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Antenna Organization in Green Photosynthetic Bacteria. 1. Oligomeric Bacteriochlorophyll *c* as a Model for the 740 nm Absorbing Bacteriochlorophyll *c* in *Chloroflexus aurantiacus* Chlorosomes[†]

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ABSTRACT: Bacteriochlorophyll (BChl) *c* was extracted from *Chloroflexus aurantiacus* and purified by reverse-phase high-pressure liquid chromatography. This pigment consists of a complex mixture of homologues, the major component of which is 4-ethyl-5-methylbacteriochlorophyll *c* stearyl ester. Unlike previously characterized BChls *c*, the pigment from *C. aurantiacus* is a racemic mixture of diastereoisomers with different configurations at the 2a chiral center. Diluting a concentrated methylene chloride solution of BChl *c* with hexane produces an oligomer with absorption maxima at 740–742 and at 460–462 nm. Both the absorption spectrum and the fluorescence emission spectrum (maximum at 750 nm) of this oligomer closely match those of BChl *c* in chlorosomes. Further support for this model comes from the ability of alcohols, which disrupt BChl *c* oligomers by ligating the central Mg atom, to convert BChl *c* in chlorosomes to a monomeric form when added in low concentrations. The lifetime of fluorescence from the 740 nm absorbing BChl *c* oligomer is about 80 ps. Although exciton quenching might be unusually fast in the in vitro BChl *c* oligomer because of its large size and/or the presence of minor impurities, this result suggests that energy transfer from the BChl *c* antenna in chlorosomes must be very fast if it is to be efficient.

Bacteriochlorophyll (BChl)[1] *c* is a family of green pigments that occur only in green photosynthetic bacteria. These pigments resemble pyrochlorophyll *a* in being 7,8-dihydroporphyrins that have a central Mg atom but lack a 10-(carboxymethyl) group. Although its synthesis is repressed at high light intensities, BChl *c* is usually the major chlorophyll in green bacteria and can constitute 90–95% of the total, the rest being BChl *a* (Pierson & Castenholz, 1978; Olson, 1980). Except in the case of green sulfur bacteria (Chlorobiaceae), in which a small portion of the BChl *c* (or a structurally modified form) appears to function as the primary electron acceptor (Nuijs et al., 1985; Braumann et al., 1985), all of the BChl *c* occurs in chlorosomes, antenna structures attached

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[1] Abbreviations: BChl, bacteriochlorophyll; BPh, bacteriopheophytin; *C.*, *Chloroflexus*; Chl, chlorophyll; HPLC, high-pressure liquid chromatography; NMR, nuclear magnetic resonance; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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to the cytoplasmic membrane of the cell. In *Chloroflexus aurantiacus*, chlorosomes are about 110 nm long, 30 nm wide, and 10 nm thick (Staehelin et al., 1978). Their function is to absorb light energy, which is then transferred to the adjacent photosynthetic membrane.

Two interesting features of BChl *c* in chlorosomes are that its red absorption maximum is shifted from 660–669 nm (the range for BChl *c* dissolved in various organic solvents) to 735–760 nm (Gloe et al., 1975; Gloe & Risch, 1978) and that it is very strongly oriented. Measurements of linear dichroism performed on oriented chlorosomes have shown that the Q_y electronic transition dipole of BChl *c* makes an angle of about 37° with the long axis of *C. aurantiacus* chlorosomes (Betti et al., 1982; van Dorssen et al., 1986a). BChl *c* in chlorosomes from the green sulfur bacterium *Chlorobium limicola* is also oriented, having its Q_y electronic transition dipole parallel to the long axis of the chlorosome (Fetisova et al., 1986; van Dorssen et al., 1986b).

Proteolytic digestion of *C. aurantiacus* chlorosomes provided evidence that BChl *c* is associated in an approximately 7 to 1 molar ratio with a low molecular weight protein in the chlorosome interior (Feick & Fuller, 1984). The molecular weight of this protein is 5600, and its amino acid sequence has been determined (Wechsler et al., 1985). Given a molecular weight of 840 for BChl *c* (Gloe & Risch, 1978) and the 7 to 1 ratio of BChl *c* to protein molecules, the BChl *c*–protein complex is 51% BChl *c* by weight. Electron microscopic examination of chlorosomes in freeze-fractured cells reveals rodlike structures in the chlorosome interior that run the length of the chlorosome (Staehelin et al., 1978; Sprague et al., 1981). These rods are thought to consist almost entirely of the BChl *c*–protein complex. Similar, but thicker, longitudinal rods have been observed in *Chlorobium* chlorosomes (Staehelin et al., 1980). These are also thought to be composed of BChl *c* and protein (Olson, 1980).

Interestingly, BChl *c* extracted from Chlorobiaceae spontaneously forms oligomers with red absorption maxima at 745–748 nm when concentrated solutions in CCl_4 or CH_2Cl_2 are diluted in hydrocarbon solvents, and it has been proposed that these oligomers are a good model for BChl *c* in the antennae of green bacteria (Bystrova et al., 1979; Smith et al., 1983a). These studies demonstrated that both the 2-(hydroxyethyl) group and the central Mg atom are involved in oligomer formation, and likely structures for the oligomer were proposed. [A detailed comparison of the structures proposed by Bystrova et al. and by Smith et al. was presented by Olson et al. (1985).] The essential feature of both oligomer structures is that the constituent BChl *c* molecules are arranged in chains in which the 2-(hydroxyethyl) group of each molecule ligates the central Mg atom of the next (see Figure 1). The Q_y electronic transition dipoles are oriented along the chain of BChl *c* molecules, and it is easy to imagine that, in a chlorosome, these chains might be bound and oriented roughly parallel to the long axis of the chlorosome by the protein molecules that, together with BChl *c*, make up the longitudinal rods seen by electron microscopy.

Similar oligomers using BChl *c* from *C. aurantiacus* have not been reported. *C. aurantiacus* belongs to a taxonomic group (Chloroflexaceae) that is only distantly related to the Chlorobiaceae (Gibson et al., 1985), and BChl *c* from *C. aurantiacus* is structurally different from BChl *c* in the Chlorobiaceae (Caple et al., 1978; Gloe & Risch, 1978; Kemmer et al., 1979; Risch et al., 1979; Smith et al., 1980, 1983b). Specifically, BChl *c* from *C. aurantiacus* reportedly contains only ethyl and methyl substituents at positions 4 and 5, respectively, and is primarily esterified to stearol via its 7-propionic acid group, although small amounts are esters of phytol and geranylgerniol (Figure 1) (Risch et al., 1979). In contrast, BChl *c* from *Chlorobium limicola* and *Prosthecochloris aestuarii* has both ethyl and *n*-propyl groups at position 4 (with small amounts of isobutyl) and is almost entirely ethyl substituted at position 5 (with small amounts of methyl). The esterifying alcohol is mostly farnesol (>90% in *Chlorobium limicola*; Caple et al., 1978). Furthermore, the absorption spectrum of BChl *c* in vivo is somewhat different in *C. aurantiacus* from that in the Chlorobiaceae. Because of these differences, preparation of an oligomer of BChl *c* from *C. aurantiacus* appeared to be a useful way to test and extend the proposal that the in vitro BChl *c* oligomer is a good model for BChl *c* in chlorosomes.

