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Structure and Interactions of the Chlorophyll *a* Molecules in the Higher Plant Lhcb4 Antenna Protein

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Received: April 20, 2000; In Final Form: July 20, 2000

A study of the vibrational bands of the red-most pigments in the minor photosystem II antenna protein, **Lhcb4**, is presented. The two vibrational techniques used, resonance Raman and fluorescence line-narrowing (FLN) spectroscopies, are shown to give essentially the same information on the pigments investigated, with the exception that FLN provides information on the red-most, fluorescence-emitting species only. Fluorescence emission at 4.2 K is shown to originate from two pools of chlorophylls one of which, contributing to 50% of the emission, corresponds to only one chlorophyll molecule per monomer. This opens the way to the identification of the emitting species within the three-dimensional model of **Lhcb4**.

Introduction

The first step of photosynthesis is the light-harvesting process, in which the energy of light is absorbed by antenna proteins and transferred, in the form of excitation energy, to the reaction centers where primary charge separation occurs. In higher plants and green algae this process is extremely complex, mobilizing a large number of chromophores and polypeptides. Both photosystems have a double light-harvesting system, made up of inner and outer antennae. The inner antenna system involves subunits of the reaction center itself in the case of PSI[†], and CP43 and CP47 for PSII. The outer antenna proteins of both PSI and PSII, namely LHCI and LHCII, form part of the LHC multigene family.^{1,2} Most of these proteins bind both chlorophyll (chl) *a* and *b* and a number of xanthophyll molecules. The structure of the bulk LHC of PSII, LHCIIb, which binds 65% of PSII chlorophyll, has been solved to 0.34 nm resolution by electron diffraction of 2-D crystals, and an atomic model could be derived from such studies.³ However, LHCIIb is the most complex protein of the PSII antenna system, as it is constituted of a trimer of subunits each binding 5–6 chl *b* and 7–8 chl *a* molecules, and 3–4 xanthophylls.^{4,5} Among these, only 12 chl molecules and two xanthophylls could be distinguished in the atomic model derived from 2-D crystallography, and neither the chemical nature of individual chls and xanthophylls nor the orientation of the chls in the protein, could be precisely determined.³

Owing to their sequence homologies, the other LHCII proteins, namely CP29, CP26, and CP24 (or LHCII *a*, *c* and *d*), are predicted to form the same basic protein fold.⁴ However,

they are present as monomers, and their pigment binding stoichiometry is simpler than that of LHCIIb. CP29 or **Lhcb4**, which is encoded by the nuclear *Lhcb4* gene, is the largest of these antenna proteins, and it binds six chl *a*, two chl *b*, and two carotenoid molecules.^{6,7} Due to its relative simplicity, spectroscopic data on **Lhcb4** are often more easily interpretable. Because of this, and as it is relatively easy to purify in large amounts,⁷ this protein has been the object of many studies, although no direct structural data are as yet available for this particular protein.^{8–10} Recently, it was shown that it is possible to reconstitute this protein from either WT or mutated apoproteins and its different cofactors.⁶ **Lhcb4** thus constitutes a model of choice for understanding both the structural and functional aspects of the proteins belonging to the LHC family.

Understanding the function of an antenna protein requires not only a full description of the energy transfer processes which occur in this protein, but also why a given cofactor acts at a defined point of the energy transfer cascade, i.e., how the protein structure tunes those physicochemical properties (such as the excited state energy) that influence energy transfer phenomena to and from the other pigments. For antenna proteins from purple bacteria, vibrational spectroscopy, and more particularly resonance Raman spectroscopy, has proved invaluable for describing the molecular mechanisms tuning the electronic properties of bacteriochlorophyll molecules, but this method has not yet been extensively applied to plant systems. For chl-containing proteins, another vibrational method can be used to obtain submolecular information, namely fluorescence line-narrowing (FLN) spectroscopy. This method was recently applied to LHCIIb,¹¹ PSII reaction centers,¹² and the cytochrome *b₆f* complex,¹³ and it is expected that it will yield even more selective structural information than resonance Raman. Indeed, FLN gives access to vibrational information only on those molecules involved in the fluorescence process, i.e., the terminal emitters of a given protein. A drawback is that it can be applied only at extremely low temperatures (below 15 K), and on the red-most absorbing chromophores. In this paper, we compare fluorescence line-narrowing spectra to Fourier transform preresonance Raman

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† Abbreviations: chl, chlorophyll; CCD, charge-coupled device; FLN, Fluorescence line-narrowing; FWHM, full width at half maximum; LHC, light-harvesting complex; OD, optical density; PS, photosystem; THF, tetrahydrofuran.

