Chlorophyll Binding to Monomeric Light-harvesting Complex
A MUTATION ANALYSIS OF CHROMOPHORE-BINDING RESIDUES

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The chlorophyll binding properties of the higher plant light-harvesting complex II have been studied by site-directed mutagenesis of pigment-binding residues. Mutant apoproteins were overexpressed in Escherichia coli and then refolded in vitro with purified chromophores to yield holoproteins selectively affected in chlorophyll-binding sites. Biochemical and spectroscopic characterization showed a specific loss of pigments and absorption spectral forms for each mutant, thus allowing identification of the chromophores bound to most of the binding sites. On these bases a map for the occupancy of individual sites by chlorophyll a and chlorophyll b is proposed. In some cases a single mutation led to the loss of more than one chromophore indicating that four chlorophylls and one xanthophyll could be bound by pigment-pigment interactions. Differential absorption spectroscopy allowed identification of the Qy transition energy level for each chlorophyll within the complex. It is shown that not only site selectivity is largely conserved between light-harvesting complex II and CP29 but also the distribution of absorption forms among different protein domains, suggesting conservation of energy transfer pathways within the protein and outward to neighbor subunits of the photosystem.

In green plants light energy for photosynthesis is collected by an antenna system, made of many homologous proteins belonging to the Lhc multigene family (1). These pigment proteins are organized around photosynthetic reaction centers to form supramolecular complexes embedded into the thylakoid membranes. Lhc proteins bind about 70% of the pigments involved in plant photosynthesis. Understanding of energy transfer processes in the antenna and reaction centers requires recognition of the topological organization of subunits (2–4) and knowledge of three major parameters, namely: (i) the distances between chromophores; (ii) the mutual orientation of dipole transition moments; and (iii) the absorption/fluorescence energy levels. Although the elucidation of LHCII structure at 3.4 Å resolution (5) has allowed localization of chlorophyll-binding sites and of their relative distances, identification of transition dipole orientation and energy levels are precluded by insufficient resolution of the structure so far obtained or are not accessible by structural methods. Among Lhc proteins the most abundant is LHClI, which can be isolated as an heterotrimeric complex of the Lhcb1–3 gene products (6). LHCII binds 7 Chl a, 5 Chl b, and three xanthophyll molecules/mol of polypeptide (1.6–1.8 mol of lutein, 0.2–0.4 mol of violaxanthin, and 1.0 mol of neoxanthin) and is the best characterized Lhc polypeptide. (5, 7–10). Knowledge of the energy transfer factors for this protein would be a major step toward elucidation of light harvesting function. In this study we have used mutation analysis with the aim of the identification and characterization of the chromophores bound to each site; a series of mutant apoproteins was constructed by overexpression in bacteria of the Lhcb1 gene in which individual chlorophyll-binding residues (5) were substituted for by residues unable to coordinate porphyrins. Upon in vitro refolding with purified pigments, proteins missing specific chromophores were obtained in their monomeric form, which could be trimerized by addition of lipids (11). In this work we focus on the monomeric form of LHCII to avoid the effect of inter-subunit interactions on the spectral properties of individual chlorophylls that could complicate the attribution of spectral forms to individual sites. Biochemical analysis and differential absorption spectroscopy allows a proposed map for the chemical nature and the absorption properties of chlorophylls within individual sites.

EXPERIMENTAL PROCEDURES

DNA Constructions—Plasmids were constructed using standard molecular cloning procedures (12). Bacterial hosts were Escherichia coli TG1 strain (13) and SGI3009 strain (14). cDNA of lhc1 from Zea mays was a kind gift of Dr. Matsouka (15). Mutations were obtained according to the method of Yukenberg et al. (16). The sequence was determined by the dideoxy method (17) by an automated apparatus (Applied Biosystems model 377).

Isolation of Overexpressed Lhcb1 Apoprotein from Bacteria—LHCII was isolated from the SGI3009 strain transformed with the lhc1b construct following a protocol previously described (10, 18, 19).

Reconstitution of LHCII-Pigment Complexes—Purification was performed as described in Giuffra et al. (20) with the modifications reported in Croce et al. (10).

Purification of Reconstituted LHCII—Was performed by ion exchange chromatography (20). For determination of pigment to protein stoichiometry, it was necessary to obtain fully purified complexes that did not contain any residual contamination by bacterial proteins. The reconstituted LHCII was thus purified by preparative isoelectrofocusing (21) followed by ultracentrifugation in glycerol gradient (15–40% including 0.06% dodecylmaltoside and 10 mM Hepes, pH 7.6; run was for 12 h at 60,000 rpm in SW60 Beckman rotor) to eliminate amphyloses.

Protein and Pigment Concentration—The concentration of the LHCII apoprotein purified from E. coli inclusion bodies was determined by the bicinchoninic acid assay (22). For stoichiometric (pigments/protein ratio) determination, the protein concentration was determined by the ninhydrin method (23). Chlorophyll concentration was determined by the method of Porra et al. (24). HPLC analysis was as in Ref. 25. Chlorophyll to carotenoid ratio and Chl a/b ratio was independently

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measured by fitting of acetone extract spectra with the spectra of individual purified pigments as described previously (9).

