Functional architecture of photosynthetic light harvesting complexes
Mozzo, Milena

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The absorption properties of chlorophylls are modulated by the local environment. Chlorophylls \( a \) and \( b \), the cofactors of antenna complexes from green plants, are mainly coordinated by aminoacidic ligand located in the proteic scaffold. Site direct mutagenesis on photosynthetic apoproteins is used as a tool for modifying the affinity binding of the cofactors to the complex, thus perturbing pigment-pigment and pigment-protein interactions and/or the three-dimensional conformation of the complexes. Several aspects are investigated in this section using the mutagenesis tool:

- Identification of the energy levels of coordinated chlorophylls
- Probing the structure of antenna complexes
- Identifying fingerprints domains
Chapter 2

PIGMENT-PIGMENT INTERACTIONS IN LHCA4 ANTENNA COMPLEX OF HIGHER PLANTS PHOTOSYSTEM I*

ABSTRACT

The red-most fluorescence emission of Photosystem I (733 nm at 4K) is associated to the Lhca4 subunit of the antenna complex. It has been proposed that this unique spectral feature originates from the low energy absorption band of an excitonic interaction involving Chlorophyll A5 and a second Chlorophyll a molecule, probably B5 (Morosinotto et al., JBC 2003). Due to the short distances between chromophores in Lhc proteins, the possibility that other pigments are involved in the red-shifted spectral forms could not be ruled out. In this study, we have analyzed the pigment-pigment interactions between nearest neighboring chromophores in Lhca4. This was done by deleting individual Chlorophyll binding sites by mutagenesis, and analyzing the changes in the spectroscopic properties of recombinant proteins refolded in vitro. The red-shifted (733 nm) fluorescence peak, the major target of this analysis, was lost upon mutations affecting sites A4, A5 and B5 and was modified by mutating site B6. In agreement with the shorter distance between Chlorophylls A5 and B5 (7.9 Å) vs. A4 and A5 (12.2 Å) in Lhca4 (Ben-Shem et al., Nature 2003), we conclude that the low energy spectral form originates from an interaction involving pigments in sites A5 and B5. Mutation at site B6, although inducing a 15 nm blue-shift of the emission peak, maintains the red-shifted emission. This implies that chromophores responsible for the interaction are conserved and suggests a modification in the pigment organization. Besides the A5-B5 pair, evidence for additional pigment-pigment interactions between Chlorophylls in sites B3-A3 and B6-A6 was obtained. However, these features do not affect the red-most spectral form responsible for the 733 nm fluorescence emission band.

* This chapter is based on the article: T. Morosinotto, M. Mozzo, R. Bassi and R. Croce, published in Journal of Biological Chemistry (2005), 280, 20612-10619
INTRODUCTION

The low-temperature fluorescence emission spectrum of leaves shows two major components, peaking at 685 nm and 735 nm. Fractionation of the thylakoid membranes showed that the former emission is associated to Photosystem II, located in the grana stacks and the latter to Photosystem I, in stroma membrane domains. In each Photosystem, light is absorbed by antenna Chls and efficiently transferred to the reaction center, where charge separation takes place. In Photosystem II, the reaction center absorbs around 680 nm and it is isoenergetic with its antenna (1). In Photosystem I, instead, a significant Chls pool, responsible for the 735 nm emission, absorbs at wavelengths longer than 700 nm, the absorption maximum of the primary donor. This causes most of the energy to be stored at energies lower than that of the reaction center, implying up-hill energy transfer prior to charge separation (2).

The presence of Chls absorbing at energy lower than the primary donor is the fingerprinting of Photosystem I in all organisms (3). In higher plants, however, Photosystem I is enriched in low-energy forms, which extend the absorption into the far-red region of the spectrum, providing an advantage for light absorption of shaded leaves in dense canopies (4). These spectral forms are concentrated in the LHCl complex (5), which is composed of 4 subunits, organized in a half-moon shaped structure, located on one side of the PSI-core complex (6). The four Lhca complexes, namely Lhca1 to 4, belong to the Lhc multigenic family, which contains also all the antenna proteins of Photosystem II (7). Lhca proteins have molecular weights between 21 and 24 kDa, they coordinate Chl \( \alpha \), Chl \( \beta \), lutein, violaxanthin and small amounts of \( \beta \)-carotene and they are found in dimeric form upon purification in mild conditions.

