

A Structural Investigation of the Central Chlorophyll *a* Binding Sites in the Minor Photosystem II Antenna Protein, **Lhcb4**[†]

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ABSTRACT: Mutant proteins from light-harvesting complexes of higher plants may be obtained by expressing modified apoproteins in *Escherichia coli*, and reconstituting them in the presence of chlorophyll and carotenoid cofactors. This method has allowed, in particular, the engineering of mutant LHCs in which each of the residues coordinating the central Mg atoms of the chlorophylls was replaced by noncoordinating amino acids [Bassi, R., Croce, R., Cugini, D., and Sandonà, D. (1999) *Proc. Natl. Acad. Sci. U.S.A.* 96, 10056–10061]. The availability of these mutants is of particular importance for determining the precise position of absorption bands for the different chlorophyll molecules, as well as the sequence of energy transfer events that occur within LHC complexes, provided that the structural impact of each mutation is precisely evaluated. Using resonance Raman spectroscopy, we have characterized the pigment–protein interactions in the minor photosystem II antenna protein, **Lhcb4** (CP29), in which each of three of the four central chlorophyll *a* molecules has been removed by such mutations. By comparing the spectra of these mutants with those of the wild-type protein, the state of interaction of the carbonyl group, the coordination state of the central magnesium ion, and the dielectric constant (polarity) of the immediate environment in the binding pocket of the chlorophyll *a* molecule were defined for each cofactor binding site. In addition, the structural impact of the absence of one chlorophyll *a* molecule and the quality of protein folding were evaluated for each of these mutated polypeptides.

The harvesting of light energy and its delivery to reaction centers is the essential first step driving photosynthesis in photosynthetic eukaryotes and prokaryotes. In green plants, this function is carried out principally by members of the LHC¹ multigene family, a set of homologous pigment-binding proteins which are organized around the reaction centers of both photosystems I and II to form large supramolecular complexes in the thylakoid membrane. An atomic model of the most abundant LHC protein, the bulk photosystem II LHC antenna or LHCIIb, was generated from a 3.4 Å electron diffraction map of two-dimensional crystals. This model presents the overall protein fold of the protein along with the position of most of the bound cofactors (chlorophylls *a* and *b* and xanthophylls) and, in some cases, the coordinating residue for each (*I*). In the absence of a more detailed structure, however, a less direct approach to defining the identities, orientations, and pigment–protein interactions of these cofactors has been used—that of

reconstitution of wild-type and mutated polypeptides where pigment binding has been perturbed (2). These studies are very well advanced for one of the minor LHCII proteins, **Lhcb4**, in which reconstituted mutant pigment–protein complexes have been produced lacking each of seven of the eight bound chl molecules (3). Interestingly, in these reconstituted proteins, of the seven binding sites investigated (and, by extension, the eighth), four exhibited mixed chl *a*/chl *b* specificity, which obviously further complicates interpretation of spectra of the wild-type and mutant proteins. However, the binding sites in the center of the structure, called *a1*, *a2*, *a4*, and *a5*, appear to be specific for chl *a* (although site *a1* could not be studied directly).

Resonance Raman spectroscopy has been used extensively in the characterization of physicochemical mechanisms underlying the absorption properties of bacteriochlorophyll cofactors in bacterial light-harvesting proteins (4). In particular, this technique allowed a quantification of the role of the different types of interactions in tuning the electronic properties of these molecules. Up to now, it has seen only limited use for chl-containing light-harvesting proteins, in particular because of the difficulty of identifying, in the resonance Raman spectra, the individual contributions of each of the bound chls *a* or *b* in the different LHCs. However, this method can yield the same specific information for chl molecules as for bacteriochlorophylls. Indeed, a number of Raman bands have been shown to be sensitive to the conformation of the chl macrocycle (5). As the molecular

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¹ Abbreviations: chl, chlorophyll; CCD, charge-coupled device; FCP, fucoxanthin–chlorophyll *a/c*-protein; fwhm, full width at half-maximum; LHC, light-harvesting complex; OD, optical density; PSII, photosystem II.

