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The Nature of a Chlorophyll Ligand in Lhca Proteins Determines the Far Red Fluorescence Emission Typical of Photosystem I*

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Photosystem I of higher plants is characterized by a typically long wavelength fluorescence emission associated to its light-harvesting complex I moiety. The origin of these low energy chlorophyll spectral forms was investigated by using site-directed mutagenesis of *Lhca1–4* genes and *in vitro* reconstitution into recombinant pigment-protein complexes. We showed that the red-shifted absorption originates from chlorophyll-chlorophyll (Chl) excitonic interactions involving Chl A5 in each of the four Lhca antenna complexes. An essential requirement for the presence of the red-shifted absorption/fluorescence spectral forms was the presence of asparagine as a ligand for the Chl *a* chromophore in the binding site A5 of Lhca complexes. In Lhca3 and Lhca4, which exhibit the most red-shifted red forms, its substitution by histidine maintains the pigment binding and, yet, the red spectral forms are abolished. Conversely, in Lhca1, having very low amplitude of red forms, the substitution of Asn for His produces a red shift of the fluorescence emission, thus confirming that the nature of the Chl A5 ligand determines the correct organization of chromophores leading to the excitonic interaction responsible for the red-most forms. The red-shifted fluorescence emission at 730 nm is here proposed to originate from an absorption band at ~700 nm, which represents the low energy contribution of an excitonic interaction having the high energy band at 683 nm. Because the mutation does not affect Chl A5 orientation, we suggest that coordination by Asn of Chl A5 holds it at the correct distance with Chl B5.

Photosystem I is a multisubunit pigment-protein complex of the chloroplast membrane acting as a plastocyanin/ferredoxin oxido-reductase in oxygenic photosynthesis. One important spectroscopic feature of PSI[1] is the presence of Chls absorbing at energy lower than the PSI primary electron donor, P700. Although these spectral forms account for only a small percentage of the total absorption, their effect in the energy transfer and trapping of PSI is very prominent (1), with at least 80% of excitation in the complex transiting through them on their way to P700 (2). It has been widely proposed that these forms represent the low energy contributions of excitonic interactions, which involve two or more Chl molecules (3–5); however, the identity of the chromophores involved and the details of the interaction are still unknown.

Although the presence of low energy-absorbing Chls is ubiquitous in the PSI of different organisms, their amounts and energies appear to be highly species-dependent (1). In the PSI of higher plants, the red forms are associated with the outer antenna, LHCI (6, 7). LHCI is composed by four pigment-binding proteins, namely Lhca1–4 (6, 8). These complexes, localized on one side of the core complex (9, 10), are organized in dimers with 10 Chl molecules per subunit (11).

As for their properties, Lhca2 and Lhca4 differ from Lhca1 and Lhca3 in many respects. The first two have higher Chl b content with respect to Lhca1 and Lhca3 and bind two carotenoid molecules per polypeptide rather than three (12). For their folding, Lhca2 and Lhca4 require both Chl *a* and Chl *b*, whereas Lhca1 and Lhca3 are stable with Chl *a* only (12, 13). Circular dichroism spectra, yielding information of pigment organization within the complex, also show strong similarities between Lhca2 and Lhca4 on one hand and Lhca3 and Lhca1 on the other (12). The close relation between Lhca2 and Lhca4 is confirmed by sequence analysis, which shows 55% identity and 75% homology (14). Surprisingly, the distribution of red forms does not fit into the picture, because these are associated mainly with Lhca3 and Lhca4 (fluorescence emission at 725–730 nm), whereas Lhca1 and Lhca2 have emission at higher energy (702 nm) (12, 13, 15). Based on high homology between proteins with different content in red forms, a sequence motif associated with the presence of lowest absorption band, common to Lhca3 and Lhca4 and not present in Lhca1 and Lhca2, should possibly be detected. In fact, Lhca3 and Lhca4 have Asn as the ligand for Chl A5 instead of His (Fig. 1), which is in this position in all other components of Lhc family, including Lhca1 and Lhca2 (14), which show no or a low amount of red-shifted spectral forms. It should be noted that Chl A5 has been involved in red forms together with Chl B5 (16). In this work, the effect of the presence of Asn as ligand for Chl A5 in Lhca complexes was analyzed in relation to the appearance of the red emission, which characterizes the antenna complexes of Photosystem I. We show that an essential requirement for the presence of the red-shifted absorption/fluorescence spectral forms was the presence of Asn as ligand for the Chl *a* in the binding site A5 of Lhca complexes. Conversely, substitution of

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[1] The abbreviations used are: PSI/II, Photosystem I/II; CD, circular dichroism; Chl, chlorophyll; FWHM, full-width half-maximum; HN, His → Asn mutation; LD, linear dichroism; LHCI, light-harvesting complex of PSI; LHCII, light-harvesting complex of PSII; NH, Asn → His mutation; WT, wild type.

