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Isolation and characterization of carotenosomes from a bacteriochlorophyll *c*-less mutant of *Chlorobium tepidum*

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Abstract

Chlorosomes are the light-harvesting organelles in photosynthetic green bacteria and typically contain large amounts of bacteriochlorophyll (BChl) *c* in addition to smaller amounts of BChl *a*, carotenoids, and several protein species. We have isolated vestigial chlorosomes, denoted carotenosomes, from a BChl *c*-less, *bchK* mutant of the green sulfur bacterium *Chlorobium tepidum*. The physical shape of the carotenosomes (86 ± 17 nm × 66 ± 13 nm × 4.3 ± 0.8 nm on average) was reminiscent of a flattened chlorosome. The carotenosomes contained carotenoids, BChl *a*, and the proteins CsmA and CsmD in ratios to each other comparable to their ratios in wild-type chlorosomes, but all other chlorosome proteins normally found in wild-type chlorosomes were found only in trace amounts or were not detected. Similar to wild-type chlorosomes, the CsmA protein in the carotenosomes formed oligomers at least up to homo-octamers as shown by chemical cross-linking and immunoblotting. The absorption spectrum of BChl *a* in the carotenosomes was also indistinguishable from that in wild-type chlorosomes. Energy transfer from the bulk carotenoids to BChl *a* in carotenosomes was poor. The results indicate that the carotenosomes have an intact baseplate made of remarkably stable oligomeric CsmA–BChl *a* complexes but are flattened in structure due to the absence of BChl *c*. Carotenosomes thus provide a valuable material for studying the biogenesis, structure, and function of the photosynthetic antennae in green bacteria.

Introduction

Photosynthetic organisms have evolved a multitude of distinctively different light-harvesting antenna structures (Green 2003; Green et al. 2003). All of these antennae contain chlorophyll species or linear tetrapyrroles as the primary chromophores; many but not all chlorophyll-based antennae additionally contain carotenoids. The major antenna in photosynthetic green bacteria is the chlorosome, an unusual structure that contains the largest number of chlorophylls known for any antenna (Blankenship et al. 1995; Blankenship and Matsuura 2003; Frigaard and Bryant 2004). Although the green bacteria comprise of two types of organisms that are very different phylogenetically and physiologically, these organisms share the obvious similarities of possessing chlorosomes and relying on phototrophic metabolism. The green sulfur bacteria are strict anaerobes and