From X-ray structural analysis, 13-14 Chls molecules have been found to be coordinated to each Lhca subunit (6); nevertheless, measurements on purified complexes showed that only 10-11 Chls are bound to the apoprotein upon purification or reconstitution (8). All LHCl components have red-shifted emission forms, albeit at different energies: Lhca1 and Lhca2 emit at 701 nm, while Lhca3 emits at 725 nm and Lhca4 at 733 nm, as revealed from the analysis of recombinant proteins (8-11). It has been shown that an excitonic interaction between Chl \( \alpha \) molecules is responsible for the large shift in the absorption (12) and that this is associated with the presence of an asparagine residue as a ligand for Chl A5 (12) (Chl binding sites nomenclature from (13), suggesting that this Chl is directly involved in the interaction. One of the major requirements for strong pigment-pigment coupling is a short distance between the interacting chromophores.
In this work, we have used the recent structural data of the Lhca4 protein (6) as a guidance for mutation analysis of residues coordinating chlorophylls in the Lhca4 complex. The analysis of these mutants provides information on the presence of pigment-pigment interactions within this PSI subunit, which hosts the red-most shifted spectral forms, and provides evidence for the Chl \( a \) molecule bound to site B5 being the interacting partner of Chl\( A5 \).

**EXPERIMENTAL PROCEDURES**

**SAMPLE PREPARATION**

cDNAs of Lhca4 from *Arabidopsis thaliana* (8) were mutated with the QuickChange© Site directed Mutagenesis Kit by Stratagene©. WT and mutants apoproteins were isolated from the SG13009 strain of *E. coli* transformed with constructs following a protocol described previously (14;15). Reconstitution and purification of protein-pigment complexes were performed as described in (16) with the following modifications: the 1.1 ml reconstitution mixture contained 420 \( \mu \)g of apoprotein, 240 \( \mu \)g of chlorophylls and 60 \( \mu \)g of carotenoids. The Chl \( a/b \) ratio of the pigment mixture was 4.0. The pigments used were purified from spinach thylakoids.

**PROTEIN AND PIGMENT CONCENTRATION**

HPLC analysis was performed as in (17). Chlorophyll to carotenoid ratio and Chl \( a/b \) ratio were measured independently by fitting the spectrum of acetone extracts with the spectra of individual purified pigments (18).

**SPECTROSCOPY**

The absorption spectra at RT and 77K (LT) were recorded using a SLM-Aminco DK2000 spectrophotometer, in 10 mM Hepes pH 7.5, 20% (w/v) glycerol (70% at LT) and 0.06% \( \beta \)-DM. Wavelength sampling step was 0.4 nm, scan rate 100 nm/min, optical pathlength 1 cm. Fluorescence emission spectra were measured using a Jasco FP-777 spectrofluorimeter and were 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 about 0.02 \( \mu \)g/ml in 60% glycerol and 0.03% \( \beta \)-DM.
The CD spectra were measured at 10°C on a Jasco 600 spectropolarimeter. Wavelength sampling step was 0.5 nm, scan rate 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 were normalized to the polypeptide concentration based on the Chl binding stoichiometry.

RESULTS

Mutation analysis was performed on Chl binding residues of Lhca4 complex. The putative binding ligands of Chls A3, A4, A5, B3, B5 and B6 (nomenclature from (13)) were substituted with residues that could not coordinate the central Mg of the Chls. All mutations are indicated in Table I, where the correspondence with Chl binding residues nomenclature in (6) is also reported. After expression in bacteria and in vitro refolding with purified pigments, the reconstituted complexes were purified by sucrose gradient ultracentrifugation and anionic exchange chromatography. All mutants yielded stable reconstituted monomeric complexes, as shown from their mobility in glycerol gradient (data not shown). The only exception was mutant A3: in this case, no stable pigment-protein complex was obtained. This mutation most probably affects the stability of site L1, as was previously shown for the homologous protein Lhca1 (19), thus preventing the correct folding of the complex (20). The pigment composition of each reconstituted complex is reported in Table I.

Recombinant WT Lhca4 bound Chl a and b in a ratio of 2.5 plus lutein and violaxanthin in a ratio of 5.0, in agreement with previous results (8). On the basis of the differences in Chl a/b ratio, it may be suggested that mutants A4 and B3 lose preferentially Chl a as compared to the WT, while mutants A5, B5 and B6 lose preferentially Chl b. Mutant B3 showed an increased xanthophyll content with respect to Chl a+b, but the ratio between lutein and violaxanthin was unchanged as compared to the WT. In all other mutants the Chl/Car ratio was very similar to the WT, but they all showed higher lutein/violaxanthin ratio, indicating a partial loss of violaxanthin.
Table I. Pigment composition of WT and mutant Lhca4 complex.