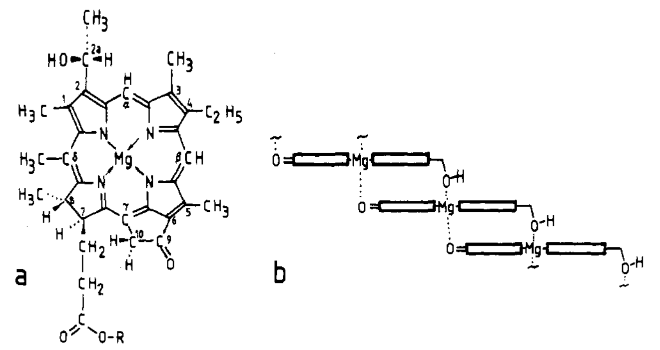

FIGURE 1: Structures of monomeric and oligomeric bacteriochlorophyll *c*. (a) BChl *c* from *C. aurantiacus* according to Gloe and Risch (1978) showing the labeling system used here. The configuration at the 2a chiral center is *R*. The esterifying alcohol (*R*) on the 7-propioniate side chain is mostly stearol. (b) Oligomeric BChl *c* according to Smith et al. (1983a) and Bystrova et al. (1979).

The results presented here show that BChl *c* from *C. aurantiacus* is a more complex mixture of homologues than was found by Risch et al. (1979), although those authors correctly identified the major component of that mixture. A comparison of the absorption and fluorescence spectra of an oligomer of this pigment with those of BChl *c* in chlorosomes shows that they are practically identical. Furthermore, both BChl *c* in chlorosomes and oligomeric BChl *c* can be converted to monomeric forms by titration with small amounts of alcohol. These results are consistent with the hypothesis that BChl *c* molecules in chlorosomes are held together in an arrangement similar to that of BChl *c* molecules in the in vitro oligomer.

MATERIALS AND METHODS

Chloroflexus aurantiacus strain J-10-F1 was grown either in 1-L screw-capped bottles as described previously (Betti et al., 1982) or in a 16-L fermenter (Labline Type L 1523) with illumination provided by two banks of three 100-W tungsten bulbs placed about 5 cm from the fermenter on opposite sides. Cells were harvested by centrifugation and stored at −20 °C.

Chlorosomes were prepared as described by Feick and Fuller (1984). BChl *c* was extracted and precipitated by a modification of the procedure of Watanabe et al. (1984) and purified by reverse-phase HPLC. This modified procedure is as follows: Bacterial cells were extracted 3 times with methanol (approximately 2 mL/g wet cells in each extraction) and the extracts decanted after centrifugation. The extracts were combined, and 1/10 volume of *p*-dioxane was added. Carotenoids were precipitated by slow addition of H_2O (0.5–1 mL/10 mL of extract) and removed by centrifugation. Addition of H_2O was continued to give a methanol:dioxane:H_2O ratio of 30:3:10 and the sample kept overnight at −20 °C to precipitate BChl *c* and *a*. The precipitate was collected by centrifugation, and the supernatant (which contained mostly

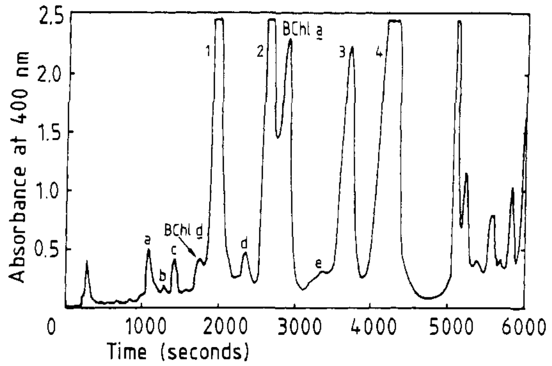

FIGURE 2: Separation of bacteriochlorophylls from *C. aurantiacus* by HPLC. A 1-mL sample in methanol was loaded onto a 9.6 × 500 mm preparative column and chromatographed at a flow rate of 5 mL/min with 92.5% methanol as the eluting solvent. The four major BChl *c* peaks are numbered 1–4, and minor BChl *c* peaks are lettered a–e. Peaks marked BChl *d* and BChl *a* were identified from their absorption spectra. After peak 4 eluted, the solvent was changed to pure methanol. The later peaks were mostly carotenoid with small amounts of bacteriopheophytin *c*.

BPh *c*, judging from its absorption spectrum) was discarded. All solvents were deaerated by several cycles of evacuation and flushing with argon and stored under argon. All operations were performed in dim light to further inhibit photooxidation of BChl *c*.

The collected BChl was dissolved in a minimum volume of methanol, filtered through a 0.2-μm syringe filter, and purified by HPLC. The HPLC system consisted of an ISCO Model 2300 pump and either a 4.8 × 250 mm analytical column or a 9.6 × 500 mm preparative column packed with C-18 derivatized silica (Whatman ODS 3). The absorbance of the eluate was monitored at 400 nm in a flow cell in a Shimadzu UV-160 spectrophotometer. The eluting solvent was 92.5% methanol–7.5% H_2O. [Equivalent resolution could be obtained with 80% acetone–20% H_2O, a solvent system recommended by Smith & Goff (1985) for separation of BChls *d*.] Different fractions were collected separately, identified from their absorption spectra, and dried in vacuo on a rotary evaporator. As a final purification step, BChl *c* fractions were dissolved in a minimal volume of methylene chloride and precipitated by 50-fold dilution with petroleum ether. The precipitate was collected by centrifugation, dissolved in a minimal volume of methanol, dried in vacuo, and stored under argon at −20 °C.

Because our chromatographic system was different from that used for separation of BChls *c* from *C. aurantiacus* by Risch et al. (1979), the BChl *c* fraction used for oligomer formation was examined by NMR and by mass spectroscopy for structural determination. NMR samples were dissolved in CD_3OD and spectra obtained with a Bruker AM 400 spectrometer. Mass spectra were acquired with a Varian-MAT 312 mass spectrometer in the electron impact mode (70 eV, source temperature 125 °C).

