Resonance Raman Spectroscopic Evidence for the Identity of the Bacteriochlorophyll *c* Organization in Protein-Free and Protein-Containing Chlorosomes from *Chloroflexus aurantiacus**

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Protein-free and protein-containing chlorosomes from *Chloroflexus aurantiacus*, strain Ok-70fl, were studied by resonance Raman (RR) spectroscopy. Both preparations gave the same spectra of the bacteriochlorophyll *c* (BChl *c*) chromophores in the range of 1200–1750 cm^{-1}. This strongly corroborates previous evidence [Griebenow *et al.*, Z. Naturforsch. **45c,** 823–828 (1990), and references therein] that the three-dimensional structure of the antenna complexes is *not* determined by direct interaction with protein but rather is due to BChl *c* self-organization. The analysis of the coordination-sensitive marker bands of the chlorin macrocycle reveals a mixed six- and fivefold ligation of the Mg ion. Based on two C=O stretching vibrations originating from a free and a Mg-bound C-9 keto group, it is concluded that only in the six-coordinated state the keto group serves as an axial ligand to the Mg ion of a neighbouring chlorin. The second permanently bound axial ligand is attributed to the C-2a hydroxyl group.

Introduction

The light-harvesting antenna complexes of photosynthetic organisms contain a large number of chromophores whose spectra and spatial arrangement are optimized for efficient transfer of light energy to the reaction centers. In most cases, the important three-dimensional structural template of these complex systems are proteins which bind the chromophores [1–3]. It has been suggested, however, that in some cases also in the absence of protein stable antenna complexes may be formed by self-organization [4, 5].

The first experimental evidence for such a protein-free antenna system has recently been provided by our group in the case of *Chloroflexus aurantiacus* [6–9]. Chlorosomes, the main antennae of this organism, include up to 16,000 BChl *c* molecules as well as a minor fraction of BChl *a* and carotenoids [10]. Contrary to previous findings by Feick and Fuller [11] and Wechsler *et al.* [12], we demonstrated that there is no fixed protein-BChl *c* ratio, ruling out the idea that these antennae are constituted by specific protein-BChl *c* complexes [7]. This conclusion is supported by a comparative study of protein-containing (MIR) and protein-free (GEF) chlorosomes [6–9]. Linear and circular dichroism spectroscopy, picosecond energy transfer measurements, and electron micrographs revealed far-reaching similarities between both types of chlorosome preparations.

In the present work, these studies are complemented by resonance Raman (RR) spectroscopy. This technique probes the vibrational pattern of the chromophores and thus can provide detailed conformational informations [13, 14]. RR spectroscopy has been widely employed to study protein-Chl and Chl-Chl interactions. It is therefore a valuable tool in the search for the debated functional role of proteins in stabilizing the chlorosome structure in *C. aurantiacus*, as well as for further details of the BChl *c* aggregation.

Materials and Methods

Cells of *C. aurantiacus* strain Ok-70-fl were grown according to Griebenow and Holzwarth

Abbreviations: GEF chlorosomes, protein-free chlorosomes isolated by gel-electrophoretic filtration; MIR chlorosomes, protein-containing chlorosomes isolated with miranol as detergent; BChl, bacteriochlorophyll; Chl, chlorophyll; RR, resonance Raman; 5c, fivecoordinated Mg configuration; 6c, six-coordinated.

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[15]. The isolation of the MIR and GEF chlorosomes is described in detail elsewhere [7, 15]. For RR measurements the optical densities of the samples were ~2.0 at 460 nm [20 mM (Tris-hydroxymethyl-amino)methane buffer at pH 8.0]. RR experiments were carried out using an optical multichannel detection system (Triplemate 1877, Spex Instruments, equipped with an O-SMA intensified diode array, Spectroscopy Instruments). The spectral band width was ~5 cm^{-1} and the resolution per diode ~1 cm^{-1}. The 413 nm and 468 nm lines of a Kr^+ laser (model 171, Spectra Physics) were used for excitation. The power was ~10 mW focused by an 8 cm lens onto the sample. All RR experiments were carried out at ambient temperature with a total accumulation time of about ~1 h. The samples were deposited in a rotating cuvette to avoid photodegradation by the exciting laser beam. No time-dependent changes of the RR spectra were observed during the measurements and the absorption spectra of the samples before and after the experiment were identical. For both the MIR and GEF preparations, different sample preparations gave identical RR spectra which were finally added to improve the signal-to-noise ratio. In all RR spectra displayed in this paper the strong scattering background, which was about 5 times as high as the strongest RR band of BChl *c*, was substracted.