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<tr>
<th>Sample</th>
<th>Mutations</th>
<th>Chl a/b</th>
<th>Chl a</th>
<th>Chl b</th>
<th>Viola</th>
<th>Lut</th>
<th>Lut/Viola</th>
<th>Chl/Car</th>
<th>Binding site from (6:13)</th>
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<td>Lhca4-WT</td>
<td>-</td>
<td>2.5±0.1</td>
<td>71.9±1.6</td>
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<td>Lhca4-A4</td>
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<td>5.45</td>
<td>5.9±0.5</td>
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</table>

The pigment analysis of Lhca4 WT and all mutants is reported. Values are normalized to 100 Chl $a+b$ molecules.

**EFFECT OF MUTATIONS ON THE FLUORESCENCE EMISSION SPECTRA**

The effect of each mutation on the red forms of Lhca4 was assessed from the analysis of the fluorescence emission spectra of the complexes, measured at 77K, as shown in Figure 1. The emission spectrum of the B3 mutant is identical to that of WT, implying that this chlorophyll is not involved in the red-most emission.

On the contrary, complete depletion of the red forms is observed in mutants A5 and B5. Mutant A4 also loses the 733 nm emission, but retains emission around 700 nm, detectable from the asymmetric broadening of the low-energy side of the 685 nm peak. Finally, in mutant B6 red forms are still present, but the peak is blue-shifted by 15 nm as compared to WT.
**Figure 1.** Fluorescence emission spectra of Lhca4 WT and mutants at 77K. Spectra were recorded upon excitation at 500 nm and 475 nm and normalized to the maximum of the emission. (A) A5 (dashed line), B5 (dotted line), B6 (dash-dotted line) and WT (solid line); (B) A4 (dashed line), B3 (dotted line) and WT (solid line)

**ABSORPTION AND CIRCULAR DICHROISM SPECTRA**

In order to get information on the spectral characteristics of the Chls affected by the mutations, the absorption spectra of WT and mutant complexes were measured at 77K (Figure 2). Three major features were visible in the WT spectrum: the peak at 645 nm due to Chl b, the bulk Chl a absorption with maximum at 674 nm and the red tail at wavelengths >700 nm. Consistent with pigment analysis, the spectra of mutants A5, B5 and B6 showed a strong decrease of the absorption in the Chl b region as compared to WT (Figure 2A). Mutants A4 and B3, instead, did not show significant differences in this region (Figure 2B). The bulk Chl a peak was essentially conserved in the spectra of all mutants, with limited blue-shifts (around 2 nm) in mutants A4, A5 and B5. On the contrary, the absorption component at >700 nm was affected in all samples but B3 mutant, which was identical to the WT in this spectral region. Mutations at sites A5, A4 and B5, e.g., completely abolished this spectral feature. In the B6 mutant the absorption above 700 nm, although present, was clearly shifted to shorter wavelengths, consistent with the observed blue shift in fluorescence emission from 733 to 718 nm (Figure 2C).
In order to detect pigment-pigment interactions, the CD spectra of all complexes were measured and they are shown in Figure 3. The negative signal in the Chl $b$ absorption region (630-655 nm) was strongly affected in mutants B5 and B6, where the signal was respectively missing or strongly reduced in amplitude. Mutants B3 and A4 did not show detectable differences in this region, in agreement with pigment composition and absorption spectra. In this respect, mutant A5 was an exception since, despite losing Chl $b$, it conserved a Chl $b$ CD signal identical to WT, indicating that the Chl $b$ lost upon mutation at this site is probably not involved in interactions with other chromophores or that the interaction is CD silent.

In the Chl $a$ region, the main negative band was shifted to shorter wavelengths in mutants A5 and A4. This shift has been reported to be associated to the loss of the pigment-pigment interaction responsible of the red forms (12). The main difference of mutant B6 as compared to WT was detected in the 670-675 nm range, where the spectrum of the mutant showed negative component, opposite to the WT. The CD spectrum of the B3 mutant was identical to the WT but for the absence of a positive contribution at 660 nm. The CD spectrum of B5 mutant was completely different from the spectrum of the WT thus suggesting that the Chl organization in this complex is strongly affected.
Figure 2. Absorption spectra (77K) of WT and mutant Lhca4 complexes. The spectra were normalized to the same area in the 620-740 nm spectral region. (A) A5 (dashed line), B5 (dotted line), B6 (dash-dotted line) and WT (solid line); (B) A4 (dashed line), B3 (dotted line) and WT (solid line). C) comparison of red absorption of Lhca4 WT (solid line) and mutants A5 (dashed line) and B6 (dash-dotted line).
DISCUSSION

