Pigment Binding Site Properties of Two Photosystem II Antenna Proteins

**A RESONANCE RAMAN INVESTIGATION**

Received for publication, January 27, 2000, and in revised form, May 2, 2000
Published, JBC Papers in Press, May 9, 2000, DOI 10.1074/jbc.M000658200

Andy Pascal‡§, Ulrich Wacker¶***, Klaus-Dieter Irrgang¶**, Peter Horton**, Gernt Renger†, and Bruno Robert‡

*From the §Section of Biophysique des Protéines et des Membranes, Département de Biologie Cellulaire et Moléculaire, Commissariat à l’Energie Atomique and URA 2069, Centre National de la Recherche Scientifique, CE-Saclay, F-91191 Gif-sur-Yvette, France, the ¶Max-Volmer Institut für Biophysikalische Chemie und Biochemie, Technische Universität Berlin, Straße des 17. Juni 135, D-10623 Berlin, Germany, and the Robert Hill Institute, Department of Molecular Biology and Biotechnology, University of Sheffield, Sheffield S10 2UI, United Kingdom

Two light-harvesting proteins associated with photosystem II of higher plants, namely the major antenna complex LHCIIb and the minor Lhcb4 protein (CP29), have been investigated by resonance Raman spectroscopy. One of the two chlorophylls b and up to five of the six chlorophylls a present in Lhcb4 are shown to adopt similar binding conformations to the (presumably) corresponding molecules in LHCIib, whereas at least two chlorophylls in the former protein assume unique conformations relative to the bulk complex. The overall conformation of bound xanthophyll molecules is identical in the two antenna proteins, although some small differences are apparent. The pigment binding properties of these two LHCIIs are discussed, with particular reference to possible structural motifs within this extended family of proteins.

The light-harvesting proteins of higher plants form part of the extended LHC1 gene family, which also includes similar proteins in algae as well as other more distantly related members of unknown function. These antenna complexes are responsible for the absorption of visible light and its transfer, in the form of singlet excitons, to reaction center proteins, where primary charge separation takes place. This series of events constitutes the light reactions of photosynthesis, being the first step in the production of chemical energy and reducing equivalents required by the plant for survival. The antenna of photosystem II in green plants is made up of a number of LHCIIs: the major (trimeric) protein LHCIIb, plus at least three other monomeric proteins named CP29, CP26, and CP24 (1) or, alternatively, LHCl1a, LHCI1c, and LHCI1d (2). Note that a more recent nomenclature for the LHC genes (3) can be extended to their corresponding proteins, so that the latter three (minor antenna complexes would be the Lhcb4–6 proteins, respectively (Lhcb1–3) gene products constitute the polypeptides of the bulk complex, LHCIIb). Over recent years, a large body of biochemical and spectroscopic data has been accumulated on LHCIIb, and attempts have been made to relate these results to the three-dimensional structure obtained to 3.4 Å resolution by electron crystallography (4). At the same time, the apparent similarity of such features for the minor complexes has been explained in terms of a common folding pattern for the protein backbone, with only minor changes in pigment binding properties. Such assumptions are consistent with the high degree of sequence homology observed across the LHC family (5). In particular, the minor antenna Lhcb4 protein (CP29) has been the subject of intense investigation, evidencing a similar but less complicated pigment structure (6, 7). Thus, it is of interest to compare results between Lhcb4 and the major protein LHCIb for measurements giving specific information on pigment structure and binding site properties.