Oligomeric BChl *c* was prepared by diluting a concentrated methylene chloride solution of BChl *c* with *n*-hexane in the sample cuvette (Smith et al., 1983a). Steady-state fluorescence spectra were obtained on a home-built fluorometer incorporating an RCA C31034 photomultiplier as the detector. Fluorescence lifetime measurements were made at the Center for Fast Kinetics Research in Austin, TX, as described elsewhere (Brune et al., 1987).

RESULTS

Chromatographic separation of pigments via HPLC gave four major and several minor BChl *c* fractions (Figure 2). The absorption spectra of these fractions were practically identical, having maxima at 669 and 434–436 nm in methanol. One minor fraction contained a pigment judged to be BChl *d* from its absorption spectrum (maxima at 659 and 433 nm in methanol). Since BChl *d* differs from BChl *c* only in that it lacks a methyl group at the δ-position, it may be a biosynthetic precursor of BChl *c*. The BChl *c* fraction labeled 4 in Figure 2 was obtained in the largest amounts and unlike fractions 1 and 2, which were contaminated with small amounts of carotenoid and BChl *a*, respectively, contained only BChl *c*. Therefore, this fraction was selected for oligomer formation, and unless stated otherwise, all experimental results presented in this paper were obtained with this component of the mixture of BChl *c* homologues.

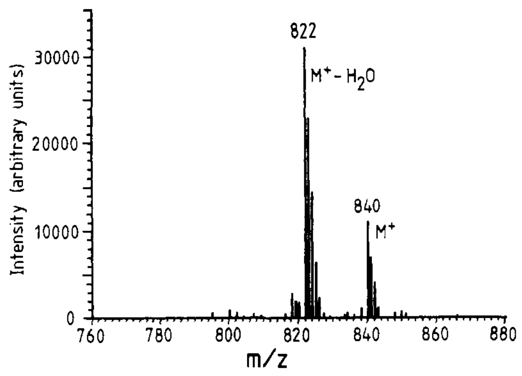

FIGURE 3: Mass spectrum of the major BChl *c* (peak 4 in Figure 2) from *C. aurantiacus* (upper mass range).

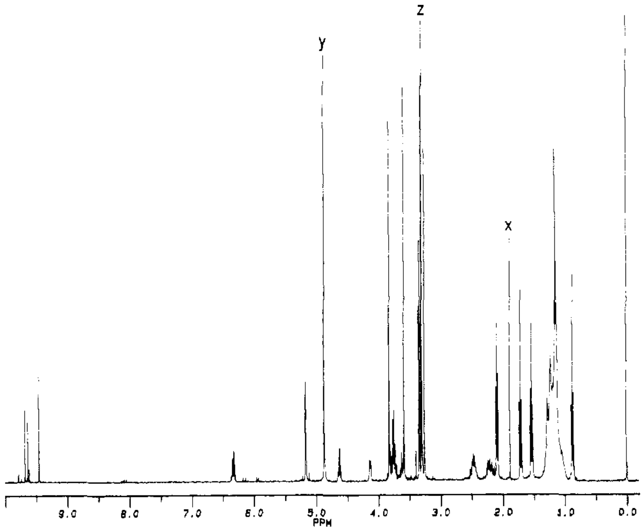

FIGURE 4: The 400-MHz proton NMR spectrum of BChl *c* (peak 4 in Figure 2) from *C. aurantiacus*. Tetramethylsilane (TMS) was present as an internal standard. The solvent was CD_3OD. The peak marked x was not observed in samples without TMS and is therefore attributed to an impurity in the TMS. Peaks marked y and z are due to water and nondeuteriated methanol impurities in the sample.

A mass spectrum of fraction 4 (Figure 3) showed the molecular ion to have a mass of 840 daltons. This is the molecular weight expected for the stearyl-esterified, 4-ethyl-5-methyl-substituted form of BChl *c* found to predominate in *C. aurantiacus* by Gloe and Risch (1978) (see Figure 1). This structure is also consistent with the proton NMR spectrum of fraction 4 (Figure 4). Assignments of proton resonance peaks (Table I) were made by comparison with previously published data on BPh *c* (stearyl ester) from *C. aurantiacus* (Risch et al., 1979) and on the basis of a 2-D (COSY) spectrum, which revealed couplings between protons assigned to adjacent carbons (data not shown).

A conspicuous feature of the NMR spectrum is the splitting of the α-H resonance into two approximately equal peaks. The

Table I: Proton NMR Data on BChl *c* in CD_3OD

proton	chemical shift (ppm)	structure[a]
α-H	9.685 (*S*?), 9.640 (*R*?)	2 s
β-H	9.460	s
2a-H	6.365, 6.348, (6.331, 6.327), 6.311, 6.294	dq
10-CH_2	5.216, 5.167, 5.160, 5.110	AB
8-H	4.652, 4.634, 4.617, 4.599	m
7-H	4.143, 4.122	m
δ-CH_3	3.823	s
4a-CH_2	3.787, 3.767, 3.748, 3.732	q
5-CH_3	3.587	s
1-CH_3	3.347, 3.338	2 s
3-CH_3	3.257	s
7b-CH_2	2.535–2.432	m
7a-CH_2	2.261–2.143	m
2b-CH_3	2.105, 2.089, 2.072	dd
4b-CH_3	1.729, 1.710, 1.691	t
8-CH_3	1.549, 1.532, 1.514	dd
stearyl protons	1.349–1.025	m
	0.888, 0.871, 0.853	t

[a] Abbreviations: AB, AB doublet; dd, doublet of doublets; dq, doublet of quartets; m, multiplet; q, quartet; s, singlet; t, triplet.

same phenomenon was observed by Smith and Goff (1985) in diastereoisomeric mixtures of methylbacteriopheophorbide (Bmph) *d* with different configurations at the 2a chiral center. (With Bmph *d*, the lower field α-H resonance was assigned to the *S* and the higher field one to the *R* diastereoisomer.) The approximately equal size of the two α-H resonances in Figure 4 indicates that BChl *c* from *C. aurantiacus* is (approximately) a racemic mixture of *R* and *S* isomers. The presence of equal amounts of the *R* and *S* forms explains several other features of the NMR spectrum, namely, the slight splitting of the 1-methyl resonance and the unusual structures of the 2a-H and 2a-methyl resonances (the former is a pair of overlapping quartets and the latter a pair of overlapping doublets having the appearance of a triplet). It may also cause the 8-methyl resonance to appear as a triplet instead of a doublet. A spectrum taken at 90 MHz (not shown) separated both the 2a- and 8-methyl resonances into pairs of doublets. While effects of the configuration about the 2a chiral center on the resonances of the 2a-methyl and the nearby α-H and 1-methyl groups were expected, the effect on the resonance of the more remote 8-methyl group was surprising.

