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Carotenoid stoichiometry in the LH2 crystal: No spectral evidence for the presence of the second molecule in the α/β-apoprotein dimer

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Abstract In this work we have investigated the carotenoid–protein interactions in LH2 complexes of *Rhodopseudomonas acidophila* both in "free in solution" mixed-micelles and in three-dimensional crystals by Raman spectroscopy in resonance with the carotenoid (Car) molecules. We show that the Car molecules when bound to their binding pockets show no significant differences when the complexes are "free in solution" or packed in crystalline arrays. Furthermore, there is no significant wavelength dependence in the Raman spectrum of the Car molecules of LH2. This indicates that there is only one Car configuration in LH2 and thus only one molecule per α/β-heterodimer.

Keywords: Bacteriochlorophyll; Carotenoid; Crystallography; Photosynthesis; Pigment–protein; Raman spectroscopy

1. Introduction

In photosynthetic purple bacteria the primary function of the peripheral light-harvesting (LH2) complex is to capture solar energy and funnel that excitation energy, via the core light-harvesting (LH1) complex, to the photochemical reaction centre (RC). The first step of this process is the absorption of solar energy by the protein-bound chromophores, carotenoid (Car) and bacteriochlorophyll (Bchl) molecules that are non-covalently bound to the LH antenna apoproteins. The latter consists of two low-molecular weight apoproteins (α and β), each spanning the membrane once [1]. In 1995 the X-ray structure of the LH2 complex from *Rhodopseudomonas* (*Rps.*) *acidophila* (now formally reclassified as *Rhodoblastus acidophilus* [2]), purified in the presence of detergent, was elucidated at a resolution of 2.5 Å [3].

This structure (RCSB PDB ID code 1KZU) revealed that the α/β-apoprotein dimers associate to form a nonameric ring-like structure within the photosynthetic membrane. Each αβ-heterodimer binds, non-covalently, three Bchl molecules two of which are strongly interacting (termed Bchl-B850) and located inside the membrane phase and a monomeric one (termed Bchl-B800), which is located at the interface between the membrane and the periplasm [3]. Each α/β-heterodimer also contains a single Car molecule called rhodopin-glucoside that is designated RG1, the glucosyl group of which is located in a hydrophilic pocket on the cytosolic side of the complex. The RG1 polyene chain runs perpendicular to the macrocycle of the Bchl-B800 (the closest approach is 3.42 Å), progresses into the neighbouring α/β-dimer, and then passes over the face of the macrocycle of the α-Bchl-B850 (at a closest distance of 3.57 Å). The long axis of RG1 runs parallel to the transmembrane helices of the α and β polypeptides. The various intermolecular interactions experienced by this Car molecule with either the α and β polypeptides and/or the bound BChl molecules result in it adopting a semi-helical structure, when viewed along its long axis. This twisted conformation of the all *trans*-Car was previously predicted from resonance Raman experiments [4–6].

Recently, an improved LH2 crystal structure at a resolution of 2.0 Å was obtained (RCSB PDB ID code 1NKZ) in which the structure of the C-terminal region that was previously lacking was elucidated [7]. In this region, from incomplete electron density maps, the presence of a second Car molecule (designated RG2) per α/β-heterodimer was postulated [7]. More precisely, this carotenoid was located between neighbouring α-apoproteins on the exterior surface of the nonameric structure, and its structure was proposed to be in a double-*cis* configuration.

Among the spectroscopic methods which can be used for studying protein-bound carotenoid molecules, resonance Raman spectroscopy is one of the most sensitive, as it yields direct information on their molecular structure. It was in particular shown that each carotenoid configuration could be discriminated using this vibrational method [8,9]. In this study we used resonance Raman spectroscopy to probe for the presence of a double-*cis*-Car in the LH2 complex from *Rps. acidophila* and found no evidence for this second Car population, whether LH2 are in mixed detergent-protein micelles or in crystals or as a function of detergent environment.

