Probing the binding sites of exchanged chlorophyll *a* in LH2 by Raman and site-selection fluorescence spectroscopies

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Abstract In this work we have selectively released the 800 nm absorbing bacteriochlorophyll *a* molecules of the LH2 protein from the photosynthetic bacterium *Rhodopseudomonas acidophila*, strain 10050, and replaced them with chlorophyll *a* (Chl*a*). A combination of low-temperature electronic absorption, resonance Raman and site-selection fluorescence spectroscopies revealed that the Chl*a* pigments are indeed bound in the B800 binding site; this is the first work that formally proves that such non-native chlorins can be inserted correctly into LH2.

Key words: Bacteriochlorophyll; Chlorophyll; Fluorescence line-narrowing spectroscopy; Raman spectroscopy; Photosynthesis

1. Introduction

In free solution, the Q_y absorption maximum of monomeric bacteriochlorophyll *a* (Bchl*a*) is located at ~770 nm and is red-shifted to ~800 nm or ~850 nm when bound to the light-harvesting complex 2 (LH2). Relatively simple calculations have shown that the wavelength of absorption by any given bound Bchl*a* molecule is determined by its specific micro-environment and is affected by both pigment–pigment and pigment–protein interactions [1]. Resonance Raman (RR) spectroscopy has proved invaluable for describing the molecular mechanisms in tuning the electronic properties of Bchl*a* molecules in the LH proteins from purple bacteria [2]. In fact, it has been especially useful when combined with site-directed mutagenesis in the bacterium *Rhodobacter sphaeroides* [3–7].

The determination of the crystal structure at 140 K of the LH2 membrane protein from *Rhodopseudomonas acidophila* 10050 to a resolution of 2.1 Å has revealed the arrangement of the pigments within that complex [8,9]. The light-harvesting pigment cofactors, Bchl*a* and carotenoid molecules, are non-covalently attached to an apoprotein scaffold. It has a nonameric ring structure. Each monomeric unit contains an α- and β-apoprotein, one carotenoid (rhodopin glucoside) [10] and three Bchl*a* molecules. The nine α-apoproteins form a hollow cylinder with the nine β-apoproteins arranged radially outside [11]. The complex contains two discrete pools of Bchl*a* molecules. Eighteen of the Bchl*a* molecules are sandwiched between the α- and β-apoproteins and form a ring of nine overlapping Bchl*a* dimers. These molecules absorb prominently at 850 nm and are known as the Bchl*a*-B850 molecules. The other nine Bchl*a* molecules lie towards the cytoplasmic side of the membrane and are located between the transmembrane helices of the β-apoproteins. These pigments absorb at 800 nm, which most often exhibit spectroscopic properties typical of monomeric Bchls not involved in strong excitonic interactions [12] and are denoted Bchl*a*-B800.

Recently, it has been shown that Bchl*a*-B800 molecules within the LH2 protein from *Rps. acidophila* can be selectively released and replaced with native Bchl*a* (esterified with phytol) with near 100% efficiency [13]. By using Raman spectroscopy in resonance with the Soret transitions of the Bchl*a* molecules, it was shown that these molecules are correctly bound within the reconstituted complex [14]. When the conserved βHis21 residue was changed for a Ser in the related bacterium *Rb. sphaeroides* it resulted in a Bchl*a*-B800-deficient LH2 with perturbations in the electrochromic behaviour of carotenoid molecules [15,16]. This did not interfere with energy transfer between the carotenoids and Bchl*a*-B850 pigments nor did it cause significant structural changes to the B850 site [16]. Similar results were obtained when the Bchl*a*-B800 was biochemically extracted from the LH2 from *Rps. acidophila*. [13,14,17].

More recently it was shown that the B800 binding sites can be reconstituted with a range of modified (bacterio)chlorin molecules, including chlorophyll *a* (Chl*a*) [17]. Using these modified complexes, the B800 → B850 energy transfer mechanism was investigated to evaluate the Förster theory [18]. Although suggested by circular dichroism measurements [13], it has still not been formally proved that the reconstituted LH2s containing non-native (bacterio)chlorin molecules in the B800 sites are correctly incorporated into the protein. This is especially true for those pigments, such as Chl*a*, that are not naturally found in purple bacteria. Few spectroscopic methods, e.g. fluorescence line narrowing (FLN) and RR, can provide such conclusive information.