Fraction 4 from *C. aurantiacus* formed an oligomer in *n*-hexane with absorption maxima at 740–742, 460–462, and 324–325 nm. Compared to BChl *c* in methylene chloride (absorption maxima at 668, 435, and 335 nm), the red and Soret bands undergo bathochromic shifts, and the UV band undergoes a hypsochromic shift (Figure 5). Oligomer formation was also accompanied by an increase in intensity of the red absorption maximum and a decrease in intensity of the Soret peak. An attempt to determine the extinction coefficient of aggregated BChl *c* at 740 nm gave values of 83–102 mM^{-1} cm^{-1}, with an average (from four determinations) of 91 mM^{-1} cm^{-1}, assuming the value in methanol to be 74 mM^{-1} cm^{-1} at 670 nm, as reported by Stanier and Smith (1960) for BChl *c* from *Chlorobium limicola*. An exact value for the extinction coefficient of BChl *c* in the oligomer is difficult to obtain because of the volatility of the solvents (hexane and methylene chloride) and because the oligomer tends to precipitate and to become adsorbed on the inner surfaces of the cuvette, resulting in an inhomogeneous mixture.

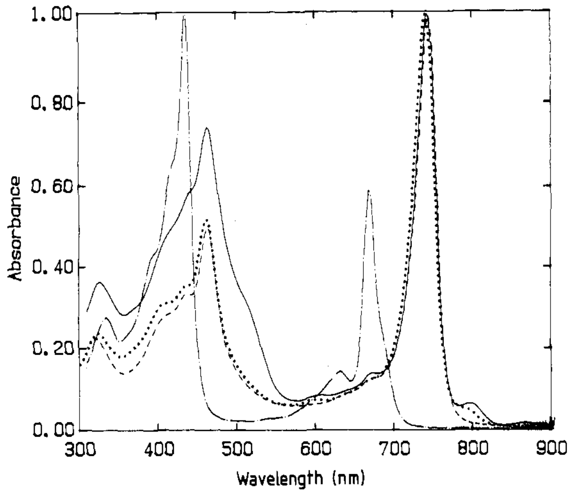

FIGURE 5: Absorption spectra of chlorosomes in 10 mM Tris-HCl, pH 8.0 (—), a dried film of chlorosomes after extraction with hexane (···), oligomeric BChl *c* (– –), and BChl *c* dissolved in CH_2Cl_2 (–·–). The BChl *c* oligomer occurred as a thin film precipitated on the inner faces of the sample cuvette. Traces of monomeric BChl *c* were removed by decanting the hexane–methylene chloride solvent from the cuvette and refilling with fresh hexane. This procedure had no effect on the absorption spectrum of the sample other than slightly decreasing the absorbance at ~670 and ~435 nm by removing monomeric BChl *c*. The shoulder on the long-wavelength side of the red absorption peak of BChl *c* in CH_2Cl_2 is probably due to absorption by a dimeric form of the pigment (see text). All spectra were normalized to have the same maximum absorbance.

The absorption maxima of oligomeric BChl *c* correspond very closely to the absorption maxima of chlorosomes. However, chlorosomes have additional contributions to their absorption spectrum from carotenoids and a small amount of BChl *a*. When a concentrated aqueous suspension of chlorosomes was dried on an inner face of a spectrophotometer cuvette, the absorption spectrum (not shown) was identical with that of chlorosomes in aqueous suspension. Filling the cuvette with hexane and decanting 3 times extracted carotenoids from the chlorosome film without otherwise affecting its absorption spectrum. After extraction, the relative intensities as well as the positions of the absorption bands of chlorosomes matched those of oligomeric BChl *c* extremely well, as shown in Figure 5. The presence of BChl *a* (about 1 per 25–30 BChl *c*) (Feick et al., 1982; van Dorssen et al., 1986a) with absorption maxima at 792, 600, and about 370 nm accounts for the difference between the spectra of extracted chlorosomes and aggregated BChl *c*. [There is also a slight difference between the red absorption maxima of the aggregated BChl *c* sample (742 nm) and the extracted chlorosome film (740 nm) shown in Figure 5, but maxima at both wavelengths (and those in between) were observed in different oligomer and chlorosome preparations.] The reported extinction coefficient of BChl *c* in chlorosomes at 742 nm is 102 ± 2 mM^{-1} cm^{-1} (van Dorssen et al., 1986a), which, although larger than the average value, is within the range found for BChl *c* in the oligomer.

Oligomeric BChl *c* is weakly fluorescent and has the fluorescence emission spectrum shown in Figure 6. The single maximum at 750 nm corresponds well to a fluorescence emission maximum at 748–751 nm from isolated chlorosomes at room temperature that has been ascribed to BChl *c* (Betti et al., 1982; van Dorssen et al., 1986a). In some oligomer preparations, a second fluorescence maximum was observed at 730 nm. This correlated with minor amounts of a more strongly fluorescent (and probably smaller) aggregate absorbing maximally at 710–715 nm (see below).

Fluorescence lifetime measurements (Table II) gave values of 55–135 ps for the 740 nm absorbing oligomer in solution and somewhat shorter values for the oligomer as a precipitated film on the inner faces of a cuvette. This compares with a lifetime of 6.4–6.5 ns for monomeric BChl *c* in methylene chloride plus 0.3% methanol. In pure methylene chloride,

Table II: Fluorescence Lifetimes for Monomeric and Oligomeric BChl *c* from *C. aurantiacus*[a]

	wavelength (nm)		lifetime		
sample	excitation	emission	T_1	T_2	$R(2/1)$
BChl *c* in CH_2Cl_2	600	>650	1.2 ns	6.4 ns	3.1
	630	690	1.0 ns	6.3 ns	1.1
BChl *c* in CH_2Cl_2 + 0.3% CH_3OH	630	690	6.5 ns	none	
BChl *c* oligomer in hexane + 5% CH_2Cl_2	727	760	81 ± 17 ps[b]	2.3 ± 0.2 ns[c]	0.06 ± 0.03[c]
	712	760	135 ps	2.5 ns	0.45
BChl *c* oligomer in hexane, washed film	727	760	37 and 47 ps	none	
BChl *c* oligomer in hexane + 10% CCl_4	700	760	80 ps	2.1 and 3.0 ns	0.19
	700	730	220 ps	2.1 ns	2.1
BChl *c* oligomer in 50% hexane–50% CCl_4	700	730	2.9 and 3.0 ns	none	

[a] T_1 and T_2 are the lifetimes of the first and second components of fluorescence emission and $R(2/1)$ is the ratio of the amplitude of the second component to that of the first when more than one component was observed. The error limits for each calculated lifetime were always less than ±10%. [b] Mean and standard deviation from four measurements. [c] Mean and standard deviation from three measurements.