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Lhca1 RYKESELIHCRWAMLAVPGILVPEALGYGNWV
Lhca2 WFVQAELVHGRWAMLGVAGILIPEXLGKIGII
Lhca3 WLAYGEIINGRFAMLGAAGAIAPEILGKAGLI
Lhca4 WFVQAELVNGRWAMLGVAGMLLPEVFTKIGII
      .*::: *:***...* : ** :  . :
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FIG. 1. **Sequence comparison between the B helices of the four Lhca complexes.** The *bold* characters indicate the ligand for Chl A5. An asterisk (*) stands for identity, a colon (:) indicates strong homology, and a period (.) stands for weak homology.

the His ligand with Asn produces a shift of Lhca1 fluorescence emission to lower energy. We conclude that His *versus* Asn binding of Chl A5 controls the strength of the interaction with Chl B5 by changing the interchromophore distance.

EXPERIMENTAL PROCEDURES

DNA Constructions and Isolation of Overexpressed Lhca Apoproteins from Bacteria—cDNAs of Lhca1–4 from *Arabidopsis thaliana* (11, 12) were mutated with QuikChange© site-directed mutagenesis kit, Stratagene. WT and mutant apoproteins were isolated from the SG13009 strain of *Escherichia coli* transformed with constructs following a protocol described previously (17, 21–23). Reconstitution and purification of protein-pigment complexes were performed as described (11, 12).

Protein and Pigment Concentration—High performance liquid chromatography analysis was as described (18). The chlorophyll-to-carotenoid ratio and the Chl *a/b* ratio was measured independently by fitting the spectrum of acetone extracts with the spectra of individual purified pigments (19).

Spectroscopy—The absorption spectra at room temperature and 100 K were recorded using an SLM-Aminco DK2000 spectrophotometer in 10 mM Hepes, pH 7.5, 20% glycerol (60% at low temperature) and 0.06% *n*-dodecyl-β-D-maltopyranoside. The wavelength sampling step was 0.4 nm, the scan rate was 100 nm/min, and the optical pathlength was 1 cm. Chlorophyll concentration was ~10 μg/ml. Fluorescence emission spectra were measured using a Jasco FP-777 spectrofluorometer and corrected for the instrumental response. The samples were excited at 440, 475, and 500 nm. The spectral bandwidth was 5 nm (excitation) and 3 nm (emission). Chlorophyll concentration was ~0.02 μg/ml in 60 and 80% (Lhca1 only) glycerol and 0.03% *n*-dodecyl-β-D-maltopyranoside. LD spectra were obtained as described by using samples oriented uniaxially by the polyacrylamide gel-squeezing technique (20).

The CD spectra and denaturation temperature measurements were measured at 10 °C on a Jasco 600 spectropolarimeter. The wavelength sampling step was 0.5 nm, the scan rate was 100 nm/min, and spectra were recorded with eight accumulations. The OD of the samples was 1 at the maximum in the Qy transition for all complexes, and the samples were in the same solution described for absorption measurements. All spectra presented were normalized to the polypeptide concentration based on the Chl binding stoichiometry as described (16).

Denaturation temperature measurements were performed by following the decay of the CD signal at 459 nm while increasing the temperature from 20 to 80 °C with a time slope of 1 °C per min and a resolution of 0.2 °C. The thermal stability of the protein was determined by finding the $t_{1/2}$ of the signal decay.

RESULTS

Mutation Asn-His in Lhca3 and Lhca4—Lhca proteins are strongly conserved, and residues coordinating Chl ligands are identical in the four members of the sub-family. The only exception is the ligand of Chl A5, a His in Lhca1 and Lhca2, which is substituted by Asn in Lhca3 and Lhca4. Because this difference correlates with the presence of a strong red-shifted fluorescence emission band at ~730 nm, we proceeded to mutate residues Asn-62 (Lhca3) and Asn-47 (Lhca4) into His. After overexpression in bacteria and reconstitution *in vitro* with purified pigments, mutant Lhca3 and 4 complexes were obtained having a monomeric aggregation state as determined by gradient ultracentrifugation (not shown).

The pigment composition of the Asn → His (NH) mutant (Lhca3-N62H and Lhca4-N47H), as compared with the WT complexes reconstituted in the same conditions, is reported in Table I. It is worth noting that the conservative mutation leaves the number and relative ratio of the chromophores in the complexes unchanged with respect to their WT counterparts. Nevertheless, spectroscopic analysis shows that Lhca3-N62H and Lhca4-N47H have lost their low energy absorption features present in WTs (Fig. 2). This is confirmed by low temperature fluorescence emission spectra (Fig. 3). In Lhca3, the emission peak is shifted from 725 nm in the WT to 686 nm in the mutant. In WT Lhca4 the emission maximum is at 730 nm, whereas the mutation yields a complex with a 686-nm peak, although an additional emission is present at around 702 nm. The difference between WT and mutant absorption spectra showed that the loss of absorption in the red tail of the Q_y transition was compensated by an increase at around 675 nm (676 in Lhca3 and 674 in Lhca4). A second positive contribution in the difference absorption spectrum was visible at 683 nm. No significant difference is observed in Chl *b* and carotenoid absorption regions, thus indicating that the mutation does not affect the energy levels of these pigments. It has been suggested that the red forms are the result of strong excitonic interactions between antenna Chls (3, 16). To analyze this aspect, the CD spectra of the four samples were recorded (Fig. 4). Both Lhca3 and Lhca4 WT were characterized by the presence of a large negative contribution at wavelength longer than 700 nm. Upon mutation, this signal disappeared, together with a positive contribution at 680–681 nm. The shape of the signal in the CD difference spectra (Fig. 4, *dash-dot lines*) suggests the loss in the mutants of an excitonic interaction with the low energy contribution at 700–705 nm and the high energy band around 682–683 nm, in complete agreement with the absorption spectra in which two positive contributions at these wavelengths were lost upon mutation.