conformation of chls depends in particular on the coordination state of the central Mg atom, the number of axial ligands can be deduced from the position of these Raman bands. For example, the band which arises from the stretching mode of the methine bridges of the chlorin macrocycle is observed at ca. 1600 cm^{-1} when the central Mg atom is six-coordinated, and around $1610\text{--}1615\text{ cm}^{-1}$ when this atom binds only one axial ligand (i.e., when it is five-coordinated). In the higher frequency region of the spectra, bands are seen which arise from the stretching modes of carbonyl groups conjugated with the chl macrocycle, that is, the C₉-keto (chl *a* and *b*) and C₃-formyl (chl *b* only) groups. In apolar solvents and in the absence of hydrogen-bonding interactions, the keto carbonyl stretching mode of chl *a* is located at 1695 cm^{-1} , while that of chl *b* is observed at 1701 cm^{-1} ; the chl *b* formyl carbonyl group vibrates at 1663 cm^{-1} under these conditions (6). Intermolecular interactions with any of these groups results in their stretching mode being downshifted by up to 40 cm^{-1} , the extent of the shift being indicative of the strength of the interaction involved (7). If, on the other hand, the polarity of the immediate environment of the carbonyl group is increased, then its stretching frequency is shifted down by $5\text{--}10\text{ cm}^{-1}$. Thus, measurements of the stretching mode frequencies of these groups can be used to define the state of interaction of the bound chl molecules present (ligation state, H-bonding interactions), as well as the dielectric constant (polarity) of their binding pocket. Such parameters are of central importance to structure and function in photosynthetic proteins as they serve to tune the vital physicochemical properties (absorption, fluorescence, etc.) of these pigment cofactors, which in turn determine their energy collection and transfer characteristics. On the other hand, any perturbation of the structure of the chl-binding sites, induced by a chemical modification or a mutation, will result in dramatic shifts of these frequencies. Resonance Raman spectroscopy may thus also be used for assessing the quality of folding of modified proteins, particularly when they bind, as LHCs from higher plants do, a large number of chl and carotenoid cofactors.

Recently we presented resonance Raman spectra for two of the photosystem II LHC proteins, LHCIIB and **Lhcb4** (8). While these data allowed us to define binding site properties for populations of bound chl molecules, it was not possible to identify these populations with the supposed structural positions of each chl in the atomic model (1). The availability of mutant **Lhcb4** proteins in which individual chl molecules are missing (3) permits us to continue this work by carrying out such an identification. In the same way, as the resonance Raman technique is ideally suited to the study of the precise conformation of protein-bound carotenoids (9), the method can give information on how the latter cofactors are affected by the mutation and/or reconstitution procedure. At the same time, studying these mutants by resonance Raman spectroscopy provides a measure of any overall structural perturbation that may have been caused by the mutation. A resonance Raman investigation of these different mutants, yielding precise information on their structure, is thus an important step for understanding the impact of the different mutations on the function of the wild-type protein.

We concentrate in this paper on those sites which indicated absolute specificity for the bound chl—i.e., the central, chl *a* binding sites *a2*, *a4*, and *a5*. We show that removal of a

single bound cofactor from each site results principally in the loss of bands corresponding to contributions of one single chl molecule. Characterization of these contributions thus gives access to the individual properties of those chlorophylls that have been selectively removed by the mutations. At the same time, smaller overall effects on Raman spectra of the remaining pigments indicate small changes in binding site properties of these latter chromophores. While in some cases these may be direct effects on neighboring pigments, more general changes in the overall protein folding conformation to accommodate the loss of a chlorophyll molecule in the structure should result in larger spectral perturbations (e.g., widening and/or shifting of most bands). In addition, as site *a5* is neighbored by only one chl *b* binding site (the mixed site *b6*), while *a2* and *a4* are neighbored by other chls *a* only (1, 3), chl *b* Raman spectra can more easily be used to interpret changes in terms of local or larger-scale effects. We can thus define to what extent the structure of these different mutants has been perturbed by the mutation and the concomitant loss of the chlorophyll, an important piece of structural information for interpreting the observed changes in electronic and functional properties of these mutants.