It has previously been suggested that the red absorption typical for Lhca complexes represents the low-energy band of an excitonic interaction having the high-energy term at 683 nm (12). Site-selected fluorescence measurements indicate that the red-most absorption band of Lhca4 peaks at 708 nm at 4K (21), thus yielding a value of 260 cm$^{-1}$ for the interaction energy. On this basis, the calculation of the distance between the interacting Chls, assuming the best possible geometric arrangement, yields to a value of 8.8 Å or below. From the structure of Lhca4 (6), four pairs of Chls can accomplish this requirement: A5-B5, whose center to center distance is 7.9 Å, A3-B3 (8.13 Å), B6-A6 (8.35 Å) and A4-B4 (1031) (8.33 Å) (see Table II, listing the center-center distance of all Chls as calculated from the structure of (6)). The analysis of mutant proteins described here is meant to reveal which of these chromophores are responsible for the red forms.
Table II. Distance between Lhca4 Chls. Center to center distances (Å) were calculated from the structure (6). The value below 8.8 Å are in bold.

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<th>A3</th>
<th>A4</th>
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**CHANGES IN PIGMENT COMPOSITION INDUCED BY MUTATIONS**

Recombinant monomeric Lhca4 binds 10±1 Chls with a \(a/b\) ratio of 2.5 and two xanthophyll molecules: lutein in site L1 and both lutein and violaxanthin in site L2 (8). Ideally, mutation at each Chl binding site would yield loss of only one Chl molecule. However, previous analysis of several Lhc complexes showed that, besides the target Chl, additional chromophores might be lost, in particular chlorophylls and xanthophylls bound to neighboring sites, due to the highly cooperative nature of pigment binding in this protein family. The occupancy of Chl binding site A1 and xanthophyll binding site L1, respectively hosting Chl \(a\) and lutein, were shown to be essential for protein folding in all Lhc proteins analyzed so far. In fact, mutants affected in these sites in most cases were unable to fold *in vitro* (19;22;23).

Lhca4 mutants A4 and B3 exhibit a lower Chl \(a/b\) ratio as compared to the WT, suggesting loss of Chl \(a\) molecules. B3 mutant shows lower Chl/Car ratio as compared to the WT, but the same values of lutein/violaxanthin ratio, indication that there is no loss of carotenoids. By normalizing to the Car content, it can thus be concluded that this mutation affects the binding of one Chl \(a\) molecule. In the case of A4 mutant, the Chl/Car ratio is identical to the WT, but the amount of violaxanthin is decreased, indicating a partial loss of carotenoids. No changes
in the Chl $b$ region were observed in the absorption and CD spectra, suggesting that no Chl $b$ is lost upon mutation at site A4. Normalization to the Chl $b$ content indicates that this mutation affects the binding of one Chl $a$ molecule and partially destabilizes one carotenoid binding site. It is most likely that the site affected is L2, which is located in the proximity of Chl A4 and which accommodates violaxanthin and lutein. The remaining three mutants, A5, B5 and B6 had a Chl $a/b$ ratio higher than the WT, suggesting preferential loss of Chl $b$. In the case of mutants A5 and B5 the Chl/Car ratio is similar to the WT, but the lutein/violaxanthin ratio is higher, again suggesting loss of carotenoids from site L2, consistent with the results of the mutational analysis in Lhca1, Lhca2 and Lhca3 (19).

Table III: Pigment content of Lhca4 mutated complexes.

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<th>Sample</th>
<th>Mutation</th>
<th>$\Delta$Chl $a$</th>
<th>$\Delta$Chl $b$</th>
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<th>$\Delta$Lutein</th>
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<td>A5 –11015</td>
</tr>
<tr>
<td>Lhca4-B3</td>
<td>H185F</td>
<td>-1</td>
<td>-1</td>
<td>-0.1</td>
<td>-0.3</td>
<td>B3 –11023</td>
</tr>
<tr>
<td>Lhca4-B5</td>
<td>E102V/R105L</td>
<td>-2</td>
<td>-2</td>
<td>-0.2</td>
<td>-0.6</td>
<td>B5 –11025</td>
</tr>
<tr>
<td>Lhca4-B6</td>
<td>E94V</td>
<td>-1</td>
<td>-1</td>
<td>-0.1</td>
<td>-0.3</td>
<td>B6 –11026</td>
</tr>
</tbody>
</table>

Values are expressed as a difference with respect to the pigment composition of the WT complex.