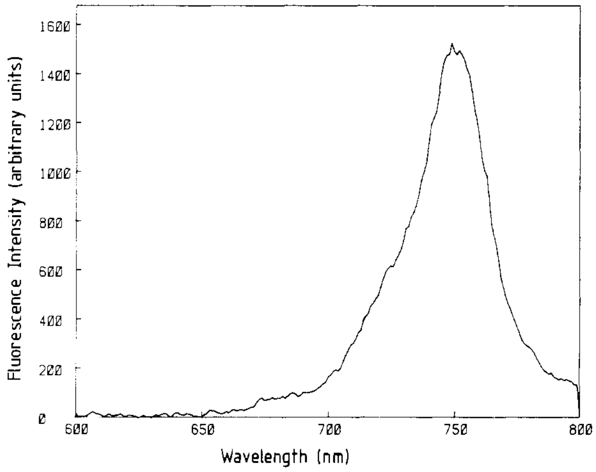

FIGURE 6: Fluorescence emission spectrum of oligomeric BChl *c*. The absorption spectrum of the oligomer BChl *c* sample used for this measurement is shown in Figure 5. The sample was illuminated with 460-nm light at an angle of about 30° to the surface of the sample cuvette, and fluorescence was measured along an axis perpendicular to the illuminated surface.

BChl *c* exhibited a broadening on the long-wavelength side of the 668-nm absorption band that disappeared on addition of 0.3% methanol and may be due to absorption by a BChl *c* dimer. This broadening was observed previously by Smith et al. (1983a). A 1.2-ns component of fluorescence from BChl *c* in methylene chloride also disappears on addition of 0.3% methanol, indicating that it most likely arises from the postulated dimer. A possibly analogous dimer of methyl bacteriochlorophyllide *d* that forms in chloroform was recently described in detail by Smith et al. (1986), but they did not investigate its fluorescence.

In most of the samples used for fluorescence lifetime measurements on oligomeric BChl *c*, precipitation of the oligomer was inhibited either by increasing the amount of methylene chloride in the solvent to 5% or by including 10% CCl_4 (the remainder of the solvent being *n*-hexane). The latter resulted in a more rapid and more nearly complete conversion of monomeric BChl *c* to the 740 nm absorbing oligomer. However, small amounts of a more highly fluorescent and probably smaller oligomer persisted in both solvent systems. Further studies on this smaller oligomer are in progress, but preliminary results indicated that it is the predominant product of BChl *c* aggregation in 50% *n*-hexane–50% CCl_4 and that it has an absorption maximum at 710–715 nm, a fluorescence emission maximum at 730 nm, and a fluorescence lifetime of 2–3 ns. Thus a comparison of the amplitudes of the fast and slow components of fluorescence emission using excitation flashes at 700, 712, or 727 nm shows that the contribution of the slow component was greatest with 712-nm excitation (see Table II). Because excitation at 700 nm was performed on a different sample in a solvent slightly different from that used for 712- and 727-nm excitation, the results are only qualitatively comparable. Nevertheless, the expected spectral dependence for excitation of the fast and slow components of fluorescence emission was observed. When the 740-nm oligomer was precipitated on the inner faces of a cuvette and washed with hexane, more soluble BChl *c* oligomers and residual monomers were removed as indicated by the loss of long-lived fluorescence components, and a slight decrease in the lifetime of the fast component was noted. (This decrease in lifetime may be largely due to changing the orientation of the sample cell in order to collect fluorescence from an oligomer precipitated on a surface instead of suspended in solution.) Keeping excitation at 700 nm but decreasing the measuring wavelength from 760 to 730 nm increased the contribution of the 2–3-ns component of fluorescence from 0.19 times that of the fast component to 2.1 times that of the fast component. (The 220-ps lifetime of the fast component in the latter experiment may have been distorted to this rather long value by incomplete separation from the slow component.) Excitation of BChl *c* in 50% *n*-hexane–50% CCl_4 at 700 nm produced 730-nm fluorescence that decayed as a single exponential with a 3-ns lifetime.

Smith et al. (1983a) noted that aggregated BChl *c* could be converted to a monomeric form by titration with methanol, and Bystrova et al. found similar oligomer disruption by traces of pyridine. Our titrations showed that 0.3% methanol (10 μL/3 mL) was adequate to convert oligomeric BChl *c* completely to a monomeric form with absorption maxima at 663 and 431 nm (Figure 7a). Adding a similar, but slightly higher, concentration of methanol (0.5%) to hexane in a cuvette with dried chlorosome films on the inner faces likewise disrupted the 740 nm absorbing form of BChl *c* and extracted both BChl *c* and BChl *a* into the hexane solution (Figure 7b).

More interestingly, it was possible to convert the 740 nm absorbing form of BChl *c* in an aqueous chlorosome suspension selectively to a monomeric form with absorption maxima at 671 and 438 nm by addition of small amounts of hexanol or octanol (Figure 8). Presumably, these rather lipophilic alcohols partitioned into the chlorosomes and disrupted the interaction between adjacent molecules. When the alcohol additions were done gradually to avoid overshooting the end point of the titration, BChl *a* in the chlorosomes was not affected, judging from the fact that its near-infrared absorption maximum remained at 795 nm. A 10-fold dilution of hexanol-treated chlorosomes brought about an immediate shift of the BChl *c* absorption maximum to 729 nm (not shown). The extent of the recovery increased when the chlorosomes were washed to remove hexanol by several cycles of concentration by centrifugation in a Centracon 30 ultrafiltration unit (Am-

