH 7.5 and suspended at 1–3 mg chl/ml in 10% glycerol. Then 10% DM stock solution was added to yield a final concentration of 1% and the sample was incubated on ice, with occasional stirring, for 30 min. The surfactant extract was spun at 12 300 *g* for 5 min to remove insoluble residue and the resolution of the sample was performed by non-denaturing IEF. A vertical slab gel contained 7% acrylamide, 0.19% N,N′-methylenebisacrylamide, 2% ampholine carrier ampholites (pH 3.5–5.0), 0.375% DM and 0.00075% riboflavine as polymerizing agent. The Hoefer Mighty Small II (Hoefer Scientific Instruments) units and 0.5 mm-thick gels (for analytical purposes) or 1.5 mm gels (for preparative purposes) were used. The cathode solution was 20 mM NaOH and the anode solution was 20 mM H_3PO_4. The samples were applied to the wells of precooled gels and focused for a total of 5–6 h at constantly increasing voltage (50–300 V in 50 V increments and 300-500 V in 100 V increments). The gel was protected from light with an aluminum foil cover. pH of the resulting bands was determined with respect to the series of pI marker proteins (IEF mix 3.6–6.6, Sigma). The green subcomplexes were electroluted from the gel using Model 422 Electroeluter (BioRad, Italy) filled with 20 mM Tris/maleate, pH 7.0. DM and glycerol were added to the eluates to yield 1% (DM) and 10% (glycerol) final concentrations, the samples were solubilized on ice for 30 min and purified by rerunning on a second IEF gel followed by a second electroelution.

SDS-PAGE and denaturing IEF

The polypeptides of the LHC II subcomplexes were precipitated by adding 5 volumes of 80% acetone. The pellets, obtained by centrifugation at 12 300 × *g* for 10 min, were dissolved and the polypeptides fractionat-

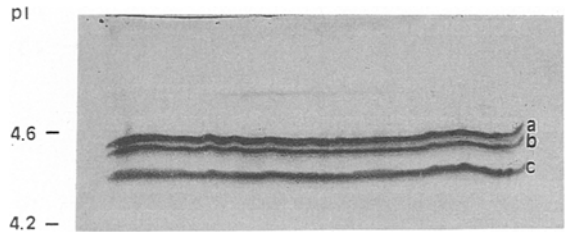

Fig. 1. Fractionation of LHC II by non-denaturing IEF. 500 μg chl of LHC II resolved in a 1.5 mm-thick, preparative, vertical 7% polyacrylamide gel containing 0.75% DM (pH 3.5–5.0). The symbols of the subcomplexes are indicated.

ed by SDS-PAGE using the buffer system of Laemmli (1970). The electophoreses were run on 14% acrylamide gels at 25 mA/gel for 3 h. The gels were fixed at 12% TCA and stained with 0.08% CBB G-250 by an improved, highly-sensitive method (Neuhoff et al. 1988).

For denaturing IEF the pellets of polypeptides of the LHC II subcomplexes were solubilized in a buffer containing 9.5 M urea, 2% NP-40, 5% mercatptoethanol and 2% ampholine carrier ampholites (3.5/10 pH and 3.5/5.0 pH blended at 1:4 stoichiometry). The samples were focused by denaturing IEF in tube gels according to O'Farrell (1975) and the gels were stained with CBB G-250 according to Blakesley and Boezi (1977). To perform a relative quantitation of the polypeptide bands SDS-polyacrylamide and denaturing IEF gels were scanned using Shimadzu CS-9000 Flying Spot Dual-Wavelength Scanner with on-board integration system.

Spectroscopic analyses

Steady-state absorption spectra were recorded at room temperature on Shimadzu UV-160A spectrophotometer. Low temperature (77 K) fluorescence emission spectra were recorded with a Carl Zeiss Jena SPM-2 fluorimeter using 430 nm as an excitation wavelength.

Other methods

Chl was determined in 80% acetone by the procedure of Arnon (1949).

Results

The structural organization of LHC II can be analyzed by non-denaturing isoelectrofusing (IEF), the technique which was shown to resolve chl-proteins without the removal of pigments from them (Mullet 1983; Spangfort et al. 1987; Dainese et al. 1990). The existing approaches, however, are hampered by the incomplete resolution of the fractions (Giardi et al.