Raman spectroscopy has been widely used for determining the interactions assumed by and the conformation of pigment molecules in photosynthetic proteins of purple and green bacteria (8, 9). More recently, this method was applied to the fucoxanthin-chlorophyll a/c-protein from brown algae (10) and to LHCIIb (11), both of which are members of the LHC family, and the results indicated that such measurements may be even more suited to the study of chlorophyll-containing proteins. Indeed, for such pigment-protein complexes, the resonance phenomenon (whereby the excitation light coincides with an electronic transition of the chromophore) may be used to full effect. This can lead to a 106-fold increase in the Raman signal of the chromophore relative to scattering due to any other molecules present (12). Additionally, as most members of the LHC family contain more than one type of chlorophyll molecule (a and c in the case of fucoxanthin-chlorophyll a/c-protein; a and b for LHCIIb and Lhcb4), the matching of the excitation light with the Soret electronic transition of one or other chl type allows a near-selective excitation of each of these molecules (10). Moreover, it is expected that shifting the excitation wavelength within the Soret transition of one type of chl contained in these proteins may increase the contribution of a subset of these chls. Thus, excitations at 406.7 and 413.1 nm, both located within the Soret transition of chl a, should lead to different Raman contributions of the equivalent chls a bound to the protein. On the other hand, resonance Raman spectroscopy can provide precise information on the molecular conformation and configuration of carotenoid molecules, the spectra of which...
may be selectively obtained when exciting at wavelengths longer than 460 nm. It is thus possible, from a well chosen set of Raman spectra of a protein from the LHC family, to obtain information on the ensemble of cofactors bound to this protein. We present an investigation by resonance Raman spectroscopy of the higher plant antennae, LHClIb and Lhcb4, in an attempt to relate apparent similarities in overall protein structure to pigment binding configurations.

**EXPERIMENTAL PROCEDURES**

LHCIIb was isolated by non-denaturing isoelectric focusing of spinach photosystem II membranes as described previously (13, 14); preparation of the Lhcb4 protein was by column chromatography of Tris-washed photosystem II (7), using a modification of the procedure described by Henrysson et al. (15). Absorption spectra were recorded at 77 K in an SMC-TBT flow cryostat (Air Liquide, Sassenage, France) on a Varian Cary E5 double-beam scanning spectrophotometer.

Protein samples for resonance Raman spectroscopy were concentrated in Microcon-30 concentrators (Amicon) to an A in the Soret region of 50–100 (~1 mg 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 charge-coupled device detector (Spectrum One, Jobin-Yvon, France), as described previously (16). 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).

**RESULTS AND DISCUSSION**

**Resonance Raman Spectra of Bound Carotenoids**—Resonance Raman spectra of the bound xanthophyll molecules in LHClIb and Lhcb4 have been recorded using a wide range of excitations between 457.9 and 530.9 nm (data not shown). Fig. 1 displays such spectra using excitation at 488.0 nm. Three carotenoids are present in these proteins in differing stoichiometries, namely lutein, neoxanthin, and violaxanthin. The spectra obtained are essentially identical to those of \( \beta \)-carotene, exhibiting bands grouped into four regions (called \( \nu_i \) to \( \nu_4 \)). The most intense band, \( \nu_2 \), is indicative of the xanthophyll conformation. This band is present at 1530 cm\(^{-1}\) for all-trans \( \beta \)-carotene, whereas upon trans \( \leftrightarrow \) cis isomerization it is upshifted, this shift being more than 10 cm\(^{-1}\) for 13- or 15-cis \( \beta \)-carotene (17). In addition, the presence of carotenoid molecules possessing a central cis bond (\( i.e. \) either 13- or 15-cis) generally results in the appearance of additional bands, arising from modes allowed by the lower symmetry of these molecules. These bands are absent in both the spectra in Fig. 1, and we did not observe them in any other resonance conditions (data not shown), whereas the \( \nu_2 \) band is at 1530 cm\(^{-1}\) for both LHClIb and Lhcb4 and is not inhomogeneously broadened (again, this is the case for all excitation conditions, though see below). This indicates that all xanthophylls in these two proteins have a central trans configuration. It must be noted that the same conclusions could be drawn whether LHClIb was extracted from dark-adapted or light-adapted organisms. In particular, no evidence was found for the presence of the 15-cis carotenoid forms hypothesized by Gruszecki et al. (18) using HPLC analysis. The presence of neoxanthin in the 9-cis configuration (19) could, however, be responsible for excitation-dependent variations in \( \nu_2 \) of up to 5 cm\(^{-1}\) (not shown).