2. Materials and methods

2.1. Cell culturing, LH2 purification and crystallisation

Rhodopseudomonas acidophila 10050 cells were grown photoheterotrophically at 28 ± 2 °C [10]. The glass culture bottles were located between banks of incandescent lamps that provided an average

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Abbreviations: Bchl, bacteriochlorophyll; LH, light-harvesting; RC, reaction centre; *Rps.*, *Rhodopseudomonas*

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illumination intensity of 10 Wm^{-2}. The LH2 complex was purified in the presence of the detergent LDAO (Fluka, Sigma–Aldrich Inc.) and crystallised in the same detergent, or after detergent exchange into βOG (Anatrace Inc., Maumee, USA) as previously described [3,7,11,12]. For clarity, the LH2 pigment–protein complex encompassed by a micelle of LDAO or βOG are termed $LH2_{LDAO}$ and $LH2_{\beta OG}$, respectively. The same cryoprotective protocol [7,12] was used that was employed prior to the X-ray diffraction experiments, therefore, ensuring that the crystals were not damaged upon freezing prior to the spectroscopic measurements.

2.2. Spectroscopy

Low-temperature absorption spectra of detergent-purified LH2 were collected using a Varian Cary E5 Double-beam scanning spectrophotometer. Resonance Raman (RR) spectra were recorded with a Jobin Yvon U1000 spectrometer equipped with a liquid nitrogen cooled CCD camera (Jobin Yvon Spectrum One). Excitation was provided by Coherent Argon (Innova 100) laser 496, 501, 514.5 and at 528 nm. The RR spectra were recorded with a 90-degree geometry, and the samples were maintained at 77 K in a SMC-TBT flow cryostat cooled with liquid nitrogen (Air Liquide, Sassenage, Fr.). For each excitation wavelength, no evolution of the Raman signal was observed during data acquisition. The averaged RR spectra are plotted without any spectral smoothing.

3. Results and discussion

Shown in Fig. 1 is the 77 K absorption spectrum in the carotenoid molecule absorption range of the detergent-purified $LH2_{LDAO}$ complex from *Rps. acidophila* 10050. The solid arrows represent the location of the excitation wavelengths of 496, 501, 514.5 and 528 nm that were used to probe the conformation of the carotenoid molecules using resonance Raman spectroscopy.

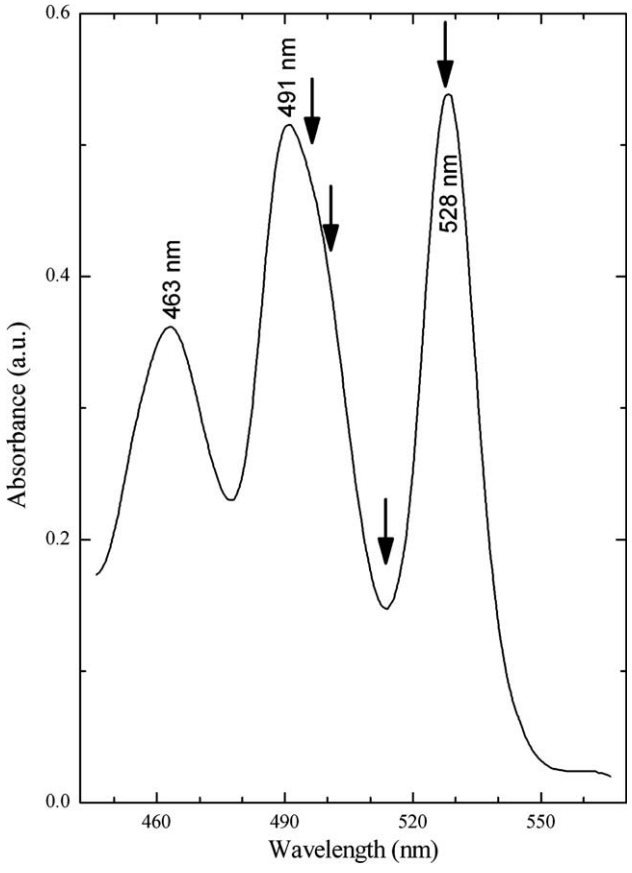

Fig. 1. Low-temperature (10 K) absorption spectrum in the carotenoid region of the $LH2_{LDAO}$ from *Rps. acidophila* in "free in solution" mixed-micelles. The arrows represent the position of the excitation wavelengths (496, 501, 514.5 and 528 nm) used in Figs. 2 and 3.