Moreover, normalization to the Car content would give a Chl $a$ content higher than in the WT for both complexes, contrasting with the loss of Chl $a$ absorption forms observed in the spectra. On this basis, we suggest that mutation at site A5 actually induces the loss of one Chl $a$ and one Chl $b$ with a concomitant partial loss of the xanthophyll in site L2, thus accounting for the change in Chl $a/b$ and in lutein/violaxanthin ratio. The effect of the mutation at site B5 is stronger, as suggested by the large changes in the CD spectrum and by the strong reduction of the thermal stability of the complex (60% of WT, data not shown). Furthermore, the lutein/violaxanthin and Chl $a/b$ values are strongly increased with respect to the WT. In order to explain these data, we suggest that this mutation leads to the loss of four Chls (2 Chl $a$ and 2 Chl $b$) and of the carotenoid in site L2. The mutant at site B6 shows a higher Chl/Car ratio and a slightly higher lutein/violaxanthin ratio as compared to WT, indicating xanthophyll loss, consistent with the phenotype of the same mutants in other Lhc complexes (22-24). From these data we suggest that mutant B6 loses one Chl $b$ molecule and has reduced occupancy of the L2 carotenoid binding site. Table III summarizes the pigment binding properties of WT and mutants.
PROPERTIES OF INDIVIDUAL CHROMOPHORES IN LHCA4

The fluorescence emission spectra of the complexes show that the Chl in site B3 is not involved in the interaction leading to the red forms, the emission spectrum of this mutant at low temperature being identical to that of the WT. All other mutants are instead affected, although to a different extent, on their red emission component. In the following, we discuss the properties of the individual Lhca4 chromophores and the effects of the mutation of the corresponding binding sites.

MUTANT B3

The H185F mutant loses one Chl $a$ molecule thus suggesting that a Chl $a$ is bound to site B3 as was the case in the homologous Lhca1 complex (19).

In the CD spectrum the main difference with respect to WT is the disappearance of the 660 nm (+) signal. This feature suggests that Chl $a$ in site B3 interacts with a neighboring pigment. Unfortunately, no clear changes can be observed in other spectral regions: possibly the expected negative component of the interaction is hidden by other signals. Since the nearest neighbor of Chl B3 is Chl A3 (8.13Å), the most likely hypothesis is that the positive CD band at 660 nm is the signature of an interaction between Chls A3 and B3.

The absorption difference spectrum shows three positive bands with maxima at 660 nm, 672 nm and 680 nm (Figure 4). The first band corresponds to the signal lost in the CD spectrum and likely represents the high-energy term of the Chl-Chl interaction. The remaining two peaks are possibly a combination of the low-energy band of the interaction, which is lost in the mutant, and of the absorption of the now monomeric Chl A3, still present in the complex.

The absence of negative components in the spectrum can be explained by proposing that the redistribution of the oscillator strength between the two interacting Chls favours the low energy band and that the shift induced by the interaction is small. In this case, the expected negative contribution in the difference absorption spectrum, deriving from the now monomeric Chl A3 can be hidden by the positive signal of the low energy band of the dimers. This suggestion is supported by the observation that the amplitude of the 660 nm absorption band accounts for less than one Chl molecule. Moreover, the Gaussian deconvolution of the difference spectrum (data not shown) suggests that the low-energy absorption form peaks at 677 nm, while the monomer absorption is blue shifted by only 1-2 nm. This can in fact be expected for an interaction between pigments with very different site energies, as it is the case. Furthermore, a very similar situation was previously described for mutants A3 and B3 of Lhca1 (19), where the picture was clearer thanks to the possibility of analyzing both mutants. Although in the case of Lhca4 the impossibility of reconstituting mutant A3 does not allow obtaining further details, the data are consistent with the hypothesis that the two
interacting monomers are not isoenergetic and that the Chl monomer in site A3 absorbs at lower energy than B3. Moreover, the data clearly indicate that Chl B3 is not involved in the red emission of Lhca4 complex. The small difference above 700 nm represents less than 6% of the absorption of the WT in this region and it is not significant as shown by the fact that the fluorescence emission is identical to the one of the WT.