EXPERIMENTAL PROCEDURES

Preparation of wild-type reconstituted **Lhcb4** and mutants deficient in individual chlorophyll *a* binding sites was carried out by a combined mutation/reconstitution procedure, as described elsewhere (3). Samples for resonance Raman spectroscopy were concentrated in Microcon-30 concentrators (Amicon) to an OD in the Soret region of $20\text{--}100$ ($\sim 1\text{ mg}$ of chl/mL). Absorption spectra were taken before and after Raman measurements to verify sample integrity. Resonance Raman spectra at 77 K were obtained in an SMC-TBT flow cryostat using a Jobin-Yvon U1000 Raman spectrophotometer equipped with an N₂-cooled, back-thinned CCD detector (Spectrum One, Jobin-Yvon, France), as described previously (10). Excitation was provided by Coherent Argon (Innova 100) and Krypton (Innova 90) lasers (457.9 and 488.0 nm and 406.7 and 413.1 nm , respectively) and a Liconix Helium–Cadmium laser (441.6 nm). Approximate normalization of the Raman spectra for comparison and calculation of differences was carried out using the carotenoid ν_1 and chl *a* methine bridge modes ($\sim 1530\text{ cm}^{-1}$ and $1600\text{--}1615\text{ cm}^{-1}$, respectively), taking into account the known pigment stoichiometry (3).

RESULTS

Carotenoids in Mutant Proteins. Recently, resonance Raman spectra of native **Lhcb4**, along with those of the related protein LHCIIB, have been reported (8). Presented in Figure 1 are spectra of the bound xanthophylls in the wild-type and mutant-reconstituted proteins, obtained by excitation at 488.0 nm . The spectra are predominantly identical to those of β -carotene, exhibiting bands grouped into four regions (called $\nu_1\text{--}\nu_4$), and moreover they show negligible differences from each other. Thus, it can be concluded that the xanthophyll configuration remains essentially identical for the three mutant proteins under investigation. This is as expected given that the mutants exhibited identical xanthophyll-binding properties to the wild type (3). There are,

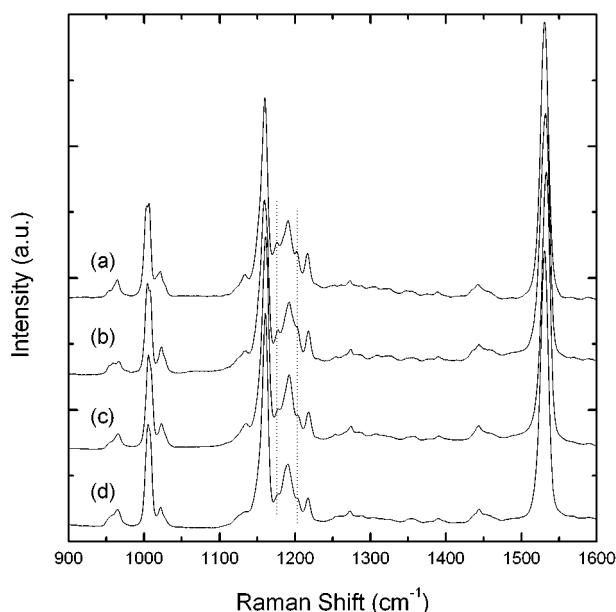


FIGURE 1: 77 K carotenoid resonance Raman spectra of recombinant **Lhcb4** proteins, excited at 488.0 nm (10). Proteins were reconstituted from wild-type (a), E111V/R218L (b), H216F (c), and H114F (d) **Lhcb4** polypeptides (b–d correspond to sites *a4*, *a2*, and *a5*, respectively). Dotted lines indicate minor differences between the spectra, as discussed in the text.