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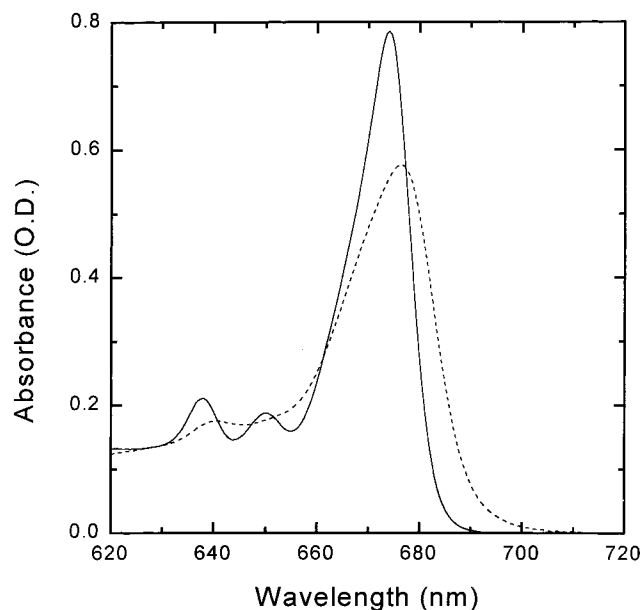


Figure 1. Absorption spectra of **Lhcb4** at room temperature (dashed line) and 77 K (full line).

spectra obtained at several temperatures from the same **Lhcb4** samples. From this study, the structure of, as well as the interactions assumed by, the different chl *a* molecules in **Lhcb4** may be precisely described, in particular those which act as terminal emitters at low temperature.

Materials and Methods

Lhcb4 preparation was by column chromatography of Tris-washed PSII.⁷ Protein samples for FLN measurements were diluted to an OD in the Soret region of around 0.5 (~0.5 mg chl/mL) in 80% glycerol, 0.06% dodecyl maltoside, 20 mM HEPES pH 7.5; for FT preresonance Raman spectroscopy they were concentrated in Microcon-30 concentrators (Amicon) to an OD of 100–500. Spectra were recorded at various temperatures in an SMC-TBT flow cryostat (Air Liquide, Sassenage, France) or a helium bath cryostat (Utreks). Absorption spectra were measured on a Varian Cary E5 double-beam scanning spectrophotometer.

FT Raman spectra were recorded at 4 cm⁻¹ resolution using a Bruker IFS 66 interferometer coupled to a Bruker FRA 106 Raman module equipped with a continuous Nd:YAG laser. All spectra were recorded with backscattering geometry from concentrated samples held in standard aluminum cups (room-temperature experiments). For low temperature measurements, samples were deposited on a glass plate prior to freezing and held at 77 K in a TBT cryostat in a flow of liquid nitrogen, as described previously.¹⁴ Spectra were the result of 1000 to 10 000 co-added interferograms. Spectra of chls *a* and *b* in diethyl ether and/or tetrahydrofuran (THF) were recorded as previously described by Nèveke et al.¹⁵

FLN experiments on **Lhcb4** were recorded at 4.2 K in a Utreks cryostat, as extensively described in ref 11, with a Chromex 500I spectrograph equipped with a Chromex Chromcam 1 CCD detector (spectral bandwidth 5 cm⁻¹). Excitation light was provided by a dye laser (CR599, Coherent, spectral bandwidth 1 cm⁻¹) pumped by an argon laser (Coherent Innova 310). The power of the excitation laser was kept below 2 mW/cm² to minimize the effects of spectral hole-burning below 30 K. Spectra are the mean of 11 different excitations in the range 679.2–683.6 nm. FLN spectra of isolated chl *a* were recorded