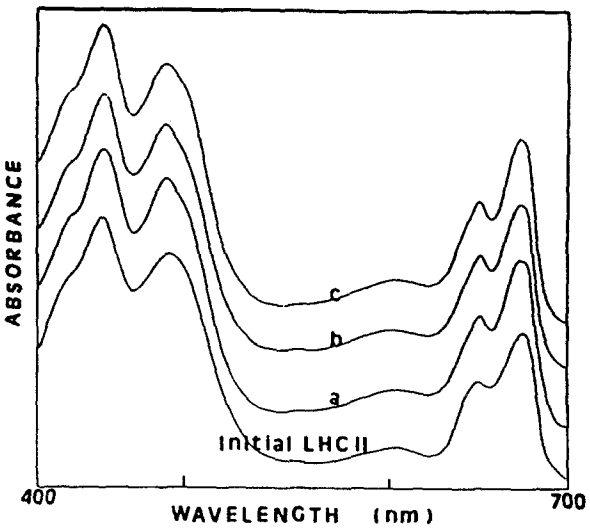

Fig. 2. Room temperature absorption spectra of LHC II and its subcomplexes a, b and c.

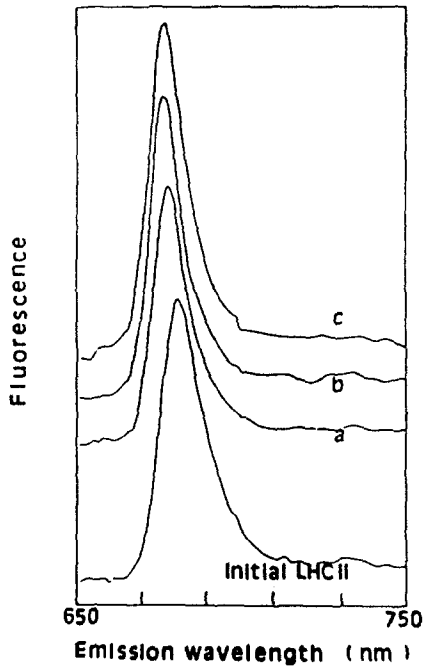

Fig. 3. Low temperature (77 K) fluorescence emission spectra of LHC II and its subcomplexes a, b and c, obtained using 430 nm as an excitation wavelength.

1990; Bassi et al. 1993) as well as the long time needed to perform the separation (Spangfort and Andersson 1989; Dainese et al. 1990). In order to improve the performance of non-denaturing IEF the vertical polyacrylamide slab gel was used as a separation medium in conjunction with different types and amounts of detergents as solubilizing agents. The attempts with LHC II subjected to IEF in the presence of OG both in polyacrylamide gel and the sample did not give satisfactory results, regardless of the running conditions applied. The high resolution of LHC II was found to be strictly related to the use of DM in combination with the gels of suitable acrylamide concentration (7%), formed by riboflavine-dependent polymerization. The gels produced using ammonium persulphate as a polymerizing agent gave severely distorted bands while the application of inadequate acrylamide concentration (under or above 7%) led either to the lack of LHC II resolution or the inefficient penetration of the gel by an LHC II extract. When the initial LHC II sample was treated with 0.5–3.0% DM and the solubilization mixture fractionated by IEF in 7% acrylamide vertical slab gel containing 0.20–0.75% DM, three prominent green bands were resolved with no signs of the disruption of pigment-protein interaction (the omission of DM from the gel resulted in the focusing of LHC II extract as a single, broad band). pI of the LHC II subcomplexes, designated a, b and c in Fig. 1, were identified to be 4.52 (fraction a), 4.47 (fraction b) and 4.35 (fraction c). As the subcomplexes could not be resolved in a pure form directly from a single IEF separation, all the investigations were performed on the LHC II subcomplex samples that underwent two consecutive IEF runs. The LHC II subcomplexes were associated at a proportion of 1.60 ± 0.19:1:1.43 ± 0.22 (a:b:c) as judged by chl distribution. They have almost identical chl *a*/chl *b* ratios of 0.99 ± 0.11 (a), 0.98 ± 0.09 (b) and 1.08 ± 0.14 (c) and almost identical absorption spectra in 400–700 nm range with dominating maxima at 674.5, 651, 473 and 437.5 nm (Fig. 2). The red maximum of chl *a* is clearly blue-shifted 2–4 nm when compared with initial LHC II most probably reflecting the removal of contaminating CP29 and CP26 during IEF. Low temperature (77 K) fluorescence emission spectra of the subcomplexes were identical, with a distinct peak at 677–678 nm, 4–5 nm blue-shifted in comparison with initial LHC II (682 nm) (Fig. 3). Thus the spectroscopic data demonstrate a close similarity among the LHC II subcomplexes concerning the content and organization of their chlorophyll.