The strength of a Chl-Chl interaction depends on both the distance between the chromophores and the orientation of their dipole moments. To check whether the Asn *versus* His exchange had affected the orientation of the Chls, linear dichroism spectra of the complexes were measured (Fig. 5).

The spectra of WTs are identical to those of the corresponding mutant proteins in the 460–520 nm region, an indication that no changes occur in the carotenoid orientation. In the mutants, the positive contribution at long wavelengths is substituted by a new absorption around 676 nm, which still exhibited positive LD. The comparison of the difference absorption and LD spectra (Figs. 2 and 5) suggests that Chl A5, although changing its ligand residue, maintains the same orientation as in the WT. We therefore exclude the proposal that the loss of the excitonic interaction might be due to a change in the orientation of Chls as a consequence of the mutation.

Mutation His → Asn in Lhca1 and Lhca2—The above results clearly demonstrate that the presence of Asn as a ligand for Chl A5 is a requirement for the presence of the red-most forms. To test the hypothesis that this feature is also sufficient for the presence of this spectroscopic property, Lhca1 and Lhca2, which have His ligand at their A5 site (H47 and H52, respectively) and show emission at 702 nm (13), were mutated to Asn-47 and Asn-52. Both mutant proteins yielded a refolded monomeric product. In the case of Lhca2, the recombinant complex was unstable as judged from heat denaturation data (Table I). This suggests that the His → Asn exchange is affecting the folding of Lhca2, thus preventing a selective evaluation of the effect of ligand exchange. On this basis, this sample will not be analyzed any further in this work.

In the case of Lhca1, a stable mutant complex was obtained. The fluorescence spectra was shifted by 11 nm toward the red compared with the WT (Fig. 6*A*). Consistently, the absorption and LD spectra showed an increase in the red-most absorption region of Lhca1-H47N as compared with WT.

Additional features are the loss of the 684 nm (positive LD)

TABLE I
Pigment composition of reconstituted complexes

Abbreviations used in this table include the following: Car, carotenoids; Car_T, carotenoid total; Chl_T, chlorophyll total; Viola, violaxanthin; Lute, lutein; β-Car, β-carotene (Lhca3); Neo, neoxanthin (Lhca1); and DT, denaturation temperature.

Sample	Chl *a*/*b*	Chl/Car	Chl_T	Car_T	Chl *b*	Viola	Lute	β-Car/Neo	DT
Lhca1-WT	4.02 ± 0.13	3.45 ± 0.14	10	2.90 ± 0.13	1.99 ± 0.07	1.09	1.52	0.28	54.2 ± 1.5
Lhca1-HN	4.1 ± 0.06	3.2 ± 0.16	9.5	2.96 ± 0.07	2.04 ± 0.07	1.08	1.63	0.26	51.2 ± 1.8
Lhca2-WT	1.85 ± 0.15	5.02 ± 0.18	10	1.99 ± 0.07	3.52 ± 0.19	0.47	1.50		53.3 ± 1.9
Lhca2-HN	2.43 ± 0.05	4.60 ± 0.11	9	1.96 ± 0.05	2.63 ± 0.03	0.48	1.48		43.2 ± 1.6
Lhca3-WT	5.50 ± 0.13	3.70 ± 0.09	10	2.70 ± 0.06	1.54 ± 0.03	0.63	1.70	0.37	45.6 ± 1.4
Lhca3-NH	5.60 ± 0.29	3.68 ± 0.19	10	2.72 ± 0.14	1.52 ± 0.07	0.65	1.74	0.33	39.5 ± 1.5
Lhca4-WT	2.38 ± 0.11	4.83 ± 0.20	10	2.07 ± 0.09	2.96 ± 0.09	0.36	1.72		47.2 ± 0.8
Lhca4-NH	2.36 ± 0.09	4.91 ± 0.08	10	2.04 ± 0.09	2.97 ± 0.12	0.35	1.68	0.08	49.5 ± 1.9

