The Oligomeric Arrangement of the Light-Harvesting Chlorophyll *a/b*-Protein Complex of Photosystem II

Grzegorz Jackowski and Ewa Kluck

Department of Plant Physiology, Adam Mickiewicz University, Al. Niepodległości 14, 61-713 Poznań, Poland

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Carnation, Light-Harvesting Chlorophyll *a/b*-Protein Complex, Oligomer, Polypeptide, Stoichiometric Ratio

The light-harvesting chlorophyll *a/b*-protein complex of photosystem II (LHC II) was isolated from carnation (*Dianthus caryophyllus* L.) leaves by K^+-induced aggregation of *n*-heptylthioglucoside-treated photosystem II particles. When solubilized with a mixture of lithium docedyl sulphate, octyl-β-D-glucopyranoside and dodecyl-β-D-maltoside the LHC II was resolved by mild sodium dodecyl sulphate-polyacrylamide gel electrophoresis into four oligomeric forms and a monomeric one. LHC II contained five major polypeptides only two of which (27 and 26 kDa) were found to be its authentic components. The oligomeric forms of LHC II were found to differ in the stoichiometric ratios of the polypeptides present. The 26 kDa polypeptide was enriched in the largest oligomeric forms while the 27 kDa polypeptide tended to form a monomer or to assemble as lower oligomeric states of LHC II.

Introduction

The main light-harvesting chlorophyll *a/b*-protein complex *i.e.* LHC II, or LHC II b according to the nomenclature of Thornber's group (Peter and Thornber, 1991) is an exceptionally complicated system with a structural heterogeneity recognized at the levels of DNA, protein and pigment-protein holocomplex. The heterogeneity at pigment-protein level stems from the existence of subpopulations, *i.e.* LHC II subunits differing with respect to the strength of an association with the PS II reaction centre core (Staehelin and Arntzen, 1983; Larsson *et al.*, 1987; Spangfort and Andersson, 1989). When analyzed by the most widely used mildly denaturing SDS-PAGE systems ("green" gels) thylakoid membranes are resolved into 7–8 Chl-proteins, two of which are thought to be LHC II (Anderson *et al.*, 1978; Camm and Green, 1980; Green, 1988). They are generally interpreted as monomeric and trimeric forms of LHC II (Anderson *et al.*, 1978) thus the oligomerization is an additional source of heterogeneity of the LHC II holocomplex. The trimeric form seems to be the stable, functional unit of LHC II *in vitro* and probably *in vivo* (Kuhlbrandt, 1984, 1987) while a monomer is thought to be an unstable, partially decomposed form (Ide *et al.*, 1987). As the existence *in vivo* of large complexes, most probably multiple trimers, containing LHC II subunits has been suggested recently (Peter and Thornber, 1991), it was the aim of our study to elucidate the oligomeric arrangement of LHC II in terms of the multiplicity and the polypeptide composition of the oligomeric forms. Our data indicate that by using a unique mixture of detergents in conjunction with a mild electrophoretic system the resolution of a highly purified LHC II preparation into four oligomeric and one monomeric form can be achieved. The oligomeric forms are shown to have different polypeptide compositions.

Abbreviations: Chl, chlorophyll; CP24 and CP29, chlorophyll-protein complex 24 and 29; DM, dodecyl-β-D-maltoside; LDS, lithium dodecyl sulphate; LHC II, light-harvesting chlorophyll *a/b*-protein complex of photosystem II; LHCP II, apoprotein of LHC II; OG, octyl-β-D-glucopyranoside; PS II, photosystem II; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; TCA, trichloroacetic acid.

Reprint requests to G. Jackowski.
Telefax: 0 10-48 61-52 36 15.

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Materials and Methods

LHC II isolation

LHC II was isolated from the leaves of carnation (*Dianthus caryophyllus* L. *cv.* Rosalie) as a by-product in the procedure of PS II complex purification described by Enami *et al.* (1989). The procedure was modified in the following way:

300 mM KCl was used during *n*-heptylthioglucoside treatment of PS II instead of 40 mM $MgCl_2$ and the pellet of aggregated LHC II was washed with 100 mM KCl and H_2O as described by Kuhlbrandt (1987). Finally LHC II was suspended in 2 mM Tris/maleate, pH 7.0, 10% glycerol (TMG buffer) and stored in dry ice.