**Spectroscopy**—Absorption spectra were obtained using a Shimadzu UV-2450 spectrophotometer at room temperature. Fluorescence excitation and emission spectra were obtained by using a Jasco FP-777 spectrophotometer. Samples were in 10 mM Hepes, pH 7.6, 0.06% dodecyl maltoside, 20% glycerol. Chlorophyll concentration was about 10 μg/ml for CD and absorption measurements and 0.01 μg/ml for fluorescence measurements. Analysis of spectra by Gaussian deconvolution and second-derivative analysis was performed by using the (Origin™ MicroCal, Soft. Inc.) software package.

**RESULTS**

Mutations were performed on the nine amino acid residues in the LHCII sequence that have been proposed from the structure to provide ligation of Chl molecules (5). In the case of Chl A6 ligand proposed to be the peptide carbonyl group of glycine 78, which is made available for coordination by being not involved in intra-helix H-bonds with the α-amino group of the proline residue 82, we have changed proline 82 into valine to avoid a LHCII protein binding 11 Chl rather than 12 (one Chl a/b ratio of WT LHCII differed from 1.4 by more than 0.03 were discarded. The data reported here refer to three independent reconstitution experiments for each mutant protein.

**Reconstitution and Stability of Mutant Proteins**—WT LHCII was reconstituted with a yield of 35% on a protein basis (Fig. 1). Mutant proteins were reconstituted with a similar yield with some exceptions; the A1 mutant (E180L/R70I) had a yield of 10%, suggesting that this ionic pair is important in stabilizing the structure, whereas, interestingly, mutants in the other intra-helix ion pair (A4 site, E65V/R185L) did not show a decrease in reconstitution yield. Three mutations in helix C, disrupting the intra-helix ion pair (B5.1 and B5.2) or the Chl binding (B6.1) decreased the reconstitution yield to 25, 12, and 25%, respectively. Stability of the complexes was checked by keeping the samples in ice or at room temperature and repeating absorption spectra during the following 24 h. WT and all mutants but A1 showed no shift in the wavelength of the Qy transition peak within 0.2 nm for 24 h in ice or 6 h at RT. The A1 mutant was stable for 4–6 h in ice.

**Fluorescence Emission Spectra**—The function of LHCII is the light harvesting by the three different chromophore types present in the complex and excitation energy transfer to Chl a prior to further transfer to photosynthetic reaction centers. High efficiency of energy transfer can only be obtained if the relative distances and orientation between chromophores is maintained. This can be assessed by fluorescence emission analysis using three different wavelengths of excitation (440, 475, and 500 nm) specifically absorbed by Chl a, Chl b, and xanthophylls. Emission from impaired individual or groups of chlorophyll will show up at different wavelengths. For WT LHCII and most of the mutant proteins, fluorescence emission spectra typically showed a single major peak at 681 nm. The spectra were essentially identical irrespective of excitation wavelength, implying energy transfer and equilibration between all the bound pigments (Fig. 2). This indicates that mutations did not significantly disrupt protein structure or disturb pigment-pigment interactions between chromophores not specifically affected by the mutation. Nevertheless, small differences in peak emission wavelengths indicate that the relative distribution of the Chl absorption forms was modified. Some Chl b emission at 660 nm was observed in the helix C mutants B5.1, B5.2, and B6.1, indicating that a fraction of the energy absorbed by Chl b could not be transferred to Chl a. However, even in the case of B5.1 and B6, most of energy was equilibrated as shown by the identical shape of the spectra above 670 nm. The only clear exception to this pattern was the B5.2 mutant, in which energy from carotenoid excitation at 500 nm was transferred to a different Chl a pool emitting at higher wavelengths with respect to the overall Chl a emission excited at 440 nm. This implies that energy transfer between two different Chl a groups was impaired.

The A2 mutant was equilibrated, but its emission peak was blue shifted by 4 nm, indicating the Chl affected by the mutation is probably the chromophore with the lowest energy level in the complex. Site A2 was suggested to be important for energy transfer toward neighboring antenna proteins on the basis of its protruding position in LHCII structure (5).

| LHCII mutants | A1, E180V/R70I | A2, N183V | A3, Q197V | A4, E65V/R185L | A5, H68I |
|---------------|----------------|-----------|-----------|----------------|----------|
| A6, P82V      | B3, H212V      | B5.1, E139L | B5.2, F139L/R142L | B6.1, Q131E |
| B6.2, Q131E   |               |           |           |                |          |

Each mutant is designated by the site as assigned by Kuhlbrandt and co-workers (11). The targeted amino acid residues and their substitutions are shown.
Absorption Spectra—To verify whether mutations have affected selectively different Chl absorption forms, we have analyzed WT and mutant LHCII proteins by room temperature absorption spectroscopy. The Q<sub>r</sub> region of the spectra is shown in Fig. 3 with their second derivative analysis. WT monomeric recombinant LHCII shows two broad peaks respectively at 652 and 674 nm (Fig. 3) as previously shown for the native complex purified from thylakoids (27, 28). This protein could be made into trimers by incubation with lipid extract leading to the purified from thylakoids (27, 28). This protein could be made into trimers by incubation with lipid extract leading to the changes on absorption and CD spectra previously reported to be due to trimerization in native LHCII (27, 28). Here we report on monomeric proteins.