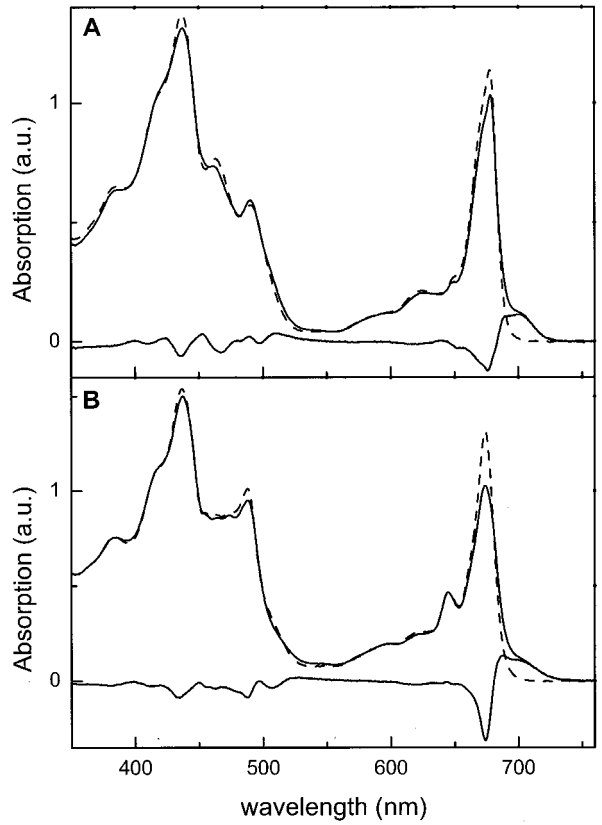

FIG. 2. **Absorption spectra at 100 K of Lhca3 and Lhca4 WT and NH mutant.** WT (*upper solid line*), NH mutant (*dashed line*), and difference (*lower solid line*) spectra of Lhca3 (*A*) and Lhca4 (*B*) are shown. All the spectra are normalized to the total absorption (see "Experimental Procedures"). *a.u.*, arbitrary units.

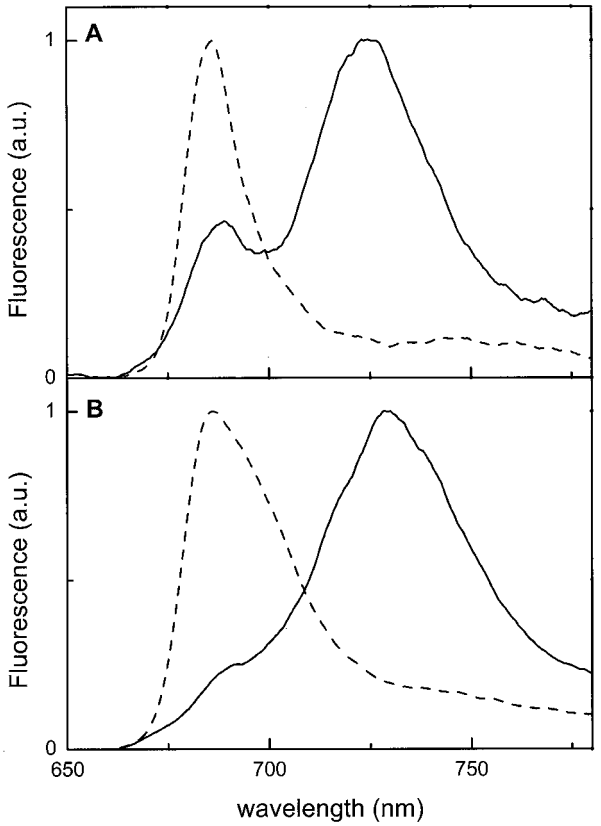

FIG. 3. **Fluorescence emission at 77 K of Lhca3 and Lhca4 WT and NH mutant.** Spectra of WT (*solid line*) and NH mutant (*dashed line*) of Lhca3 (*A*) and Lhca4 (*B*) are shown. The spectra are normalized to the maximum (see "Experimental Procedures"). *a.u.*, arbitrary units.

and of the 678 nm (negative LD) absorption bands (Fig. 5, *B* and *D*), probably due to small differences in pigment content. Moreover, the CD spectrum of the mutant is red-shifted and shows increased negative signal above 700 nm (Fig. 5*C*). We can therefore conclude that the substitution of His with Asn as ligand for Chl A5 in Lhca1 allows the interactions that yield the red forms to be established.

DISCUSSION

Chlorophyll a is essential for plant photosynthesis. This single molecular species catalyzes a number of functions including light harvesting, excitation energy transfer, charge separation, and electron transport. Such a unique performance depends largely on the modulation of its physico-chemical properties by the environment provided by specific binding proteins. In reaction centers, the presence of dimers of the Chl molecule confers the "special" nature of a primary donor. In antenna proteins, energy levels of each individual chlorophyll chromophore are modulated by interactions with amino acid groups and carotenoid molecules to optimize both energy transfer and photoprotection (21–23). In Photosystem II, the protein environment also provides modulation of chlorophyll fluorescence lifetime and, thus, of the efficiency of energy transfer to P680 by undergoing conformational changes induced by the binding of violaxanthin *versus* zeaxanthin to an allosteric site called L2 (24). The most dramatic example of modulation of the spectroscopic properties of Chl is found in PSI with the presence of long wavelength absorption forms extending light harvesting to the low energy region of the spectrum not absorbed by PSII pigments of the shading canopy (25). Although PSII fluoresces in the 675–695 nm range, PSI exhibits fluorescence emission at ~735 nm, thus implying a 60 nm shift with respect to fluorescence emission of Chl *a* in solution. These characteristics have been attributed to the LHCI complex and, more recently, associated with the two *Lhca* gene products Lhca3 and Lhca4 (12). Nevertheless, the structural features responsible for the origin of red forms are still unknown, although mutation analysis of Lhca1 has suggested Chls A5 and possibly B5 to be involved in