LHC II solubilization

LHC II prepared as described above was pelleted by centrifugation at 40,000×*g* for 10 min and the pellet was resuspended in TMG buffer. For most preparative and analytical work the mixture of 1.2% LDS, 2.4% OG and 1.2% DM containing 1.2 mg Chl per ml was applied. In some experiments LHC II was solubilized in the mixture of these detergents used at lower concentrations. After stirring for 30 min on ice the insoluble residue (exclusively containing starch), was pelleted at 15,000×*g* for 5 min and the samples were subjected to electrophoresis on precooled gels.

Electrophoresis

The oligomeric forms of LHC II were resolved under mildly denaturing SDS-PAGE conditions. Electrophoresis was carried out at 4 °C for 20 h using tube gels of 9% acrylamide (0.2 mA/tube) and the buffer system of Kirchanski and Park (1976) (SDS omitted in the gels). For Ferguson analysis the gels were run in the same manner except that the LHC II was fractionated on gels of four acrylamide concentrations (6.0–10.5%). The green bands of oligomeric forms of LHC II were cut out from the gel, the slices were dispersed by squeezing through a syringe and the slurry incubated at 4 °C in TMG buffer. The acrylamide residue was removed by centrifugation. It was found that although microelution of oligomeric forms of LHC II from acrylamide was not 100% efficient none of the polypeptides was preferentially trapped within the acrylamide residue. To analyze the polypeptide composition of the bands proteins were precipitated (from the supernatants) with 5 volumes of 80% acetone and resolved by denaturing electrophoresis performed at room temperature for 3–6 h using slab gels of 14% acrylamide (25 mA/gel) and the buffer system of Laemmli (1970). The gels were fixed for 1 h at 12% TCA, stained with Coomassie Brilliant Blue G-250 by an improved, highly sensitive method (Neuhoff *et al.*, 1988) destained and scanned by an LKB Ultroscan XL laser densitometer with on-board integration system.

Other

Chl concentration was assayed according to Arnon (1949).

Results

In order to separate and analyze the oligomeric states of LHC II different methods of bulk LHC II isolation were tested in combination with several mixtures of various detergents and electrophoretic separation. The most effective solubilization and stabilization of the products was attained with a mixture of LDS (1.2%), OG (2.4%) and DM (1.2%) which will be called "standard" herein, the result of the solubilization being independent of the sequence of the detergents' additions. When the bulk LHC II preparation, obtained by K^+-induced aggregation of *n*-heptylthioglucoside-treated PS II particles, was solubilized under standard conditions and the mixture fractionated by a mildly dissociating electrophoresis a pattern of five green bands and a band of free pigments was obtained. Very similar patterns of the green bands were found for three higher plant species, *i.e.* carnation, spinach and pea (Fig. 1) demonstrating that the multiplicity of LHC II classes is a general phenomenon.

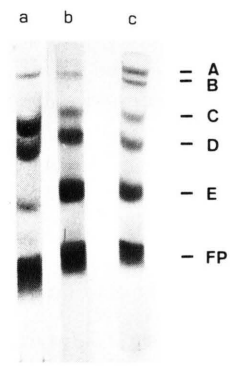

Fig. 1. LHC II of three plant species separated by mildly denaturating electrophoresis. LHC II was isolated by K^+-induced aggregation of *n*-heptylthioglucoside-treated PS II particles from carnation (a), spinach (b) and pea (c). Prior to electrophoresis LHC II (70 µg Chl) was solubilized under standard conditions. The gels were not stained. FP, free pigments.