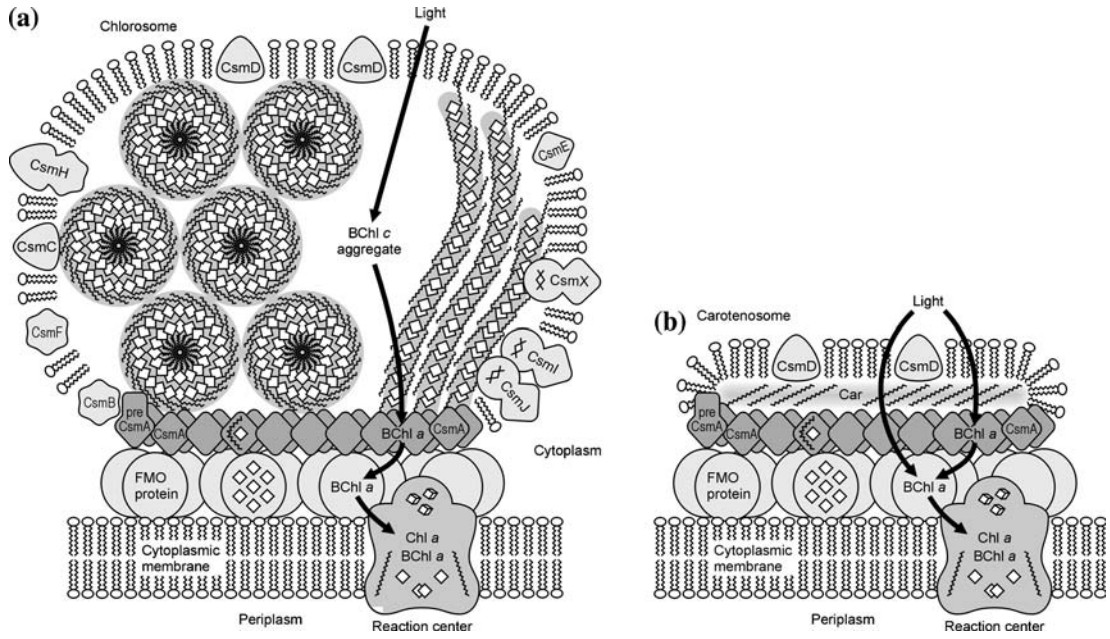

Figure 1. Simplified model of (a) the chlorosome structure in wild-type *Chlorobium tepidum* and (b) the carotenosome structure in the *bchK* mutant of *Chlorobium tepidum*. (a) depicts both the commonly favored rod model (left side of the chlorosome interior; Nozawa et al. 1994) and the recently proposed alternative lamellar model (right side of the chlorosome interior; Pšenčík et al. 2004) of BChl *c* aggregation. Light and excitation energy transfer is shown with thick arrows.

obligately phototrophic (Garrity and Holt 2001a), whereas the green filamentous bacteria (a subgroup of the filamentous anoxygenic phototrophic bacteria) can grow aerobically and chemotrophically in the dark or phototrophically in the absence of oxygen (Garrity and Holt 2001b). Most of the research on chlorosomes has been conducted with various strains of green sulfur bacteria (*Chlorobi*) and the green filamentous bacterium *Chloroflexus aurantiacus* (*Cfx*).

In contrast to most other antenna complexes, chlorosomes do not have a fixed size or stoichiometric composition. They are typically about 100–200 nm long and 30–100 nm wide in green sulfur bacteria but are a little smaller in *Cfx. aurantiacus* (Oelze and Golecki 1995). Chlorosomes mostly consist of large aggregates of BChl *c* molecules (or BChl *d* or BChl *e* molecules depending on the organism), which function as the primary light-harvesting antenna pigment; smaller amounts of BChl *a*, carotenoids, and isoprenoid quinones are also present (Blankenship et al. 1995; Blankenship and Matsuura 2003). The ratio of BChl *a* to BChl *c* in chlorosomes isolated from the green sulfur bacterium *Chlorobium tepidum* is typically about 0.01 (Frigaard et al. 2003); the BChl *a* component is therefore hardly detectable in the absorption spectrum due to masking by BChl *c*. However, it is clearly detectable in fluorescence emission spectra and many optical kinetic studies and other spectroscopic evidence show that this BChl *a* is an important intermediate species that participates in transferring excitation energy from the BChl *c* antenna out of the chlorosome (Blankenship et al. 1995; Blankenship and Matsuura 2003). The ratio of BChl *a* to BChl *c* in whole cells of *Chl. tepidum* is typically about 0.03; most of this BChl *a* is associated with the FMO protein (~60%), the remainder being distributed between the chlorosomes (~30%) and the reaction centers (~10%) (Frigaard et al. 2003).

A lipid- and protein-containing envelope, which is usually described as a monolayer membrane, surrounds the chlorosome (Figure 1a). This envelope has a high content of glycolipids in both green sulfur and green filamentous bacteria (Blankenship et al. 1995). Glycolipids are rare in other anoxygenic phototrophs but are common in oxygenic phototrophs. Ten chlorosome proteins have been identified in *Chl. tepidum* (CsmA, CsmB, CsmC, CsmD, CsmE, CsmF, CsmH, CsmI, CsmJ, CsmX), and all are located in the envelope (Chung et al.

1994; Chung and Bryant 1996a, b; Vassilieva et al. 2002b). All 10 proteins species seem to be conserved in *Chl. vibrioforme* strain 8327 and *Chl. phaeobacteroides* strain 1549 (Frigaard et al. 2001, Vassilieva et al. 2002a). Three chlorosome proteins have been characterized in *Cfx. aurantiacus* (CsmA, CsmM, CsmN; Feick and Fuller 1984; Niedermeier et al. 1994) but there probably are at least three more chlorosome proteins in this organism (Frigaard and Bryant 2004).