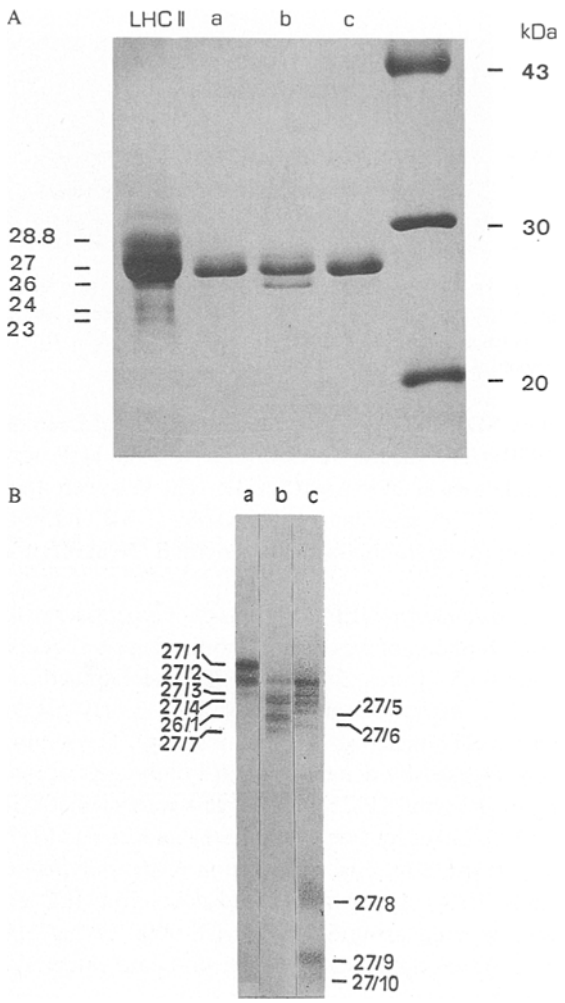

Fig. 4. Polypeptide composition of LHC II and its subcomplexes. 2 μg chl of LHC II and the subcomplexes a, b and c were separated by SDS-PAGE (A) or denaturing IEF (B). The gels were stained with CBB G-250

To determine their polypeptide composition the LHC II subcomplexes were electroeluted from non denaturing IEF gels and subjected to one-dimensional SDS-PAGE or denaturing IEF. The pattern of staining the gels with CBB G-250 can be treated as a measure of relative abundancies of individual LHCP II as the binding characteristics of CBB G-250 to defined LHCP II are identical or very similar (Dainese and Bassi 1991). The initial LHC II sample could be resolved by SDS-PAGE into five major polypeptides of 28.8, 27, 26, 24 and 23 kDa (Fig. 4A) associated at 2.8:10.3:1:1:1.1 stoichiometry as judged by an integrating densitometry of CBB-stained bands. Only two of the polypeptides found in initial LHC II, i.e. 27 and 26 kDa, were shown

Table 1. Distribution of pI isoforms of LHCP II in LHC II subcomplexes

pI isoform (PI)		LHC II subcomplex		
		a	b	c
		% recovered in each isoform		
27/1	(5.90)	53	-	-
27/2	(5.82)	34	16	36
27/3	(5.76)	13	-	7
27/4	(5.68)	-	37	19
27/5	(5.65)	-	-	5
26/1	(5.61)	-	31	-
27/6	(5.57)	-	-	2
27/7	(5.50)	-	16	-
27/8	(4.70)	-	-	18
27/9	(4.32)	-	-	10
27/10	(4.20)	-	-	3

The isoforms were quantitated by an integrating densitometry of CBB G-250-stained denaturing IEF gels. The data represent one typical experiment (out of four).