Although the carotenoid spectra of the two proteins are very similar, some small differences can be observed. The bands discussed below are indicated in Fig. 1 by dotted lines. Around 1200 cm\(^{-1}\) in the \( \nu_2 \) region, the minor bands at 1176 and 1203 cm\(^{-1}\) are more intense for Lhcb4. Bands in the \( \nu_2 \) region (1000 cm\(^{-1}\)) show differing relative intensities when the two proteins are compared, and an additional mode occurs at 1028 cm\(^{-1}\) in Lhcb4. In the \( \nu_4 \) region around 960 cm\(^{-1}\), the Lhcb4 protein exhibits a small, single band with a shoulder, as is the case for isolated carotenoids (17), whereas for LHClIb this region is much more complicated, involving the presence of at least four components. In this region, out-of-plane bending modes of C-H bonds contribute, and the presence of a number of components reveals slight deviations from the planar geometry of one or more carotenoids bound to LHClIb. The trimeric protein LHClIb binds two lutein molecules, one neoxanthin, and about one-third of a molecule of violaxanthin per monomer (\( i.e. \) 6:3:1 per trimer), whereas Lhcb4 (which is monomeric) binds the same three carotenoids but in substoichiometric amounts, two molecules per monomer in total (7, 14, 20). Thus, although the precise structural significance of these differences is difficult to predict without intensive in vitro studies of the three xanthophylls present, the differences indicate either an altered pigment structure for at least one of the carotenoids, an increase in the contribution of one of them to the spectra, or (in the case of LHClIb) the appearance of modes corresponding to the additional carotenoids present.

**Resonance Raman Spectra of Bound chls a and b**—Shown in Fig. 2 are 77 K absorption spectra of LHClIb (solid line) and Lhcb4 (dashed line). As described previously (6, 7) these two spectra are globally similar, although the higher pigment content of LHClIb is reflected in the presence of a number of additional components within the absorption bands of chls a and b, the most evident of them being located on the blue side of thechl a \( Q_y \) band (around 670 nm) and throughout the chl b \( Q_y \) transition. The significance of this spectral similarity with respect to common structural features of the LHC proteins has been discussed in some length in the literature (see, for example, Refs. 6 and 7). It is of interest to see whether these similarities in absorbance properties are reflected in resonance Raman spectra of the bound chlorophyll molecules for the two proteins: \( i.e. \) whether it corresponds to common structural motifs of their chl binding sites.

Resonance Raman spectroscopy can provide important structural information on the binding site properties of (bacterio)chlorin molecules (8). For excitation conditions within the Soret electronic transition, it has been shown that resonance Raman spectra of chlorophyll a contain a number of bands that may be...
used for determining accurately the conformation of these molecules in vitro, as well as in their protein binding pocket (21, 22). As the molecular conformation of chl depends in particular on the number of axial ligands on the central magnesium atom, the coordination number of this atom may be deduced from the frequency of some of these bands (8). This is notably the case for the methine bridge stretching modes; this band, observed at about 1600 cm$^{-1}$ when the central magnesium is six-coordinated, is up-shifted to 1610–1615 cm$^{-1}$ for five-coordinated magnesium. In resonance Raman spectra of chl b molecules, this band is extremely weak at best (23, 24), and thus, determining the coordination state of the central magnesium proves much more difficult. There is an intense band in resonance Raman spectra of chl b arising from C$_a$=C$_b$ stretching modes (23) that is sensitive to the molecular core size. 2 This band is located at 1530 cm$^{-1}$ when the magnesium atom of these molecules is six-coordinated, and near 1540 cm$^{-1}$ when it binds one axial ligand only. However, proteins from the LHC family generally bind carotenoid molecules, the presence of which results, in resonance Raman spectra excited at 420–550 nm, in a less selective excitation of chl b molecules (25). On the other hand, an increase in the dielectric constant of the immediate environment (i.e., an increase in polarity) results in a downshift of 5–10 cm$^{-1}$.