CsmA was initially thought to be a BChl *c*-binding protein (Feick and Fuller 1984; Wagner-Huber et al. 1988; Blankenship and Matsuura 2003). However, recent evidence clearly point to CsmA being a BChl *a*-binding protein in both *Cfx. aurantiacus* (Sakuragi et al. 1999; Montaño et al. 2003) and *Chl. tepidum* (Bryant et al. 2002; Frigaard et al. 2004a; this work). CsmA is highly conserved within the green sulfur bacteria (more than 90% amino acid sequence identity) and is less conserved but still recognizably similar between green sulfur bacteria and green filamentous bacteria (about 30% sequence identity). The protein has a single conserved histidine residue that most likely binds BChl *a*. The *csmA* gene of *Chl. tepidum* encodes a 79-residue polypeptide denoted pre-CsmA, which typically is detected in small amounts in isolated chlorosomes. Most of this protein retains its N-terminal methionine but 20 amino acid residues are removed by C-terminal processing in the protein's mature form (Chung et al. 1994; Persson et al. 2000). Very little is known about the function of the other chlorosome proteins. CsmI, CsmJ, and CsmX from *Chl. tepidum* are iron–sulfur cluster-binding proteins (Vassilieva et al. 2001) that may participate in redox-regulation of the energy transfer in the chlorosome (H. Li et al. manuscript in preparation). To investigate the functions of the chlorosome proteins, mutants of *Chl. tepidum* have been created that lack one, two, three, or four chlorosome proteins (Chung et al. 1998; Frigaard et al. 2004a; H. Li et al. unpublished data). Currently, only the *csmA* gene has not been inactivated – an observation that demonstrates the functional importance of CsmA. Surprisingly, only the *csmC* mutant shows an obvious phenotype among the single-locus mutants. The chlorosomes from this mutant are somewhat smaller and have a blue-shifted BChl *c* absorption and fluorescence emission maximum, and the cells grow slightly more slowly under low light (Frigaard et al. 2004a). Thus, CsmC may be involved in, but is certainly not essential for, the molecular organization of BChl *c*.

Very little is known about the biogenesis of chlorosomes (reviewed in Oelze and Golecki 1995). Chlorosomes are present constitutively in green sulfur bacteria, whereas they are induced in green filamentous bacteria only under phototrophic conditions at low oxygen tension. It is clear that within any cell, both the number and size of the chlorosomes vary significantly with growth conditions. It is also clear that in both types of green bacteria, the average chlorosome size decreases with increasing light intensity. However, it is not clear in which order the chlorosome components are assembled or what starting materials are required.

A mutant of *Chl. tepidum* completely devoid of BChl *c* was recently constructed by inactivation of the *bchK* gene (Frigaard et al. 2002). This gene encodes BChl *c* synthase, which is responsible for the last step of BChl *c* biosynthesis. Chlorosomes may not be expected to form in the *bchK* mutant due to the inability of the mutant to synthesize their major component, BChl *c*. However, preliminary work with the *bchK* mutant identified a low-density, orange-colored fraction containing carotenoids, BChl *a*, and the major chlorosome protein CsmA (Frigaard et al. 2002). Because of its orange color and high content of carotenoids, this fraction was denoted 'carotenosomes'. In this work we have purified and characterized these carotenosomes, and we show that they are vestigial chlorosome-like structures (Figure 1b). It is likely that further structural and biochemical analyses of the carotenosomes from the *bchK* mutant (as well as from other mutant strains) will reveal new information on chlorosome structure, function, and biogenesis.

Materials and methods

Bacterial strains and cultivation

The wild-type strain and the BChl *c*-less *bchK* mutant of *Chl. tepidum* used were described previously (Frigaard et al. 2002). Both strains were cultivated in CL medium (Frigaard and Bryant 2001) in 2-l bottles at 47 °C under incandescent

illumination. The wild-type strain was grown at approximately 120 μmol photons s^{-1} m^{-2} whereas the *bchK* mutant was grown at approximately 400 μmol photons s^{-1} m^{-2} in a thermostatically controlled aquarium.