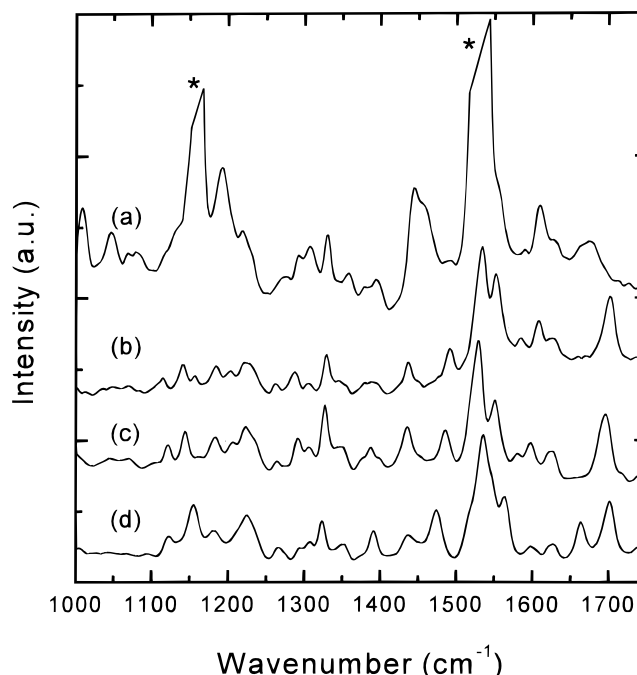


Figure 2. Room-temperature FT-Raman spectra (1000–1750 cm⁻¹) of **Lhcb4** (a), chl *a* in diethyl ether (b), chl *a* in THF (c), and chl *b* in THF (d). Excitation was at 1064 nm. Note that the major carotenoid peaks in **Lhcb4**, marked with an asterisk, have been cut for clarity.

at 10 K in a TBT cryostat in a flow of helium gas, using 676.4 nm excitation provided by a Krypton laser (Coherent Innova 90) on a Jobin-Yvon U1000 Raman spectrophotometer equipped with an N₂-cooled, back-thinned CCD detector (Spectrum One, Jobin-Yvon, France).

Results

Figure 1 displays room- and low-temperature absorption spectra in the *Q_y* region of the **Lhcb4** used in this study. These spectra are identical to those previously reported using the same preparation,⁷ and essentially identical to those for **Lhcb4** prepared by isoelectric focusing; see, e.g., ref 8

FT-Raman Spectroscopy of Lhcb4. Figure 2 displays the room-temperature FT-Raman spectrum of **Lhcb4** in the 1000–1750 cm⁻¹ range, as compared to that of chl *a* in diethyl ether and THF, and of chl *b* in THF. At room temperature, isolated chls in THF have two axial ligands to their central magnesium ion and are, therefore, six-coordinated, whereas in diethyl ether they are five-coordinated.¹⁶ The **Lhcb4** spectrum (Figure 2a) is dominated by intense contributions of the bound xanthophyll molecules at 1004, 1160, and 1530 cm⁻¹.¹⁷ It is of particular interest to determine which chlorophyll molecules contribute to this spectrum. Spectra of chl *a* and *b* contain a number of bands with similar frequencies (see Table 1). However, as already reported,¹⁸ there are two bands that may be considered as fingerprints for chl *b* contributions, located at *ca.* 1565 and 1660 cm⁻¹. The precise origin of the ~1565 cm⁻¹ band has not yet been determined, but it was clearly shown that the frequency of this band is sensitive to the coordination state of the central Mg atom of chl *b*. In Raman spectra obtained in resonance conditions with the Soret transition of chl *b*, this band is observed at 1566 or 1559 cm⁻¹ when the central Mg is five- or six-coordinated, respectively.¹⁸ In preresonance with the *Q_y* transition (Figure 2d), this band is observed at 1563 cm⁻¹ for six-coordinated chl *b*. It should thus be observed between 1563 and 1570 cm⁻¹, depending on the coordination state of the

TABLE 1: Comparison of Vibrational Bands of Lhcb4 and Isolated Chlorophylls Measured by FT Preresonance Raman (FT-R) and Fluorescence Line-Narrowing (FLN) Spectroscopies