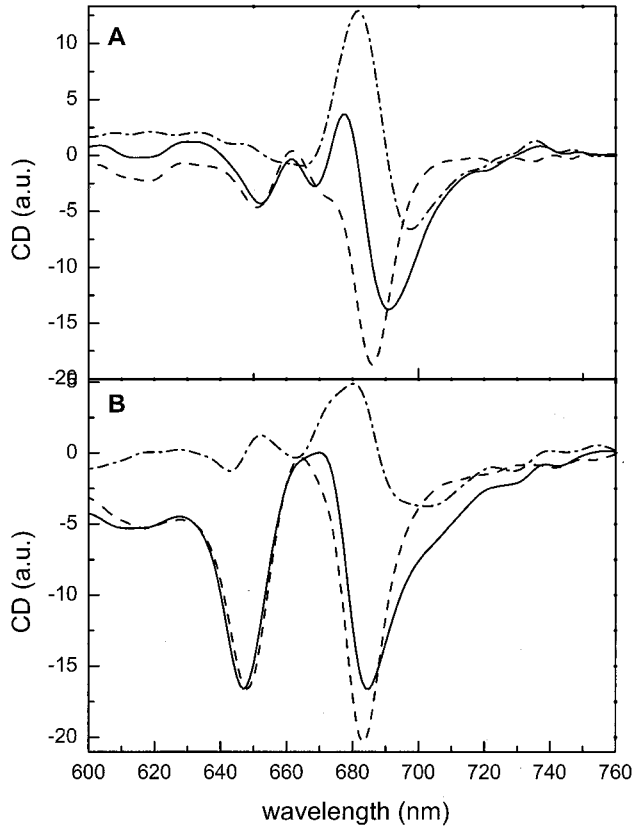

FIG. 4. **Circular dichroism spectra at room temperature of Lhca3 and Lhca4 WT and NH mutant.** WT (*solid line*), NH mutant (*dashed line*), and difference (*dash-dot line*) spectra of Lhca3 (*A*) and Lhca4 (*B*) are shown. The spectra are normalized to the same absorption (see "Experimental Procedures"). *a.u.*, arbitrary units.

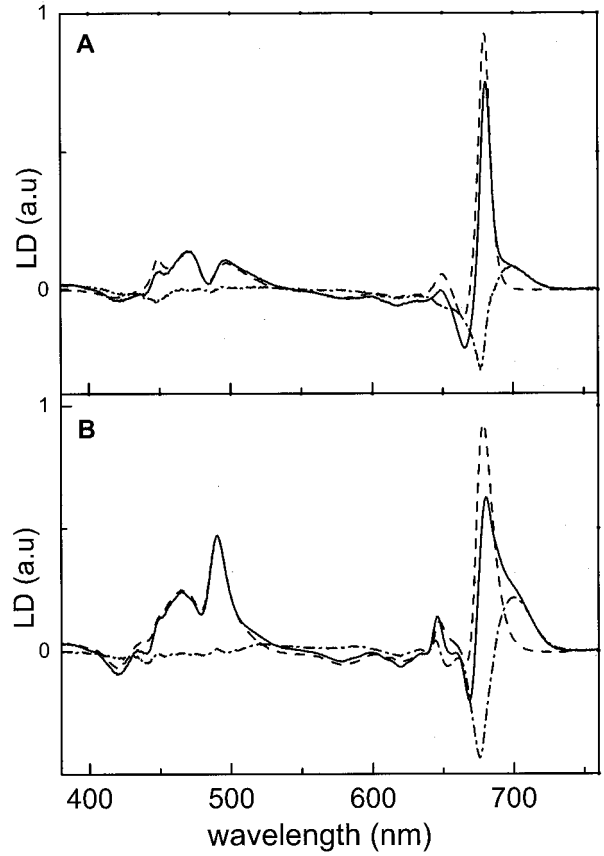

FIG. 5. **Linear dichroism spectra at 100 K of Lhca3 and Lhca4 WT and NH mutant.** Linear dichroism spectra at 100 K of WT (*solid line*), NH mutant (*dashed line*), and the difference (*dash-dot line*) spectra of Lhca3 (*A*) and Lhca4 (*B*) are shown. The spectra are normalized in the blue region (see "Experimental Procedures"). *a.u.*, arbitrary units.

the low energy absorption band (16). The involvement of Chl A5 was also suggested in the case of Lhca4, because the deletion of the N-terminal protein domain abolished the red emission (26). Analysis of the protein sequences has identified a difference in the ligand for Chl A5 in Lhca3/4 as compared with Lhca1/2. In the first two complexes, an Asn is present in this position, whereas His is the ligand in the other two, as it is in all other higher plant antenna complexes, which do not exhibit red-shifted emission. To determine the role of the ligand, this residue was mutated in all four Lhca proteins, substituting His for Asn in Lhca1 and Lhca2 and Asn for His in Lhca3 and Lhca4.

Is Asn, as Ligand for Chl A5, Necessary to Yield to the Low Energy Absorption Forms?—Although Lhc proteins differ in the number of Chl ligands, site A5 belongs to the highly conserved core domain formed by transmembrane helices A and B. The His residue in the A5 ligand position was shown to bind a Chl *a* molecule in all of the Lhc proteins analyzed to date (*i.e.* Lhcb1, Lhcb4, and Lhca1) by the loss of a Chl upon mutation of the ligand residue to a non-binding Phe residue. It is important to note that, in both Lhca3 and Lhca4, the substitution of Asn with His did not influence either the number or the composition of pigments bound to the complex.

Nevertheless, the mutation led to the loss of the red forms in both Lhca3 and 4, proving that Asn has a major role, as ligand of Chl A5, in effecting their presence. Moreover, only very specific spectral changes were obtained as a result of the mutation, whereas the spectra were unaffected at most wavelengths. Thus, the Asn to His substitution has a very specific effect, whereas it does not influence the overall properties of the pigment-protein complexes. We conclude that coordination through His induces a re-organization of chromophores involved in the interaction(s) responsible for the red-most spectral feature in the WT complexes.