Preparation of chlorosomes and carotenosomes

Chlorosomes were prepared from wild-type *Chl. tepidum* cells as previously described (Vassilieva et al. 2002b). Carotenosomes were prepared from *bchK* cells using a modified procedure. Late-exponential cultures were harvested at $4500 \times g$ for 10 min at 4 °C, washed in 10 mM KH_2PO_4, 50 mM NaCl, pH 7.0, pelleted again in small tubes at $6000 \times g$ for 10 min at 4 °C, and stored at −20 °C until use. The thawed pellet from 2 l of culture was resuspended in 50 ml of isolation buffer (50 mM Tris(hydroxymethyl)aminomethane, 2 M NaSCN, 10 mM sodium ascorbate, 5 mM Na_2EDTA, 0.5 mM phenylmethanesulfonyl fluoride, 1 mM 1,4-dithiothreitol, pH 8.0) and passed through a cooled French press three times at 19,000 lb in^{-2}. The cell extract was clarified by centrifugation at $13{,}000 \times g$ for 20 min at 4 °C and the supernatant was saved. For routine preparation and unless otherwise stated, the supernatant was supplemented with crystalline sucrose to a final concentration of 20% (w/v), transferred to ultracentrifuge tubes, and overlaid with isolation buffer containing 5% (w/v) sucrose, (the supernatant fraction constituted about half of the volume of the tubes and the fresh buffer constituted about one-third of the volume of the tubes). The tubes were centrifuged at $270{,}000 \times g$ for 2 h at 4 °C. After centrifugation the carotenosomes appeared as a dark orange band floating on top of the solution. This band was removed using a Pasteur pipette and stored in small aliquots at −20 or −80 °C until used.

Biochemical characterization of carotenosomes

SDS-PAGE and immunoblotting analyses of proteins were carried out as described previously (Vassilieva et al. 2002b). Total protein was assayed using a modified Lowry procedure as described (Procedure no. P5656, Sigma, St. Louis, MO) using bovine serum albumin as standard. Dr J. Zhao at Peking University (Beijing, People's Republic of China) determined the N-terminal sequences of proteins as described by Vassilieva et al. (2002b). The quantization of BChl *c*, BChl *a*, and carotenoids by absorption spectroscopy in methanol extracts and by HPLC analyses was carried out as described by Frigaard et al. (2004b).

Results

Changes in the proteome of bchK cells

One-dimensional SDS-PAGE analysis of whole-cell protein extracts from the wild-type and *bchK* mutant of *Chl. tepidum* showed differences in the expression level of several proteins (data not shown). Two proteins with significantly increased expression in the *bchK* mutant were identified by N-terminal amino acid sequencing as ferritin (CT1740, genome-predicted mass 23 kDa; the genome-predicted and determined N-terminal sequences were identical: MLSKTILDKL NHQVN) and a protein related to small heat-shock proteins (CT0644, genome-predicted mass 15 kDa; determined N-terminal sequence: MLVKIAIDPMGLFDD; genome-predicted N-terminal sequence: MLMKIAKDPMRLFDD).

Isolation of carotenosomes

The carotenosomes behaved similarly to wild-type chlorosomes in that they were efficiently dissociated from the cytoplasmic membrane in the presence of 2 M NaSCN, but behaved differently in that their density was significantly lower. Wild-type chlorosomes have a density corresponding to that of isolation buffer containing 2 M NaSCN and about 10–15% (w/v) sucrose. By performing ultracentrifugation of crude carotenosomes preparations, we empirically determined that the density of carotenosomes is between that of the isolation buffer with no added NaSCN or sucrose and that of isolation buffer containing 2 M NaSCN and no added sucrose. The cytoplasmic membrane fraction has a higher density corresponding to that of isolation buffer with 2 M NaSCN and about 30% (w/v) sucrose. These values suggest that carotenosomes have a lower protein-to-lipid content than wild-type chlorosomes and cytoplasmic membranes. For routine preparation of carotenosomes, 5% (w/v) sucrose was included in the isolation buffer. This

concentration allowed complete separation of the carotenosome fraction by flotation after only 2 h of ultracentrifugation.