chl <i>a</i> in THF (six-coordinated; FLN, 10 K) ^a	CP29 (FLN, 4.2 K) ^b	CP29 (FT-R, 77 K) ^c	CP29 (FT-R, 298 K) ^c	chl <i>a</i> in THF (six-coordinated; FT-R, 298 K) ^c	chl <i>a</i> in ether (five-coordinated; FT-R, 298 K) ^c	chl <i>b</i> in ether (five-coordinated; FT-R, 298 K) ^c	conformation- sensitive modes ^d
1042	1049	1043	1045	1045	1049	—	R8
1065	1068	1063/1079	1064/1076	1071	1071	1094	
1117	1111	1115	Car	1115	1118	1123	
1143	1143	Car	Car	1142	1144	1155	
1179	1184	Car	Car	1182	1185	1182	R7
1220	1224	1224	Car	1223	1224	1125	
1263	1262	1261/1272	1265/1272	1263	1263	1267	
1287	1286	1292	1295	1292	1287	1293	
1306	1306	1305	1303	1306	1306	1308	
1324	1326	1328	1325	1327	1329	1324	
1348	1344/1353	1355	1355	1351	1352	1351	
1369	1373	1375	1377	1379	1379		
1384	1387	1390	1390	1388	1388	1392	
1428	1436	1440 (Car?)	1440	1435	1437	1437	R6
1484	1490	1489		1485	1492		R5
1527	1537	Car	Car	1528	1534	1535	R4
1549	1556	1554	1554	1550	1552	1563	R3
1600	1610	1611	1609	1597	1608	1598	R1

^a 676.4 nm excitation. ^b 679.2–683.6 nm excitation. ^c 1064 nm excitation. ^d Soret excitation;¹⁸ Car, presence of carotenoid bands.

central Mg, in FT-Raman spectra recorded in our experimental conditions. For chl *a* excited in the Soret region a band is observed at 1545 or 1554 cm⁻¹, depending on the coordination state of the central Mg atom of the molecule.^{17,18} In FT-Raman spectra of six-coordinated chl *a* (Figure 2c) we observe this band at 1549 cm⁻¹, and it upshifts to 1555 cm⁻¹ upon change in its coordination state to five (Figure 2b, Table 1). In FT-Raman spectra of **Lhcb4** (Figure 2a), the only band in this region occurs in the foot of the intense 1530 cm⁻¹ band arising from the carotenoids, and its frequency is at 1554 cm⁻¹, i.e., it indicates contributions of chl *a* molecules only. The other band considered as a fingerprint of chl *b* contributions, around 1660 cm⁻¹, arises from the stretching modes of its formyl carbonyl group (this group is absent in chl *a*). The frequency of this band is sensitive to the intermolecular interactions chl *b* is involved in. It has been shown that the stretching modes of the two formyl groups of the two chls *b* present in **Lhcb4** contribute at 1630 and 1649 cm⁻¹ in resonance Raman spectra excited in the Soret region.¹⁹ In FT-Raman spectra of this protein, no band contributes at these frequencies (Figures 2a, 3a, and 4a,b). Note that the band around 1625 cm⁻¹ corresponds to stretching modes of conjugated vinyl groups, and is present in spectra of both chls *a* and *b*.^{16,17,20} Thus, none of the characteristic bands of chl *b* are present in FT-Raman spectra of **Lhcb4**, and so in these preresonance conditions (where the difference in energy between the *Q_y* of the chls and the excitation wavelength is ca. 5500 cm⁻¹), FT-Raman spectroscopy ensures a nearly complete selectivity between the two types of chl bound to **Lhcb4**, yielding information on chl *a* molecules only.

Fourier transform Raman spectroscopy has not yet been widely applied to chl *a* molecules. In particular, the precise information content is less well-characterized than that of resonance Raman spectra. From Raman spectra of a series of metal-substituted chl *a* molecules measured in resonance with the Soret transition, Fujiwara and Tasumi determined 8 modes between 1000 and 1620 cm⁻¹ whose frequency is directly related to the core-size of the molecule, i.e., to the conformation of its conjugated macrocycle.¹⁸ While different excitation conditions (in this case, in preresonance with the *Q_y* transition; Figure 2) will involve different selection rules, so that some of the bands seen by Fujiwara and Tasumi¹⁸ may be absent here (and other, new bands may appear), some of the R1–8 bands are neverthe-

less expected in the spectra shown in Figure 2 and will show similar (though not identical) dependence on conformation. Out of the eight modes quoted, four bands in equivalent positions and showing similar variation with coordination state may be observed in FT-Raman spectra of isolated chl, namely R1, R4, R5, and R8, located (for chl *a* in THF) at 1608, 1552, 1485, and 1045 cm⁻¹, respectively (see Table 1). Other bands contribute at the positions expected for the R3, R6 and R7 modes, but their frequencies appear poorly sensitive to coordination changes of the central Mg of chl *a*. It seems likely, therefore, that these bands are not equivalent to the R3, R6 and R7 modes. Thus from FT-Raman spectra of chl *a* molecules, the R1, R4, R5 and R8 modes (but these bands only) can be used for determination of molecular conformation. In addition, the intermolecular interactions assumed by these molecules can be assessed via the frequencies of their keto carbonyl stretching modes in the 1660–1700 cm⁻¹ region (see below).