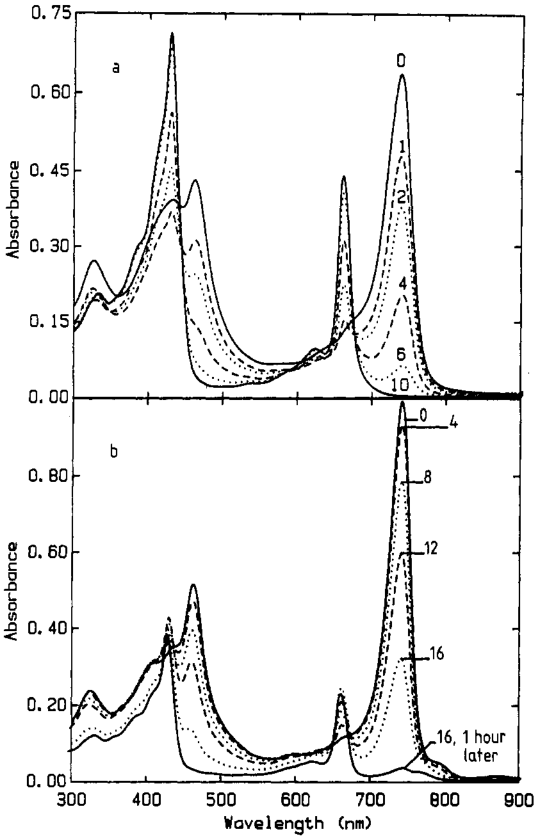

FIGURE 7: (a) Methanol titration of oligomeric BChl *c* in 3 mL of hexane. (b) Methanol titration of a dried chlorosome film in a cuvette filled with 3 mL of hexane. Numbers next to the 740-nm peak of each spectrum indicate microliters of methanol added before recording the spectrum. The absorption of the chlorosome film is exaggerated relative to that of solubilized BChl because the film was thickest at the center of the cuvette face on which it dried. As extracted BChl *c* diffused into the hexane–methanol solvent mixture, the concentration of the pigment in the path of the measuring beam decreased.

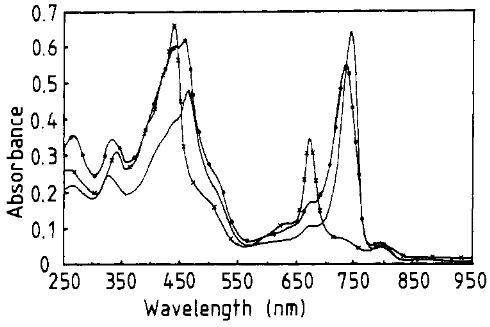

FIGURE 8: Absorption spectra of chlorosomes in 10 mM Tris-HCl, pH 8.0 (—), and the same sample after adding 1% 1-hexanol (×) and after removing 1-hexanol by several cycles of concentration by ultrafiltration and dilution with fresh buffer (O).

icon Corp.) followed by dilution with fresh buffer. After this procedure, the near-infrared and Soret maxima of BChl *c* in chlorosomes appeared at 733 and 456 nm.

DISCUSSION

Analysis of BChl *c* from *Chloroflexus aurantiacus* by reverse-phase HPLC and by NMR spectroscopy yielded two unexpected results. First, BChl *c* from this organism is a rather complex mixture of homologues, containing four major components and several minor ones. This is inconsistent with the report by Risch et al. (1979) that BChl *c* from *C. aurantiacus* consists of only three components, differing in the alcohols esterified to the 7-propionic acid side chain. In agreement with Gloe and Risch (1978) and Risch et al. (1979), analysis by NMR and mass spectroscopy indicated that the major BChl *c* homologue has ethyl and methyl substituents at positions 4 and 5, respectively, and is esterified to stearyl alcohol. Second, the major BChl *c* homologue from *C. aurantiacus* is a racemic mixture of *R* and *S* diastereoisomers, with different configurations at the 2a chiral center in the hydroxyethyl side chain. NMR spectra of the other three major BChl *c* fractions obtained by HPLC also showed them to be approximately equimolar mixtures of *R* and *S* diastereoisomers (data not shown). This may indicate that biosynthesis of BChl *c* in *C. aurantiacus*, unlike that in the Chlorobiaceae (Smith et al., 1983b; Smith & Goff, 1985), is not stereoselective at this position. Although it is possible to create a racemic mixture chemically (Smith & Goff, 1985), this requires treatments much more severe than any to which the pigments were exposed during our isolation procedure. It is not clear why Risch et al. (1979) did not find a mixture of *R* and *S* isomers in their preparations of BPh *c* from *C. aurantiacus*.

Diluting a concentrated solution of BChl *c* in methylene chloride with *n*-hexane produced an oligomer with absorption maxima at 740–742, 460–462, and 324–325 nm. These maxima differ slightly from those for the oligomers described by Smith et al. (1983a) (maxima at 748 and 452 nm) and by Bystrova et al. (1979) (red maximum at 745 nm in the comparable aggregate; Soret maximum not stated but about 450 nm in the spectra shown). These differences are probably a consequence of differences between the structures of the BChl *c* molecules from *C. aurantiacus* used in our experiments and the mixtures of BChl *c* homologues from Chlorobiaceae used in their experiments. One of the other BChl *c* homologues separated by HPLC (peak 3 in Figure 2) was investigated and found to form an aggregate with an absorption spectrum indistinguishable from that shown for oligomeric BChl *c* in Figure 5. It is noteworthy that while the absorption spectrum of our oligomer closely matches that of BChl *c* in the *Chloroflexus* antenna, the spectra of the oligomers of Bystrova et al. (1979) and of Smith et al. (1983a) match the in vivo spectra of BChl *c* in the Chlorobiaceae rather well. Smith et al. (1983a) found a lower near-infrared extinction coefficient (about 55 mM^{-1} cm^{-1} at 748 nm for BChl *c* in their oligomer vs 91 mM^{-1} cm^{-1} at 742 nm in our oligomer). While some of this discrepancy may be due to pigment structural differences, incomplete aggregation, as indicated by a shoulder at about 710 nm in the absorption spectrum of their oligomer, and the inherent difficulty of accurately measuring extinction coefficients of rather insoluble oligomers may also account for it.

Both the absorption and fluorescence emission spectra of oligomeric BChl *c* are practically identical with those of BChl *c* in *C. aurantiacus* chlorosomes. This strongly suggests that oligomeric BChl *c* is a good model for BChl *c* in chlorosomes. Further evidence in support of this model comes from the observation that BChl *c* is extracted from a dried chlorosome film by 0.5% methanol in hexane. This is only slightly higher than the concentration of methanol in hexane (0.3%) required to convert oligomeric BChl *c* completely into a soluble monomer. Similarly, adding 1% hexanol to a suspension of chlorosomes in aqueous buffer selectively converted the BChl *c* absorbing at 740 nm to a (monomeric) form absorbing maximally at 671 nm. Removing hexanol partially reversed this effect, shifting the absorption maximum of BChl *c* to 733 nm. Changes in position or increased hydration of BChl *c* molecules in the chlorosomes during addition and removal of hexanol might account for the incomplete recovery. Bystrova et al. (1979) noted that the absorption maximum of aggregated BChl *c* in dry CCl_4 was more red shifted than that of BChl *c* in wet CCl_4. Alcohols should disrupt the structure of the oligomer shown in Figure 1 by displacing BChl *c* hydroxyethyl groups as ligands to the Mg atoms of adjacent molecules. Evidence of a different nature for the importance of ligation of Mg in red shifting the absorption of BChl *c* in chlorosomes is provided by the observation of Gerola and Olson (1986) that pheophytinization of BChl *c* in chlorosomes from *Chlorobium limicola* shifts the 748-nm absorption band to 673 nm.