Carotenoid molecules generally yield very intense resonance Raman signals, and this spectroscopy has been extensively used for probing the structure and geometry of Car molecules in photosynthetic LH and RC complexes [5,8,9,13,14]. Resonance Raman spectra of Car molecules are dominated by four regions of relatively high intensity bands at approximately 950, 1010, 1160 and 1520 cm^{-1}. These have been attributed to out-of-plane C–H modes (ν_4 modes), the stretching modes of C–CH_3 bonds between the main chain and side methyl carbons (ν_3 modes), C–C bond stretching modes mixed with C–H bond bending modes (ν_2 modes), and the stretching modes of conjugated C=C bonds (ν_1 modes), respectively [15,16]. *cis–trans* isomerisation affect the ν_1 and ν_2 modes, and the change in molecular symmetry they induce results in the appearance of new bands in the spectra, which may be used as fingerprints for determining the position of the *cis* conformation [8,13]. Using these properties of resonance Raman, it was concluded as early as in 1970s that the RC-bound carotenoid was in an unusual conformation, which was later shown to be 15-15′ *cis* [17,18]. More recently, this spectroscopy helped in detecting very small structural changes in the protein-bound Car molecule in the bacterial RC from *Rhodobacter sphaeroides* in which the pigment binding site had been altered by site-selected mutagenesis [19,20]. Although these changes were small enough not to affect the optical properties of the Car cofactor, and beyond the limits of detection (ca. 2.0 Å) of X-ray crystallography [19,20], all of the mutations caused clear alterations in the carotenoid resonance Raman spectra [21]. This illustrates the extreme sensitivity of this spectroscopic method in characterising pigment conformational and configurational changes.

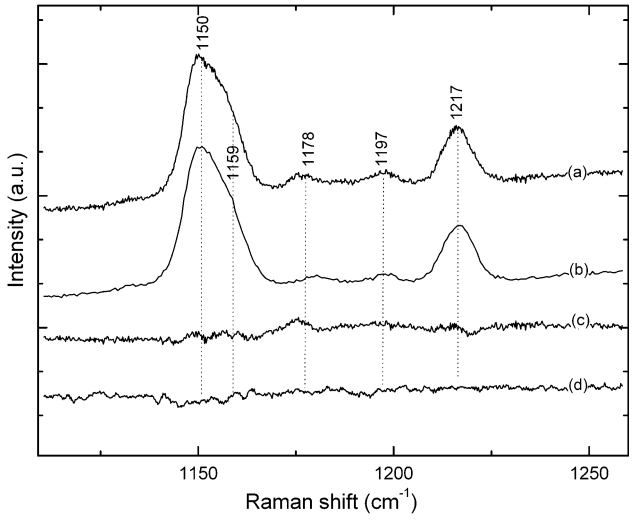

Fig. 2. 77 K resonance Raman spectra of the ν_2 region of the Car molecules from LH2 as a function of detergent environment. (a) $LH2_{\beta OG}$ in "free-in-solution" mixed micelles, (b) $LH2_{\beta OG}$ crystals, and (c) the difference spectrum of (a)–(b). (d) As (c) except where the LH2 complexes are in the presence of LDAO. λ_{ext} = 496 nm.