Mutations at sites A1 and A4 affect Glu/Arg ionic pairs in the helix A/helix B cross. The spectrum of the A4 mutant shows a red shift of the major (Chl a) peak by 1.2 nm with respect to the WT and higher amplitude of the 652 nm shoulder (Fig. 3A). The second derivative analysis showed a decreased amplitude of a spectral form around 675 nm (Fig. 3A.1). Mutation at site A1 (E180L/R70I) yielded a large blue shift (4 nm) of the Chl a peak, and the ratio of the amplitudes of the Chl a versus Chl b peaks was lower with respect to the WT. Second derivative analysis clearly shows perturbation of the red-most signal at around 680 nm (Fig. 3, A and A.1). Mutations at sites A2 and A5 also affect two Chls close to the center of the LHCII structure (Fig. 1). The A2 mutant spectrum (Fig. 3B) shows a blue shift of 2 nm with respect to the WT because of the complete loss of the red most signal at around 680 nm (Fig. 3B.1). The mutation in the A5 site (H681) yields a spectrum rather similar to that of the A4 mutation showing a decrease in the ratio between the Chl a and Chl b peaks with respect to the WT, because of the loss of a spectral form absorbing at 674–675 nm (Fig. 3, B and B.1).

The Chl bound to site A6 has been proposed to be coordinated through the Gly<sup>78</sup> peptidyl-carbonyl. The absorption spectrum of the P82V mutant is identical to that of WT, suggesting that if this is the ligand of Chl in site A6, the availability of this group is not essential to Chl binding (Fig. 3, C and C.1).

Mutations B5.1, B5.2, B6.1, and B6.2 are targeted to ligands on helix C. Their absorption spectra show similar characteristics, suggesting that the chromophores affected by these mutations might be part of a common pool (Fig. 3, D and D.1). The main feature is a decrease of the Chl b peak at around 652 nm with the amplitude of the effect being in the order B5.1 (E139L) < B6.1 (Q131L) < B5.2 (E139L/R142L). Differences with respect to the WT spectrum are also evident in the Chl a region, where blue shifts of the peak, by 2, 2, and 3 nm, respectively, were observed. The second derivative analysis also shows that upon removal of bulk 652 nm absorption, a 646 nm signal is revealed in the B5.2 (E139L/R142L) mutant, suggesting that this absorption form is associated to a site located in a protein domain not affected by helix C mutations (Fig. 3D). Differences are also detected in the Chl a spectral region as changes in the relative amplitude of the different absorption forms (Fig. 3D.2). The B6.2 (Q131E) mutation is intended to substitute a Chl-binding residue with another putative one present, in the same position, in homologous proteins like CP29, CP26, or LHCI. This mutation leads to a decreased Chl b absorption (652 nm) accompanied by increased absorption at around 675 nm (Fig. 3D), suggesting that the B6 site decreases its affinity for Chl b in favor of Chl a. The spectrum is otherwise similar to WT.

The targets of B3 and A3 mutations are located in the D helix domain. The absorption spectra of both mutant proteins show a decreased Chl b (652 nm) absorption (Fig. 3E). Nevertheless, the trough between Chl a and Chl b peaks became more evident, suggesting that blue Chl a absorption forms at around 663–665 nm are depleted as indicated by the second derivative analysis (Fig. 3E). The spectral effects displayed by mutant proteins clearly indicate that mutations are specific for different absorption forms covering the whole of the LHCII spectrum. This suggests that these constructions can be used for correlating the absorption forms with specific chromophores in LHCII.

Pigment Composition—Previous work on carotenoid-binding sites of LHCII showed that maize Lhcb1 has three sites tightly binding xanthophylls in agreement with the results with LHCl from thylakoid membranes (8–10). Two of them, located in the helix A/helix B cross as detected by electron crystallography (5), can bind either lutein or violaxanthin in a 1.8 to 0.2 ratio. In this work, because of the slightly different conditions of reconstitution we obtained 1.68 ± 0.02 lutein and 0.32 ± 0.03 violaxanthin for WT LHCII. The third site, not resolved in the structure, is selective for neoxanthin. Pigment composition of WT and mutant proteins was determined by HPLC analysis and extrapolation from the absorption spectra of the acetone extracts. This combined approach proved to be effective in minimizing errors in the Chl/car ratio (9). Because the lutein-binding sites are located in the loops between α-helices (29), which are not targeted by mutations, we tentatively used lutein (1.68 mol/mole of protein) as a reference. The results are shown in Table II. The validity of the assumption of 1.68 luteins/polyepitope was verified by direct measurement of the Chl to protein stoichiometry as previously performed in the homologous protein CP29 (26). However, the amount of material and the number of repetitions needed for this determination allowed reliable results only on mutant proteins obtained with high yield (Table III). These results support the validity of the data in Table II, although a discrepancy was found in the case of the A4 mutant.