An alternative explanation for these results is that addition of alcohols or removal of Mg disrupts interactions between ligands on the BChl *c* binding protein and the Mg atoms of the bound BChl *c* molecules. These interactions are a central feature of the model of the BChl *c*–protein complex proposed by Wechsler et al. (1985), and their disruption might also convert BChl *c* to a form with an absorption spectrum like that of monomeric BChl *c* in solution. The complete disruption of BChl c_{740} in chlorosomes by 1% hexanol without affecting BChl a_{792} implies, however, that the interactions responsible for red shifting the absorption maximum of BChl *c* are different from those responsible for red shifting that of BChl *a*.

Other researchers (Gottstein & Scheer, 1983; Scheer et al., 1985; Scherz & Parson, 1984a; Scherz et al., 1985) have reported that BChl *a* and BPh *a* form oligomers in organic solvent–water mixtures and in detergent micelles with spectral features analogous to those noted here for BChl *c* in hexane. These spectral features include large bathochromic shifts accompanied by increased extinction coefficients for the near-infrared absorption maximum and decreased extinction coefficients for the Soret transition. The best studied of these is a small BPh *a* oligomer (possibly a dimer) that forms in detergent micelles (Scherz & Parson, 1984a,b). Scherz and Parson (1984b) calculated electronic absorption and circular dichroism spectra of BPh *a* dimers as a function of the arrangement of the component pigments and noted that the calculated values matched the observed spectra best when the BPh molecules were stacked so that pyrrole rings I and III overlapped with angles of 15° between the Q_y electronic transition dipoles and 20° between the planes of the overlapping porphyrin rings. This arrangement is similar to that shown in Figure 1 for BChl *c* molecules in the oligomer and thus is indirect evidence that that arrangement can account for the observed absorption spectrum of the BChl *c* oligomer that forms in *n*-hexane.

Olson et al. (1985) calculated spectral properties of BChl *c* oligomers assuming structures similar to that shown in Figure 1 and concluded that the model of Bystrova et al. (1979), in which the Mg atoms of BChl *c* molecules are ligated on opposite sides by a 9-keto group from one adjacent molecule as well as a 2-(α-hydroxyethyl) group from the other adjacent molecule, predicts the observed spectral properties better than a model without the 9-keto–Mg interaction. The calculated red shifts of the Q_y transitions were too small, possibly because these calculations did not consider mixing of nondegenerate as well as degenerate excited states, as did the calculations of Scherz and Parson (1984b). Furthermore, point dipole calculations such as those used by Olson et al. (1985) do not correctly predict spectral properties of closely interacting molecules; point monopole calculations may be more accurate (Warshel & Parson, 1987).

As noted previously, Wechsler et al. (1985) have proposed an alternative model for the arrangement of BChl *c* molecules in chlorosomes on the basis of the amino acid sequence of the BChl *c* binding protein. In this model, the protein is arranged as an α-helix, and seven BChl *c* molecules are bound to it by ligation of their central Mg atoms by glutamine and asparagine amide groups. The BChl *c* molecules in this proposed arrangement do not overlap, although some of them are in contact via hydrogen bonding between the 2-(hydroxyethyl) and 9-keto groups of adjacent molecules. The angle between the Q_y transition dipoles of adjacent BChl *c* molecules [which is important for determining the magnitude of the bathochromic shift of the red absorption maximum (Scherz & Parson, 1984b)] is quite variable in the Wechsler et al. model. While it is possible that BChl *c* molecules on adjacent pigment–protein complexes could interact to form dimers with a structure similar to that proposed by Scherz and Parson (1984b) for BPh *a*, the placement of BChl *c* molecules on the surfaces of adjacent α-helices that curve away from one another makes it extremely unlikely that all of the BChl *c* molecules could interact this way. Intuitively, one would expect the heterogeneous arrangement of BChl *c* molecules suggested by this structure to result in a broad near-infrared absorption band formed from the Q_y transitions of BChl *c* molecules in slightly different environments. Fourth-derivative spectra of isolated chlorosomes (and of aggregated BChl *c* in hexane) revealed only a single sharp peak in the 740-nm region (data not shown) and therefore do not support this suggestion.

The short fluorescence lifetimes of aggregated BChl *c* (81 ± 17 ps in hexane plus CH_2Cl_2 or CCl_4 and 35–50 ps in a precipitated film) indicate a quenching of excited states that is about 100 times faster than that for monomeric BChl *c* in solution (lifetime $\approx$ 6.5 ns). This rapid quenching presents a problem for accepting oligomeric BChl *c* as a model for BChl *c* in chlorosomes. To account for the high efficiency of excitation transfer out of the BChl *c* portion of the antenna (Brune et al., 1987), excitation transfer must be at least an order of magnitude faster than quenching within the antenna. It is not clear that excitation transfer out of a BChl c_{740} oligomer in chlorosomes can be fast enough to prevent substantial losses.

Extremely fast excitation transfer is less necessary if secondary factors cause quenching in the in vitro oligomer to be faster than that in the in vivo oligomer. One such factor might be oligomer size. Experimentally determined excited singlet-state lifetimes of a hydrated chlorophyll *a* aggregate absorbing at 740 nm are in the 40–60-ps range (Alfano et al., 1985; Malý et al., 1987), and Alfano et al. (1985) have argued that fluorescence lifetimes in oligomers decrease in proportion to the number of molecules in the oligomer. The large size of BChl *c* oligomers in hexane is indicated by their tendency to precipitate and by the fact that they can be filtered from solution with a 0.2-μm filter. [For comparison, chlorosomes are only about 0.11 μm long (Staehelin et al., 1978)]. It has been suggested that oligomers in chlorosomes contain only 13–14 closely interacting BChl *c* molecules (Smith et al., 1983; Feick & Fuller, 1984), but definitive information on this point is lacking.