Orientation and distance are the major determinants in excitonic interactions. In this case, LD analysis shows that the orientation of the Chls is not affected by the mutation. On this basis, we suggest that the main effect of the mutation is to increase the distance between the two interacting Chls. This is realistic considering the structure of the two residues, with Asn having smaller steric hindrance as compared with His (Fig. 8). It has to be considered that the His *versus* Asn substitution appears to be a usual tool for modulation of Chl-Chl interactions in Lhc proteins; Chl A2, when coordinated by Asn in Lhcb1, establishes excitonic interactions with neighbor pigments, namely Chl B2 and Chl A4. This interaction is absent in Lhcb4 (CP29) in which His is the ligand for Chl A2 (21, 22) and again present in the Asn-bearing Lhcb5 (CP26), whose overall biochemical and spectral characteristics are very close to those of CP29 (19).

Is the Presence of Asn as Ligand for Chl A5 Sufficient to Lead to the Red Forms?—A complete mutation analysis of Lhca1 complex showed that the red-most spectral form in this complex, emitting at 702 nm, originates by an interaction between Chls in sites A5 and B5 (16). Upon mutation of the His ligand for Chl A5 in Lhca1, the fluorescence emission of Lhca1-H47N showed a 11-nm red shift as compared with the WT, a clear indication of the formation of a new red spectral form. The absorption and LD spectra display a small but clearly detectable increase in the absorption at wavelengths of >700 nm. Gaussian analysis of the difference LD spectra, where the

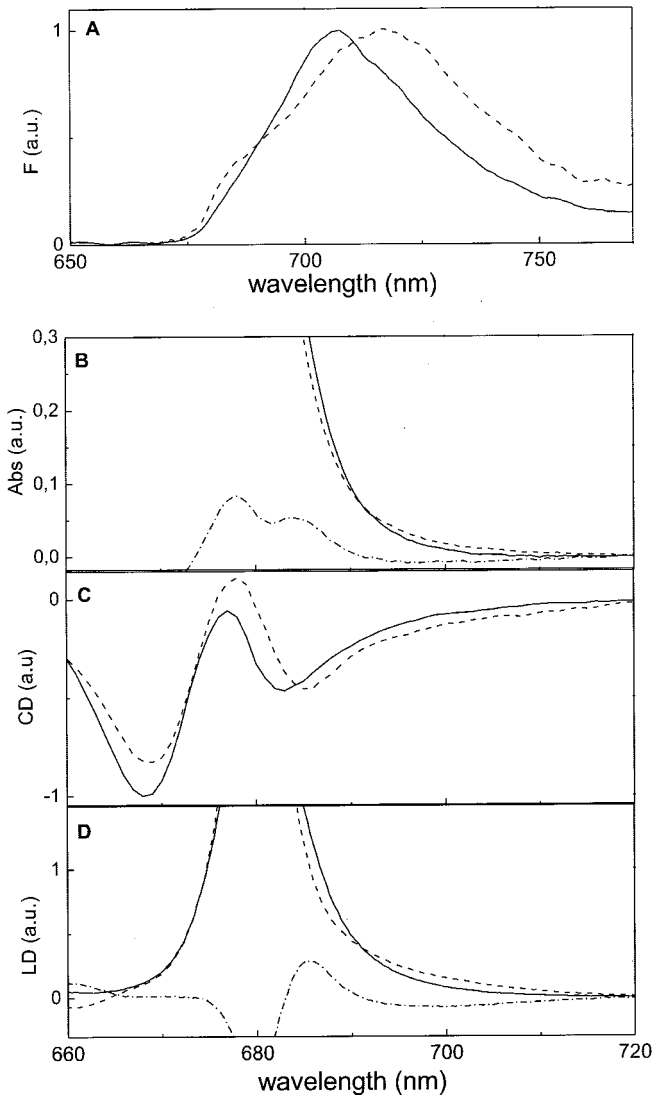

FIG. 6. **Spectroscopic analysis of Lhca1.** Spectroscopic analysis of Lhca1 WT (*solid line*) and Lhca1-HN mutant (*dashed line*) is shown. *A*, fluorescence (*F*) emission spectra upon excitation at 440 nm at 77 K. *B*, absorption (*Abs*) spectra at 100 K. *C*, CD spectra at 10 °C. *D*, LD spectra at 100 K. The difference spectra are also presented in most cases (*dash-dot line*). *a.u.*, arbitrary units.

signal is more clearly detectable, indicates that the newly formed band absorbs with a maximum at 700 nm. The pigment complement of Lhca1-HN is similar to that of the WT, although not identical, as was the case for Lhca3-NH and Lhca4-NH. By normalizing the number of Chls to the carotenoid content, a value of 9.5 Chls in the mutant *versus* 10 in the WT is obtained. It therefore appears that the Asn introduced in Lhca1 as Chl A5 ligand, although able to coordinate Chl, cannot produce 100% occupancy of the site as is the case in the WT. We conclude that the Lhca1 H47N mutant yields a mixed population in which ~50% of the complexes lose the Chl A5 ligand. This is confirmed by preliminary results showing incomplete energy equilibration among Chl *a* chromophores, although Chl *b* to Chl *a* energy transfer is complete. Nevertheless, this experiment shows that the presence of Asn is sufficient to generate the red forms in Lhca1. Our failure to obtain a similar result with Lhca2 suggests, however, that the overall structure of the complex must be suited to host the Chl A5 in the new location. The Lhca2-HN mutant, in fact, looses pigments and does not exhibit red-shifted fluorescence.