All the mutant proteins showed a lower Chl content per 1.68 luteins than the WT protein with the exception of the P82V site A7), which had the same composition as the WT, and the Q131E (site B6), which showed a higher Chl a content, suggesting the mutation increased the affinity for Chl a in site B6. The A4 mutant showed very little change in pigment composition on a lutein basis. However, strong effects on the absorption spectrum (Fig. 3B) and the Chl/protein stoichiometry of 10 (Table III) consistently indicated the loss of two Chl. We therefore recalculated the pigment composition of the A4 mutant on
the basis of 10 Chl (a+b); these values are given as (*A4) in Table II. From this new normalization it appears that this mutant lost part of its lutein, whereas the neoxanthin content was not affected. Three mutant proteins were found to bind 11 chlorophylls; the A5 mutant lost Chl a only, whereas the A3 and B3 mutants appear to lose both Chl a and Chl b, thus

Fig. 2. Fluorescence emission spectra of recombinant WT LHCII and mutants. Excitation was at 440 nm for Chl a (solid line), 475 nm for Chl b (dashed line), and 500 nm for xanthophylls (dotted line). For other conditions see “Experimental Procedures.”
leading to the tentative conclusion that site A5 binds Chl a, while B3 and A3 sites can be occupied by either Chl a or Chl b with roughly equal probability. Somewhat similar is the case of mutant A1, which appears to lose Chl a only in the amount of 1.5 mol/mole of protein. This result suggests not only that site A1 is occupied by Chl a but also that a neighbor site occupied by Chl a is affected. This is probably site B1, which is the closest one for which a specific binding residue was not detected. These results are in agreement with data obtained in the homologous protein CP29 (26). The most striking result, however, was the
Measurements.

The mutants lost close to half of their neoxanthin, whereas the mutants targeted to helix C residues. Thus B5.1 and B6.1 mutants, neoxanthin content was strongly decreased in the region is enriched in Chl b species. Although a lutein content obtained with mutations on the helix C, indicating that this specific ligand residue could not be identified in the protein might be important in the binding of chromophores for which a stabilization of the hydrophobic core of the pigment protein. The mutation analysis is now extended to LHCII, whose binding of three xanthophylls and 12 Chl molecules, although only 8–9 binding residues were identified, makes resolution of the complete chromophore map more difficult. Consistently one of the most striking effects was the loss of more than one chromophore upon removal of a single residue in several mutant proteins. This result contrasts with the case of CP29, although in most cases a single Chl was lost upon each mutation (26, 30). In the following we discuss individually the results described above for each of the mutant LHCII proteins in the attempt to determine for each site: (i) whether it binds Chl a, Chl b, or either and (ii) which is the wavelength of absorption for the bound chromophore. To these aims we calculate difference absorption spectra. The spectra were normalized to the area of absorption in the Q_y region on the basis of the number of bound Chl as determined in Tables II and III, taking into account the ratio of 0.7 in the extinction of Chl b and Chl a in this region (31, 32). The analysis of difference spectra is complicated by the loss of more than one Chl molecule. On the hypothesis that Chl bound to the nearest neighbor site lacking a specific ligand residue was the one most likely to be affected by mutations, we have used the edge to edge inter-chromophore distances calculated from the LHCII structure (5) for attribution of additional sites beside those defined by mutations. The distance values are listed in Table IV.

**TABLE II**

Pigment composition of WT and mutant LHCII proteins as determined by HPLC analysis and acetone extract fitting

| Protein          | Chl a | Chl total | Chl a | Chl b | Lutein | neo | viola | ΔChl a | ΔChl b | ΔChl  |
|------------------|-------|-----------|-------|-------|--------|-----|-------|--------|--------|-------|
| WT               | 1.4   | 12        | 7     | 5     | 1.68   | 1.05| 0.32  |        |        |       |
| A1 (E180L/R70I)  | 1.08  | 10.5      | 5.45  | 5.05  | 1.65   | 1.08| 0.07  | −1.5   | −1.5   | −1.5  |
| A2 (N183L)      | 1.44  | 9.4       | 5.54  | 3.84  | 1.68   | 0.84| 0.07  | −1.5   | −1.2   | −2.7  |
| A3 (Q197L)      | 1.45  | 11        | 6.51  | 4.49  | 1.72   | 1.72| 0.2   | −0.5   | −0.5   | −1.0  |
| A4 (E65L/R185L) | 1.51  | 11.7      | 7.07  | 6.47  | 1.68   | 1.17| 0.19  | −0.3   | −0.3   | −0.3  |
| A5 (H68I)       | 1.19  | 11        | 6     | 5     | 1.67   | 1.1  | 0.12  | −1     | −1     | −1.0  |
| A7 (P82V)       | 1.4   | 12        | 7     | 5     | 1.68   | 0.85| 0.2   |        |        |       |
| B3 (H212V)      | 1.43  | 11.7      | 6.47  | 4.53  | 1.68   | 0.83| 0.16  | −0.5   | −0.5   | −1.0  |
| B5.1 (E139L)    | 1.57  | 10        | 6.1   | 3.9   | 1.68   | 0.64| 0.1   | −0.9   | −1.1   | −2.0  |
| B5.2 (E139L/R142L) | 3.71 | 8        | 6.3   | 1.7   | 1.66   | 0.04| 0.09  | −0.7   | −3.3   | −4.0  |
| B6.1 (Q131L)    | 1.82  | 10        | 6.45  | 3.54  | 1.67   | 0.48| 0.06  | −0.5   | −1.5   | −2.0  |
| B6.2 (Q131E)    | 1.18  | 12        | 6.5   | 5.5   | 1.68   | 1.0 | 0.15  | −0.5   | +0.5   |       |