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Abbreviations: (B)Chl*a*, (bacterio)chlorophyll *a* (esterified with phytol); (B)ChlX, (bacterio)chlorophyll absorbing at X nm; B800, B850, binding sites for Bchl*a* absorbing at 800 and 850 nm, respectively; FLN, fluorescence line narrowing; LH, light-harvesting; *Rps.*, *Rhodopseudomonas*; *Rb.*, *Rhodobacter*; RR, resonance Raman; THF, tetrahydrofuran

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FLN spectroscopy gives, in essence, vibrational information similar to that obtained by RR [19]. An advantage with FLN is that it is more selective than RR in that it gives access to vibrational information only on the red-most absorbing chromophores involved in the fluorescence process, i.e. the terminal emitters of the protein. Thus, FLN has been very useful in obtaining related information in chlorophyll-containing proteins [19–21] where information obtained by RR spectroscopy is more complicated to interpret, due to the numerous overlapping chromophore environments.

In this work, we compare FLN and RR spectra obtained at 10 K from the LH2 protein from *Rps. acidophila* where the B800 sites do not contain the native Bchl*a* but rather have Chl*a* molecules instead. We demonstrate for the first time that non-native chlorophyll molecules can be correctly inserted into modified LH2s, and that the chlorin pigments are subject to globally the same localised environment as the native Bchl*a* pigments.

2. Materials and methods

2.1. Isolation of LH2 proteins

Liquid cultures of *Rps. acidophila* 10050 were grown anaerobically at 30°C in Pfenning's medium [22]. Cells were harvested by centrifugation. Photosynthetic membranes were then isolated by rupturing the whole cells in a French press. Finally, the membranes were solubilised using LDAO and LH2 proteins purified as previously described [13,17].

2.2. Pigment exchange procedure

The pigment exchange protocol has been extensively described elsewhere [13,17]. In short, all of the Bchl*a*-B800 molecules were released from their binding sites by incubating a LH2 sample in buffer containing Triton BG-10 at a pH of 4.75 at 30°C for 1 h. B850 complexes lacking Bchl*a*-B800 were then purified by ion exchange chromatography using phosphocellulose as the absorbent. Optimal reconstitution of the B800 sites was achieved by incubating the B850-only sample with a three-fold excess of Chl*a* for 2 h at a pH of 8 and at room temperature.

2.3. Spectroscopy

During spectroscopic measurements the temperature was maintained at 10 K (unless specifically mentioned) in a SMC-TBT flow cryostat (Air Liquide, Sassenage, France) cooled with a flow of helium gas. The absorption spectra were collected using a Cary E5 double-beam scanning spectrophotometer (Varian Plc, Sydney, Australia). The RR and FLN spectra were recorded with a 90° geometry using a Jobin Yvon U1000 spectrophotometer equipped with a N_2-cooled back-thinned charge-coupled device detector (Jobin Yvon Spectrum ONE). The (bacterio)chlorin molecules were excited at 363.8 and 413.1 nm using a Coherent Innova 100 argon laser. A Coherent Innova 90 krypton laser was used to probe the Chl*a* molecules at 676.4 nm. The FLN and RR spectra were obtained from the same protein sample. Absorption spectra were taken before and after vibrational measurements to verify sample integrity. Where appropriate, the background fluorescence was removed using GRAMS32 Spectral Notebase (Galactic Industries, Salem, NH, USA).

3. Results

The 10 K electronic absorption spectrum of the Q_x- and Q_y-Bchl*a* electronic transition regions of the native LH2 protein is shown in Fig. 1a. At this temperature, the Q_y absorption transitions of the Bchl*a*-B800 and Bchl*a*-B850 molecules have maxima at 803 and 869 nm respectively. The absorption band at 590 nm is attributed to the Q_x transitions of the three Bchl*a* molecules in each α/β-heterodimer. The absorption spectrum of the LH2 reconstituted with Chl*a* in the B800 site is shown

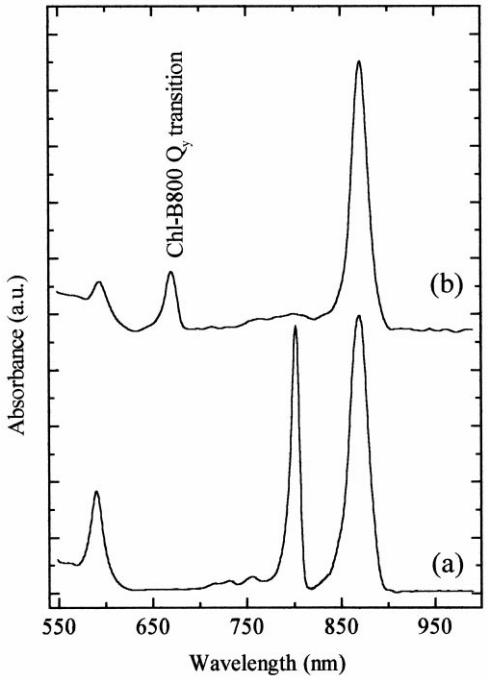

Fig. 1. Low-temperature (10 K) absorption spectra of (a) native and (b) Chl-B800-reconstituted LH2 proteins from *Rps. acidophila* 10050.

