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The Association of the Antenna System to Photosystem I in Higher Plants

COOPERATIVE INTERACTIONS STABILIZE THE SUPRAMOLECULAR COMPLEX AND ENHANCE RED-SHIFTED SPECTRAL FORMS*

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We report on the association of the antenna system to the reaction center in Photosystem I. Biochemical analysis of mutants depleted in antenna polypeptides showed that the binding of the antenna moiety is strongly cooperative. The minimal building block for the antenna system was shown to be a dimer. Specific protein- protein interactions play an important role in antenna association, and the gap pigments, bound at the interface between core and antenna, are proposed to mediate these interactions Gap pigments have been characterized by comparing the spectra of the Photosystem I to those of the isolated antenna and core components. CD spectroscopy showed that they are involved in pigment-pigment interactions, supporting their relevance in energy transfer from antenna to the reaction center. Moreover, gap pigments contribute to the red-shifted emission forms of Photosystem I antenna. When compared with Photosystem II, the association of peripheral antenna complexes in PSI appears to be more stable, but far less flexible and functional implications are discussed.

Photosystem I (PSI)[1] in higher plants mediates the light-driven electron transport from plastocyanin to ferredoxin. It is composed by two moieties; (i) a core complex, binding chromophores involved in charge separation and electron transport; (ii) an antenna system (LHCI) responsible for increasing the absorption cross-section. In higher plants the core complex is composed of 14 subunits (1, 2), and it binds about 100 Chl a and 22 β-carotene molecules (3, 4). The four major polypeptides of higher plant LHCI are encoded by the nuclear genes *Lhca1–4* (4, 5). Two other Lhca genes, *Lhca5* and *Lhca6*, have been identified in *Arabidopsis thaliana* as well as in other plant species (5). The Lhca5 polypeptide has been recently detected in thylakoids where it appears to be part of the PSI·LHCI supercomplex although in substoichiometric amounts (6, 7). The *Lhca6* open reading frame encodes a protein homologous to Lhca2, and it was assumed to be a pseudo-gene (5) although it might be expressed under specific conditions.[2] Thus, Lhca5 and Lhca6 seem to be only minor components of higher plant LHCI under standard laboratory growth, but they could play a role in specific environmental conditions.

Recently, the structure of PSI·LHCI complex from *Pisum sativum* has been resolved by x-ray crystallography, showing the presence of one copy each of the Lhca1–4 polypeptides bound to one side of the PSI core (4). The structure also revealed the presence of several chlorophyll molecules bound at the interface between antenna subunits and between LHCI and the PSI core complex, defined as "linker" and "gap" chlorophylls, respectively (4). This finding was rather unexpected, although it was known that selected pigment spectral forms in LHCI depend on the direct contact between two subunits (8, 9). The fractionation of the PSI·LHCI complex into antenna and core moieties allowed for the isolation of an interfacial pigment pool composed by Chl a, Chl b, and carotenoid molecules (10). The analysis of plants lacking individual Lhca proteins is also consistent with the location of Chls in between protein subunits (11).

LHCI subunits form dimers, as suggested by the *in vitro* reconstitution of Lhca1 with Lhca4 (9, 12) and by the dimeric aggregation state of native LHCI, including Lhca2 and Lhca3 (12). Composition of PSI·LHCI preparations from Lhca mutants is also consistent with Lhca1 interacting with Lhca4 and Lhca2 with Lhca3 (11, 13, 14).

In this work the association of LHCI polypeptides to PSI core was analyzed. The biochemical analysis of a set of plants lacking each individual Lhca1–5 gene products showed that the interaction between Lhca subunits and PSI core is strongly cooperative. In fact, when one subunit is missing, the whole LHCI system is de-stabilized. On this basis, protein-protein interactions mediated by gap pigments are suggested to play an important role in LHCI binding to PSI core. Gap pigments were also spectroscopically characterized, evidencing their involvement in specific pigment-pigment interactions and their influence on red forms.

EXPERIMENTAL PROCEDURES

Plant Material and Thylakoids Purification—The *A. thaliana* plants used in this study lack individual Lhca1–5 proteins (Δa1-a5) and were

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[1] The abbreviations used are: PSI and PSII), Photosystem I and II, respectively); α(β)-DM, *n*-dodecyl-α(β)-D-maltoside; Chl, chlorophyll; Lhca and Lhcb, light harvesting complex of Photosystem I (II); LHCI, antenna complex of Photosystem I; WT, wild type; HPLC, high performance liquid chromatography.

[2] F. Klimmek, submitted for publication.

obtained after T-DNA insertion (Δa1, Δa4, and Δa5) or antisense RNA inhibition (Δa2 and Δa3) in the Colombia background (WT col-0), as described before (13, 14). Growth of plants and isolation of thylakoids from dark-adapted plants was as in Haldrup *et al.* (15).

Purification of Photosystem I Particles and LHCI—Non-denaturing Deriphat-PAGE was performed following the method developed by Peter and Thornber (16) with the following modifications; the stacking gel had 3.5% (w/v) acrylamide (38:1 acrylamide/bisacrylamide). The resolving gel had an acrylamide concentration gradient from 4.5 to 11.5% (w/v) stabilized by a glycerol gradient from 8 to 16% (w/v). 12 mM Tris and 48 mM glycine, pH 8.5, were also included in both gels. Thylakoids, at a Chl concentration of 1 mg/ml, were solubilized with an equal volume of 1.2 or 2% α- and β-DM, respectively (Anatrace©, Maumee, OH). Thus, final concentrations of, respectively, Chls and α and β-DM were 0.5 mg/ml and 0.6 and 1%. Solubilized thylakoids were vortexed for 1 h, left for 10 h in ice and then centrifuged at 13,000 rpm for 15 h to pellet the unsolubilized material. 30 μg of chlorophylls were loaded per each gel lane.

Purification of PSI-LHCI particles from thylakoids was performed by sucrose gradient ultracentrifugation upon solubilization of membranes with 1% β-DM as described in Croce *et al.* (17). PSI core and LHCI moieties were dissociated through a further solubilization with β-DM and zwittergent (18). Lhc monomers, dimers, and trimers, fractions were concentrated, reloaded on a 0.1–1 M sucrose gradient, and centrifuged in a SW60 Beckman rotor for 16 h at 55,000 rpm for further fractionation.

Spectroscopy and Pigment Analysis—The absorption spectra were recorded using a SLM-Aminco DK2000 spectrophotometer, in 5 mM Tricine, pH 7.8, 0.2 M sucrose, and 0.03% β-DM. The CD spectra were measured at 10 °C on a Jasco 600 spectropolarimeter. The chlorophyll to carotenoid ratio and Chl a/b ratio were independently measured by fitting the spectrum of acetone extracts with the spectra of individual purified pigments (19) and by HPLC analysis as in Gilmore and Yamamoto (20).