**TABLE III**

Stoichiometry of chlorophyll to protein as determined by the ninhydrin method

The measurements are the averages of 12 measurements on three preparations. Values are expressed in moles (see “Experimental Procedures”).

| Protein          | Chl/polypeptide |
|------------------|-----------------|
| WT               | 12.0 ± 0.4      |
| A2 (N183L)      | 9.5 ± 0.6       |
| A3 (Q197L)      | 11.1 ± 0.3      |
| A4 (E65L/R185L) | 9.8 ± 0.3       |
| A5 (H68I)       | 11.0 ± 0.3      |
| A7 (P82V)       | 11.5 ± 0.5      |
| B3 (H212V)      | 11.2 ± 0.4      |
| B6.2 (Q131E)    | 11.5 ± 0.2      |

loss of more than one and up to four Chl molecules in the case of five mutant proteins (B5.1, B5.2, B6.1, A2, and A4). This is evident not only from the stoichiometry on lutein or protein basis but also from the Chl a/b ratio. The expected values in the case of a single Chl a or Chl b loss are respectively of 1.2 and 1.75, whereas values up to 3.7 from the WT result of 1.4 were obtained. This indicates that pigment-pigment interactions might be important in the binding of chromophores for which a specific ligand residue could not be identified in the protein structure (5).

Chl a/b ratio higher than the WT value of 1.4 were mostly obtained with mutations on the helix C, indicating that this region is enriched in Chl b species. Although a lutein content similar to that of WT was essentially conserved in all the mutants, neoxanthin content was strongly decreased in the mutants targeted to helix C residues. Thus B5.1 and B6.1 mutants lost close to half of their neoxanthin, whereas the double mutant only bound traces of it, thus indicating that the neoxanthin-binding site is also located in this protein domain characterized by high Chl b content.

**DISCUSSION**

LHCII is the most abundant membrane protein on earth with the major function to harvest light through its bound chlorophyll and xanthophylls. In a previous work we have identified the xanthophyll-binding sites (10). In this study we have attempted the determination of one of the major parameters of energy transfer in this protein, i.e., the identification of the chromophore noncovalently bound to each of the 12 Chl sites as Chl a or Chl b and of their absorption transition characteristics, which are determined by pigment-protein and pigment-pigment interactions in each site. The approach was to first remove specific Chl ligands from the apoprotein by site-directed mutagenesis and refold the pigment-protein complex in vitro from its components. This approach was first used for CP29, a less structurally complex member of the Lhc protein family binding eight Chl and two xanthophylls (26). LHCCII, although more complex, offers a major advantage with respect to CP29 in that it appears to be more stable; double mutations on Arg/Glu ligands of sites A1 and B5 inhibited pigment binding to CP29, whereas the homologous mutants could be isolated and characterized in LHCII. This is probably due to the higher number of chromophores contributing to the stabilization of the hydrophobic core of the pigment protein. The mutation analysis is now extended to LHCII, whose binding of three xanthophylls and 12 Chl molecules, although only 8–9 binding residues were identified, makes resolution of the complete chromophore map more difficult. Consistently one of the most striking effects was the loss of more than one chromophore upon removal of a single residue in several mutant proteins. This result contrasts with the case of CP29, although in most cases a single Chl was lost upon each mutation (26, 30). In the following we discuss individually the results described above for each of the mutant LHCII proteins in the attempt to determine for each site: (i) whether it binds Chl a, Chl b, or either and (ii) which is the wavelength of absorption for the bound chromophore. To these aims we calculate difference absorption spectra. The spectra were normalized to the area of absorption in the Q_y region on the basis of the number of bound Chl, as determined in Tables II and III, taking into account the ratio of 0.7 in the extinction of Chl b and Chl a in this region (31, 32). The analysis of difference spectra is complicated by the loss of more than one Chl molecule. On the hypothesis that Chl bound to the nearest neighbor site lacking a specific ligand residue was the one most likely to be affected by mutations, we have used the edge to edge inter-chromophore distances calculated from the LHCII structure (5) for attribution of additional sites beside those defined by mutations. The distance values are listed in Table IV.

A1 (E180L/R70I)—This mutation involves the ion pair proposed to coordinate the Chl in site A1. The Chl a/b ratio in the reconstituted mutant protein is 1.08, indicating a decrease in the Chl a content respect to the WT. Although the low reconstitution yield did not allow determination of the Chl/protein stoichiometry, the carotenoid content of this mutant seems to be identical to the WT. Assuming 1.68 lutein/polypeptide, it is possible to conclude that this mutant binds 10.5 Chl molecules: 5.5 Chl a and 5 Chl b. Two Chl molecules are thus affected by the mutation in the sites A1 and, probably, B1, respectively. B1 is the nearest neighbor for which a binding residue was not identified. Both sites A1 and B1 are thus concluded to bind Chl a. The WT minus A1 difference absorption spectrum is shown in Fig. 4. A major peak at 679 nm is detected in the Chl a absorption region together with minor components around 651...
and 663 nm. The fluorescence emission spectra (Fig. 2) indicate that a fraction of Chl b is not able to transfer energy to the complex. This may be due to a Chl b (possibly B5 or B6) connected through Chls in site A1 or B1, the loss of which prevents excitation energy equilibration. It cannot be excluded that Chl b disconnection is due to a destabilized complex. However, apart from the Chl b emission, the fluorescence spectrum is very similar to WT.