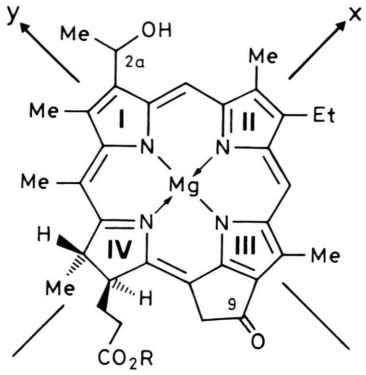

Fig. 1. Structure of BChl *c*. The chlorosomes of *Chloroflexus aurantiacus* contain a mixture of 2a-epimeric bacteriochlorophyllides *c* with R = geranylgeranyl, cetyl, oleyl, phytyl, and stearyl [24].

Results and Discussion

Except for the lack of the carbomethoxy group at C-10 and the substitution of the vinyl group at C-2 by a hydroxyethyl group, the structure of BChl *c* is very similar to that of Chl *a* (Fig. 1). It is therefore not surprising that the RR spectra of the monomeric forms of both compounds are nearly the same [16]. This agreement considerably facilitates the interpretation of the RR spectra of the MIR and GEF chlorosomes, since well established relationships between structural and spectral parameters of the chlorin, derived from extensive studies of Chl *a* and related compounds, can be applied [13, 14, 17–19]. Like other chlorin chromophores, the electronic absorption spectrum of BChl *c* exhibits two components in the Soret band region attributable to the B_y and B_x transition. Their maxima are at ~420 and 460 nm, respectively, in the GEF and MIR chlorosomes. Both transitions were used to excite the RR spectra.

Figure 2 shows the RR spectra of the GEF and MIR chlorosomes in the frequency range between 1580 and 1730 cm^{-1}. While no chlorin fundamentals are expected above 1630 cm^{-1}, this region includes the C=O stretching vibration of the C-9

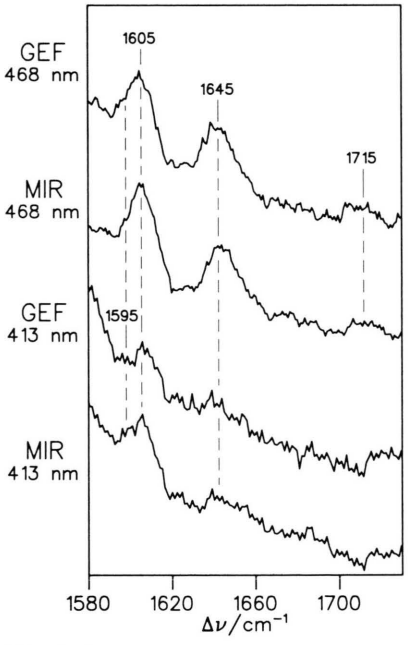

Fig. 2. RR spectra of GEF and MIR chlorosomes between 1580 and 1730 cm^{-1} excited at 468 and 413 nm.

keto group which, in contrast to the ester group of the C-7 side chain, is conjugated to the delocalized π electron system of the chlorin ring and therefore may exhibit a considerable resonance enhancement. Most surprisingly, the RR spectra excited at 468 nm display two bands in this region, a relatively sharp band at 1645 cm^{-1} and a weaker, but broader peak centered at 1715 cm^{-1}. This suggests that in both chlorosomes the keto group exists in two conformational states (the spectra of both preparations do not differ in any relevant respect; *vide infra*).