however, some small differences that can be discerned in the spectra. The major ν_1 band, around 1530 cm^{-1} , is seen to shift slightly in mutants E111V/R218L and H216F (missing chls *a2* and *a4*, respectively; Figure 1b,c). In addition, the relative sizes of the small bands at 1176 and 1203 cm^{-1} , in the central ν_2 region (indicated by dotted lines), show some variation for all three mutants. Such small changes have already been observed in bacterial reaction centers, mutated in the protein-binding pocket of the carotenoid cofactor (11). Crystal structures of the different reaction center mutants revealed no significant changes in the carotenoid configuration, within the accuracy of the structure derived from crystallography ($\sim 2\text{ \AA}$), and it was concluded that the different mutations were inducing very small deviations ($\ll 1\text{ \AA}$) of the carotenoid from its wild-type configuration. It is highly likely that the variations observed in the resonance Raman spectra of the different mutants obtained in these excitation conditions arise from a similar phenomenon. In short, they reveal that the carotenoid molecules are able to feel the structural reorganization of the protein induced by the mutations, but they do not imply that these molecules undergo any significant changes in their molecular configuration. Note in particular that the position and structure of the ν_4 band, which has been shown to be particularly sensitive to the carotenoid configuration (i.e., the planarity of the molecule along the C–C bonds; 12), show only slight variation from the wild-type spectrum in the three mutants studied here.

Chlorophylls in Mutant Proteins. As well as carotenoids, resonance Raman spectra can be obtained for the other pigments bound by LHC proteins, chlorophylls *a* and *b*. By varying the precise position of the exciting laser line, and using the fact that the Soret electronic transitions of the chlorophyll *a* and *b* molecules are shifted relative to each other, it is possible to obtain resonance Raman spectra containing selectively the contribution of one or other type

of chlorophyll present (8). In the simplest case, where the mutation has no or very little impact on the other bound chlorophylls, a comparison of such spectra for mutants missing chls from individual binding sites with those of the wild-type protein should give the Raman contributions of the missing chl only. However, if the mutation has an effect on the other bound chl molecules, more than one type of spectral change can be expected. In the case of structural perturbations of the binding site(s) of nearby chls, resonance Raman spectra of the mutant should contain, in addition to major losses corresponding to the missing chl, smaller variations due to limited reorganization of the binding sites of these nearby chls. However, if the loss of a chl molecule induces even limited perturbation of the electronic transitions of the neighboring chls, the contributions to the overall spectra of the latter may vary dramatically due to the changes in resonance properties. Such effects have been extensively documented in the case of bacterial reaction centers excited in the Bchl Soret transition (13, 14). It is somewhat time-consuming to scan the Soret transition with a continuous, tuneable laser between 400 and 440 nm, so as to obtain spectra with many excitations in this spectral region. In this latter case, we cannot formally conclude on the state of the mutant protein from Raman spectra alone. However, as the pigment content has been precisely determined for every mutant and one chl only is lost in each case (3), it is possible to describe from the data, even in these difficult cases, both the impact of the mutation and the contribution of the lost chl to the spectrum.

Chl *a4*. As the three mutants studied here only exhibit loss of chl *a*, spectral differences are expected when in resonance with chl *a* only, and not chl *b*. This is the case for mutant E111V/R218L, in which the coordinating residue at position *a4* has been mutated (Figure 2). As described by Pascal et al. (8), excitations at 406.7 and 413.1 nm mainly ensure resonance with chl *a* molecules, while excitation at 457.9 and 441.6 nm ensures nearly selective resonance with chl *b*. In Figure 2a,b, spectra obtained in chl *b* resonance conditions are essentially the same as for the wild type, with only small changes in the overall shape of the envelope of bands that can be assigned to minor changes in the protein fold. In spectra obtained in resonance with the chl *a* Soret transition (Figure 2c,d), apart from similar changes in overall shape, the principal difference is a loss of contributions on the low-frequency side of the envelope of keto carbonyl modes. The difference spectra (dotted lines) indicate loss of a chl *a* molecule with a methine bridge stretching mode at 1616 cm^{-1} and a keto carbonyl vibrator at ca. 1660 cm^{-1} . Thus, the chl *a* bound to site *a4* is five-coordinated, based on the position of its methine bridge mode, and its keto carbonyl stretching frequency around 1660 cm^{-1} indicates a carbonyl taking part in a strong H-bond with its protein host (around 25 kcal; 7). On the other hand, we may conclude from the Raman data that the mutation E111V/R218L, and the ensuing loss of chl *a4*, does not dramatically affect either the binding sites of the other chls in the protein or their electronic properties in the Soret region. The **Lhcb4** apo-protein thus accommodates the loss of the chl bound to site *a4* without a sizable structural reorganization.