Gaussian deconvolution of the difference absorption spectrum shows three bands: a major component peaking at 679 nm (79% of total absorption) and other two small bands (21%) at higher energy. At least part of the absorption at lower wavelength can be attributed to the vibrational sublevels of the main Chl a transition. The full width half-maximum of this band is 12 nm, which is in good agreement with the expected value for Chl a band in protein (33). We therefore propose that Chl in sites A1 and B1 has similar absorption wavelengths at 679 nm.

**A2 (N183L)**—The Chl/protein stoichiometry of the A2 mutant indicates that 10 Chls are still bound to the complex. Consistent with the Chl a/b ratio of 1.44, this mutant binds 6.0 Chl a and 4.0 Chl b, implying one Chl a and a Chl b site. The nearest neighbor site to A2 lacking an identified ligand residue is B2. Because site A2, which is located in the most conserved domain of Lhc proteins, was shown to be a Chl a site in CP29 (26), it is suggested that Chl b is rather bound to site B2.

The difference absorption spectrum (Fig. 5) shows a major band at 681 nm (75% of the absorption from Gaussian deconvolution). This is consistent with the fluorescence emission spectrum characterized by a blue shift of 4 nm with respect to the WT and implies the loss of the red-most Chl absorption form. Two minor bands at lower energy, peaking at 662 and 645 nm, are also detected, complicating the attribution of the energy level to the Chl b in site B2. The amplitude of the two peaks is comparable. Although only the sum of their amplitudes can account for the absorption strength of a Chl b, the explanation for such energy splitting is not straightforward. Further analysis is in progress to determine whether excitation interactions are involved (see also below for A4 mutation). We tentatively attribute both 662 and 645 nm signals to Chl B2 (Table V).

**A4 (E65V/R185L)**—The mutation on this ion pair proposed to bind Chl in the site A4 yields a Chl a/b ratio of 1.51. The Chl to apoprotein stoichiometry indicates that 10 Chl are bound to the complex: 6 Chl a and 4 Chl b, implying that Chl a and a Chl b sites are affected. The nearest neighbor site, lacking an identified ligand residue, to A4 is B2. Therefore A2 and A4 mutations consistently indicate Chl b occupancy for site B2.

The difference absorption spectrum between WT and A4 shows a quite complex pattern (Fig. 5B). Three bands can be identified by second derivative analysis (not shown) and Gaussian deconvolution peaking at 674, 660, and 643 nm. Comparison with the WT minus A2 difference spectrum suggests that the two bands at higher energy might derive from the involvement of the same (B2) chromophore allowing for the small wavelength differences possibly because of the different energy of vibrations related to the major Chl a peak. On this basis interaction between Chl in A4 and B2 can be proposed despite their dis-

**TABLE IV**

| Site | Ligand | A1  | A2  | A3  | A4  | A5  | A6  | A7  | B1  | B2  | B3  | B5  |
|------|--------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| A1   | E180/R70 | 5.7 |     |     |     |     |     |     |     |     |     |     |
| A2   | N183   |     | 19  | 10.9|     |     |     |     |     |     |     |     |
| A3   | Q197   |     |     |     |     |     |     |     |     |     |     |     |
| A4   | E65/R185 | 14.7| 13.7| 11.6|     |     |     |     |     |     |     |     |
| A5   | H68I   | 15.4| 20.0| 12.9| 6.4 |     |     |     |     |     |     |     |
| A6   | 12.2   | 12.2| 19.6| 20.7| 13.5|     |     |     |     |     |     |     |
| A7   | 17.4   | 20.7| 15.8| 16.9| 7.9 | 8.0 |     |     |     |     |     |     |
| B1   | 5.4    | 15.8| 24.5| 15.5| 13.4| 11.1| 15.3|     |     |     |     |     |
| B2   | 10.1   | 4.0 | 12.3| 10.3| 19.6| 20.9| 24.7| 20.7|     |     |     |     |
| B3   | H212   | 23.9| 14.1| 4.2 | 16.6| 21.6| 23.4| 23.6| 30.9| 13.5|     |     |
| B5   | E138   | 11.3| 18.9| 20.1| 10.1| 4.9 | 11.9| 9.7 | 8.1 | 21.6| 28.5|     |
| B6   | Q131   | 15.5| 19.9| 22.1| 19.2| 10.5| 4.0 | 4.0 | 8.3 | 25.6| 29.3| 7.4 |

**Fig. 4.** WT minus A1 difference absorption spectrum. Solid line, difference absorption spectrum; dotted line, Gaussian components; dashed line, sum of Gaussians.
This is strongly supported by the analysis of CD spectra (Fig. 5C); the 683 nm (−) signal completely disappears in both A2 and A4 mutants involving loss of Chl B2. This implies that this large red-most signal is not the sum of two or more distinct contributions and therefore is likely to derive from exciton interaction.

Direct interaction between Chls in sites A2 and A4 is unlikely because of the long distance and the presence of protein.
structures in between, the most likely origin of the 683 nm CD signal is an interaction between Chls in sites A2 and B2 in close proximity to each other. The common characteristics of A2 and A4 mutants therefore derive from the common loss of Chl B2.