The Soret electronic transitions of chls $a$ and $b$ differ by more than 1000 cm$^{-1}$, their maxima being at around 430 and 450 nm, respectively. Due to these different positions, the resonance Raman spectra of chls $a$ and $b$ can be observed selectively by making use of the appropriate excitation wavelength. We have used the 457.9 nm line of an Argon laser to obtain selectively the chl $b$ Raman signal (23). As discussed by Feiler et al. (24), in these conditions of excitation, the resonance is mainly with the $B_a$ component of this electronic transition, thus resulting in a high activity of the formyl carbonyl stretching modes, whereas the keto carbonyl stretching modes are low in intensity. The latter may be more easily observed using the 441.6 nm of a helium-cadmium laser; however, these excitation conditions result in a less selective excitation of chl $b$, and chl $a$ molecules then also contribute to the resonance Raman spectra (26). Shown in Fig. 3 are Raman spectra of LHCIIb and Lhcb4 in the 1580–1720 cm$^{-1}$ region obtained using both of these excitations. Although the two sets of spectra differ markedly, some similarities can be discerned. The spectra for Lhcb4 are fairly simple, exhibiting mainly three bands at 1615, 1630, and 1649 cm$^{-1}$. The bands at 1630 and 1649 cm$^{-1}$ may be readily attributed to formyl C=O stretching modes. These two bands thus evidence two chl $b$ populations having formyl groups vibrating at different frequencies: one, at 1649 cm$^{-1}$, corresponding to a formyl group either involved in a weak molecular interaction or in a polar environment, and another, at 1630 cm$^{-1}$, indicating a formyl C=O involved in a medium strength hydrogen bond with its peptide environment ($\sim$14 kJ/mol) (25). Given that pigment analysis indicates only two chl $b$ per Lhcb4 poly peptide (7, 27), it can be assumed that the two chl $b$ molecules correlate with these two populations observed in the Raman spectra.

The attribution of the 1615 cm$^{-1}$ band requires comparison of the chl $b$ resonance Raman spectra of Lhcb4 with those of other pigment-binding sites.

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2 A. Pascal, unpublished results.
LHClIib. In the spectra of the latter complex, the relative intensity of this band to that of the bands arising from the formyl carbonyl stretching modes is much lower. As the relative chl a/b stoichiometry in the Lhcb4 protein is more than twice that in LHClIib (14, 28), we conclude that the 1615 cm\(^{-1}\) band in these spectra arises from preresonance contributions of the bound chl a molecules. Comparison of these spectra reveals some important features. In Raman spectra of LHClIib, three main bands are observed in the region corresponding to stretching modes of formyl carbonyl groups. The principal band around 1630 cm\(^{-1}\) corresponds well with the band at the same position in Lhcb4 for excitation at 441.6 nm, although the increase in width of the same band at 457.9 nm excitation indicates additional contributions in the 1620–1625 cm\(^{-1}\) region. Additional formyl stretching mode contributions are observed at 1640 and 1655 cm\(^{-1}\), which are not exhibited by Lhcb4. The second band in the Lhcb4 protein (at 1650 cm\(^{-1}\)) does not appear to have an equivalent mode in spectra of LHClIib, indicating that the intermolecular interactions assumed by this pigment in Lhcb4 are likely to be altered in the equivalent chl b binding site in LHClIib. This difference between the carbonyl modes of two apparently equivalent chlorophylls in the two proteins may arise from a simple change in hydrogen bonding to the formyl group, or alternatively could reflect a change in the polarity of the binding pocket.

An estimation can be made of the number of pigment molecules contributing to each band observed in the Raman spectra. It should be noted that the relative intensities of Raman bands do not depend on the stoichiometry of the corresponding populations alone, and in particular, the extent of resonance with the excitation line for each population will also have an effect on its respective contribution to the spectra. Therefore, this estimation may be somewhat inaccurate, to plus or minus one chl for the more intense bands, but is nevertheless of interest in evaluating the spectra. Given a chl b stoichiometry of five to six for monomeric LHClIib (4, 29), and considering that the 1655 cm\(^{-1}\) band arises from at least one chl b, the main band at 1625–1630 cm\(^{-1}\) must correspond to at least two, and probably three, chl b molecules, with one or two additional chls b having their formyl carbonyl groups vibrating around 1640 cm\(^{-1}\). Thus, three to five chlorophylls b in LHClIib possess a medium to strongly bound formyl C=O.