Energy Levels of the Interacting Chls—To determine the energy levels of the interacting Chls, the difference absorption spectra (WT minus mutant) of Lhca3 and Lhca4 were analyzed in terms of Gaussian bands.

The data, for Lhca4 only, are reported in Fig. 7 and are fully consistent with the data for Lhca3. Three Gaussian components are required for a satisfactory description of the difference spectrum, *i.e.* two positives at 683 nm (FWHM = 15.4 nm) and 700 nm (FWHM = 31.8 nm) and one negative at 675 nm (FWHM = 9.3 nm). The two positive bands are likely to represent the high and the low energy bands of the excitonic interaction, respectively. This proposal is supported by the analysis of the CD spectra; the mutation led to the loss of one negative component at around 703–705 nm and of a positive component at 680 nm. The negative band represents the absorption of the monomers in the absence of the interaction. Considering that the 675-nm band is the only negative contribution observed in the spectra, it can be concluded that the monomers are isoenergetics. Similar values for the absorption of monomeric Chl A5 were found in LHCII and CP29 (21, 22), thus supporting the present conclusion. We have to highlight the fact that the bandwidth of the red-most transition is very large, as is expected in the case of a Chl dimer because of strong electron-phonon coupling, whereas the absorption at 675 nm has the FWHM typical of a Chl monomer.

The low energy transition responsible for the fluorescence emission at 730 nm in a native LHCI preparation composed of Lhca heterodimers was proposed to be lying at 711 nm and to be associated with Lhca4 (27). Such a 711-nm band is not detectable in our difference spectra. We notice, however, that in order to describe the red absorption tail of both Lhca4 and Lhca3 with a form peaking at 709–711 nm having an FWHM of ~30 nm (as the fluorescence line narrowing data suggest; Ref. 28) a second Gaussian band with maximum around 697 nm and FWHM of 25 nm would be required. This model with two red-shifted forms, however, would imply an oscillator strength of <1 Chl per Lhca4 monomer associated with the 711-nm form in both Lhca3 and Lhca4 (data not shown). Although a scenario implying more than one red-shifted form in Lhca proteins cannot be discarded without complementary experiments, at present we favor the simpler hypothesis that the 730-nm fluorescence emission band originates from a single absorption form with the maximum of ~700 nm on the basis of the following considerations.

First, the values of the Huang-Rhys factors and the mean frequencies of the phonon distribution for the red band of LHCI, as calculated from fluorescence line narrowing measurements, are 2.7 and 0.2 and 110 cm^{-1} and 190 cm^{-1}, respectively (28). Thus, a value of 670 cm^{-1} for the Stokes shift ($2S_1v_1$ + $2S_2v_2$) can be proposed, localizing the absorption band responsible for the 730-nm fluorescence emission in Lhca4 (735 nm in LHCI) at 700–705 nm. Second, the band shape of the red-most part of the absorption and LD spectra are identical, implying that if two components are present, then they must have the same dipole orientation of their Q_y transition, which seems unlikely. Third, the CD spectra show the red-most minimum at 703–705 nm, in agreement with the data obtained in the native LHCI preparation (minimum at 706 nm) (27). The absorption maximum associated to this CD signal is thus expected to be shorter than 705 nm.

It has also been proposed that Chl *b* participates in the interaction yielding the red forms in Lhca4 (29). The absorption difference spectra for Lhca3 and Lhca4 do not show any contribution in the Chl *b* region. If a Chl *b* was directly involved in the interaction, a shift of this band upon loss of the interaction is expected. On the basis of the present data we can then exclude a direct involvement of Chl *b* molecules in the red emission forms. This conclusion is in agreement with the pre-

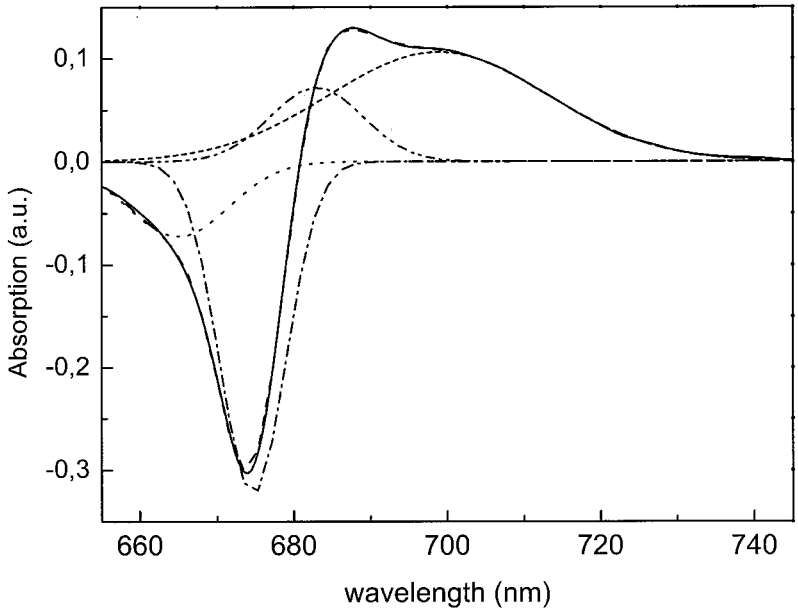

FIG. 7. **Gaussian description of the difference absorption spectra.** Difference absorption spectra of Lhca4-WT and Lhca4-N47H (*solid line*) and its Gaussian description is shown. *a.u.*, arbitrary units.

vious suggestion that Chl *b* involvement was indirect and due to a structural role for Chl B6 (12, 16).