To test whether LHC II subclasses (designated A–E in order of increasing mobility as indicated in Fig. 1) differ by molecular weight or by surface charges the subclasses were separated on PAGE of four acrylamide concentrations and subjected to Ferguson plot analysis as described by Allen and Staehelin (1991) (Fig. 2). The plots of log 100 R_f (determined with respect to the band of free pigments) against acrylamide concentration were traced for carnation LHC II subclasses. The plots for C and D subclasses have the same y-intercept (which is a measure of net surface charge) and the plot for E form consistently focus (on log 100 R_f-axis) to the point slightly different from that found for C and D. On the basis of these data we reasoned that the subclasses C through E were separated, in general, due to the large differences in their molecular weight, the Chl-binding proteins are thus most probably oligomeric forms of LHC II. A small difference in surface charge seems to contribute to the separation of E-oligomer from C and D. A possible explanation for this difference is that E-oligomer does not have the same polypeptide composition as the C and D forms which, in turn, would have identical polypeptide composition.

The E and D subclasses most probably represent monomeric and trimeric forms of LHC II, respectively, as they comigrated (data not shown) with the analogous forms of LHC II isolated by a traditional, mildly denaturing electrophoresis of OG/SDS-solubilized carnation thylakoids (Dunahay and Staehelin, 1986). The C form is probably a double trimer (hexamer) as estimated from the migration of high molecular weight standard proteins. The Ferguson relationship data for A and B subclasses proved not to be reliable due to small R_f values. It was not possible to determine precisely whether net surface charge of these subclasses differed from those of C, D and E forms. The A and B forms could be large oligomers representing multiple trimers of unknown multiplicity.

The identification and characterization of the stoichiometric ratios of polypeptides of bulk LHC II and its oligomeric forms – eluted from the gels after mildly denaturing electrophoresis – were based on denaturing SDS-PAGE combined with integrating laser densitometry of the resolved polypeptide bands. The bulk carnation LHC II was resolved into five polypeptides of 28.8, 27, 26, 24 and 23 kDa (Fig. 3) in a stoichiometric ratio of 2.1: 7.6:1:1:0.8 (28.8:27:26:24:23 kDa). Three of them (27, 26 and 23 kDa) have previously been described as constituents of carnation LHC II (Jackowski and Kluck, 1993) and the 28.8 kDa polypeptide was identified as contaminant from CP29 (Jackowski and Kaźmierczak, unpublished data).

The oligomers A–D contained only the polypeptides 27 and 26 kDa as their constituents (Fig. 3). Table I shows that polypeptide 26 kDa has a remarkable tendency to remain preferentially in large oligomeric forms as it occurs 1.5 times more frequently in A and B forms than in bulk LHC II. The polypeptide 27 kDa is slightly enriched in

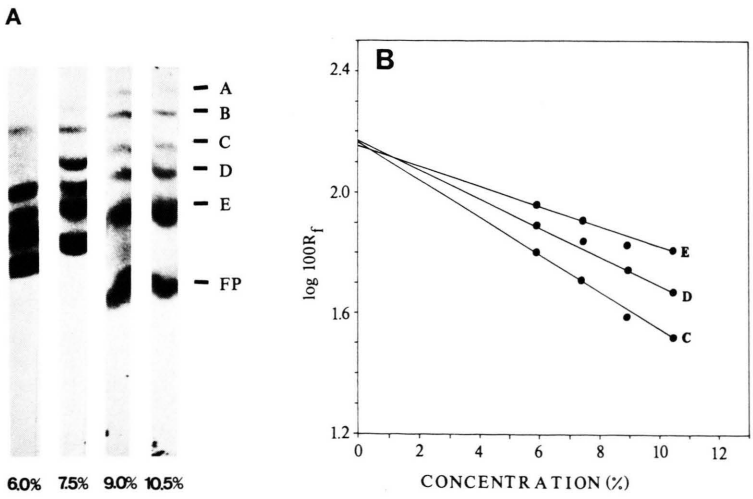

Fig. 2. Ferguson plot analysis of LHC II subclasses. LHC II (70 μg Chl) solubilized under standard conditions was separated on gels of four acrylamide concentrations (A). Relative mobilities (R_f) determined with respect to the band of free pigments were used to construct the plots of 100 R_f against acrylamide concentration (B). Acrylamide concentrations are shown at the bottom of each gel. FP, free pigments.