SDS-PAGE and Western Blotting Analysis—SDS-PAGE was performed as in Laemmli (21) but was optimized for the separation of Lhca1–4 from *A. thaliana* (10) by using an acrylamide/bisacrylamide ratio of 75:1 and a total concentration of acrylamide plus bisacrylamide of 4.5 and 15.5% for the stacking and running gel, respectively, with 6 M urea incorporated in the running gel. For gel staining, 0.05% Coomassie R in 25% isopropanol, 10% acetic acid was used to improve signal linearity with protein amount (22). Two-dimensional separation was performed using non-denaturing Deriphat PAGE in the first dimension and SDS-PAGE in the second; gel slices were equilibrated in 7 M urea, 2% SDS, and 100 mM Tris-HCl, pH 6.8, for 30 h before loading. After SDS-PAGE, polypeptides were transferred into a nitrocellulose membrane (Sartorious AG, Gottingen Germany) using a blot system from Bio-Rad. Detection of the various Lhca polypeptides with specific antibodies was performed as described in Jansson *et al.* (23).

Stoichiometry Evaluations Based on Coomassie Stain Quantification—The protein amount was evaluated after SDS-PAGE by determining the amount of Coomassie stain bound to each band by colorimetry, as described (10). The gel image was acquired by using a Bio-Rad GS710 scanner. The picture was then analyzed with Gel-Pro Analyzer© software, which quantifies the staining of the bands as IOD (optical density integrated on the area of the band).

Reconstitution in Vitro of Lhca1–4 Monomeric Complexes—cDNAs of Lhca1–4 from *A. thaliana* (12, 24) were expressed and isolated from the SG13009 strain of *Escherichia coli* following a protocol previously described (25, 26). Reconstitution *in vitro* of *Arabidopsis* Lhca1–4 was performed as in Croce *et al.* (12).

RESULTS

Three Distinct PSI Populations Can be Separated by Non-denaturing PAGE—Plants depleted in individual Lhca polypeptides are a very useful tool for the analysis of the antenna association to PSI core (11, 13). Here, the effect of the depletion of individual Lhca polypeptides on pigment binding complexes was analyzed by non-denaturing PAGE electrophoresis; thylakoids from WT and Δa1-a5 plants were solubilized under very mild conditions (0.6% α-DM) and fractionated by non-denaturing Deriphat-PAGE (16). As expected, the lack of Lhca polypeptides has no detectable effects on Photosystem II subunits, which separate in several bands ranging from monomeric Lhcb to PSII-LHCII supercomplexes. In fact, as shown in Fig. 1, the band profile from ΔLhca plants was very similar to WT but for the region corresponding to PSI-LHCI in the upper part of the gel. A close-up of this gel region is shown in Fig. 2*A*. In WT plants, only one major PSI-LHCI band is present, whereas in Lhca-deficient plants up to three bands could be resolved. To characterize these populations with different apparent size, we ran a second dimension on SDS-PAGE under denaturing conditions that enabled the separation of the component polypeptides of each green band (not shown; however, see Fig. 3 for the same kind of separation from β-maltoside-solubilized samples). The slowest migrating bands in the first dimension correspond to the complete PSI-LHCI complex with the four Lhca1–4 polypeptides. The fastest green bands instead correspond to the PSI core with no LHCI polypeptides left bound. The bands with intermediate mobility (indicated as PSI-LHCI*), consisted of PSI with a reduced antenna complement.

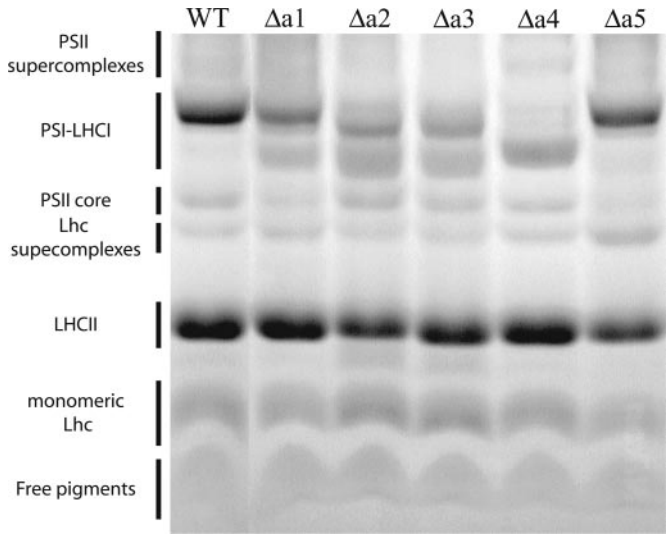

FIG. 1. **Non-denaturing gel profiles of thylakoids from WT and Δa1-a5 plants.** Thylakoids have been solubilized with final 0.6% α-DM before loading. Bands detected in WT have been identified from their mobility, and they are indicated on the *left*. 30 μg of Chls have been loaded in each lane.

A band corresponding to the PSI core was detected in all Δa1-a4 plants but not in WT under these solubilization conditions. This suggests that, when an individual Lhca polypeptide is lacking, PSI-LHCI supercomplex stability is affected, and a large fraction of PSI particles completely loses its LHCI moiety. In Δa4 plants, the band corresponding to the PSI core is the most represented, indicating a severe destabilization of LHCI in these plants, consistent with the small PSI antenna size detected in these plants (11). Instead, in Δa1 plants the main band corresponded to unaffected PSI-LHCI as in WT, whereas in Δa2 and Δa3 plants a band of PSI-LHCI* was the most abundant. The pattern of Δa5 plants was very similar to the one from WT, and only very minor differences could be detected.

In Fig. 2*A*, additional faint bands can be recognized in the different samples; by two-dimensional analysis these could be identified as co-migrating PSII supercomplexes that are partially retained with the very mild solubilization conditions used. Solubilization with 1% β-DM is a slightly harsher treatment, and it completely dissociates PSII supercomplexes but does not affect PSI-LHCI WT, as shown in Fig. 2*B* (17, 27). The same PSI bands identified upon solubilization with 0.6% α-DM are detected after solubilization with β-DM as well, but now they are cleared from the interfering PSII-LHCII bands. Nevertheless, their relative intensities are modified by the stronger solubilization treatment. In Δa1 plants, the three band classes were all detectable; PSI with a full antenna complement (PSI-LHCI), PSI core, and PSI-LHCI*, consistent with the fact

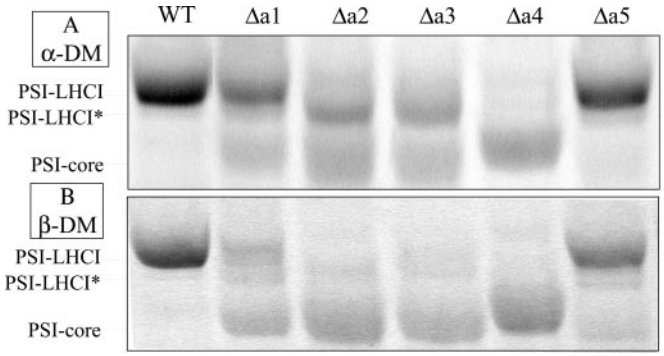

FIG. 2. **Non-denaturing gel profiles of thylakoids purified from WT and Δa1-a5 plants, particularly of Photosystem I region.** Thylakoids have been solubilized with final 0.6% α-DM (*A*) or 1% β-DM (*B*) before loading, and 30 μg of Chls have been loaded in each lane. Three different PSI bands have been identified by a second denaturing electrophoresis to be, respectively, PSI with full antenna (*PSI·LHCI*), PSI with reduced antenna (*PSI·LHCI**), and PSI without any antenna polypeptide (*PSI-core*).