Bands in the 1660–1690 cm\(^{-1}\) region for both proteins correspond to the keto groups of bound chlorophyll molecules. In spectra obtained with 457.9 nm excitation, it may be noted that the bands arising from the stretching modes of the keto carbonyl groups of chl b exhibit only low intensity. As discussed above, this excitation line induces a resonance mainly with the \(B_2\) component of the Soret electronic transition, thus favoring the contribution of stretching modes of the formyl carbonyl of these molecules, and not of the keto carbonyl group (24). The increase in intensity of these bands for 441.6 nm excitation may reflect an increase in chl a resonance and/or the location of this wavelength within the \(B_2\) absorption band of chl b. Given the similarity of these spectra with those in Fig. 4 (discussed below), it seems likely that they mainly contain contributions of chls a.

Resonance Raman spectra in conditions favoring chl a excitation (i.e. 406.7 and 413.1 nm) are shown in Fig. 4 for the two antenna proteins. Modes corresponding to the methine bridges appear at around 1612 cm\(^{-1}\) for both proteins and do not appear inhomogeneously broadened. This indicates that the central magnesium atoms of most if not all chl a molecules are five-coordinated in each case. In the region corresponding to bands arising from the stretching modes of the keto carbonyl groups, Lhcb4 exhibits a broad band around 1665–1680 cm\(^{-1}\), together with a shoulder at 1659 cm\(^{-1}\). The intensity of the latter, along with the known chl a stoichiometry of six (7, 27), probably indicates the presence of two chls a with their keto carbonyls vibrating at 1659 cm\(^{-1}\) and thus involved in a strong intermolecular hydrogen bond. Among the four remaining chls a, three probably contribute in the 1665–1675 cm\(^{-1}\) region. The keto C=O groups of these molecules thus take part in medium strength hydrogen bonds. The sixth chl a molecule may be responsible for a further widening of this main band toward higher frequencies at 406.7 nm excitation; it would thus have a free-from-interaction keto carbonyl in a polar environment, vibrating near 1685 cm\(^{-1}\). (Note the possible inaccuracies in these estimations discussed above.)

Although LHClIib is believed to contain only one or two more chl a molecules than Lhcb4, the region of the keto carbonyl stretching modes of this protein in these excitation conditions exhibits much more structure than that of the Lhcb4 protein. No fewer than five distinct components may be observed, at 1659, 1670, 1678, 1685, and 1700 cm\(^{-1}\). The component at 1659 cm\(^{-1}\) is very similar to that observed in Lhcb4; in particular, it appears to gain intensity in resonance conditions at 406.7 nm for both proteins. Some of the modes contributing to the central keto carbonyl region (1665–1680 cm\(^{-1}\)), particularly those more intense for excitation at 413.1 nm, may correspond to those of Lhcb4 around 1670 cm\(^{-1}\). However, the main band in the keto stretching mode region in LHClIib, observed at 1685 cm\(^{-1}\), does not exist in Lhcb4 spectra. Based on seven or eight chlorophylls a per monomer (4, 29), this region may be analyzed as follows. There are probably two chls a whose keto carbonyls are strongly hydrogen bonded, vibrating at 1659 cm\(^{-1}\), and corresponding to two chls a in Lhcb4; three whose keto C=O groups participate in medium to strong hydrogen bonds (1670–1680 cm\(^{-1}\), one or two of which may correspond to chls a in Lhcb4; and probably two chl a molecules whose keto groups are free from interactions but in a polar environment (1685 cm\(^{-1}\)), as for one apparent chl a molecule in Lhcb4. The component at 1700 cm\(^{-1}\) arises from the stretching mode of keto carbonyl groups free from intermolecular interactions, located in a rather apolar environment; this band corresponds to one chlorophyll molecule in LHClIib (no equivalent in
Lhcb4). The possible variation in these numbers due to the inaccuracy discussed above is reflected in the range given for some of the contributions.