Chl *a2*. In Figure 3 are compared the resonance Raman spectra of the mutant protein missing the chl in site *a2* (H216F) to those of the wild type. The main difference

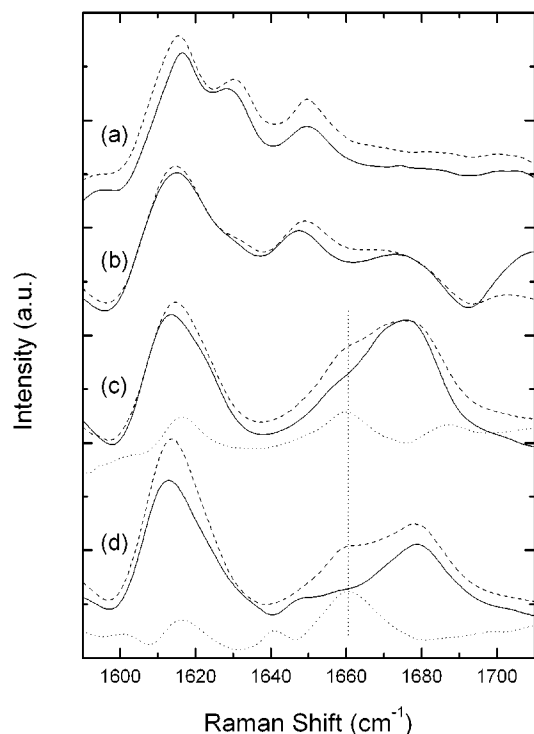


FIGURE 2: Chl *a4* site. 77 K chl resonance Raman spectra of wild-type **Lhcb4** (dashed line) and mutant E111V/R218L missing chl *a4* (solid lines), excited at 457.9, 441.6, 413.1, and 406.7 nm (a–d, respectively). Difference spectra are also presented (dotted lines).

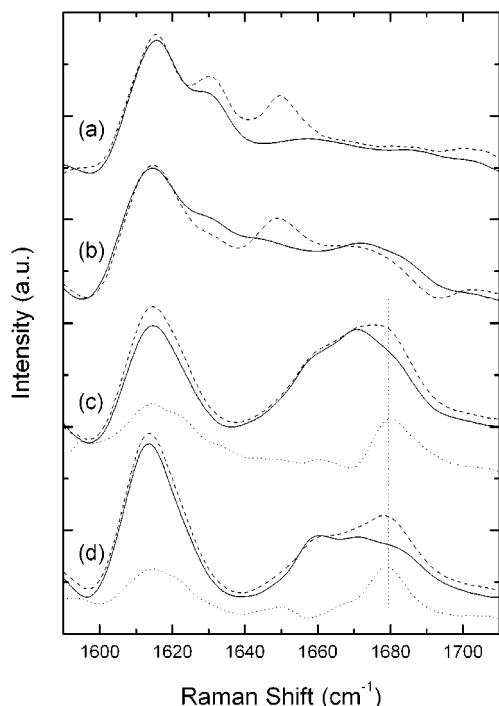


FIGURE 3: Chl *a2* site. 77 K chl resonance Raman spectra of the H216F mutant (see Figure 2 for further details).