To evaluate the effect of lowering the temperature on the structure of **Lhcb4**, FT-Raman spectra of this protein were recorded at 77 K (Figure 3a). None of the bands observed in these spectra experience shifts greater than 2 cm⁻¹ (Table 1). In particular, none of modes R1, R4, R5, and R8 exhibit large frequency changes, and neither is any splitting of these bands observed upon lowering the temperature. It is thus possible to conclude that temperature affects neither the average conformation nor the coordination state of the Mg atoms of the different chl *a* molecules present in **Lhcb4**. In the high-frequency region, above 1600 cm⁻¹, are seen contributions from stretching modes of the conjugated keto carbonyl groups of chl *a* molecules. These modes have been extensively studied,²¹ and it was clearly demonstrated that their frequency reflects the strength of intermolecular interactions the molecules are involved in. Lowering the temperature has little if any effect on the frequency of these modes (Figures 2a, 3a; see also Figure 4a,b), most of the changes observed being attributable to the increase in resolution induced by the temperature change. This indicates that the intermolecular interactions assumed by the different chls *a* in **Lhcb4** are not deeply modified by cooling from 298 to 77 K.

In the keto carbonyl stretching region, at least four different components may be observed, at ca. 1655, 1664, 1672–8, and 1691 cm⁻¹ (Figure 4a,b). These components reflect the presence

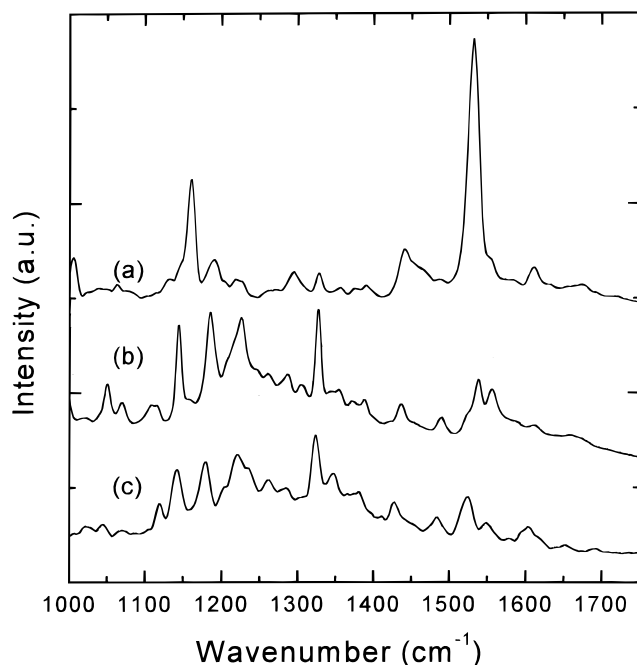


Figure 3. Vibrational spectra of **Lhcb4** (1000–1750 cm^{-1}): (a) 77 K FT-Raman spectrum, excited at 1064 nm; (b) 4.2 K FLN spectrum, average of 11 excitations from 679.2 to 683.6 nm. Also shown is a 10 K FLN spectrum of chl *a* in THF, excited at 676.4 nm (c).

of different populations of chl *a* molecules whose keto carbonyl groups are involved in different types of intermolecular interactions with their surrounding protein environment. As these spectra were measured in preresonance conditions (1064 nm excitation), it is likely that the contributions of the different chlorophyll *a* molecules present in **Lhcb4** will all have the same or very similar relative intensities, so that the intensity of each component should reflect the number of chl *a* molecules in that population. Deconvolution of this spectral region indicates that the components at 1691, 1655, and 1664 cm^{-1} each arise from one chl *a* molecule only, while the other three chls *a* present contribute to the unresolved cluster located between 1670 and 1680 cm^{-1} (data not shown). Note that while there may be some uncertainty in the exact positions and relative amplitudes of these contributions, the overall pattern of positions and number of contributing chls is nevertheless well within the limits of these measurements.