in Fig. 1b. The position of the peak of the Q_y absorption transition of the Bchl*a*-B850 molecules is red-shifted by some 26 cm^{-1} to 871 nm. Residual absorption intensity between 750 and 800 nm corresponds to only a few per cent of that observed in the native complex. The position of the Q_y absorption band of the Chl molecules is at 670 nm in the Chl-B800-reconstituted LH2. In this protein, the intensity of the Q_x-Bchl transition is reduced somewhat. This is due to the removal of one third of all the Bchl*a* pigments, and their replacement with Chl*a* which has its Q_x-Chl transition slightly to the blue of that of Bchl*a*, and accounts for the slight increase in intensity in this region.

Shown in Fig. 2 are the FLN spectrum (Fig. 2a, $\lambda_{ext} = 676.4$ nm) and the RR spectrum of the Chl*a* molecules, under resonance conditions with the Soret electronic transition (Fig. 2b, $\lambda_{ext} = 413.1$ nm), in the Chl*a*-reconstituted LH2 protein. The native and reconstituted LH2s were also studied by RR spectroscopy (not shown), under resonance conditions ($\lambda_{ext} = 363.8$ nm) with the Soret electronic transition of the Bchl*a* pigments bound to these two complexes. Without subtracting any underlying fluorescence baseline, it is obvious that RR and FLN give essentially the same vibrational modes for the Chl-containing protein, and for some of these bands the signal-to-noise level is superior under FLN conditions. Shown in Table 1 is a summary of the major FLN and RR vibrational modes of Chl*a* obtained from the Chl-B800-reconstituted LH2.

Shown in Fig. 3 are the RR and FLN shifts in the high-frequency carbonyl region (1575–1725 cm^{-1}) for the native (Fig. 3a) and the Chl-B800-reconstituted LH2 (Fig. 3b). The RR spectrum of the native LH2 protein excited at 363.8 nm is dominated by a strong signal at 1610 cm^{-1} arising from the stretching modes of the Bchl*a* methine bridges. This band has been cropped in Fig. 3a,b to permit an enhanced view of the

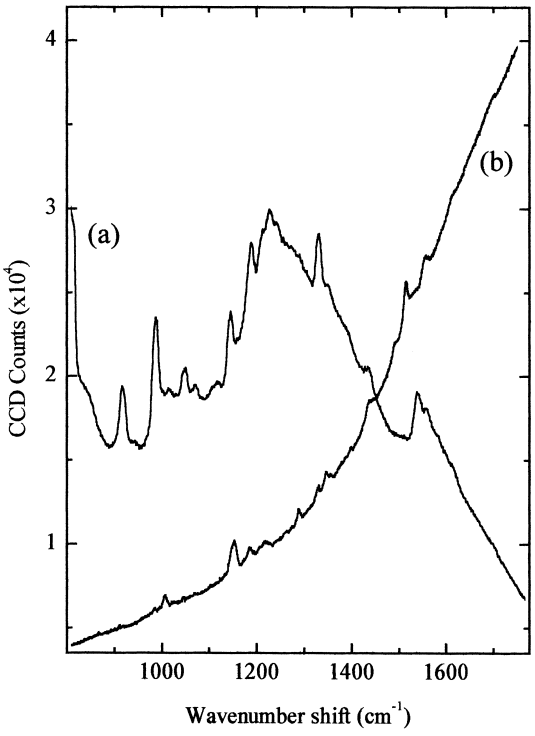

Fig. 2. Comparison of the signal-to-noise ratio in the FLN (a) and RR (b) spectra of the Chl chromophores in the Chl-B800-reconstituted LH2 from *Rps. acidophila* measured at 10 K.