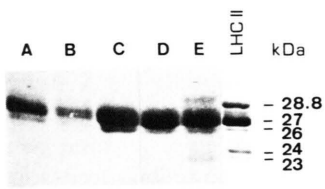

Fig. 3. The identification of polypeptides of bulk LHC II and its oligomeric forms. LHC II was solubilized under standard conditions and subjected to mildly denaturing electrophoresis. The oligomers A, B, C, D and E were eluted from the gel and their polypeptide composition analyzed by fully denaturing SDS-PAGE along with that of bulk LHC II. The lanes were loaded with 8–12 μg Chl of the respective samples and the gel was stained with Coomassie Brilliant Blue G-250.

forms C, D and E as compared with bulk LHC II and large oligomers (A and B). Neither oligomers nor the monomer exhibited any sign of the presence of 24 kDa polypeptide and this is apparently due to the fact that this subunit is not associated with pigments and therefore represents a contamination of LHC II. The polypeptide 23 kDa exists as a monomer only, however even in monomeric form the relative abundance of the polypeptide is lower than in bulk LHC II (Table I) and it suggests that a fraction of this polypeptide loses its pigments during solubilization/electrophoresis and does not comigrate with any of the forms A–E. When the bulk LHC II was solubilized with a mixture of LDS, OG and DM used at lower concentrations (0.3%, 0.6%, 0.3%, respectively) than in the standard solubilization, the 23 kDa polypeptide was found in the A–D forms although being present in strongly reduced amounts when compared with bulk LHC II (Table I). Thus it can be concluded that this polypeptide may be assembled only into very unstable oligomers (or loosely associate as a monomer with stable oligomers formed by 27 and 26 kDa polypeptides) and it is likely that this is a contaminant co-purifying with LHC II.

Discussion

It has been known for several years that conventional mildly denaturing "green" gel systems separate LHC II into two bands which were originally thought to be the monomeric and trimeric forms of the complex based on their molecular weight (Anderson *et al.*, 1978; Camm and Green, 1980; Ryrie *et al.*, 1980). Structural *in vitro* studies performed on two-dimensional as well as three-dimensional crystals of LHC II revealed that the complex had 3-fold symmetry (Kuhlbrandt, 1984, 1987) and the final confirmation of the aggregate being a trimer came from the analytical centrifugation of OG-solubilized LHC II (Butler and Kuhlbrandt, 1988). The trimer is, therefore, thought to be the preferred form of LHC II also inside the thylakoid membrane although the monomeric state can not be ruled out (Ide *et al.*, 1987). However, large complexes containing only LHC II subunits have recently been described in barley (Peter and Thornber, 1991) and this raised the question of a presence *in vivo* of complex oligomeric forms representing multiplicity higher than three.

Table I. Stoichiometric ratios and relative abundance of polypeptides 27, 26 and 23 kDa in bulk LHC II and its oligomeric forms. The ratios were determinated by integrating laser densitometry of the gels after fully denaturing electrophoresis of polypeptides of bulk LHC II. The relative abundance of each polypeptide is shown in parentheses as percentages of total polypeptide peak area. The data represent the mean for 4–5 determinations (oligomeric forms) and 14 determinations (bulk LHC II).

Solubilization LDS, OG, DM [%]	LHC II bulk	Oligomeric forms A	B	C	D	E
		Stoichiometric ratios of peak areas (relative abundance of peak areas) 27:26:23				
None	7.6:1:0.8 (80.9:10.6:8.5)	–	–	–	–	–
0.30, 0.60, 0.30 (mild)	–	5.2:1:0.2 (81.3:15.5:3.1)	5.2:1:0.3 (81.3:15.6:3.1)	6.5:1:0.2 (84.4:13.0:2.6)	7.3:1:0.2 (85.9:11.8:2.4)	7.4:1:0.7 (81.3:11.0:7.7)
1.20, 2.40, 1.20 (standard)	–	5.2:1:0 (83.9:16.1:0)	5.2:1:0 (83.9:16.1:0)	6.4:1:0 (86.5:13.5:9)	6.4:1:0 (86.5:13.5:0)	8.7:1:0.2 (87.9:10.1:2.0)