observed in chl *a* resonance Raman spectra of the mutant (Figure 3c,d) is the loss of a contribution on the high-frequency side of the envelope corresponding to keto carbonyl modes of these molecules ($1650\text{--}1685\text{ cm}^{-1}$). From the difference spectra (dotted lines), it can be seen that the position of the missing band is at ca. 1679 cm^{-1} . This loss is accompanied by a decrease in the intensity of the band

corresponding to methine bridge modes at 1614 cm^{-1} . Other smaller variations are observed in the spectra but are sufficiently minor to be explained by rearrangements in the overall protein folding pattern to accommodate loss of the chl *a* in site *a2*, or by direct effects on the chls *a* neighboring the *a2* site. Note that these smaller changes in band shape mean that here, and for the other mutants, the exact position of the bands corresponding to the missing chl molecule may vary by a few wavenumbers from the observed peaks in the difference spectra. More significant changes are seen in chl *b* excitation conditions, although the binding stoichiometry of this pigment was the same as that of the wild type (3). It is interesting to note that the spectra shown here of the H216F mutant resemble more closely those of the native protein than do the wild-type reconstituted product. In particular, the intensity of the second chl *b* formyl carbonyl vibrator at 1649 cm^{-1} is very much reduced with respect to the 1630 cm^{-1} band (15). The band can nevertheless be observed (most obvious for 441.6 nm excitation; Figure 3b), and pigment analysis clearly demonstrates that no chl *b* is lost in this mutant. This effect could be accounted for by a small twisting of the formyl group out of plane of the macrocycle, such as seen associated with redox changes of the haem *a3* of cytochrome *c* oxidase (16), although such a conformational change would be highly unfavorable energetically for a chlorophyll molecule. Indeed this phenomenon has only been seen once before for (bacterio)chlorophyll-proteins, in the rare case of a specific mutant resulting in steric effects around an (acetyl) carbonyl group (17). It thus seems more likely that this change is due to a resonance effect. If the Soret electronic transition of the chl *b* corresponding to this formyl mode shifts slightly away from the excitation lines used, such a decrease of its overall contribution to the spectra will be observed. A similar conclusion was possible for wild-type reconstituted **Lhcb4** as compared to the native protein (15). We can nevertheless conclude that the chl *a* molecule present in site *a2* has its methine bridge stretching mode around 1615 cm^{-1} , corresponding to a central magnesium with one axial ligand. The keto carbonyl group of this chl *a* has a stretching frequency around 1680 cm^{-1} , indicating either that this group is free from interactions but in a polar environment, or that it is involved in a weak H-bond with the surrounding amino acids.

Chl *a5*. Mutant H114F, in which a chl *a* molecule is missing from site *a5*, shows the largest overall changes in chl resonance Raman spectra of the three mutants studied here (Figure 4). Spectra corresponding to chl *b* excitation (Figure 4a,b) show significant broadening of bands as well as a reduction in the intensity of the formyl carbonyl band at 1649 cm^{-1} (although the latter is less drastic than for the *a2* mutant, H216F). However, both formyl carbonyl bands are still present, consistent with the measured chl *b* stoichiometry of 2 for this mutant (3). This band broadening could indicate a perturbation of the chl *b* binding site(s). Alternatively, if structural perturbations in the mutant complex result in some chl *b* molecules binding to sites normally selective for chl *a*, it could also result in the same effect. Chl *a* spectra are also altered quite significantly (Figure 4c,d), and this is reflected in the difference spectra (dotted lines), which give tentative values for the positions of methine bridge and keto carbonyl stretching modes. The methine bridge mode of the chl *a* in *a5* is clearly in the $1612\text{--}1620\text{ cm}^{-1}$ region, while

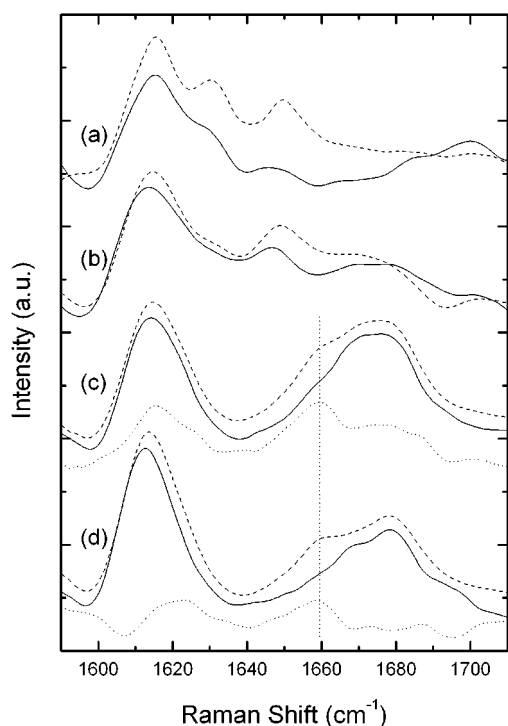


FIGURE 4: Chl *a5* site. 77 K chl resonance Raman spectra of the H114F mutant (see Figure 2 for further details).