Figure 4. Difference absorption spectrum between Lhca4 WT and Lhca4-B3 mutant. The area of each absorption spectrum was normalized to the Chl content (Table III) prior to subtraction.
Mutant E94V loses one Chl b molecule, suggesting that a Chl b is accommodated in site B6. The fluorescence emission spectrum of the mutant is 15 nm blue-shifted as compared to WT, implying a change in the environment of the “red” pigments. Although a loss of Chl a molecules was not detected in the complex, the absorption spectrum of the mutant differs from the absorption spectrum of the WT in the Qy Chl a absorption region (Figure 5A). A decrease in intensity above 700 nm (max 712 nm) is observed in the absorption, together with a gain around 693-694 nm. This difference explains the fluorescence shift: the red-most absorption in the mutant is blue-shifted and this new spectral form is responsible for the 718 nm emission.

To get information on the new absorption band present in the mutant, the absorption spectrum at low temperature of the Lhca4-B6 complex was described in terms of Gaussian components (Figure 5B). In order to fit the spectrum, a wide band peaking at around 694 nm is required (FWHM: 26 nm), while in WT the red most band was detected at 708 nm⁻¹. However, the 694 band in the B6 mutant has width similar to the absorption band of the red pigments, suggesting a similar origin. This implies that the chromophores involved in the interaction are still in place, although somehow disturbed in their organization. In addition, the results clearly imply that the Chl located in site B6 is not directly responsible for the low-energy emission, while its role seems to be important in maintaining the right geometry between the interacting Chls, as it was previously suggested in the case of Lhca1 (19).

The Chl b in site B6 absorbs at 642/483 nm as can be judged by the absorption difference spectrum (Figure 5A). Loss of (-) amplitude in the CD spectrum at the same wavelengths (Figure 3E) suggests that this Chl is involved in pigment-pigment interactions. The positive term of the interaction is not present in the Chl b region of the CD spectrum, thus suggesting that it should be searched for in the Chl a region, where, in fact, loss of a positive 672 nm signal can be detected. Although changes of different origin in this region cannot be excluded, due to the effect on the interaction leading to the red-most form, it is likely that the 672 nm component represents the signal of the second term of the interaction involving Chl B6. The structure of Lhca4 shows that the nearest neighbor of Chl B6 is Chl A6 (6). We thus suggest that the A6 site accommodates a Chl a molecule in Lhca4 and that this Chl interacts with Chl B6. The oscillator strength associated to the 642 nm absorption band in the WT minus B6 difference spectrum corresponds to slightly less than one Chl. This implies that the energy distribution slightly favours the low-energy band of the interaction. In the difference spectrum, a small negative signal is observed around 668 nm and a positive one at 673 nm (Figure 5A). These two signals possibly represent a combination of the loss of absorption of the dimer and the gain of absorption of monomeric Chl A6, which is still present in the mutated B6 complex.
In conclusion, it appears clear that Chl B6 is not directly responsible for the low-energy spectral form in Lhca4. The Chl coordinated to this site absorbs at 642 nm and seems to interact with Chl A6. We propose that Chl B6 plays a role in keeping the conformation of the protein which leads to the red forms, as previously suggested for Lhca1 (19). This effect is possibly mediated by the xanthophyll molecule in site L2, which is partially lost in the mutant. Chl B6 has been previously shown to be a ligand for the xanthophyll molecule in site L2 (22).

![Figure 5. Spectral analysis of Lhc4-B6 mutant. (A) Difference absorption spectrum between Lhca4-WT and Lhca4-B6 mutant. Before subtraction, the spectra were normalized at the Chl content. (B) Gaussian deconvolution of the absorption spectrum at 77K of Lhca4-B6 mutant.](image)

**MUTANT A4**

Chl A4 is co-ordinated by an Arg/Glu ionic pair. The E44V/R158L mutant loses one Chl \( \alpha \) molecule, thus suggesting that site A4 accommodates Chl \( \alpha \), as in all Lhc complexes analyzed so far (19;22;23;25). The fluorescence emission spectrum of the A4 mutant complex does not show the 733 nm peak, but it still conserves emission around 700 nm (see in Figure 1 the
comparison with A5 and B5 mutants). The loss of red forms upon mutation at the A4 site may have at least two different origins:

- Chl A4 is directly involved in the red absorption and the mutation has a direct effect on the low-energy form.
- Mutation of this site changes the conformation of the protein, thus having an indirect effect on the red forms.