that these plants retain a residual level of Lhca1, and thus, a fraction of WT PSI·LHCI is still present (14). Instead, in Δa2 and Δa3 plants only bands corresponding to PSI core and PSI·LHCI* were visible. The amount of PSI·LHCI* is clearly decreased upon solubilization with β-DM as compared with the pattern with α-DM, thus suggesting that PSI·LHCI* is susceptible to detergent treatment and that, with β-DM, a larger part dissociates into PSI core and free Lhca components. In Δa4 plants the main band is again the PSI core, as previously shown with α-DM solubilization, but a weak band corresponding to PSI·LHCI* is still visible. Even if the pattern obtained from Δa5 plants is very similar to that obtained from WT, the amount of the faint bands with higher mobility (PSI·LHCI* and PSI core) is slightly higher when compared with the WT. This difference might indicate that the absence of Lhca5 affects at least a small fraction (maximum 5%) of PSI particles.

To analyze the polypeptide composition of green bands from Deriphat PAGE we ran a second dimension on a denaturing SDS-PAGE. In Fig. 3, a detail of the part involving antenna polypeptides is shown. The gel system used here as the second dimension has been previously shown to be able to separate each Lhca1–4 polypeptide from the others (10). The identification of Lhca1–4, based on gel mobility and confirmed by antibodies detection (not shown), is reported on the left. One PSI core subunit, PsaD, is also detected in the same region of the gel and was used as a reference for the PSI core. With this analysis, we could identify the polypeptides contained into the three classes of PSI bands described on Deriphat-PAGE, allowing the identification of the PSI·LHCI* band as a PSI complex with a reduced Lhca content. Moreover, although the two-dimensional resolution was not excellent due to some interference with non-ionic detergents used in the first dimension, we could also obtain information on the identity of the Lhca polypeptide retained in PSI·LHCI* from different mutant plants. In fact, in PSI·LHCI* from Δa2 and Δa3, the main bands corresponded to Lhca1 and Lhca4, whereas only Lhca2 and Lhca3 were retained in PSI·LHCI* from Δa4. The Δa1 profile is instead less clear due to the presence of the residual PSI·LHCI with full antenna. Despite this limit, however, these results suggest a reciprocal dependence of Lhca2 and Lhca3 (and Lhca1 and -4) for their stable binding to the PSI core.

LHCI Binding Stability Is Reduced in Plants Depleted in Lhca1–4 Polypeptides—We noted above that the PSI·LHCI* green band in Δa2 and Δa3 plants is susceptible to detergent treatment since its abundance is reduced under stronger solubilization conditions, whereas the amplitude of the PSI core band is increased. This effect should correspond to a release of Lhca from PSI upon β-DM solubilization. To verify this hypothesis, we performed a sucrose gradient ultracentrifugation of thylakoids membranes solubilized with 1% β-DM (Fig. 4*B*). The dissociated Lhca proteins should migrate in the upper part of the gradients together with the antenna complexes of PSII. To detect their presence, we analyzed the corresponding fractions (bands B2, B2-3, and B3) by Western blotting using specific antibodies against Lhca1–4 polypeptides (Fig. 4*A*). The presence of Lhca2 and -3 is detected in both Δa1 and Δa4 plants, suggesting that a fraction of these polypeptides is detached from the PSI core upon solubilization. Symmetrically, Lhca1 and -4 are found in the upper band from both Δa2 and Δa3 plants. It is worth noting that, instead, in WT plants Lhca are stably retained bound to PSI in these solubilization conditions. Instead, quite surprisingly, Δa5 plants showed a release of Lhca2 and Lhca4 polypeptides.

It is also interesting to determine whether Lhca polypeptides are released from PSI individually as monomers or dimers. To this aim we collected the hypothetical dimeric Lhc population (B2-3 in Fig. 4*B*) and loaded it into a second sucrose gradient for a longer separation time to increase resolution; as an example, the result in the case of Δa2 plants is shown in Fig. 4*C*. A band with an intermediate mobility between monomeric and trimeric Lhcb was resolved. Although it was not completely pure, this fraction contained dimeric Lhca complexes, as detected by the presence of a red absorption tail at wavelengths >700 nm, a spectroscopic fingerprinting of PSI antenna complexes (not shown). This finding confirms that a secondary loss of LHCI polypeptides is induced when one Lhca is missing and also shows that the Lhca polypeptides are lost as dimers, thus confirming their capacity of interacting with each other within Lhca2/3 and Lhca1/4 pairs.

Lhca Protein Stoichiometries in the Different Complexes—The sucrose gradient centrifugation after 1% β-DM solubilization of WT and mutant thylakoids also allowed the isolation of PSI particles (*B5* in Fig. 4). This separation method, however, has lower resolution with respect to the non-denaturing green gels seen above, so that PSI complexes with reduced antenna complement (PSI·LHCI*) cannot be fully separated from the PSI core (or PSI·LHCI). In an attempt to increase the purity of this complex, we harvested several fractions from band 5 (in Fig. 4) and analyzed them by green gel electrophoresis. The fast migrating part of band 5 was shown to be enriched in PSI·LHCI* with little PSI core contamination. This fraction was used for all further analyses of PSI particles presented below.

The presence of the Lhca polypeptides in these particles was first detected with immunoblotting with specific antibodies (not shown). This analysis showed a general reciprocal dependence between the presence of Lhca1 and Lhca4 on one hand and Lhca2 and Lhca3 on the other hand, in full agreement with the previous work performed on PSI particles from the same *Arabidopsis* lines (11) and with all results presented above. In addition, Western blotting detected residual amounts of Lhca3 left in Δa2 PSI particles and vice versa, in apparent contradiction with the above exposed dimeric state of Lhca polypeptides. To interpret these results, however, it must be considered that immunoblotting is a very sensitive technique for polypeptides detection, but the linearity range of the signal is limited, and stoichiometric relationships between different polypeptides cannot be determined with accuracy. For a more accurate quantitative evaluation we, thus, also determined the Lhca polypeptide content by measuring the amount of Coomassie Blue bound to each Lhca1–4 band after SDS-PAGE separation of PSI particles, as in Ballottari *et al.* (10) (Fig. 5*A*). The amount of each Lhca was then related to the amount of PsaD

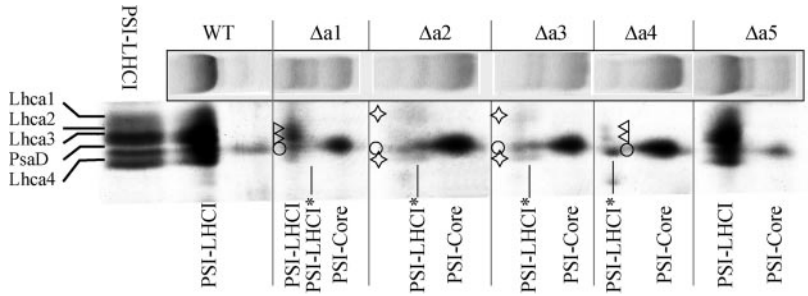

FIG. 3. **Two-dimensional SDS-PAGE of PSI bands from WT and Δa1-a5 plants (particular of region concerning Lhca polypeptides).** Gel regions containing PSI complexes from the first dimension separation Deriphat PAGE (shown on the *top*) were loaded on denaturing SDS-PAGE. On the *left*, the mobility of individual Lhca polypeptides as resolved in the lane loaded with PSI·LHCI complex from WT is indicated. *Vertical lines* indicates the position of PSI·LHCI* complexes in the first dimension. *Symbols* indicates the positions of residual Lhca and psaD polypeptides in PSI·LHCI* complexes from mutants as follows: ✧, Lhca1, Lhca4; ◁, Lhca2, Lhca3; ○, PsaD.