its keto carbonyl group probably has its stretching mode around 1660 cm^{-1} . However, it is also possible that this mode is present instead at higher frequency ($1673\text{--}1688\text{ cm}^{-1}$; Figure 4c,d). Chlorophyll *a* in site *a5* is thus five-coordinated, and its keto carbonyl group most likely takes part in a strong H-bond with its binding pocket, although the formal characterization of the interactions assumed by this group will require the study of additional mutants (see discussion, below).

DISCUSSION

The use of resonance Raman spectroscopy has allowed us to investigate the effect of mutations of chl-ligating residues on detailed structural properties of the **Lhcb4** antenna protein. In mutants in which particular binding sites no longer contain their bound cofactors, the Raman band shape of the remaining pigments provides a measure of the effect of the loss of the cofactor molecule on the overall protein-folding pattern. For all the three mutants studied, only very small deviations in xanthophyll conformation were observed, of the order of 0.1 Å or less. Mutant E111V/R218L, which lacks a chl *a* molecule in position *a4*, exhibits only minor alterations in chlorophyll configuration for the other, remaining chl molecules. Thus, aside from the loss of chl *a4*, this mutant presents a largely identical protein fold to the wild-type protein. The mutant lacking chl *a2* (H216F) also shows little changes in resonance Raman spectra of the remaining chls *a*. It does, however, exhibit significant variation for excitation conditions resonant with chl *b*. It thus appears that loss of the chl *a* molecule in the *a2* site results in the perturbation of a chl *b* binding pocket, which affects its absorption properties. As a result, the relative sizes of the contributions of the different chls *b* present are altered due to variations in their resonance with the excitation lines used. The largest differences are seen in spectra of mutant

Table 1: Frequencies (cm^{-1}) of Chl *a* Methine Bridge and Keto Carbonyl Stretching Modes for Chls *a4*, *a2*, and *a5* in **Lhcb4**^a

	methine bridge	keto C=O
chl <i>a4</i>	1616	1660.5
chl <i>a2</i>	1614	1679.5
chl <i>a5</i>	1615–1622	1659.5 (?)

^aMeasured by resonance Raman spectroscopy of mutant proteins lacking the corresponding chl molecule.

H114F, which is unable to bind a chl *a* at site *a5*. Here, not only is the chl *b* spectral band shape affected, but also the chl *a* spectra exhibit dramatic variation from the wild-type protein. Note that site *a5* is neighbored only by *a1*, *a2*, *a4*, and *b5* (with mixed chl *a/b* specificity indicated for the latter; 1, 3), and so these cannot be only local effects. It is difficult to quantify these changes in terms of small structural perturbations or alterations of electronic properties of each chlorophyll molecule involved, but it is clear that the loss of chl *a5* results in a significant reorganization of the three-dimensional structure of the mutant protein.

It is of interest to consider the factors affecting the refolding of **Lhcb4** mutants lacking a single chlorophyll molecule. The results presented here indicate that it is not at all simple to predict the quality of refolding of such mutants. Thus, the E111V/R218L and H216F mutants (*a4* and *a2* sites, respectively) show little or no perturbation of their remaining bound pigments, while mutations at symmetrically equivalent positions (*a1* and *a5*, respectively) have a significant effect on the overall structure—drastic in the case of site *a1*, as no refolded product is formed (3). Thus, control of these mutants by detailed structural measurements (such as those presented here) is essential before conclusions are drawn on the specific site targeted by the mutation. The results do, however, raise the possibility of assessing which factors have the most effect on the refolding process for these mutants. One similarity between the two sets of mutants on either hand is their position in the overall structure of the complex. Sites *a4* and *a2* are situated toward the outer side of the assembly (1) and so their absence may have a smaller effect on the folding of the protein. Sites *a1* and *a5*, on the other hand, are found toward the center of the complex, between the crossbrace (formed by helices A and B and the two xanthophylls) and the C helix (along with chl *b5*; 1). Thus, the absence of a chlorophyll at either of these sites may be expected to have a greater effect both on chl *b5* itself and on the overall folding pattern.