The Raman band arising from strongly bonded keto carbonyls in Lhcb4 (at 1659 cm\(^{-1}\)) is also present in LHCIb. Moreover, it shows a similar behavior with respect to resonance conditions in both proteins, i.e., its intensity increases at 406.7 nm excitation. It thus appears that in both of these proteins, a pair of chl molecules is present, sharing similar protein environments, as well as absorption properties in the Soret electronic transition. A similar band has also been observed in a related antenna protein from brown algae (fucoxanthin-chl a/c-protein; 10), having the same frequency and the same behavior relative to excitation conditions, which was similarly attributed to two chl a molecules. It should be noted that this population has a strongly bonded keto C=O group, and it is unlikely that such an interaction would be reproduced by chance. It is therefore tempting to conclude that this band corresponds to chlorophylls a, assuming the same conformation in equivalent chl binding pockets in these three LHC proteins, and that they represent a common structural motif of this extended gene family. Additional measurements on other LHCs will confirm whether this motif is conserved across this phylogenetically diverse set of proteins.

Conclusions—Carotenoid resonance Raman spectra indicate similar configurations of these pigments in the two photosystem II antenna proteins, Lhcb4 and LHCIb. All xanthophylls present exhibit a central trans conformation, as has already been described for the two central xanthophylls L1 and L2 in the atomic model (4). Some small differences in the spectra for the two proteins may reflect, on the one hand, the presence in LHCIb of additional bound carotenoid molecules that appear to be in a less planar configuration, giving rise to additional modes in the \(v_2\) region. On the other hand, bands in the spectra of Lhcb4 that do not appear for LHCIb either indicate slightly different properties of equivalent carotenoid binding sites in the two proteins or that the same modes are present in spectra of LHCIb but are masked by contributions of the additional xanthophylls present.

The chl carbonyl bands in LHCIb and Lhcb4 are summarized in Table I. Chlorophyll b spectra for Lhcb4 exhibit two formyl stretching modes at 1630 and 1649 cm\(^{-1}\), indicating two chl b populations that correspond to the two chl b molecules present in this protein. LHCIb spectra show one main band at 1630 cm\(^{-1}\) corresponding to two or three chlorophyll molecules, one of which is probably equivalent to a chl b in Lhcb4, plus bands at 1640 and 1655 cm\(^{-1}\). The chl b molecule in LHCIb equivalent to that in Lhcb4 having a 1649 cm\(^{-1}\) formyl vibration has an altered chl binding pocket. Two explanations could account for this difference. If the 1649 cm\(^{-1}\) band in Lhcb4 is due to a free-from-interaction formyl group in a polar environment, the equivalent chlorophyll in LHCIb may be present in a less polar environment and would thus have its formyl carbonyl stretching mode at 1655 cm\(^{-1}\). Alternatively, the chl molecule in LHCIb may be involved in a hydrogen bond at the position of its formyl group, which is not the case in Lhcb4, so that its carbonyl stretching mode would be shifted down to lower frequencies (i.e., 1640 or even 1630 cm\(^{-1}\)). Clearly the two chl b molecules present in the LHCIb atomic model (4) but not in the model of Lhcb4 (30), i.e., b1 and b2, correspond to the two additional chl b formyl vibrators seen for the former protein around 1625–1630 cm\(^{-1}\) (Table I).