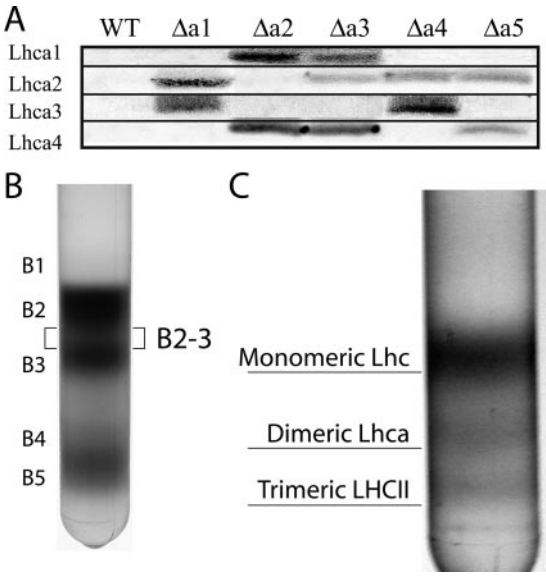

FIG. 4. **Analysis of Lhca polypeptides released upon thylakoids solubilization with 1% β-DM.** *A*, Western blotting analysis of the free Lhc complex fraction after solubilization of thylakoids membranes. *B*, sucrose gradient ultracentrifugation of Δa2 thylakoid membranes solubilized with 1% β-DM. Five bands are indicated (B1-B5) and have been identified to be, respectively, free pigments, monomeric Lhc, trimeric LHCII, PSII core, and PSI·LHCI. Note that B4 and B5 are not completely resolved. This usually occurred in mutants, and to avoid as much as possible B5 contamination from B4, only the lowest part of the B5 band was used for analyses. The hypothetical dimeric fraction between B2 and B3 (B2–3) is also indicated. *C*, second sucrose gradient ultracentrifugation of the B2–3 fraction. Bands corresponding to the different aggregation states are indicated.

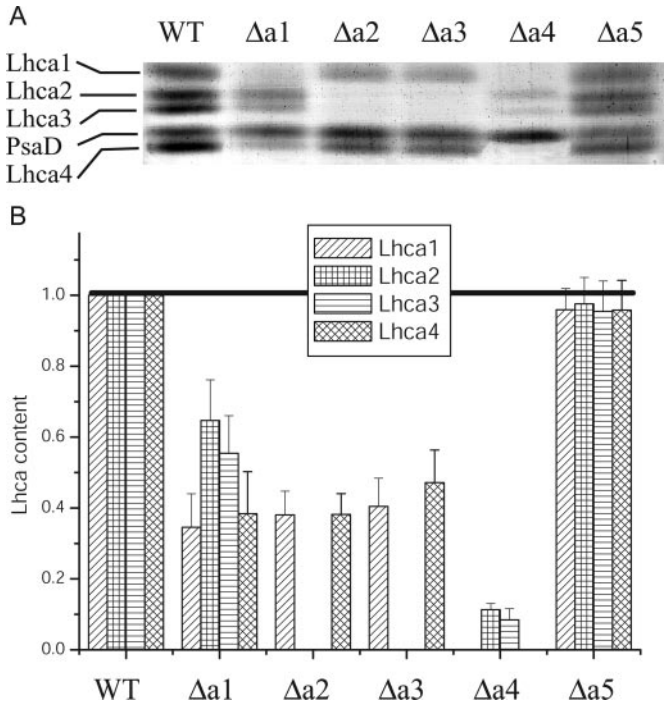

FIG. 5. **SDS-PAGE analysis of PSI particles purified from WT and Δa1-Δa5 plants.** *A*, the SDS-PAGE region, where Lhca polypeptides migrate. *B*, quantitative evaluation of Lhca1–4 polypeptides per PSI core in all samples. The stoichiometry was obtained by evaluation of Coomassie bound to each Lhca and normalization to PsaD and PsaF content.

and PsaF, two polypeptides that we used as internal standards for the quantification of PSI core. Based on the known stoichiometry of one copy of each Lhca1–4 per PSI core (4, 10), we calculated the amount of Lhca polypeptides per PSI core present in each preparation (Fig. 5*B*). In Δa1 plants, Lhca1 and Lhca4 are reduced to 35–40%, whereas ~60% of Lhca2 and Lhca3 is retained. This means that ~40% of PSI population without Lhca1 retains Lhca2 and Lhca3. The absence of Lhca2 and Lhca3 causes a similar destabilization, and in fact in Δa2 and Δa3 plants, about 40% of Lhca1 and Lhca4 is found, whereas Lhca2 and Lhca3 are below the detection limit for Coomassie stain. The Lhca2 and Lhca3 polypeptides detected with antibodies in Δa3 and Δa2 PSI, thus, should be considered as trace amounts. In Δa4 plants, around 10% of Lhca2 and Lhca3 are retained whereas Lhca1 and Lhca4 are completely absent. Instead, in Δa5 plants PSI antenna content is 96% of the WT, but this difference is not significant since it is under the experimental error of the quantification.

These data suggest two main conclusions; first, in PSI·LHCI* from the different plants Lhca1–4 and Lhca2–3 were always found in similar amounts. This illustrates the reciprocal dependence of each member of these two couples from the companion for the association to PSI core. Therefore, when one polypeptide is absent, its partner is not found associated to PSI core in significant amounts. Second, the absence of one polypeptide affects the binding stability of all the others, although to a different extent. Thus, the whole LHCI system is affected by the lack of each single Lhca.

Biochemical Properties of PSI Particles—PSI particles from each genotype, purified as described in the previous paragraph, were also analyzed for their pigment content, with a combined approach of HPLC analysis and fitting of the acetone extracts spectra with the spectra of individual pigments. The results are summarized in Table I. In PSI from Δa2, Δa3, and Δa4, a significant decrease of the Chl b content was observed, as reported in Klimmek *et al.* (11). This is in agreement with the stoichiometry results presented above; in fact, these genotypes undergo loss of antenna proteins to the larger extent where Chl b is mainly localized. Another confirmation comes from the fact

TABLE I
Pigment binding properties of PSI particles

Pigment binding properties of PSI particles purified from WT and Δa1–5 mutant, calculated per 100 Chl. S.D. was <1 for Chl a, Chl b, violaxanthin, lutein, and β-carotene and <2 in the case of the total number of carotenoid molecules. Car, carotenoid.