These measurements have also given access to the stretching frequencies of modes of specific chl *a* molecules in the atomic model. In particular, we have studied those modes that are widely used in the investigation of chlorophyll structure and interactions in vivo (4). These modes are summarized in Table 1. In each case, the methine bridge stretching frequency is higher than 1610 cm^{-1} , indicating that all three chls *a* have only one external ligand to their central magnesium. Two of the three chls (*a4* and *a5*) have their keto carbonyl vibrators around 1660 cm^{-1} (although the assignment for *a5* is somewhat tentative), and so these C=O groups take part in a strong intermolecular interaction with their protein environment. The chl *a2* keto group contributes around 1680 cm^{-1} , and so it is either weakly H-bonded or is free but present in a polar environment. In a

previous report on the Raman spectra of **Lhcb4** chlorophylls (8), the chl *a* molecules were seen to be present in three different populations. Two chl *a* molecules had their keto stretching frequencies around 1660 cm⁻¹, one had a keto vibrator around 1685–1690 cm⁻¹, and the other three chls *a* contributed in the 1665–1675 cm⁻¹ region. The carbonyl bands at 1660 cm⁻¹ are of particular interest. First, these modes have been observed in all LHC-type proteins measured to date, including the relatively distant fucoxanthin–chlorophyll *a/c*-protein (6)—indeed, the two corresponding chlorophylls were suggested to represent a conserved structural motif throughout the LHC multigene family. It would therefore appear that *a4* and possibly *a5* show a high degree of homology in the structure of their binding pockets for most if not all LHC-like proteins. In addition, a recent study of **Lhcb4** by fluorescence line-narrowing (18) has indicated that around 50% of the fluorescence of this protein at low temperature (<20 K), for excitations on the red side of the Q_y transition, was emitted by a chl *a* molecule whose keto C=O group vibrates near 1660 cm⁻¹. The data presented here suggest that this chlorophyll is in position *a4* or *a5*. This would seem to conflict with a separate study assigning chl *a2* as the red-most absorbing species at 100 K (19). However, the remainder (~50%) of the fluorescence at the lower temperatures is emitted by chl(s) *a* having their keto carbonyl modes in the 1670–1680 cm⁻¹ region, and so one of these could well be chl *a2*. Significant shifts in the Q_y electronic transitions of this and/or other chlorophylls below 100 K could easily explain this apparent discrepancy. Further fluorescence line-narrowing measurements on the appropriate mutants should determine which particular chlorophyll in the structure is responsible for the majority of the fluorescence emission at low temperature.

In this work we have, for the first time, characterized the resonance Raman contributions of specific chlorophyll molecules in a member of the LHC family, namely, **Lhcb4**. This constitutes a vital first step in defining those pigment–protein interactions responsible for tuning the physicochemical properties of these pigments, and thus ensuring the correct functioning of the protein, in terms of both light capture and energy transfer. The other **Lhcb4** mutants currently available, missing a chlorophyll molecule in sites *a5*, *b3*, *b5*, and *b6*, will also be studied by resonance Raman spectroscopy in an attempt to get a complete picture both of the effect of these mutations on the folding process and of the binding site properties for each position. However, given the observed “promiscuity” of these sites, showing affinity for both chls *a* and *b* upon refolding (3), it is unclear whether

these results will give unequivocal information on these parameters. Future production of mutants in which chl–protein interactions are perturbed (removal of H-bonding residues, alteration of dielectric constant) will allow us to determine their respective effects on the absorption, fluorescence, and energy transfer characteristics of chlorophyll molecules in vivo, properties which are essential for the functioning of these proteins as light-harvesting antennae.

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