less intense Raman modes in the 1620–1700 cm^{-1} range. The RR spectrum of the native LH2 complex is identical to that previously reported [14]. The bands at 1623 and 1696 cm^{-1} have been ascribed to the H-bonded and free-from-interaction acetyl and keto carbonyl of Bchl*a*-B800 molecules, respectively [23–25]. Both the acetyl and the keto groups of the Bchl*a*-B850 contribute at ca. 1625 cm^{-1} and 1670 cm^{-1}, respectively [2,24]. With respect to that of the native LH2, the RR spectrum of the Chl-B800-reconstituted LH2 excited at 363.8 nm also shows a band at 1610 cm^{-1} (Fig. 3b). As may be expected, the mode at 1623 cm^{-1} shifts to 1624/1625 cm^{-1} upon the removal of the Bchl*a*-B800 molecule [14]. It is worth noting that the RR spectrum of Chl-B800-reconstituted LH2 contains a weak band at ca. 1695–1699 cm^{-1}, which is not observed in the spectra of complexes devoid of the Bchl*a*-B800 molecule [14]. This weak band may arise from the keto carbonyl group of the Chl*a* molecule bound to the Chl-B800-reconstituted LH2, excited in post-Soret resonance. However, experiments on isolated Chl*a* molecules excited at 363.8 nm would be necessary to firmly establish this attribution.

In order to observe the vibrational contributions of Chl*a* pigments bound to these complexes, RR experiments were performed with excitation in resonance with the Soret transition of Chl*a* (Fig. 3c,d, $\lambda_{ext} = 413.1$ nm) [26]. The high-frequency region of spectra obtained in these conditions is shown, after removal of their fluorescence background. These conditions of excitation also correspond to pre-resonance conditions with the Soret transition of Bchl*a* molecules, however, the signal arising from these molecules is expected to be weaker that those of Chl*a*, and Raman spectra of Bchl*a* molecules obtained in these conditions, arising essentially from resonance with the B_x component of the Soret transition, do not contain contributions in the carbonyl region (Fig. 3c). However, in the Chl-B800 LH2 (Fig. 3d) the band at 1696 cm^{-1} corresponds to the stretching modes of the C_9-keto grouping of the Chl*a* bound to the complex, whilst the 1611 cm^{-1} band may arise from both its Chl*a* and Bchl*a* pigments. The position of the band arising from carbonyl stretching mode suggests that it is free from interactions [2]. However, because of the extensive fluorescence background underlying the Chl-B800-reconstituted LH2 spectrum, the signal-to-noise ratio decreases in the higher-frequency range. Due to the rather poor signal-to-noise ratio, the band at 1696 cm^{-1} is quite broad and unresolved.

Table 1
Comparison of vibrational modes of the Chl chromophores of the Chl-B800-reconstituted LH2 from *Rps. acidophila* measured by FLN and RR spectroscopies

Mode	Chl*a* in tetrahydrofuran (THF) (six-coordinated; FLN, $\lambda_{ext} = 676.4$ nm) [19]	Chl-B800-reconstituted LH2 (RR, $\lambda_{ext} = 413.1$ nm)	Chl-B800-reconstituted LH2 (FLN, $\lambda_{ext} = 676.4$ nm)
R8	1042		1048
	1065		1071
	1117		1116
	1143	1151	1145
R7	1179	1186	1188
		1207/1218	1212
	1220		1227
	1263		
	1287	1287	1285
	1306		
	1324	1328	1332
	1348	1345/1354	1351
	1369		
	1384	1397	1393
R6	1428	1436	1439
R5	1484	1490	1488
R4	1527		1540
		1515	
		1518	
R3	1549	1555/1559	1560
R1	1600	1611	1610
C_9-keto	–	1696	1697