	Chl a/b	Chl a	Chl b	Chl/Car	Total Car	Violaxanthin	Lutein	β-Carotene
ΔLhca1	8.5	89.4	10.6	5.5	18.1	1.4	5.2	11.6
ΔLhca2	10.6	91.3	8.7	6.1	16.6	1.1	3.1	12.5
ΔLhca3	10.7	91.4	8.6	5.8	17.4	1.2	3.3	12.8
ΔLhca4	18.0	94.6	5.4	5.2	19.5	1.1	2.9	15.4
ΔLhca5	8.3	89.2	10.8	5.0	19.9	1.9	5.5	12.6
WT	9.1	90.1	9.9	4.7	21.1	1.9	4.7	14.5

that β-carotene, which is mostly bound to the core complex, is relatively increased in these genotypes (Tables I and (11)). As for violaxanthin and lutein, they are decreased in all genotypes but Δa5; however, the lutein/violaxanthin ratio is not significantly affected. The conservation of this ratio suggests that all Lhca polypeptides bind these carotenoid species in approximately the same amount.

The most interesting information, however, can be obtained from the analysis of Δa4 plants. In fact, their PSI·LHCI particles, according to stoichiometry determinations, contain a very small residual antenna moiety, ~5% of WT. Despite this small antenna, significant amounts of Chl b, violaxanthin, and lutein were observed (Table I and Ref. 11). Although their content is reduced with respect to WT, these pigments are significantly more abundant in PSI-Δa4 than in the PSI core, where Chl b is barely detectable and xanthophylls are completely absent (28). The small residual antenna in Δa4 particles is not sufficient to account for this difference. The most likely hypothesis, thus, is that the extra Chl b and xanthophyll molecules belong to the "gap pigments" pool, which are bound at interfaces between the PSI core and LHCI (4). At least a fraction of these pigments could remain bound even in the absence of LHCI polypeptides in the mild solubilization conditions employed during PSI purification. On the contrary, they are lost in the PSI core from WT because of the harsher conditions used for dissociation of the PSI·LHCI complex from its LHCI moiety.

Spectroscopic Characterization of Gap Pigments—Because of their location, gap pigments are likely to influence the association between core and antenna, making the analysis of their properties interesting. To determine their contribution to the absorption spectrum of PSI·LHCI complex, we mixed the isolated PSI core and a LHCI in the same ratio they are found in PSI·LHCI (4) and compared the spectrum of this sample to the one of the whole supercomplex PSI·LHCI, normalized to its Chl content. The result of this procedure is shown in Fig. 6*A*. In the Soret's region, the difference spectra showed contributions at 434, 470, and around 500 nm, corresponding to Chl a and b and carotenoids. This spectrum is, thus, consistent with the fact that among gap pigments all the three species of chromophores are found (10). In the Q_y region, in addition to the signal from Chl b at around 650 nm, we can distinguish three different contributions originating from Chl a, at 668, 683, and >700 nm. Therefore, in the gap pigments at least two different populations of bulk chlorophylls a are present as well as a contribution in the red absorption tail, which is generally believed to originate from the LHCI subunits (29). Because the calculation of the difference spectra is influenced by the values of Chl distribution used for the normalization, we repeated the procedure using slightly different figures based on reports in the literature (4, 12, 30–32). As expected, modifications in the normalization affected the resulting difference spectra; however, all the contributions mentioned above were always detected, although their relative intensities were slightly different.

CD spectroscopy in the visible range is a useful technique for detection of interactions between pigments (see Ref. 33 and citations therein). For this reason we calculated the CD spectra of gap pigments with the same method described for the absorption (Fig. 6*B*). A CD signal is detected at wavelengths corresponding to Chl a, Chl b, and carotenoids, respectively, at 670–678 (+) and 690–705 (−), 470–475 (+), and 505–510 (+). The presence of specific CD signals suggests that gap pigments are involved in interactions with the polypeptide chains and/or with neighbor pigments. Among the different signals it is worth emphasizing the detection of a shoulder at wavelengths >700 nm, which confirms the involvement of gap pigments in red forms.

*Purification of Lhca Dimers from PSI·LHCI**—When LHCI is detached from PSI core, it is usually found as dimers (12). LHCI complexes are very difficult to purify from each other due to their biochemical similarity and to the presence of strong interactions within heterodimers. Up to now only a partial purification of Lhca1–4 from Lhca2–3 dimers could be achieved (12, 31, 34), and information available on the individual properties of Lhca is derived mainly from reconstitutions *in vitro* (9, 24). The recent structure of PSI·LHCI complex showed the presence of "gap" and "linker" chlorophylls bound at interfaces between PSI core and LHCI and between individual Lhca subunits (4). These binding sites are most probably not stable in monomeric Lhca reconstituted *in vitro*, where protein-protein interactions are missing, but could be conserved in mutants lacking individual Lhca polypeptides.

We, therefore, proceeded to the isolation of LHCI from PSI·LHCI* particles from different mutants. As expected, SDS-PAGE analysis showed that LHCI fractions from WT and Δa5 contained both Lhca dimers; the one from Δa1 is enriched in Lhca2/Lhca3, and that from Δa2 and Δa3 plants is enriched in Lhca1/Lhca4 dimers (not shown). The comparison of these three populations, thus, provides information on differences between individual Lhca. Quite surprisingly, however, the only significant difference found in absorption spectra of LHCI from Δa1, Δa2, Δa3, and WT was a small alteration in the regions corresponding to absorption of Chl b (around 470 and 650 nm, Fig. 7*A*). Here, in fact, LHCI-Δa3 showed a slightly larger signal than in WT, whereas in LHCI-Δa1 it was smaller. This difference is confirmed by the analysis of pigment binding properties of LHCI populations; LHCI from Δa1 plants has a slightly higher Chl a/b ratio (4.4 *versus* 3.5 in WT). The purification of LHCI also yielded PSI core preparations from all mutant lines. As expected, these preparations did not show any genotype-related differences (not shown).

DISCUSSION

LHCI Is Co-operatively Bound to the PSI Core—In this work we analyzed the association of the LHCI to the PSI core. The biochemical characterization of *A. thaliana* plants depleted in individual Lhca complexes showed that in all Δa1-Δa4 plants the binding of the LHCI to the PSI core was severely affected, in agreement with previous characterization of these plants (11, 14). Under solubilization conditions, in which the PSI·LHCI from WT is fully stable, we detected significant