The 1715 cm^{-1} band can be assigned to the keto group free of any intermolecular interactions [14]. This frequency is higher than observed in monomeric Chl *a* or BChl *a* in aprotic solvents [16, 17]. However, it is well established that environmental effects other than hydrogen bonding can strongly influence the frequency of this mode. According to Koyama *et al.* [17] a frequency of 1715 cm^{-1} points to a highly hydrophobic environment. Furthermore, also a tilting of the keto group out of the chlorin plane, which weakens the coupling of the C=O stretching with vibrational modes of the chlorin, may lead to a frequency upshift.

The position of the 1645 cm^{-1} band indicates that in this case the keto oxygen is subject to strong intermolecular interactions which lower the C=O stretching force constant. As pointed out by Lutz and van Brakel [16], such a low frequency rules out hydrogen-bonding interactions, for example with adjacent protein side chains, but can be attributed to a keto group coordinated to the Mg ion of a second BChl *c* molecule.

A further prominent peak in the 468 nm RR spectra of Fig. 2 is located at 1605 cm^{-1}, which is assigned to the chlorin mode R1 (notation by Fujiwara and Tasumi [19]). This mode is a sensitive marker for the coordination state of the Mg ion. In a five-coordinated (5c) state of Chl *a* it is generally observed between 1604 and 1610 cm^{-1} while in a six-coordinated (6c) configuration it shifts down by about 10 cm^{-1}. Hence, the observed peak position unambiguously indicates a fivefold coordination. However, a careful inspection of this band reveals a slight asymmetry of the band shape on the low-frequency side, pointing to the presence of a second component. Upon changing the excitation wavelength to 413 nm, this component emerges more clearly. Its frequency at ~1595 cm^{-1} agrees well with the expected value of R1 for a 6c state [19]. This suggests that for a fraction of the BChl *c* molecules in the chlorosomes a second axial ligand is coordinated to Mg.

Qualitatively similar results, albeit with a slightly higher 6c content, were also obtained from aggregates prepared *in vitro* with various BChl *c* derivatives which had been isolated from native chlorosomes [20]. In the RR spectra of these artificial aggregates two components were found not only for the R1 mode but also for other coordination markers below 1590 cm^{-1}. In the case of the natural chlorosome aggregates, these latter bands were obscured by the dominant carotenoid band at 1520 cm^{-1} (C=C stretching).

The RR spectra in Fig. 3 reveal a unique enhancement pattern for the bands between 1300 and 1400 cm^{-1}. While for 468 nm excitation the dominant peak is at 1342 cm^{-1}, this band nearly disappears at 413 nm. Instead, a band at 1337 cm^{-1} emerges, in addition to the 1355 cm^{-1} band which is only detectable as a shoulder in the 468 nm spectrum. A similar intensity redistribution is found for the doublet at 1389 and 1381 cm^{-1}, with the latter band disappearing upon 413 nm excitation.

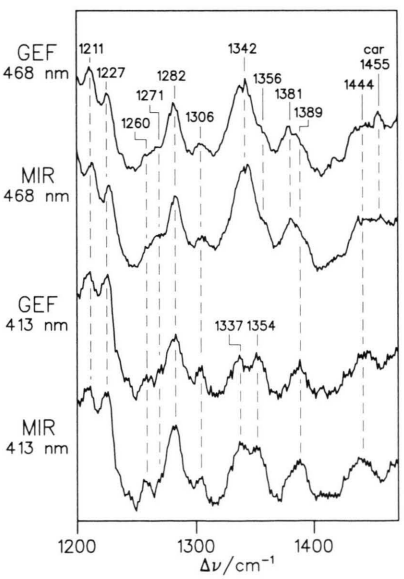

Fig. 3. RR spectra of GEF and MIR chlorosomes between 1200 and 1470 cm^{-1} excited at 468 and 413 nm (car = carotenoid band).