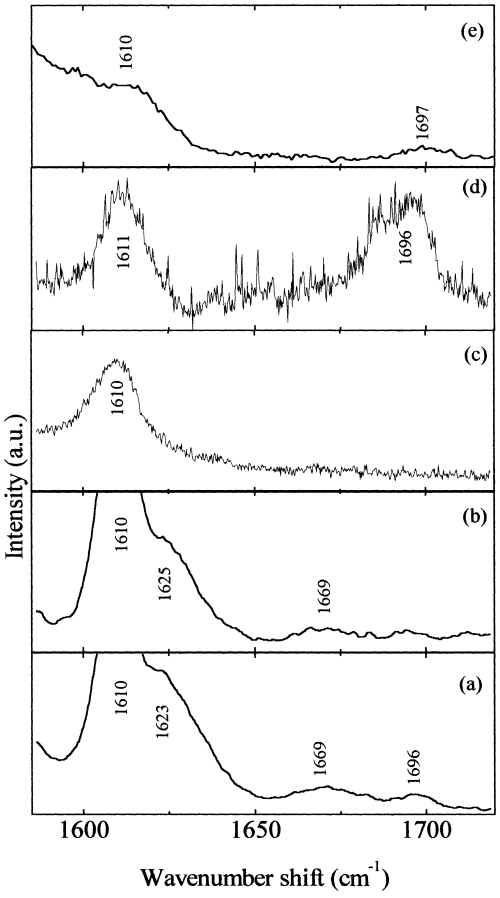

Fig. 3. Comparison of the 1575–1725 cm^{-1} Raman spectra, in resonance with the Soret transitions of (a) the native LH2, $\lambda_{ext} = 363.8$ nm; (b) Chl-B800-reconstituted LH2, $\lambda_{ext} = 363.8$ nm; (c) native LH2, $\lambda_{ext} = 413.1$ nm, 77 K; and (d) Chl-B800-reconstituted LH2, $\lambda_{ext} = 413.1$ nm. The FLN spectrum obtained at $\lambda_{ext} = 676.4$ nm, of the Chl-B800-reconstituted LH2 (e) is also shown. In all the spectra the background fluorescence has been removed.

Shown in Fig. 3e is the FLN spectrum in the high-frequency carbonyl region of the Chl-B800-reconstituted LH2 with the background fluorescence removed. The two bands present in these spectra at ca. 1611 and 1697 cm^{-1} may be easily attributed to the methine bridge and the keto carbonyl stretching modes of the Chl*a* molecules bound to the protein, respectively. The frequency of the former of these bands indicates that the central Mg ion of these molecules binds one external ligand only, even at very low temperature [26]. On the other hand, the frequency of the keto carbonyl stretching modes indicates unambiguously that it is free from any intermolecular interactions, and in an apolar environment [27].

4. Discussion

In this work, we have performed RR and FLN experiments on the LH2 protein from *Rps. acidophila* where the Bchl*a*-B800 chromophores have been replaced with Chl*a* using a biochemical extraction/reconstitution protocol [13]. Whilst RR spectroscopy yields information on the intermolecular interaction assumed by all the Bchl and Chl molecules within the sample when excited at 363.8 nm and 413.1 nm, respectively, FLN (at a suitable excitation wavelength) allows selective observation of only the Chl molecules in the protein. Moreover, it is obvious, once the background has been subtracted, that for Chl*a*-containing proteins the signal-to-noise level obtained with FLN is in generally far superior to that of RR when only the chlorin contribution is desired.

Recently, it has been demonstrated by Pascal and co-workers [19] that the FLN bands observed for Chl*a* in THF at 1042, 1179, 1428, 1484, 1549, 1527 and 1600 cm^{-1} correspond to the R_8, R_7, R_6, R_5, R_4, R_3 and R_1 modes, respectively, which are sensitive to Chl*a* conformation. Furthermore, they concluded that the Mg atoms of all the Chl*a* molecules contributing to the FLN spectra of the higher plant LH protein Lhcb4, or CP29, are five-coordinated, and the frequencies of the $R_{1,3-8}$ modes do not deviate significantly from those observed in Raman spectra of five-coordinated Chl*a*. From this, our FLN and RR experiments (see Table 1) indicate that the Chl*a* molecules, which absorb at 670 nm, have a five-coordinated Mg atom, and a free-from-interaction keto carbonyl group in an apolar environment [27]. As discussed in [28] at low temperature in aqueous solvents, the Mg ion of (bacterio)chlorin molecules becomes six-coordinated. It may stay five-coordinated only if shielded from the water molecules and/or bound to a very strong ligand, such as the imidazole side chain of a histidine molecule. Similarly, the keto carbonyl groups of Chl*a* molecules are seldom found free from interactions when exposed to water. We may thus conclude that the Chl*a* molecule bound to the Chl-B800-reconstituted LH2 is shielded from the surrounding water molecules, i.e. inserted in the protein binding site of the Bchl*a*-B800 molecule. In this binding site, it is inserted in such a way that its keto carbonyl group does not interact with neighbouring amino acid side chains, and is located in an environment of a local polarity similar to that felt by the keto carbonyl group of the native Bchl*a*-B800 molecule. We must therefore conclude that the Chl*a* is indeed inserted at the very position occupied by the Bchl*a* molecule in the B800 site. Consequently, this work has formally demonstrated for the first time that Chl-based pigments can be successfully incorporated into bacterial LH2 complexes. It must be noted that replacement of bacteriochlorine molecules by chlorins has been successfully achieved already in bacterial reaction centres, but that such replacement was impossible in the B873 binding site of LH1 antenna proteins ([29]; Lapouge, unpublished data). This is likely to indicate that constraints, for the latter site, are higher, probably due to the fact that there the replaced molecules must additionally closely interact with other (bacterio)chlorins.

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