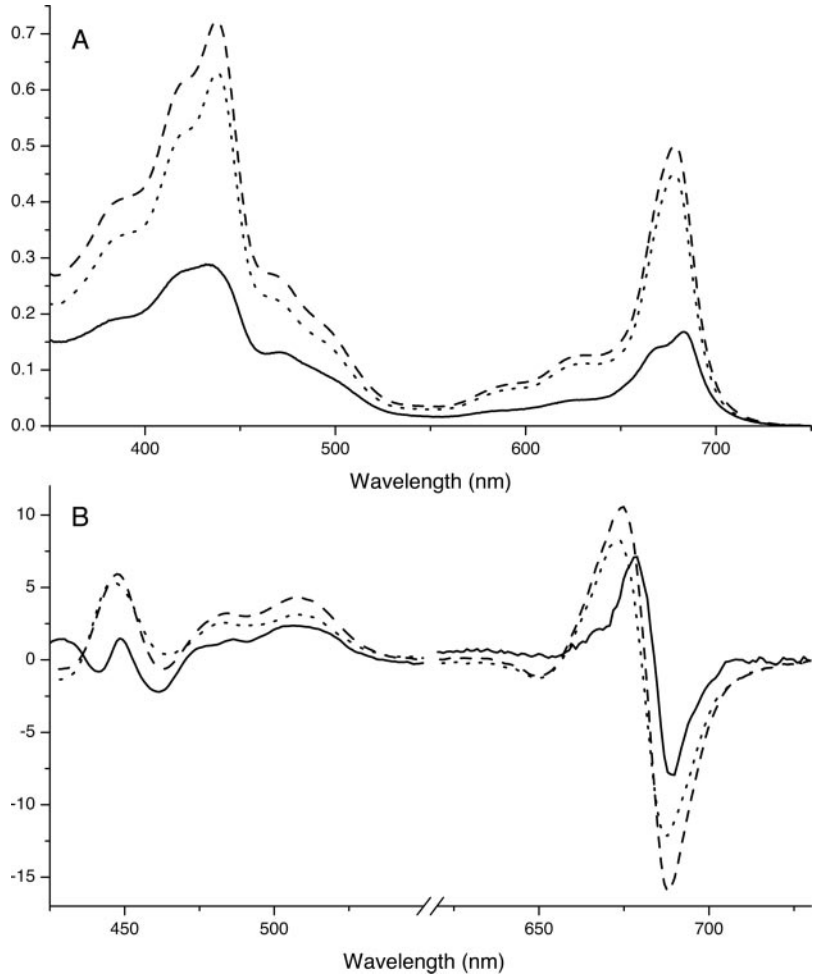

FIG. 6. **Absorption and CD spectra of gap pigments.** *A*, absorption spectrum of gap pigments (*solid*, ×3) is shown, calculated as the difference between PSI·LHCI complex (*dashed line*) and PSI core + LHCI (*dotted line*). *B*, CD spectrum of gap pigments (*solid*, ×2), calculated as the difference between PSI·LHCI complex (*dashed line*) and PSI core + LHCI (*dotted line*). *a.u.*, absorbance units.

amounts of PSI core complex having lost all the associated Lhca proteins. We conclude that, when one Lhca polypeptide is missing, the association of the remaining polypeptides to PSI core is destabilized. It is worth mentioning that the fact that some of the plants were antisense (Δa2 and Δa3) instead of knock-out plants did not affect our conclusions. In fact, any residual expression was detected, and since the protein left is below the sensitivity of the technique used for the analyses, it does not influence our results.

We also identified a PSI population with a reduced antenna content (PSI·LHCI*), which was shown to be susceptible to detergent treatment. This is evident if we compare the Δa2 and Δa3 profiles obtained upon α-DM or β-DM solubilization, the first condition being milder than the latter. With α-DM, we detected a fair amount of PSI·LHCI* that is reduced using β-DM. The loss of antenna polypeptides during solubilization was also confirmed by Western blotting analysis of free Lhc fraction (see Fig. 5). We conclude that in the different Δa1–4 plants a fraction of LHCI polypeptides is still associated to the PSI core within the thylakoid membranes, but they are easily lost upon solubilization.

All these results suggest the presence of strong interactions between all Lhca proteins and the cooperative nature for the association of LHCI to PSI core. Among the network of interactions stabilizing LHCI moiety, Lhca4 appears to play a fundamental role. In fact, when this polypeptide is missing, a very low amount of Lhca polypeptides is found associated with PSI core.

Lhca polypeptides also appear to behave like "couples," consistent with their isolation as dimers (12); in fact, Lhca1–4 and Lhca2–3 are not stably bound to PSI in the absence of each own "partner," as shown in Figs. 4 and 5. The minimal unit of PSI antenna appears, thus, to be a dimer. We can, thus, expect that stronger protein-protein interactions are found between Lhca1–4 and Lhca2–3.

Gap Pigments Mediate Intersubunit Interactions—The crystal structure of PSI·LHCI reveals a significant number of chlorophyll molecules between PSI core and LHCI and in between the individual Lhca subunits, named, respectively, "gap" and "linker" chlorophylls (4); see Fig. 8. A biochemical characterization of these chlorophylls (10, 11) and the pigment analysis of Δa4 PSI presented here suggest that not only Chl a but also Chl b and carotenoid (β-carotene, lutein, and violaxanthin) molecules are bound at the interfaces. Thus, it is more correct to speak about gap pigments rather than only gap chlorophylls.

Results presented here suggest the presence of an extended net of interactions stabilizing the binding of the antenna system to PSI, with a key role played by Lhca4. On the other hand, from the analysis of the structure of the PSI·LHCI complex, Lhca1 (and secondarily Lhca3) were proposed to be fundamental for the antenna stability. In fact, relevant interactions were identified in particular between Lhca1 and PsaG (4, 35). Lhca4 instead was only shown to have small contacts with PsaF. To explain this discrepancy, we propose that a major contribution to the association of the LHCI to the PSI core is provided by the gap pigments located in between rather than on interactions between the polypeptide chains. Two additional considerations support this hypothesis. The first is the nature of the binding of gap pigments; in fact, they are not stably coordinated either to the core nor to the antenna, but they interact with both moieties (10), and they likely could behave as "structural bridges" between different polypeptides. In addition, 6 of the 10 gap