As for the first hypothesis, three mutants show loss of the red emission in Lhca4: A4, A5 and B5. The substitution of the natural ligand for Chl A5 (Asn) with an His clearly indicates the direct involvement of Chl A5 in the low energy forms (12). In the structural model of the protein, the distance between Chl A4 and Chl A5 is too large (12.2 Å) in order to allow for the strong interaction responsible for the red form. Alternatively, the red form could originate from interactions involving all three Chls, namely A4, A5 and B5. However, in this case, mutations at sites A4 and B5 would have left at least part of the interaction in the mutants and thus part of the red-shifted emission, while this is not the case.

As for the second hypothesis, we should consider that mutation of site A4 affects the ionic bridge between E44 and R158, which stabilizes the structure of all Lhc proteins (13). The absence of the ionic pair is likely to have an effect on the packing of transmembrane helices and thus on the mutual position of the Chls coordinated to these helices. This hypothesis is consistent with the fact that the A4 mutant complex is obtained with a very low yield. Moreover, this mutation affects the xanthophyll bound to site L2, whose presence is relevant for red forms, as shown here by the B6 mutants and in previous mutation analysis. Based on these considerations, we propose that the almost complete lack of red emission in the A4 mutant is rather due to a different conformation assumed by the mutant complex than to a direct involvement of Chl A4 in the interaction yielding the red forms in WT Lhca4.

**MUTANT A5**

The mutation N47F induces the loss of two Chl molecules, one Chl \( \text{a} \) and one Chl \( \text{b} \). Site A5 has been shown to coordinate a Chl \( \text{a} \) molecule in all Lhc complexes analyzed so far (19;22;23). We thus propose that site A5 coordinates a Chl \( \text{a} \) molecule also in Lhca4, while Chl \( \text{b} \) is coordinated to a neighbor site. Although the nearest neighbor of Chl A5 is Chl B5, it is unlikely that the second Chl is lost from this site. The CD spectrum clearly shows no changes in the Chl \( \text{b} \) region upon mutation of site A5, while large changes are observed upon mutation at site B5 (see below). Unfortunately, it is presently impossible to make even a polite guess about the location of the second Chl lost upon mutation of site A5, and we prefer to leave this question open. The Lhca4-A5 complex does not show the 733 nm emission
typical for Lhca4-WT, suggesting that the A5 site is involved in the interaction leading to the red form, in agreement with previous experiments where the substitution of the Asn ligand of Chl A5 with an His (Lhca4-NH mutant) led to loss of the low-energy absorption (12).

The absorption difference spectrum WT-A5 mutant is reported in Figure 6. In the red region of the Qy Chl a absorption, at least two forms can be detected, respectively at 683 nm and above 700 nm. The same two features have previously been observed in the difference spectrum between Lhca4 WT and the mutant N47H which maintains Chl a binding to site A5 but loses the interaction leading to red-forms (12). These spectral features are thus the “markers” of the lost interaction yielding to the red forms. A negative contribution in the absorption spectrum is detected at around 670 nm. This component probably represents, at least in part, the absorption of the monomer of the Chl which in the WT interacts with Chl A5 and which is still present in the mutant complex. However, considering the low stability of the complex, this signal is probably the sum of several contributions including the absorption of partially disconnected Chls, which are present in the sample, as judged by the fluorescence emission spectrum. In fact, the analysis of the NH mutant, which has the same stability as the WT, showed the contribution of monomeric Chls at 676 nm. It should be noted that the fact that the average energy for the two excitonic levels is red shifted with respect to the absorption of the monomeric pigments is due to a change in the transition energy of the interacting pigments, an effect known as displacement energy (26).

In the Chl b region, the absorption difference spectrum shows bands at 645/473 nm (468/470 nm at RT), which can be associated with the Chl b lost in this sample. The CD spectrum in the Chl b region is identical to that of WT, indication that this Chl does not interact with other pigments or the interaction is CD silent. Whatever the reason for the absence of the CD signal is, it is clear that this Chl b does not participate in the interaction leading to the red forms, because the red-most component have a clearly negative CD signal (12). In addition, this Chl b form peaks at 645 nm, a wavelength typical for monomeric Chl b in a protein environment.

**MUTANT B5**

Mutations E102V/R105L likely yielded the loss of four Chl molecules: 2 Chls a and 2 Chls b, indicating that the substitution of the ER bridge on the C-helix strongly affects the complex as previously shown for Lhcb1 (23).