The intensity of the resonance Raman contribution of these molecules strongly depend on the precise matching between the frequencies of the excitation light used to induce resonance and the maximum of their absorption transition. This made it possible, in complex systems containing more than one carotenoid, to partially isolate the individual contribution of each of these molecules, by controlling the precise position of the exciting beam. This was achieved in particular in the major LH protein from higher plants, namely LHCII, which contains when isolated contains two luteins and one neoxanthin [22–24]. Resonance Raman spectra of LH2 over a wide range of excitation conditions were recorded, therefore, in order to try and detect the presence of the two different populations of carotenoids as suggested by the 2.0 Å structure. Shown in Fig. 2 are the v_2 region of RR spectra of the carotenoid molecules of $LH2_{\beta OG}$ in (a) free mixed-micelles, in (b) three dimensional crystals of space group *R*32 and (c) is the difference spectrum between (a) and (b). In this spectral region every carotenoid *cis–trans* isomer shows distinct differences and these are especially marked for di-*cis* carotenoids [8,13]. For example, compared to all-*trans*-β-carotene in hexane the 9-*cis*-isomer and 9-*cis*,13-*cis*-isomer are characterised by the apparition of new Raman bands at ca. 1138 and 1195 cm^{-1} of approximately equal intensity to that of the ca. 1150/1159 cm^{-1} mode while the 15-*cis*-isomer is characterised by the appearance of a major band at ca. 1240 cm^{-1} [8,13]. It is clear from Fig. 2 that, at the excitation wavelength used (496 nm) the same carotenoid molecules are present in the different samples, and that none shows evidence of sub-populations of different (*cis*-or di-*cis*) Car structures. The difference spectra (Fig. 2a and d) indicate in particular that the major spectroscopic properties of the Car molecules are the same irrespective of the detergent environment and/or protein/crystal packing. This is in complete agreement with the proposed location of the carotenoid RG1 in the 1995 X-ray crystal structure [3], and the location of the detergent micelle that surrounds the hydrophobic sections of the alpha helical transmembrane domains [25].

In order to further test the presence, or absence, of a subpopulation of Car molecules in the LH2 complexes, we have measured RR spectra using different excitation wavelengths ranging from the 0-1 to the 0-0 components of the electronic absorption spectra of the LH2-bound carotenoid molecules. Fig. 3 shows the v_2 region of $LH2_{\beta OG}$ crystals as a function of wavelength excitation. It is clear that in general the spectra are mainly invariant as a function of excitation wavelength, there is no spectral evidence for the presence of an additional single, or double-*cis* isomeric Car molecules. The very slight variation of the RR spectra in Fig. 3 is in fact due to the presence of trace amounts of other Car molecules other than Rhodopin-glucoside [26] in the LH2 complex. These minor Cars replace the RG1 molecule in the carotenoid binding pocket and do not alter the Bchl*a*:Car stoichiometry. These experiments strongly disagree with the interpretation of the 2.0 Å structure for the presence of a second di-*cis* carotenoid molecule in LH2 complexes from *Rps. acidophila*. They are, however, in accord with previous pigment analyses of the Bchl*a*:Car stoichiometry for these complexes, where it was concluded that one RG molecule was present per α/β-apoprotein dimer [27] and with the most recent crystallographic data on crystals of LH2 produced in the *meso* lipid phase, which have suggested that the putative RG2 molecule is actually a superposition of the electron density of LDAO and βOG at this site on the surface of the LH2 complex [28].

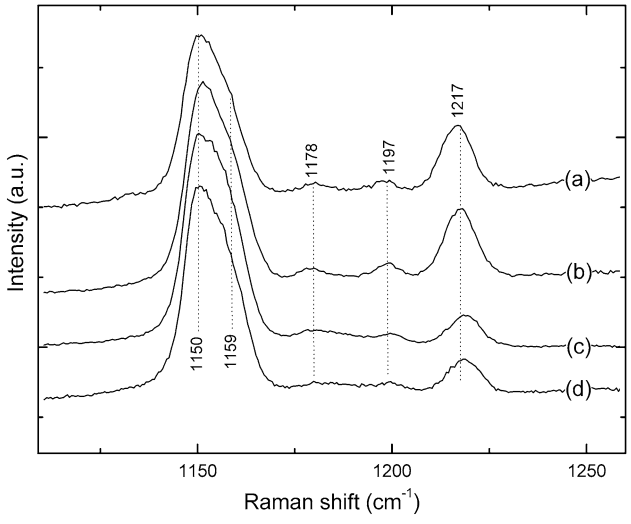

Fig. 3. 77 K resonance Raman spectra (v_2 region) of the Car molecules in $LH2_{\beta OG}$ crystals displayed as a function of excitation wavelength: (a) 496 nm, (b) 501 nm, (c) 514.5 nm and 528 nm.

4. Conclusion

From resonance Raman spectroscopy evidence, it can be concluded that the LH2 complex from *Rps. acidophila* contains only nine carotenoid molecules per ring. Each α/β-apoprotein dimer thus contains one only carotenoid molecule.

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