We have shown here that upon solubilization of LHC II from three higher plant species with a mixture of detergents and by application of a mildly denaturing "green" gel electrophoresis system run at extremely low current the separation into five LHC II subclasses occurred. The results of Ferguson plot analysis and of parallel electrophoresis of trimeric and monomeric forms of LHC II indicate that the subclasses A–D are oligomeric forms of LHC II while the E form is a monomer. The oligomers C through E carry a very similar net surface charge but it was not possible to determine reliably whether the largest oligomers (A and B forms) had a surface charge equal to that of C through E. We could not exclude the possibility that A and B carry different charges being, for instance, phosphorylated versions of large oligomers. Such a possibility would agree well with the observation that polypeptides of large barley LHC II oligomer were phosphorylated to a higher degree than those found in LHC II trimers (Peter and Thornber, 1991).

As we observed more oligomeric forms of LHC II in our gels than were previously described in many other analytical systems we tested the possibility that the large oligomers (A and B forms) are artifactually produced due to fortuitous oligomerization of trimers. To side-step this point we performed a series of control isolations keeping the LHC II sample in various detergent mixtures at all stages prior to LHC II solubilization and electrophoresis (data not shown). The A and B oligomers kept their integrity also under such conditions. Furthermore, it was shown by steady-state fluorescence measurements that the bulk LHC II kept is functional activity after the standard solubilization (data not shown). The results of the control experiments indicate that the oligomeric forms found in our study may in fact exist *in vivo*.

The apparently large oligomers of LHC II have been observed, apart from this communication, by Peter and Thornber (1991) in thylakoid extracts only when the membranes were exposed to decyl maltoside (while we used dodecyl maltoside) and it appears that the use of detergents of the alkyl maltoside type is a prerequisite to keep heavy oligomers intact during electrophoresis run. The appearance of the C oligomer (apparently a hexamer) seems to be strictly dependent on performing the electrophoresis at very reduced current for at least 20 h, as electrophoresis at shorter periods does not resolve the C and D forms (data not shown).

The bulk LHC II yielded five polypeptides of 28.8, 27, 26, 24 and 23 kDa. The analysis of the polypeptide composition of oligomeric forms of LHC II suggests, however, that only 27 and 26 kDa subunits are authentic LHC II constituents. The assignment of the 23 kDa polypeptide as the LHC II apoprotein is questioned by the inability of the polypeptide to form any stable oligomeric forms and by a strong tendency to lose pigments, even under mild solubilization conditions. We suggest that CP24 may be a candidate for being the complex housing 23 kDa polypeptide. The 28.8 kDa polypeptide was shown to be a CP29 contaminant (Jackowski and Kaźmierczak, unpublished data). As CP24 and CP29 form a supramolecular antenna complex with LHC II (Peter and Thornber, 1991; Bassi and Dainese, 1992) this would explain why apparent CP24 and CP29 apoproteins contaminate our LHC II samples. The 24 kDa polypeptide was shown not to be associated with pigments and this also excludes its assignment as LHC II apoprotein.

The analysis of the relative abundance of the two authentic LHCP in LHC II oligomers let us conclude that the polypeptide composition of the oligomers was different. The A and B forms exhibited and identical composition as well as the C and D forms while the E form had a composition different either from both the A/B and C/D forms (Table I). The observations concerning polypeptide composition of C, D and E oligomers can explain a small difference in net surface charge between C/D and E forms revealed during Ferguson plot analysis. It is polypeptide 26 kDa that is enriched in heavy oligomers (A and B) when compared with the other forms and with bulk LHC II while polypeptide 27 kDa is depleted appropriately in heavy oligomers.

The physiological significance of the heterogeneity of LHC II oligomeric forms differing in their polypeptide composition is hard to envisage. Bassi *et al.* (1991) proposed the role of two oligomeric forms of LHC II in phosphorylation-mediated state transition but our results are at present difficult to be examined in terms of this proposition. It seems, however, interesting to note that carnation LHC II subunits that are enriched

in the 26 kDa polypeptide tend to aggregate at a slower rate than the subunits enriched in 27 kDa polypeptide (Jackowski and Kluck, 1993). It is possible that the two features of the 26 kDa pigment-protein (slow rate of aggregation and tendency to form large oligomers) are interrelated and have some functional importance.

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