to be authentic carnation LHC II constituents, able to form trimers in vivo (Jackowski and Kluck 1994). The polypeptide composition of all the subcomplexes was found to be different from the composition of the initial LHC II and this observation means that the dissociation of LHC II holocomplex into the subcomplexes was complete. Namely, in the most alkaline (fraction a) and the most acidic (fraction c) subcomplexes only 27 kDa polypeptide could be detected while both 27 and 26 kDa polypeptides were found in the intermediate pI subcomplex (fraction b) (Fig. 4A). The 27/26 kDa ratio of this subcomplex was 2.20±0.20:1. A very faint band at 27.4 kDa, present among polypeptides of all the subcomplexes, corresponds most probably to the apoprotein of CP26 complex which contaminate, (along with CP29), initial LHC II samples to a small degree. In spite of differences in pI the subcomplexes a and c have seemingly the same polypeptide composition. Two explanations could be provided to solve this apparent contradiction. First, the subcomplexes could exist in various oligomeric states as it was shown (Bassi and Dainese 1992) that the difference in pI of pigment-protein complexes may reflect the differences in their oligomeric arrangement. However, all the subcomplexes were recovered almost exclusively in the same, trimeric form when resolved with the use of mildly denaturing 'green' gel electrophoresis (data not shown). Second, a and c trimers could be composed of different subsets of pI isoforms of 27 kDa polypeptide. To verify this assumption all the subcomplexes were electroeluted from non-denaturing IEF gels and their polypeptides were fractionated by fully denaturing IEF. The results are shown on Fig. 4B and Table 1. The subcomplex a was shown to contain three pI isoforms of 27 kDa polypeptide (designated 27/1, 27/2 and 27/3) covering pH range of 5.90–5.76 while the subcomplex c contained eight isoforms of 27 kDa polypeptide focusing at pH 5.82–4.20 (designated 27/2, 27/3...27/10). The subcomplex b was resolved into four polypeptide molecular species in the pH range of 5.82–5.50. The LHCP II isoforms focusing at pH 5.61 and 5.50 were observed exclusively in the b subcomplex and it implies that either one of the isoforms or both must be the molecular species of 26 kDa polypeptide. To classify the isoforms as 27 kDa or 26 kDa molecular species their relative abundance was determined by an integrating densitometry. The results are given in Table 1 – the 5.61 and 5.50 isoforms were shown to comprise 31% and 16% of the integrated area under the scan of IEF-resolved polypetide isoforms belonging to LHC II subcomplex b. Using these data the possible 27/26 kDa ratio for the entire subcomplex b can be calculated yielding the following values: 1.1:1 (when both isoforms belong to 26 kDa polypeptide), 2.2:1 (only pI 5.61 isoform belongs to 26 kDa polypeptide) or 5.3:1 (only pI 5.50 isoform belongs to 26 kDa polypeptide). As the value of 2.2:1 is identical with the 27/26 kDa ratio measured in this work for the entire subcomplex b it can be concluded that pI isoform focusing at 5.61 is a unique molecular species 26 kDa polypeptide while the total number of 27 kDa polypeptide isoforms equals ten. The isoform of 27 kDa polypeptide focusing at pH 5.82 (27/2) is the only species found in all the subcomplexes (although in different stoichiometries) the remaining ones being present in one or two subcomplexes. Only the most alkaline 27 kDa polypetide pI isoforms were found in the most alkaline subcomplex (a) with 27/1 alone standing for 53% of the integrated area under the scan of the IEF-resolved polypeptides of this subcomplex. The most acidic subcomplex (c) is, in its turn, enriched in the most acidic pI isoforms of 27 kDa polypeptide (27/8, 27/9, 27/10) together corresponding to about 31% of the integrated area. In accordance with the expectations intermediate pI polypeptide isoforms prevail in subcomplex b (Fig. 4B).

Discussion

The primary source of difficulties in the research in the area of the subcomplex organization of LHC II is the fact that a close correspondence of the LHC II populations with respect to their molecular weight makes it ineffective to apply the majority of techniques normally used to characterize chl-proteins, as mildly denaturing 'green' gel electrophoresis or sucrose gradient ultracentrifugation. Only the introduction of non-denaturing IEF, a mild technique utilizing pI as the resolving parameter, allowed the fractionation of LHC II into its subcomplexes (Mullet 1983; Spangfort et al. 1987; Bassi et al. 1988). The recent IEF procedures based on the use of preparative density gradient columns (Spangfort and Andersson 1989) or flat-bed granulated gels (Dainese et al. 1990), are, however, characterized by a limited resolution and lengthy focusing times. The development of a new IEF method able to separate LHC II subcomplexes with a better performance was therefore of particular importance in this work. We have shown that by solubilizing LHC II with DM and using a 7% polyacrylamide matrix containing the same detergent, the resolution of LHC II subcomplexes may be considerably improved and the time of separation drastically shortened to 5–6 h (vs 14–72 h according to existing protocols). We have found that high resolution of the novel IEF was critically dependent on the choice of acrylamide concentration in the gel and the use of riboflavin instead of ammonium persulphate as polymerizing agent. The solubilization and IEF running conditions used during this study let us resolve carnation LHC II into three trimeric subcomplexes focusing in the pH range 4.35–4.52. This finding is consistent with the previous reports showing that LHC II populations focus between pH 3.9 and 4.6 (Mullet 1983; Spangfort and Andersson 1989; Bassi and Dainese 1992; Andersson and Albertsson 1993). We were not able to find any major differences between the subcomplexes with respect to their chl content and organization but the subcomplexes differed in the relative amounts of their polypeptide components. Two of the subcomplexes i.e. a and c contain only the 27 kDa polypeptide while the subcomplex b contains both LHCP II – 27 and 26 kDa polypeptides in a proportion of 2.2:1. Taking into account the experimental error the latter figure may be interpreted as 2:1. As the proportion for the a, b and c subcomplexes was shown to be very close to 1.5:1:1.5 we end up with a 27/26 kDa stoichiometric ratio of 11:1 for the entire LHC II. This value is compatible with the measured ratio of 10.3:1 demonstrating the internal consistency of the data. To integrate these data into the proposal of the overall stoichiometry of LHC II subcomplexes within PS II membranes two critical assumptions, based on previous studies, had to be made. First, we assume here that PS II center core occurs in higher plants in dimeric state. This premise is supported by the data from the two-dimensional PS II and PS II center core crystals (Bassi et al 1989; Lyon et al. 1993) as well as from electrophoretic fractionation of thylakoid extracts obtained with various glycosidic surfactans (Peter and Thornber 1991b). Second, we assume that each PS II center core monomer is linked to 12 LHCP II molecules according to the stoichiomeric estimations made for soybean (Morrisey et al. 1989), maize (Dainese and Bassi 1991) and barley (Peter and Thornber 1991a; Harrison and Melis 1992). Using the above assumptions and the stoichiometries measured in our study we suggest that carnation PS II center core dimer is served by a total of eight individual trimeric subcomplexes, the a, b and c trimers' overall ratio being 3:2:3. This cannot be proven at present, however, whether all the carnation dimeric PS II holocomplex particles are served by LHC II subcomplexes associated at this ratio or there is a heterogeneity among PS II holocomplexes with respect to the arrangement of their LHC II subcomplexes.