A5 (H68I)—The Chl a/b ratio of this mutant protein is 1.19, which, together with its Chl content with respect to lutein, suggests the binding of six Chl a and five Chl b chromophores. This is supported by Chl/protein stoichiometry determination of 11. The site A5 is thus attributed to Chl a. In the WT minus A5 difference spectrum (Fig. 6), a major peak is observed at 675 nm, whereas minor differences are detected at higher energy (656 nm; 20% of the absorption), possibly because of a secondary red shifting of the absorption in surrounding chromophores with a negative component around 642 nm. A 674 nm absorption is thus proposed for Chl A5.

A6 (P82V)—The Chl bound to site A6 has been proposed to be coordinated through the Gly78 peptidyl-carbonyl, which is set free from H bonding by the presence of Pro82. Consistently pigment composition and spectral properties of the P82V mutant are identical to WT, suggesting that if Gly78 peptidyl-carbonyl is the ligand of Chl in site A6, the availability of this group is not essential to Chl binding.

A3 (Q197L)—The mutation on the residues Q197L is expected to affect the binding to site A3. The Chl a/b ratio of the reconstituted complex is 1.45, very similar to the WT protein. Because the Chl/apoprotein ratio is 11, it follows that 0.5 Chl a and 0.5 Chl b have been lost in the mutant protein. A3 is thus proposed to be a mixed site with similar affinity for Chl a and Chl b. This is consistent with the homologous mutation in CP29 (26).

The difference absorption spectrum shows different features in the Qy range. The second derivative analysis and the Gaussian deconvolution of the WT minus A3 difference spectrum (Fig. 7) shows the presence of four absorption bands peaking at 643, 653, 663, and 673 nm. The two more major of the four, 653 and 663, are proposed to derive from the A3 chromophore, and the others are from the disturbance of the neighbor B3 site (see also site B3, below for further discussion). The Chl a in A3 site is thus proposed to absorb 663 nm, whereas the Chl b band is located at 653 nm.

B3 (H212V)—The Chl in site B3 is the only one coordinated by a residue located in the amphipathic helix D. The mutant complex shows Chl a/b ratio of 1.43, whereas pigment composition and Chl/apoprotein ratio of 11 consistently suggest that 0.5 Chl a and 0.5 Chl b are lost in the mutant with respect to the WT. The WT minus B3 difference absorption spectrum shows a composite figure, similar to mutant A3, that can be deconvoluted in four bands similar to the difference spectrum of the mutant A3. The two major bands peaks are at 666 and 651 nm, and the minor peaks are at 643 and 673 nm. The B3 site is thus also proposed to be a mixed site with similar affinities for Chl a (maximum absorbance, 666 nm) and Chl b (maximum absorbance, 651 nm), which is consistent with previous finding with CP29 (26). Because of the presence of many bands in the difference spectra, the attribution of the energy levels to the Chl A3 and B3 has to be considered with caution. Sites A3 and B3 are among the most closely spaced in LHCII structure, which suggests that they can yield excitonic interactions to some extent, thus complicating the difference spectra.

B6.2 (Q131E)—Among Lhcb proteins, CP29 and CP26, characterized by lower Chl b content, have glutamate as Chl ligand in site B6, whereas LHCII and CP24, richer in Chl b, have glutamine in the corresponding position (2). The Q131E mutant was therefore designed to verify modulation of site affinity for Chl a versus Chl b. The a/b ratio of this complex is 1.65, whereas the Chl to protein stoichiometry indicates that the number of Chl bound to this mutant is 12, as in the WT, consistent with 0.5 Chl b molecule in LHCII WT being substituted by Chl a in the mutant. The glutamate residue is thus still binding Chl, but the site selectivity for Chl b is now reduced. The difference spectra show a positive band at 652 nm and a negative band at 676 nm (Fig. 8). This is in agreement with the biochemical data and implies the Chl b in site B6 absorbs at 652 nm. When in site B6 Chl a is thus absorbing at 676 nm, similar to the value determined in WT CP29 where B6 is a mixed site (26).