Fluorescence Line-Narrowing Spectroscopy of **Lhcb4**.

Figure 3 displays the FLN spectrum of **Lhcb4**, as compared to that of chl *a* in THF. As summarized in Table 1, most of the bands observed in FLN have their equivalent in FT-Raman spectra, although with differing intensities. Note that FLN has also been recorded on samples in the same conditions as for FT-Raman, i.e., at concentrations greater than 100 OD in the absence of glycerol, and the spectra were identical to those presented here (data not shown). Direct assessment of bands sensitive to the conformation of the fluorophores in FLN spectra is rather difficult for technical reasons—at the very low temperatures required to record the spectra, the central Mg atom of chl *a* molecules tends to be six-coordinated in most in vitro situations. This phenomenon has already been observed for bacteriochlorophyll molecules in vitro.²² It is thus difficult to obtain FLN spectra of five-coordinated chl *a* in vitro. However, when comparing the frequencies of the bands for **Lhcb4** and six-coordinated chl *a* in THF (Figure 3b,c), it appears that the bands which are the most shifted are those observed (for chl *a* in THF) at 1042, 1179, 1428, 1484, 1527, 1549, and 1600 cm^{-1} ,

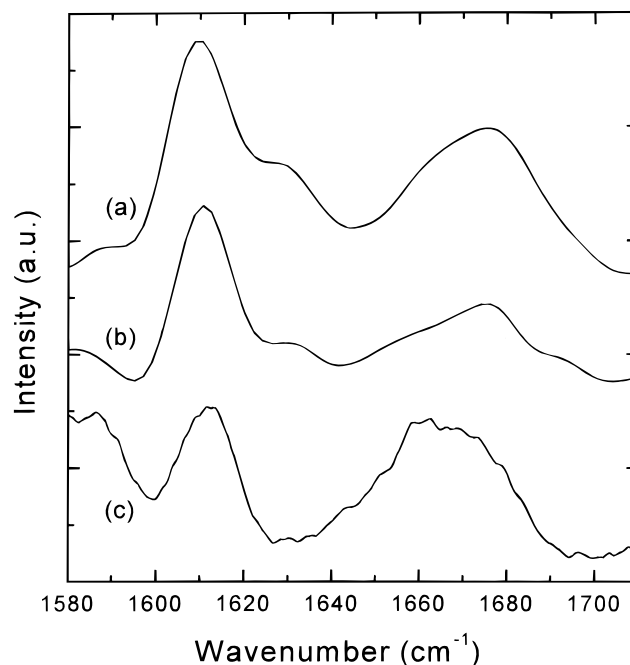


Figure 4. High-frequency region (1580–1710 cm^{-1}) of vibrational spectra of **Lhcb4**: (a) FT-Raman spectrum recorded at room temperature; (b) FT-Raman spectrum recorded at 77 K; (c) FLN spectrum recorded at 4.2 K. See Figures 2 & 3 for experimental conditions.

which correspond to the R8, R7, R6, R5, R4, R3, and R1 modes respectively, as defined by Tasumi and Fujiwara,¹⁸ all sensitive to the chl *a* core-size. It thus seems probable that these modes in FLN spectra correspond to those observed in resonance Raman spectra and that the study of the frequencies of the R1–8 modes will yield information on the conformation of those chl *a* molecules which contribute to the FLN spectra. Indeed, the use of such bands in FLN spectra of the soluble peridinin-chl *a* protein has already proved fruitful in deduction of the configuration of bound chl *a* molecules, and the conclusions were in perfect agreement with the crystallographic structure of this protein.²³ It can therefore be concluded that the Mg atoms of all the chl *a* molecules contributing to the FLN spectra of **Lhcb4** are five-coordinated, and, as the frequencies of the R1–8 modes do not deviate significantly from those observed in Raman spectra of five-coordinated chl *a* in vitro, that the conformation of these molecules is close to that adopted in organic solvents, i.e., that the protein does not induce specific distortions of these molecules upon binding.