Chlorophyll a resonance Raman spectra of Lhcb4 indicate several different populations of molecules. Two chlorophylls a have a keto carbonyl group vibrating at 1659 cm\(^{-1}\), and this group is thus involved in a strong hydrogen bond with its environment; the same two chls are evident in LHCIb, as well as the more distantly related fucoxanthin-chlorophyll a/c-protein complex from brown algae (10), and are probably a common feature of LHCs. It seems possible that these two chls correspond to two of the four centrally located molecules observed in the atomic model of LHCIb (called a1, a2, a4, and a5; Ref. 4), due to the high degree of sequence homology in this region across the LHC family. Around three chlorophyll a molecules in the Lhcb4 protein have their keto C=O stretching modes around 1665–1675 cm\(^{-1}\), indicating carotenyl groups involved in medium strength interactions. Probably, two of these three chlorophylls are equivalent to two of the three seen in LHCIb spectra in this region (note that the difference in spectral shape between the two proteins indicates that not all three chls can be equivalent). The keto group of the sixth chl a in Lhcb4 vibrates at 1685 cm\(^{-1}\), indicating that this carbonyl is probably free from interactions but is in a polar environment. In LHCIb, two chls a are indicated with their keto carboxyls in such an environment, and it would be expected that one of these is in an equivalent position in the three-dimensional structure. The final chl a molecule in LHCIb, which does not have an equivalent in Lhcb4, has a free-from-interaction keto C=O group in a rather apolar environment (vibrating at 1700 cm\(^{-1}\)). Again, the two chls a present in LHCIb but absent for Lhcb4 (a6 and a7; Ref. 4) should be responsible for the carbonyl bands observed only in spectra of the bulk complex, at 1685 and 1700 cm\(^{-1}\) (Table I). Finally, the central magnesium atom of most or all chl a molecules in both proteins are five-coordinated. This confirms the present structural data on LHCIb (4); although ligating residues were identified for only 8 of the 12 chlorophylls resolved in the atomic model of this protein, none were found to have two axial ligands.

The next step in defining the pigment binding properties of these two antenna proteins is clearly to attribute the individual Raman bands to specific binding sites. Reconstitution of LHC proteins from recombinant polypeptides expressed in Escherichia coli with isolated pigments (27, 31) allows the generation of mutant proteins in which pigment binding properties have been altered. Lhcb4 reconstituted from wild type polypeptides assumes the same pigment binding conformation as the native protein, as measured by resonance Raman spectroscopy.3 We are currently analyzing mutant Lhcb4 in which each chlorophyll molecule has been individually eliminated by mutation of its coordinating amino acid (30). This will provide direct evidence for the identity of Raman bands for each chl in the three-dimensional structure. We envisage similar measurements on reconstituted LHCIb (32), allowing us to verify (among other points) the attribution of identical Raman bands to corresponding positions in the two proteins. This will

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3 A. Pascal, D. Sandona, R. Bassi, and B. Robert, unpublished results.
also open the way to production of mutants in which pigment-protein interactions have been engineered (such as alteration of hydrogen bonding residues) so that the effect of these changes on physicochemical properties (e.g. absorption) can be determined.