Helix C Mutants: B5.1 (E139L), B5.2 (E139L/R142L), and B6.1 (Q131L)—Biochemical data and absorption spectra (Fig. 3, D and D.1) show that the three mutants B5.1, B6.1, and B5.2 share the following features: increasingly lower Chl b content and blue shift of the Chl a peak. Identification of the absorption form associated to the missing chromophores requires normalization of the spectra on the basis of the pigment/apoprotein stoichiometry, which could only be tentatively obtained on the basis of lutein content because of the low reconstitution yield of the complex. On the basis of the data in Table III, difference absorption spectra can be obtained showing two well resolved peaks at 679 and 652 nm, respectively, indicating that both Chl a and Chl b chromophores are lost in these mutant proteins.
The protein domain in between the helix C and helix A/helix B cross contains at least six Chl-binding sites and therefore shows the highest Chl density in LHCII. However, only three of the sites (A5, B5, and B6) have a specific binding residues (considering the negative result with A6 (P82V) mutant. It can therefore be hypothesized that three specifically bound Chls form a hydrophobic shell where the remaining three porphyrins and the neoxanthin, which is also lost in helix C mutants; Table III) are inserted. In this context, the increasing penetrance of the three mutations can be explained in terms of progressive destabilization of this domain. Thus B5.1 (E139L) and B6.1 (Q131L) are likely to produce a more limited effect with respect to the double mutation B5.2 (E139L/R142L), in which not only the ionic pair end capping the C-terminal of helix C is disrupted but also the highly charged R residue is substituted by hydrophobic leucine residue. This is likely to increase the length of the lipophylic sequence, allowing tilting of helix C and disruption of the hydrophobic core. This appears to lead to loss of most of the pigments therein, but the Chl A5 firmly bound to the central cross. In this context we derive from Table II that B5.1 leads to the loss of 1 Chl b and 1 Chl a bound to sites B5 and B1, respectively, because of the previous assignment of B1 to a Chl a (see A1 mutant). In the same way the B6.1 mutation leads to the loss of 1.5 Chl b and 0.5 Chl a. In the homologous protein CP29, glutamine in the B6 site led to full Chl b occupancy. We therefore propose Chl b binding in B6 and mixed a/b occupancy for one of the neighboring sites A7 or A6, both of which lack specific ligands. In addition each of the B5.1 and B6.1 mutations decreases by approximately half its neoxanthin binding. The B5.2 double mutation leads to the complete loss of neoxanthin and at least four Chl, suggesting that it adds up the effects of B5.1 and B6.1 mutations. This hypothesis leads to a hypothetical Chl a/b ratio of 2.2 for the B5.2 mutant protein versus an experimental value of 3.7, which can be matched by assuming loss of an additional Chl b from either A6 or A7 sites. From this working hypothesis (B5.1, 6.1 Chl and 3.9 Chl b; B6.1, 6.5 Chl a and 3.5 Chl b; B5.2, 5.5 Chl a and 1.5 Chl b) we can normalize the absorption spectra and calculate the differences shown in Fig. 9 to derive absorption forms associated to Chl in the helix C domain. The constancy of the wavelength on the two peaks at 652 and 679 nm in the three difference spectra strongly suggests that in this domain Chl molecules are tuned to similar wavelengths by the common environment for Chl b and Chl a, respectively. The conclusions from above discussion are summarized in Table V. This work could not be carried out in the absence of the structural model of LHCII (5), which proposed distribution of Chl a and Chl b chromophores in the complex based on their distance from central two xanthophylls acting in quenching "Chl a". The present study essentially supported the original assignment but for site B1, which we rather attribute to Chl a, and for sites A3, B3, and A6 for which we propose low selectivity,
thus binding either Chl a or Chl b. Modelling energy transfer in LHCII (34) could fit the spectroscopic properties of LHCII by locating Chl b in sites A1 and A2 and Chl a in sites B1 and B2. Although we support the occupancy of B1 site by Chl a, our data otherwise confirm the original assignments.

A Common Organization for Lhc Chromophores—Lhc proteins have their highest degree of homology in the helix A/helix B domain, whereas helix C is more divergent. This is reflected in chromophore distribution. Our best model for the LHCII chromophore map, when compared with that of CP29 (26), shows that the six chromophores directly bound to the helix A/helix B domain and to helix D (sites A1 to A5 and B3) have the same occupancy in the two proteins, whereas helix C domain appears to be characterized by its binding of an higher number of chromophores and by the higher affinity for Chl b of individual sites. This was firmly assessed in the case of site B6, for which full selectivity for Chl b is determined by the use of Gln ligand rather than Glu as in CP29. The ability of binding either Chl a or Chl b could in fact be reproduced by substitution of Gln with Glu. Similar increased selectivity for Chl b is also attributed to site B5 (a mixed site in CP29), but in this case no ligand substitution is observed by sequence comparison (26).

The protein domain including helix C and the space facing the helix A/helix B cross appears to be densely packed with Chl and xanthophyll molecules, several of which (A6, A7, B1, and neoxanthin) do not appear to be bound by specific amino acid residues but rather held in place by pigment-pigment interactions as judged from the loss of more than one chromophore upon mutation of a single residue. This is the source of some uncertainty in our attribution, particularly of sites A6 and A7 binding together 1.5 Chl a and 0.5 Chl b per polypeptide. Which of these sites is the most selective for Chl a is still open and currently being investigated. This difficulty is also reflected by the 6.5 Chl a and 5.5 Chl b yielded by summing up the attri-
bution for the different sites versus 7 Chl a and 5 Chl b bound to the WT Lhcb1. Whether this is due to some inaccuracy in the measurements or by pleiotropic effects of the mutations is not clear; however, it is quite possible that one site (possibly A6 or A7) has higher selectivity for Chl b than proposed in Table V. Nevertheless, we believe that the present map represents a good model for further analysis. Moreover, because of the very similar spectral properties of the respective Chl a and the Chl b chromophores in the helix C domain, this should not make a big difference in the energy transfer properties of the complex.

The above results suggest that a dependence of occupancy of at least one site in the helix C domain by Chl b appears to be necessary for assembly of neoxanthin consistently with earlier work with pigment-deficient mutants (35).

Chlorophyll Absorption Forms—Chl a and Chl b differ for the energy level of their Q_y transitions; moreover, these can be necessary for assembly of neoxanthin consistently with earlier work with pigment-deficient mutants (35).

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