The LHC II trimeric subcomplexes are composed of two polypeptides – 27 and 26 kDa ones – and we assume that no other chl *a/b*-binding polypeptides are able to form stable trimers. This assumption receives strong support from the fact that 27 and 26 kDa were the only polypeptides detectable in the subcomplexes a, b and c as well as in bulk LHC II trimeric form resolved by mildly-denaturing SDS-PAGE (Jackowski and Kluck 1994).

Using the denaturing IEF we have been able to resolve and identify as many as ten pI isoforms of 27 kDa polypeptide and the only isoform of 26 kDa polypeptide. Whether this complex pattern of LHCP II isoforms stems from the heterogeneity of cab genes or posttranslational modifications of pLHCP II can not be decided on the basis of the results produced in our experiments. However, the results of other studies support the concept that individual LHCP II may be different genes products (Jansson et al. 1990; Sigrist and Staehelin 1992). A large number of pI isoforms of LHCP II found in our work would thus correlate well to a large number of cab genes identified in diverse plant species (Demmin et al. 1989; Green et al. 1991). pI isoforms of 27 and 26 kDa polypeptides can represent Lhcb1 and Lhcb2 gene products (Jansson 1994)

but given a close similarity of type 1 and type 2 LHC II apoproteins with regard to their apparent molecular weight (Sigrist and Staehelin 1992, 1994) it is not possible to decide at present which carnation LHCP II isoforms can be coded for by the defined type of Lhcb sequences.

The carnation LHCP II pI isoforms focus between pH 4.2 and pH 5.9 i.e. generally at higher pH range then the LHC II subcomplexes (4.37–4.52). It must be borne in mind, however, that the pI of proteins in urea solution (and urea was used as solubilizing and dissociating agent during denaturing IEF) can not be easily correlated with those determined for LHC II subcomplexes resolved in aqueous environment, The results obtained in urea have little physical meaning and are rather of relative importance (Gelsema et al. 1979).

As expected, the most alkaline LHCP II isoforms (covering pH range of 5.82–5.90) are abundant in the most alkaline LHC II trimer (a; 4.52) while the most acidic isoforms (pH range 4.70–4.20) occur in the most acidic LHC II trimer (c; 4.37). A major point deserving further studies is the distribution of pI isoforms of LHCP II within LHC II trimeric subcomplexes. An unsolved question remains whether various pI isoforms of the 27 kDa polypeptide are assembled as homo- or heterotrimers but our data indicate indirectly that at least the 27/2 isoform, which is common for all LHC II subcomplexes, must be assembled as heterotrimers together with other LHCP II molecular species. If the 27/2 isoform were assembled exclusively as homotrimers it would be hard to understand why some of such homotrimers are recovered as the a subcomplexes while others as c subcomplexes. The deeper insight in the pattern of association of LHCP II molecular species is dependent on the establishment of yet more powerful techniques of LHC II subcomplex fractionation than that which is described in this work.

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