The fluorescence emission spectrum shows that this mutation completely abolishes the red emission forms, thus suggesting that the Chls lost upon mutation of this site are involved in the low-energy absorption. In the absorption spectra, the lack of the red-most band and of the 683 nm component is also clear (Figure 6). However, the amplitude of the main peak in the absorption difference spectrum is higher than in the case of the A5 mutant and blue-shifted by 2 nm, suggesting the loss of a second Chl a form (Figure 6). From the A5 minus B5
difference spectrum, it can be observed that the additional Chl $a$ form, lost upon B5 mutation, has absorption at 673 nm (data not shown). The amplitude of the absorption in the Chl $b$ region is reduced in agreement with the pigment analysis. The CD signal is very different from that of WT Lhca4, an indication that the mutation at this site strongly perturbs the pigment organization.

Out of the four Chls lost in this mutant, one seems to be Chl B6 due to the lack in both absorption and CD spectra of the same components that are lost for the B6 mutant. Thus, the other three sites affected by mutation E102/R105 accommodate in total 2 Chls $a$ and 1 Chl $b$. One of them is clearly bound to site B5, while for the other two we tentatively propose that they are accommodated in sites A6 and A7. The structure of LHCII shows that both Chl A6 and A7 are coordinated by water molecules and they are located in the region between the B and C helix (25). It is reasonable to predict that, if the mutation E102V/R105L strongly affects the region in between these two helices, the first effect is the loss of coordination via water molecules. Moreover, the additional Chl $a$ lost upon mutation of the B5 site, as compared to the A5 mutant, absorbs at 673 nm, which is the wavelength at which Chl A6 is expected to absorb (see above). According this hypothesis, one Chl $a$ + one Chl $b$ should be accommodated in sites B5 and A7. Both these sites are occupied by Chl $b$ in LHCII (23) and their association to the protein scaffold is stabilized by an H-bond of the formyl group of Chl $b$ with the Gln 131 (25). In Lhca4 this Gln is substituted by Glu, which is most probably not protonated at the pH present in the membrane and thus can not stabilize the Chl $b$ ligation via H-bond. It is thus possible that both sites have mixed occupancy, which would also allow explaining the two emission forms present in Lhca4-WT.

As already observed for mutant A4, the effect of the B5 mutation on the red forms may have at least two different origins:

- the Chl $a$ accommodated in B5 is the second term of the excitonic interaction involving Chl A5 (direct effect)
- mutation of site B5 induces a perturbation in the system with consequential loss of red absorption (indirect effect).

Although both hypotheses are reasonable, we favour the first one for the following motivations:

First, it has been shown that the red absorption band is the low-energy band of an excitonic interaction. The mutation analysis clearly shows that between the three candidate Chl pairs to be at the origin of the red forms, only the A5/B5 pair induces loss of the red emission.

Second, a mutation of site B5 leads to the loss of the red-most form also in Lhca1 and Lhca2 (19;24), where the impact of the mutation on the protein stability was lower than in the case of Lhca4, thus making hypothesis 2 unlikely.
CONCLUSION

In this work we have performed mutation analysis of several putative Chl binding sites of Lhca4 with the aim of understanding the origin of the red emission forms which characterize this complex. Differently from what observed in the case of Lhcb1 and Lhcb4, where most of the mutations seemed to have a local effect (22;23), in the case of Lhca4, large changes in different spectral region were observed, suggesting that in this case several mutations have the effect of perturbing the overall structure of the system. It seems that the structure of Lhca4 is more “cooperative” as compared to the structure of the antenna system of Photosystem II, possibly due to a more extended excitonic network.

Mutation analysis clearly shows that the red emission form of Lhca4 is lost upon mutation at sites A5, B5 and A4. By interpreting these results within the frame of excitonic interactions, we suggest that the two interacting Chl $a$ molecules which originate the red form are located in site A5 and B5. Loss of red emission upon mutation of site A4 is likely to be due to a different folding of the mutant, which is induced by the loss of the ionic pair E44 R158 that
stabilizes the WT structure, rather than to a direct involvement of Chl A4 in the interaction, although this possibility can not be completely ruled out. In Figure 7, the structure of Lhca4 obtained in (6) is presented. The Chls which influence the red-most absorption are indicated in colors different from green. Beside the A5/B5 pair, evidence for additional pigment-pigment interactions is obtained between Chls in sites B3-A3 and B6-A6. However, the interactions between these pigments do not lead to the formation of low-energy bands of high intensity and/or shifted to >700 nm and therefore do not contribute to the 733 nm fluorescence emission unique of this PSI subunit.

Figure 7. Structure of Lhca4. All the chlorophylls are indicated in green with the exception of those whose depletion has an effect on the red emission of Lhca4. A5 and B5 are in red, B6 in orange and A4 in yellow.
Reference List