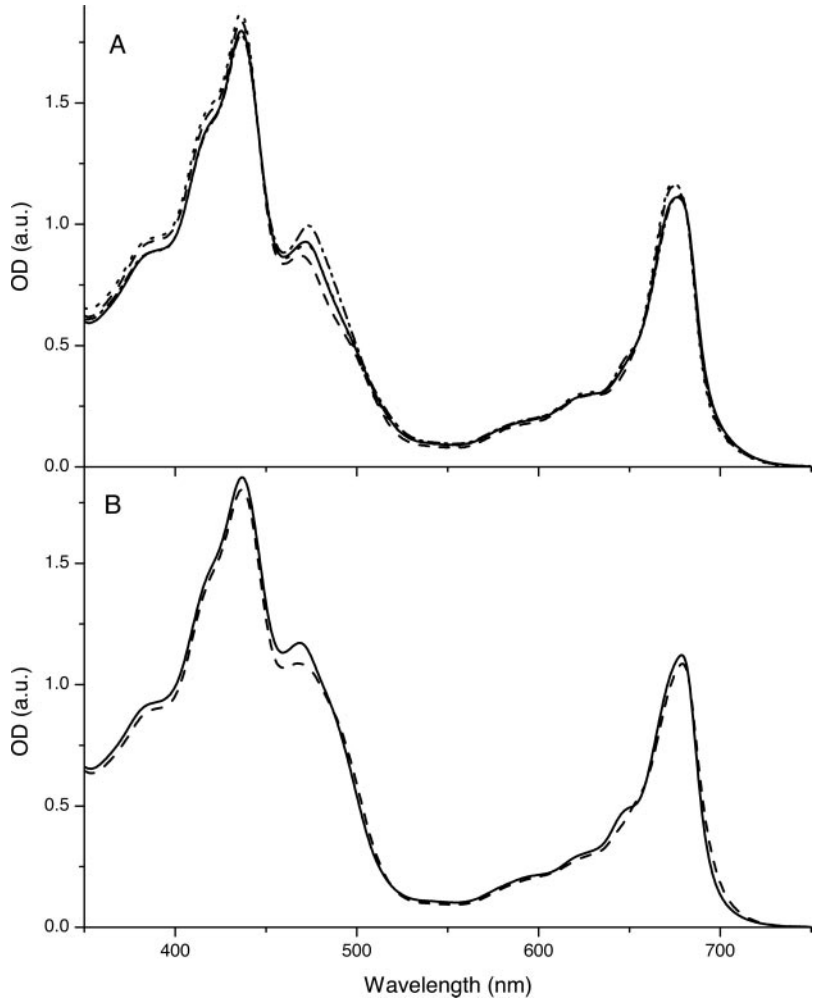

FIG. 7. **Room temperature absorption spectra of LHCI.** *A*, absorption spectra of LHCI purified from WT (*solid*) and Δa1-a3 (respectively, *dashed*, *dotted*, and *dash-dotted lines*) plants. Spectra are normalized to the total Chl content. *B*, sum of absorption spectra of Lhca pigment binding complexes reconstituted *in vitro*. Lhca1 + 4 (*solid line*) and Lhca2 + 3 (*dashed line*) are shown. Spectra are normalized to the total Chl content. *a.u.*, absorbance units.

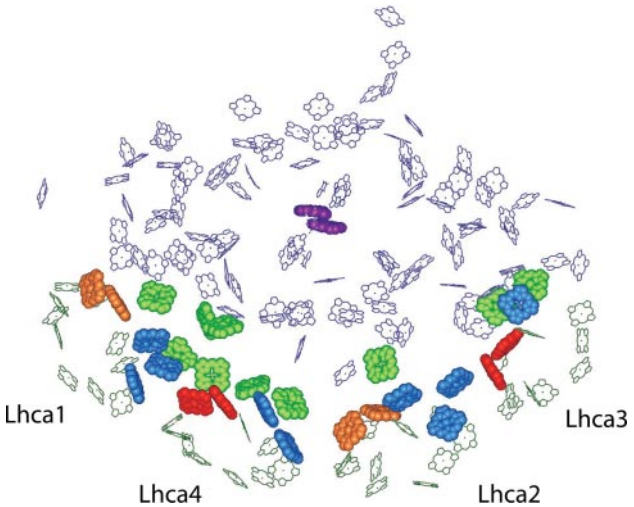

FIG. 8. **Gap, linker, and red chlorophylls in the PSI·LHCI structure.** Chl molecules of the structure from Ref. 4, deposited in the Protein Data Bank under accession number 1qzv, are shown. The Chl molecules of the core complex and antenna moieties are reported in *dark blue* and *green*, respectively, with the special pair P700 in *purple*. Gap and linker chlorophylls are evidenced in *light green* and *cyan*, respectively. Chls in sites A5 and B5 of antenna polypeptides are evidenced as well; they are in *red* in the case of Lhca3 and Lhca4 and in *orange* for Lhca1 and Lhca2.

chlorophylls identified in the structure are located close to Lhca4 (Fig. 8), which is the most important polypeptide for the stabilization of the antenna system as shown from the complete loss of LHCI in the ΔA4 mutant.

The inhomogeneous distribution of the gap pigments is also interesting for the function of the antenna and its ability to transfer energy to the reaction center. In fact, Lhca4 is the polypeptide with the red-most chlorophylls of Photosystem I (9, 36), and the major part of the excitation energy is first transferred here and then to the reaction center for charge separation (17). The presence of several nearby chlorophylls is likely to increase the energy transfer efficiency from the red Chls in the antenna to the reaction center. Consistent with this picture, chlorophylls in sites Chl A5-B5, the responsible of the red emission in Lhca4 (37), face the core complex and are very close to clustered gap chlorophylls.

Protein-Protein Interactions Enhance Red Forms—Up to now, red chlorophylls in higher plants have been identified as associated to protein subunits of the PSI core, emitting at 720 nm, and in LHCI, whose emission was at 735–740 nm, originating from Chls A5 and B5 in Lhca4 and Lhca3 (37). The spectroscopic analysis of the gap pigments instead showed a contribution in absorption and CD at wavelengths greater than 700 nm, indicating that gap pigments are also involved in the red forms typical of PSI. This red-shifted absorption may originate from additional red Chls in gap pigments or be an indirect effect on the red chlorophylls present in the antenna. Experimental evidences suggest enhancement of red forms within the antenna upon binding to the core. In fact, the isolated LHCI moiety, composed of Lhca1–4 and Lhca2–3 heterodimers, showed emissions at 735, 702, and a minor contribution at 685 nm (38). Because energy in the complexes is equilibrated and both dimers host red chlorophylls, respectively, in Lhca4 and

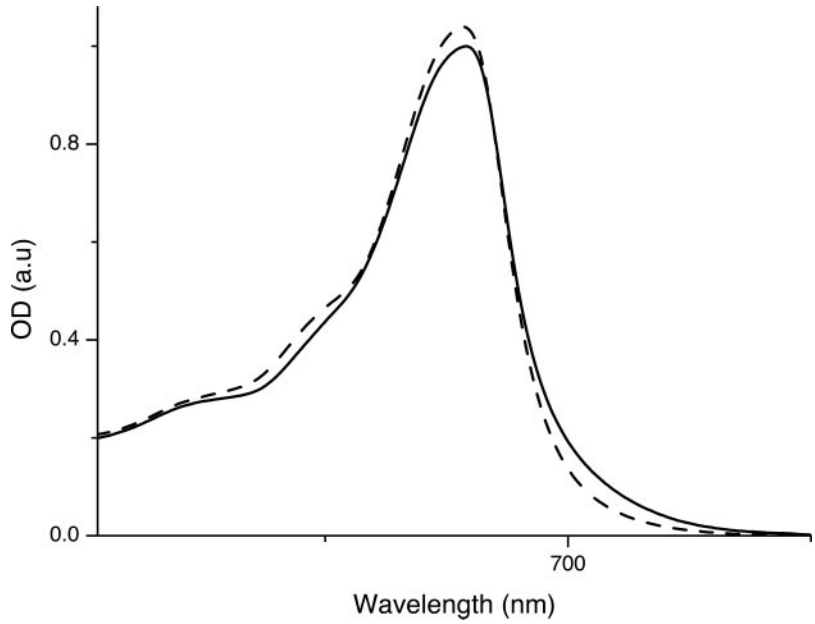

FIG. 9. **Comparison of absorption spectra between dimeric and monomeric antenna complexes.** Room temperature absorption spectra of dimeric LHCI purified (*solid line*) is compared with the sum of the monomeric Lhca1–4 complexes (*dashed line*). Spectra were normalized to the Chl content. *a.u.*, absorbance units.