Another factor that might shorten the fluorescence lifetime of oligomeric BChl *c* in vitro is the presence of trace amounts of quenching impurities. For example, BChl *c* photooxidizes easily to form a nonfluorescent product (possibly a ring-opened photobilin; Smith et al., 1980) with an absorption maximum at 755 nm (Stanier & Smith, 1960; Goedheer, 1966). This absorption maximum is ideal for an acceptor of energy from oligomeric BChl *c*. Although precautions were taken to exclude O_2 and absorption spectra rule out the presence of more than about 1% of this impurity in BChl *c* samples used for aggregate formation, even a trace of such an impurity in a large oligomer could quench efficiently. BChl *c* in vivo is protected against photooxidation both by rapid energy transfer to BChl *a* and by the presence of carotenoids. Furthermore, photosynthetic growth occurs only under anaerobic conditions. Alternative quenching mechanisms might include electron transfer between adjacent pigment molecules or internal conversion to excited vibrational levels of the ground state. The latter possibility was favored by Scherz and Parson (1984a) to explain the low quantum yield of fluorescence from BPh *a* oligomers in detergent micelles.

In conclusion, our results are most consistent with a chlorosome model in which BChl *c* exists as an oligomer with a structure like that proposed by Bystrova et al. (1979) or Smith et al. (1983a) for aggregated BChl *c* in hydrocarbon solvents. This in vivo oligomer may occur in units of 13–14 monomers (Smith et al., 1983a; Feick & Fuller, 1984) associated with a pair of protein molecules. The nature of the association between BChl *c* and protein molecules is unknown but is unlikely to be as proposed by Wechsler et al. (1985), since that structure is incompatible with the arrangement of BChl *c* molecules proposed on the basis of considerable evidence (Bystrova et al., 1979; Smith et al., 1983a) for the in vitro oligomer. Unlike BChl *c* in vivo, the in vitro oligomer is probably a long, polymeric structure composed of considerably more than 13–14 units. Extending the length of the oligomer beyond 13–14 monomers should have very little effect on its spectral properties (Olson et al., 1985). The function of the BChl *c* binding protein is thus to limit the size of the BChl *c* oligomers and to orient them in a way that facilitates rapid energy transfer within the chlorosome. Circular dichroism measurements, which can provide further information about the orientations of closely interacting pigment molecules in BChl *c* oligomers and in chlorosomes, are being initiated and will be reported elsewhere.

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Antenna Organization in Green Photosynthetic Bacteria. 2. Excitation Transfer in Detached and Membrane-Bound Chlorosomes from *Chloroflexus aurantiacus*[†]

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ABSTRACT: The photosynthetic antenna of *Chloroflexus aurantiacus* includes bacteriochlorophyll (BChl) c_{740} and BChl a_{792}, both of which occur in chlorosomes, and B808-866 (containing BChl a_{808} and BChl a_{866}), which is membrane-located (subscripts refer to near-infrared absorption maxima in vivo). BChl a_{792} is thought to mediate excitation transfer from BChl c_{740} to BChl a_{808}. Lifetimes of fluorescence from BChl c_{740} and BChl a_{792} were measured in isolated and membrane-bound chlorosomes in order to study energy transfer from these pigments. In both preparations, the lifetime of BChl c_{740} fluorescence was at or below the instrumental limit of temporal resolution (about 30–50 ps), implying extremely fast excitation transfer from this pigment. Attempts to disrupt excitation transfer from BChl c_{740}, either by conversion of part of this pigment to a monomeric form absorbing at 671 nm or by partial destruction of BChl a_{792} by oxidation with $K_3Fe(CN)_6$, had no discernible effects on the lifetime of BChl c_{740} fluorescence. Most (usually >90%) of the fluorescence from BChl a_{792} decayed with a lifetime of 93 ± 21 ps in membrane-attached chlorosomes and 155 ± 22 ps in isolated chlorosomes at room temperature. Assuming that the only difference between these preparations is the occurrence of excitation transfer from BChl a_{792} to B808-866, a 41% efficiency was calculated for this process. This value is lower than the 60% efficiency of excitation transfer from BChl c_{740} to B808-866 determined by comparison of fluorescence excitation and absorption spectra of membranes with attached chlorosomes and compares even less favorably with the 100% efficiency of excitation transfer found in whole cells by the same method. Furthermore, measurements at 77 K (on different samples) did not show an increased lifetime of BChl a_{792} fluorescence when isolated chlorosomes were compared with membrane-bound chlorosomes. These results imply either that BChl a_{792} is not an obligatory intermediate in energy transfer from BChl c_{740} to B808-866 or (more probably) that chlorosome isolation introduces new processes for quenching fluorescence from BChl a_{792}.

The light-harvesting antenna in the green photosynthetic bacterium *Chloroflexus aurantiacus* includes both a membrane-attached structure (the chlorosome) with about 10 000 bacteriochlorophyll (BChl) *c* and 350 BChl *a* molecules and a BChl *a* protein with absorption maxima at 808 and 866 nm (B808-866) that is intrinsic to the membrane (Betti et al., 1982; Feick & Fuller, 1984; van Dorssen et al., 1986a). The near-infrared absorption of BChl *c* in chlorosomes is at 740 nm (BChl c_{740}) while that of BChl *a* is at 792 nm (BChl a_{792}). Thus, the energy levels of the *Chloroflexus* antenna pigments are well spaced for the following proposed energy-transfer sequence:

$$(\text{BChl } c_{740} \rightarrow \text{BChl } a_{792})_{\text{chlorosome}} \rightarrow (\text{B808-866} \rightarrow \text{P}_{870})_{\text{membrane}}$$

where P_{870}, the reaction center BChl *a*, acts as the terminal energy acceptor. In accordance with its position in this energy-transfer sequence, it has been suggested that BChl a_{792} is located in the chlorosome baseplate (the surface through which the chlorosome is attached to the cytoplasmic membrane) (Feick & Fuller, 1984).

Linear dichroism measurements on oriented *Chloroflexus* chlorosomes have shown that BChl c_{740} is regularly arranged, with its Q_y electronic transition within 40° of being parallel to the long axis of the chlorosome (Betti et al., 1982; van Dorssen et al., 1986a). Electron microscopy of freeze-fractured chlorosomes shows internal rodlike structures that run the length of the chlorosomes (Staehelin et al., 1978; Sprague et al., 1981). These appear to be aggregates of a protein (M_r 5600) that binds and orients BChl *c* (Feick & Fuller, 1984; Wechsler et al., 1985; Brune et al., 1987).

Chlorosomes from *Chlorobium limicola*, a green sulfur bacterium, are structurally similar (Olson, 1980). They also contain internal rod elements running the length of the chlorosome (Staehelin et al., 1980) with associated BChl *c* mol-

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