Since the dominant contributor to the 413 nm and 468 nm RR spectra are the B_x and B_y transitions, respectively, it is apparent that these transitions provide different enhancement conditions for the components of the band pairs at 1342/1330 cm^{-1} and 1389/1381 cm^{-1}. The source of their enhancement are Franck-Condon overlaps. Such a mechanism is most efficient when the vibrations are aligned along the *x* and the *y* axis upon B_x and B_y excitation, respectively (Fig. 1). According to the normal coordinate analysis of octaethyl metallochlorins [21, 22], these modes include large contributions from C_a-N stretching vibrations. Hence, the major nuclear displacements associated with such modes should be parallel to the *x* axis when mostly localized in the pyrrole rings II and IV, and parallel to the *y* axis when mostly localized in I and III. The different enhancement of the Franck-Condon active modes at the two excitation wavelengths, based on the transition dipole moment directions, may well prove to provide a specific probe for particular regions of the chlorin macrocycle.

The comparison of the RR spectra in the entire frequency region between 900 and 1750 cm^{-1} reveals no differences between the GEF and MIR chlorosomes as far as the BChl *c* bands are concerned. While in the 1150–1500 cm^{-1} region (Fig. 3) upon 413 nm excitation both chlorosomes gave RR spectra which are identical in all relevant aspects, at 468 nm excitation one notes a small difference associated with the weak band at 1455 cm^{-1}. This band originates from the carotenoids. Its slightly higher intensity in the GEF samples is paralleled by a higher intensity of the C=C stretching at 1520 cm^{-1} (spectrum not shown), indicating a larger carotenoid content in the GEF than in the MIR chlorosomes.

The modes in the frequency range between 1100 and 1400 cm^{-1} involve vibrations of the C_b-C_b- and C_b-substituent bonds (C_b designates the two peripheral carbon atoms of each pyrrole-type ring). Consequently, one would expect that these bands should sensitively respond to changes in intermolecular interactions of the BChl *c* molecules. This idea is supported by previous RR studies of Chl *a* and related compounds which demonstrated a strong effect of aggregate formation and solvent-chlorin interactions on the vibrational pattern in this region [13, 14, 17, 23]. These bands appear therefore to be most appropriate for probing even subtle conformational changes due to potential protein-BChl *c* interactions. The lack of any difference between the RR spectra of the MIR and GEF chlorosomes implies that removal of the polypeptides neither modifies the conformation of the BChl *c* molecules nor their interactions within the aggregate. It confirms the conclusion, already drawn previously [6–9], that the native chlorosomes of *C. aurantiacus* are devoid of any protein determining the aggregate structure. Rather, the aggregates appear to arise from self-aggregation.

The RR spectra indicate, furthermore, the coexistence of 5c and 6c configurations. Since, in addition, the bands of a free and a Mg-coordinated C-9 keto group were detected, one has to conclude that there is yet another axial ligand which is permanently bound to the Mg ion and, hence, plays the dominant role for stabilizing the oligomeric structure of BChl *c* in the antenna complexes. The only plausible candidate is the C-2a hydroxy group in either of the two 2a configurations (2a*R*, 2a*S*) or in both [24]. In fact, NMR studies of artificial BChl *c* aggregates point to a location of this group close to the center of an adjacent BChl *c* molecule [20]. It should be mentioned in this context that the relative RR intensities of the coordination marker and the C=O stretching bands do not directly reflect the relative content of the 5c and 6c states, since the Raman cross sections may be substantially different for the bands in the two coordination configurations.

The question arises whether the two coordination states of BChl *c* found in this work are directly related to the two BChl *c* bands seen in low-temperature steady-state and room-temperature time-resolved fluorescence [9].

Finally, we should like to refer to a previous low-temperature ($T = 4$ K) RR study of these antenna complexes by Lutz and van Brakel [16]. The RR spectra presented in that work provided no evidence for a 6c state. In addition, no RR band of a free C=O vibrator was detected. The authors concluded that the Mg ion is coordinated by the C-9 keto oxygen. This discrepancy with our present study is possibly attributable to a temperature dependence of the coordination equilibrium. It may be that at low temperature the keto group is the thermodynamically preferred axial ligand. This change of the coordination pattern would require

a small reorientation of the chlorin planes with respect to each other, due to the out-of-plane tilting of the keto group. As a consequence the interactions of the C-2a hydroxyl group with the Mg ion might be sufficiently weakened leading to a 5c state.

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