Lhca3 (24), these additional emissions must be due to some kind of heterogeneity of the protein population. One likely explanation for this observation is that Lhca proteins may exist in different conformations, similar to what previously reported for PSII antenna complexes (39, 40). In the PSI·LHCI supercomplex, on the contrary, the conformation exhibiting the red-most-shifted emission appears to be stabilized, thus yielding emissions at 722, 730, and 740 nm without any emission at shorter wavelengths (17). The interactions in the PSI·LHCI, thus, can have an effect in enhancing the red forms by favoring the conformation with the lowest energy levels. This picture is consistent with the fact that Chls A5 and B5, which are responsible of the red emission in Lhca (37), face the core complex and are located close to the gap chlorophylls, and they may be influenced by their presence (see Fig. 8).

However, although the effect of the interactions on the red forms is consistent with previous data, we cannot exclude that red-shifted Chls are also present among gap pigments, directly contributing to red absorption tail or that both mechanisms contribute to the red forms enhancement. In the PSI·LHCI complex, as evidenced above, there is another set of protein-protein interactions between different antenna polypeptides responsible of the dimeric aggregation state of the LHCI. To verify if the monomer-monomer interactions have an influence in red forms, we compared the absorption spectra of the dimeric-purified LHCI with the sum of the monomeric Lhca reconstituted *in vitro* upon normalization of the spectra to the same Chl content to see the "relative content" in red forms (see Fig. 9). The spectra clearly show that in the dimeric fraction the red absorption tail is relatively enriched, whereas in the monomeric samples there is larger absorption from bulk chlorophylls at around 680 nm. It is worth mentioning that the effect is not due to the difference between the recombinant and the native sample but only to the higher aggregation state since a similar effect has already been shown comparing the monomeric Lhca1 and Lhca4 with their heterodimer reconstituted in *vitro* (12).

The effect of the protein-protein interactions in enhancing red forms is, thus, a general phenomenon and, although the origin of the red emission appears to be the same, the amplitude of red forms follows the order monomeric antenna < dimeric antenna < dimeric antenna bound to the core, according to the increased presence of intersubunit interactions.

Lhca Binding Flexibility and Comparison with PSII—In all analyses presented so far, no evidence was found for a possible compensatory replacement of missing Lhca by another polypeptide(s). In fact, Lhca1–4 appears to bind to specific sites whose occupancy is very selective. This is different from what observed in PSII, where in plants depleted in Lhcb1 and Lhcb2, Lhcb5 and Lhcb3 content increased to compensate for the missing LHCII complex. These plants even maintained the supramolecular structure of PSII supercomplexes by forming trimers with Lhcb3 (41). This suggests a different supramolecular organization of antenna systems in PSI as compared with PSII even if the components are homologous. The PSI antenna system shows stronger interactions with the core complex, as evidenced by the stability of the PSI·LHCI supercomplex to detergent treatment. Instead, PSII·LHCII complexes are only partially conserved even in the mildest conditions of solubilization (see Fig. 1). This difference in stability could be related to the presence in PSI, but not in PSII, of gap and linker pigments, which provide additional interactions between protein subunits (10).

The higher stability of PSI is accompanied by a lower level of flexibility; each polypeptide has a specific binding site that cannot be occupied by other polypeptides to a significant extent. This rigid organization of the antenna system is most probably less compatible with antenna size regulation as compared with PSII. This may be the reason why state I-state II transition mechanisms play a major role in PSI antenna size regulation (42), whereas extensive changes in the number of LHCII subunits are experienced by PSII (43).

PSI Antenna of Chlamydomonas reinhardtii Shares Organization Similarities with Higher Plants—In all eukaryotic organisms Photosystem I has an antenna system composed by polypeptides belonging to Lhc multigenic family. However, the green alga *C. reinhardtii* has a PSI with a significantly larger antenna system, estimated to contain 9–11 Lhca monomers (44, 45) and composed by 9 different Lhca polypeptides (46–49). It would, thus, be interesting to ask if the cooperative nature of the antenna association to PSI core shown here is conserved in *Chlamydomonas* as well.

In recent work LHCI supercomplexes stable to detergent treatment (0.8% β-DM) have been isolated from *Chlamydomonas* mutants depleted in the PSI core (50). This result suggests that also in LHCI from *Chlamydomonas* strong interactions between Lhca complexes are present and that the association of

antenna to the PSI core might have similar characteristics in all eukaryotic organisms (50).

How Does Lhca5 Associate with PSI·LHCI Supercomplexes?—PSI·LHCI from Δa5 plants was indistinguishable from wild type, consistent with the Lhca5 gene product being present in substoichiometric amounts (6). Nevertheless, its apparent lower stability to detergent treatment with respect to the WT complex was intriguing. In fact, a PSI core band could be detected in non-denaturing gels of Δa5 thylakoids (Fig. 2*B*) as well as the release of a fraction of Lhca2 and Lhca4 polypeptides (Fig. 4*A*) upon solubilization. These data suggest that the lack of Lhca5 affects a small fraction of the PSI population where the antenna complex is partially destabilized.

Biochemical and Spectroscopic Properties of Lhca1/4 and Lhca2/3 Dimers—We purified the LHCI moiety from plants depleted in individual Lhcas, obtaining three different populations with variable polypeptide composition. Despite the presence of different Lhca, the purified antenna complexes, when characterized, did not show any striking difference (Fig. 7*A*). This results suggests that Lhca1–4 and Lhca2–3 dimers have very similar properties, in agreement with data on isolated native LHCI populations enriched in Lhca1–4 and Lhca2–3, which also showed little difference (12). The finding is rather surprising if we consider that individual Lhca holoproteins have distinct properties (9, 24). However, we should be aware that since Lhca1–4 and Lhca2–3 strongly interact, the properties of dimers rather than their individual monomers should be considered. In Fig. 7*B* the sums of the individual absorption spectra of Lhca1 + Lhca4 and Lhca2 + Lhca43 are shown. Dimer properties are not identical to the sum of the monomers, since extra pigments are bound at the interfaces between polypeptides, and they are not stably bound in monomers reconstituted *in vitro* (4, 12). However, even taking in account these limitations, the strong similarity of the two sums confirms that dimers with essentially the same properties can originate from monomers with different characteristics and supports the resemblance between Lhca1–4 and Lhca2–3 heterodimers. A further confirmation to this hypothesis is that no large differences were evidenced in pigment binding properties of PSI particles from different mutant plants. The variations observed, in fact, were correlated with the total amount of antenna polypeptides lost rather than the loss of specific proteins. A partial reduction of Lhca1/4 with respect to Lhca2/3 did not have any relevant effect, confirming that the dimers have similar characteristics. In conclusion, the minimal unit of LHCI seems to be a heterodimeric complex composed of two polypeptides with distinct spectral properties, and it might have implications with respect to the evolution of higher plant LHCI. According to a model recently presented (51), ancient PSI-antenna might have been composed by two or more homodimers of identical Lhca-type protein subunits. The binding sites could then have been modified through co-evolution of the Lhca and the reaction center proteins, resulting into four individual Lhca-type proteins optimized in stable docking to one specific binding site.

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The Association of the Antenna System to Photosystem I in Higher Plants: COOPERATIVE INTERACTIONS STABILIZE THE SUPRAMOLECULAR COMPLEX AND ENHANCE RED-SHIFTED SPECTRAL FORMS

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