Based on the above considerations, we conclude that the two bands of the excitonic interaction lye at 683 and 700 nm and a value of 180 cm^{-1} can thus be obtained for the resonance interaction energy, V_{12}. The red shift observed for the two excitonic bands, as compared with the absorption of the monomers, is due to the displacement energy as was already observed for dimers of Chl in solution (30, 31).

An oscillator strength of 1.17 Chl molecules per monomer is associated with the 700-nm absorption band in Lhca4. From this value, the angle between the dipole transition moments of the interacting monomers can be calculated to be ~80°. A model for the organization of the two interacting Chls in WT and the mutants Lhca3 and Lhca4 is proposed in Fig. 8, which is based on the structure of LHCII (32).

In the LD spectra, the 700-nm band shows a positive contribution, which indicates that the angle between the dipole transition moment and the normal to the membrane plane is >54.7°. As for the 683-nm contribution, it does not appear in the LD spectrum, thus suggesting that the Chl responsible for this absorption has a dipole transition moment oriented at the magic angle.

Conclusion—In this work, we have identified the primary role for an Asn residue as a ligand for Chl A5 in determining the chromophore organization yielding the most red-shifted spectral forms of Lhca3 and Lhca4, from which originates the far red fluorescence emission band typical of PSI. The presence of Asn in that position is necessary for the formation of the red-shifted band. It also appears to be sufficient in complexes where Chl binding to site A5 by an Asn residue is allowed. Its role is to keep the two Chls close enough for excitonic interaction. We also suggest that the differences in transition energy and amplitude of red-most bands in the individual Lhca1–4 antenna complexes depends on the strength of the interaction most probably between the same Chl pair, namely Chl A5 and B5. The finding that the fluorescence emission spectrum of Lhca4, upon mutation of Asn-47 to His, closely resembles that of Lhca2-WT supports this conclusion. Our analysis also strongly suggests that the 730-nm emission band originates from a broad absorption around 700 nm. This transition represents the low energy contribution of an excitonic interaction, the high-energy band of which was found at 683 nm. The possibility that the 700-nm absorption contains more than a single contribution cannot be completely discarded, and this point needs to be analyzed further.

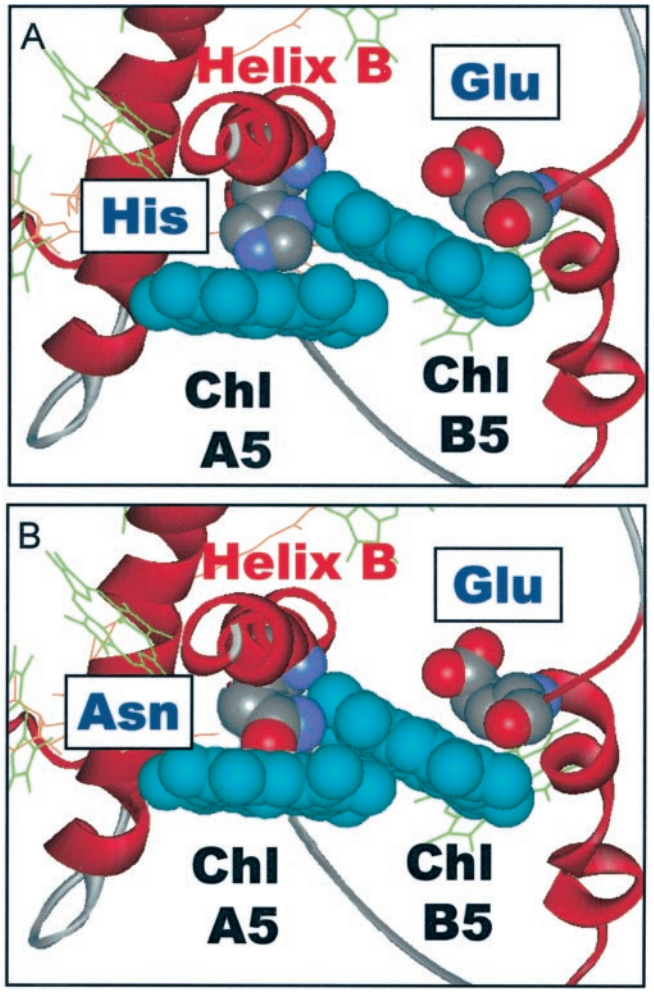

FIG. 8. **Model of Lhca3 and Lhca4 structure.** Suggested geometrical organization of the two interacting Chls responsible for the low energy absorption in Lhca3 and Lhca4. *A*, organization in the mutants in which Asn is substituted by His. *B*, organization in the WTs.

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