REFERENCES

1. Bassi, R., Rigoni, F., and Giacommeti, G. M. (1990) Photochem. Photobiol. 52, 1187–1206
2. Thornber, J. P., Peter, G. F., Chitnis, P. R., Nechustai, R., and Vainstein, A. (1988) in Light-Energy Transduction in Photosynthesis: Higher Plant and Bacterial Models (Stevens, S. E., Jr., and Bryant, D. A., eds) pp. 137–154, American Society of Plant Physiology, New York
3. Jansson, S., Pichersky, E., Bassi, R., Green, B. R., Ikeuchi, M., Melis, A., Simpson, D. J., Spangfort, M., StaeHELIN, L. A., and Thornber, J. P. (1992) Plant Mol. Biol. 10, 242–253
4. Kuhlbrandt, W., Wang, D. N., and Fujiyoshi, Y. (1994) Nature 367, 614–621
5. Pichersky, E., Subramaniam, R., White, M. J., Reid, J., Aebi, R., and Green, B. R. (1991) Mol. Gen. Genet. 227, 277–284
6. Jennings, R. C., Bassi, R., Garlaschi, F. M., Dainese, P., and Zucchelli, G. (1993) Biochemistry 32, 3203–3210
7. Pascal, A., Gradinaru, C., Wacker, U., Peterman, E., Calkoen, F., Irgang, K.-D., Horton, P., Renger, G., Van Grondelle, R., Robert, B., and Van Amerongen, H. (1998) Eur. J. Biochemistry 262, 817–823
8. Robert, B. (1996) in Biophysical Techniques in Photosynthesis (Amesz, J., and Hoff, A. J., eds) pp. 161–176, Kluwer Academic Publisher, Dordrecht, Netherlands
9. Robert, B. (1999) in The Photochemistry of Carotenoids (Frank, H. A., Young, A. J., Britton, G., and Cogdell, R. J., eds) pp. 189–201, Kluwer Academic Publishers, Dordrecht, Netherlands
10. Pascal, A. A., Caron, L., Rousseau, B., Lapouge, K., Duval, J.-C., and Robert, B. (1998) Biochemistry 37, 2450–2457
11. Ruban, A. V., Horton, P., and Robert, B. (1995) Biochemistry 34, 2333–2337
12. Albrecht, A. C. (1961) J. Chem. Phys. 34, 1476–1484
13. Dainese, P., Hoyer-Hansen, G., and Bassi, R. (1990) Photochem. Photobiol. 51, 693–703
14. Ruban, A. V., Young, A. J., Pascal, A. A., and Horton, P. (1994) Plant Physiol. 104, 227–234
15. Henrysson, T., Schröder, W. P., and Akesson, H.-E. (1989) Biochim. Biophys. Acta 977, 301–308
16. Sturgis, J. N., and Robert, B. (1984) J. Mol. Biol. 235, 445–454
17. Koyama, Y., Takii, T., Saiki, K., and Tsukida, K. (1983) Photochem. Photobiol. 5, 139–150
18. Gruszecki, W. I., Matula, M., Ke-chi, N., Koyama, Y., and Krupa, Z. (1997) Biochim. Biophys. Acta 1319, 267–274
19. Liaaen-Jensen, S. (1990) New J. Chem. 14, 747–759
20. Giuffra, E., Zhu, C., Sandona, D., Croce, R., Cugini, D., Garlaschi, F. M., Bassi, R., and Jennings, R. C. (1997) Biochemistry 36, 12984–12993
21. Fujimura, M., and Tasumi, M. (1986) J. Phys. Chem. 90, 5646–5650
22. Nave, A., Lapouge, K., Sturgis, J. N., Hartwig, G., Simonin, J., Scheer, H., and Robert, B. (1997) J. Raman Spectrosc. 28, 599–604
23. Lutz, M. (1984) in Advances in IR and Raman Spectroscopy (Clark, R. J. H., and Hester, R. E., eds) pp. 211–390, John Wiley & Sons, New York
24. Feiler, U., Aloufi, D., Lutz, M., and Robert, B. (1994) Photosynth. Res. 41, 175–180
25. Zhadorozhnyi, B. A., and Ishchenko, I. K. (1965) Opt. Spectrosc. (English translation) 19, 306–308
26. Lutz, M. (1977) Biochim. Biophys. Acta 460, 408–430
27. Giuffra, E., Cugini, D., Croce, R., and Bassi, R. (1996) Eur. J. Biochemistry 238, 112–120
28. Bassi, R., Pineau, B., Dainese, P., and Marquardt, J. (1993) Eur. J. Biochemistry 212, 297–303
29. Thornber, J. P., Peter, G. F., Morishige, D. T., Gómez, S., Anandan, S., Welty, B. A., Lee, A., Kerfeld, C., Takeuchi, T., and Freis, S. (1993) Biochem. Soc. Trans. 21, 15–18
30. Bassi, R., Croce, R., Cugini, D., and Sandona, D. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 10056–10061
31. Plumley, F. G., and Schmidt, G. W. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 146–150
32. Remelli, R., Varotto, C., Sandona, D., Croce, R., and Bassi, R. (1999) J. Biol. Chem. 274, 33510–33521