It is of particular interest to determine how many chl *a* molecules participate in the FLN spectra. Bands arising from the carbonyl stretching modes contribute very weakly in the higher frequency region (Figure 3). As seen in Figure 4, a quite complex cluster of bands is observed, with the main contribution being located at 1659 cm^{-1} . Thus, the FLN spectrum of **Lhcb4** does not arise from a single chl *a* only. However, nearly half of the area of this cluster corresponds to the 1659 cm^{-1} component, the other half corresponding to the weaker components located in the 1670–1680 cm^{-1} region. This band at 1659 cm^{-1} is probably equivalent to the band at 1655 cm^{-1} in FT-Raman spectra, and we determined that this contribution arises from one molecule of chl *a* only (see above). Note that the variation in apparent position of this band is due to the weak contribution of the carbonyl bands to the FLN spectrum, and the value determined from the Raman spectra is probably more accurate. Therefore this single chl *a* molecule contributes to half of the intensity of FLN spectra of **Lhcb4**, with the other

half probably corresponding to two (or more) other chl *a* molecules with keto carbonyl modes at higher frequencies.

Discussion

In this paper, we have applied two different vibrational techniques to study **Lhcb4**. FT-Raman spectroscopy yields an overall picture of the conformation of and the interactions assumed by the chl *a* molecules bound to this protein. This technique indicates unambiguously that all of these molecules possess a five-coordinated central Mg atom, and that they are not particularly distorted upon protein binding. It also allows a description of the intermolecular interactions assumed by each of these molecules. The keto carbonyl of only one of the **Lhcb4**-bound chls *a* vibrates a 1691 cm^{-1} , corresponding to a keto group free-from-interactions surrounded by a strictly apolar environment.²⁴ One molecule has a keto carbonyl group involved in a strong H-bond with the surrounding apoprotein, contributing at 1655 cm^{-1} , and another has a keto C=O with a medium-to-strong H-bond with its environment, contributing at 1664 cm^{-1} . Among the three other chl *a* molecules present in **Lhcb4**, at least one forms a medium strength H-bond between its keto group and the surrounding amino acids (corresponding to an unresolved component on the lower frequency side of the $1670\text{--}1680\text{ cm}^{-1}$ cluster); the two others either form weak H-bonds with the protein, or have their keto carbonyls free-from-interactions but in a polar environment.²⁴ Resonance Raman spectra of **Lhcb4** have already been presented for excitations in the Soret region.¹⁹ In conditions of chl *a* excitation, similar carbonyl contributions were observed, although the 1655 and 1664 cm^{-1} modes reported here were seen as a single, unresolved band around 1659 cm^{-1} in the previous report. Similar, blue band resonance Raman spectra have been observed for other members of the LHC multigene family, including the bulk complex LHCIIb,^{19,25} as well as a related antenna protein from brown algae, FCP.²⁰ These have all shown a similar distribution of chl *a* carbonyl contributions, consistent with their assumed structural similarity based on sequence homology.⁴ In the case of FCP, this similarity is confirmed in FLN spectra, which indicate a similar position and distribution of chl *a* carbonyl modes when in preresonance with their Q_y transition (not shown).

As documented by Peterman et al.,¹¹ FLN spectroscopy yields information on the red-most chl *a* molecules bound to the protein, which contribute to the fluorescence observed at 4.2 K. From a study of the medium frequencies of these spectra, it may be concluded that these molecules cannot be distinguished from the other chls *a* bound to **Lhcb4** on the basis of their macrocycle conformation. However, in the carbonyl stretching frequencies, these molecules may be identified. Half of the contribution to the FLN spectra (and thus half of the fluorescence excited around 680 nm at 4.2 K) arises from the chl *a* whose keto carbonyl is strongly H-bonded, while the other half arises from at least two chls *a* with keto groups contributing around $1670\text{--}1680\text{ cm}^{-1}$. Interestingly, similar spectra of the bulk PSII antenna complex LHCIIb indicate a more localized emission, with almost all of the fluorescence originating from a single chl molecule (not shown). The exact significance of

this observation is as yet unclear. Obviously it would be of some interest to identify which chl molecules in the three-dimensional model of **Lhcb4** are responsible for these contributions, and therefore for fluorescence emission at 4.2 K. The availability of mutants without each of seven of the eight chlorophylls present¹⁰ should render such an identification possible, as FLN measurements on a mutant lacking a chl molecule, which usually contributes to the emission, will produce significantly different spectra.

Acknowledgment. A.P. was supported by a FEBS postdoctoral fellowship.

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