Figure 2 shows the protein composition of the various fractions obtained during preparation of carotenosomes using a slightly modified procedure. After ultracentrifugation of the whole-cell extract (lane 2), the supernatant was collected (lane 4) and supplemented with crystalline sucrose to a final concentration of 5% (w/v). After ultracentrifugation the carotenosomes appeared as a dark orange band floating on top of the solution. This band was removed using a Pasteur pipette, diluted at least 10 times in fresh isolation buffer containing 5% (w/v) sucrose, and subjected to two additional rounds of ultracentrifugation (lanes 5 and 6). It is immediately apparent from lanes 5 and 6 that essentially no cellular proteins other than those associated with the carotenosomes were obtained in the top fraction that floated after ultracentrifugation. To demonstrate that the components obtained in this fraction are associated with a large structure, the carotenosomes loaded in lane 6 were also subjected to ultrafiltration using a 100,000 molecular-weight cut-off centrifugal device. All pigments and proteins in the carotenosome preparation were retained on the filter (lane 7).

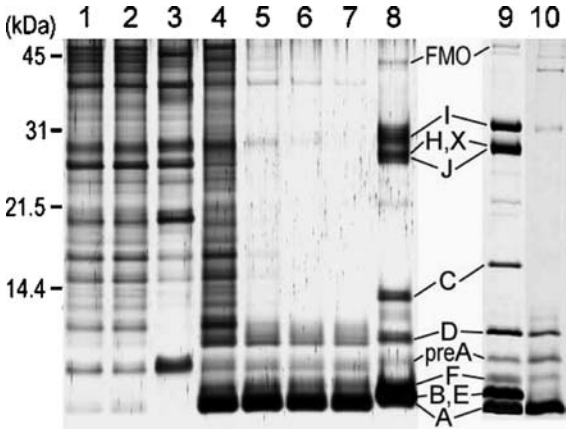

Figure 2. SDS-PAGE analysis of the protein composition of various fractions during preparation of carotenosomes: lane 1, whole cells; lane 2, clarified cell extract after French press disruption; lane 3, pellet after first ultracentrifugation; lanes 4–6, carotenosome fraction after first, second, and third ultracentrifugation, respectively; lane 7, carotenosome fraction retained on 100,000 molecular weight cut-off centrifugal filter; lane 8, chlorosomes from wild-type. Lanes 1–3, 6 and 7 correspond to 2.7 μg total protein and lanes 4–8 correspond to 2.0 μg total carotenoids. Proteins were visualized by silver staining. Numbers on the left indicate the position of molecular weight markers in kDa. Lanes 9 and 10 are isolated chlorosomes and carotenosomes, respectively, analyzed on a different SDS-PAGE gel with a higher resolution in the low-molecular region.

Electron and atomic force microscopy of carotenosomes

Negative staining and electron microscopy were used to visualize chlorosomes isolated from wild-type cells (Figure 3a). Carotenosomes could be visualized in the same manner (Figure 3b), although the quality of these images was not as high as that obtained with wild-type chlorosomes. Nevertheless, the carotenosomes appeared somewhat smaller and had a somewhat more irregular shape than wild-type chlorosomes.

Both chlorosomes (data not shown) and carotenosomes (Figures 3c and d) were also visualized with atomic force microscopy. The chlorosomes appeared as smooth, prolate ellipsoids with dimensions of approximately 212 ± 46 nm long, 122 ± 35 nm wide, and 35 ± 7 high (31 samples). The carotenosomes appeared as much more flattened structures with dimensions of approximately 86 ± 17 nm long, 66 ± 13 nm wide, and only 4.3 ± 0.8 nm high (22 samples). Based on the images obtained, it was obvious that atomic force microscopy was superior to electron microscopy with respect to identifying the carotenosomes and determining their physical dimensions.

Protein composition of carotenosomes

The ratio of protein to carotenoid in the carotenosomes was about one-third of that in wild-type chlorosomes (Table 1). The most likely reason for this is that the contents of all proteins, except CsmA and CsmD, were significantly reduced or below the level of detection. An analysis of wild-type chlorosomes (Figure 2, lanes 8 and 9) and carotenosomes (Figure 2, lanes 7 and 10) by SDS-PAGE showed that the carotenosomes contain CsmA, pre-CsmA, CsmD, and possibly some minor amounts of CsmB, CsmE, CsmF, and but that CsmI was significantly reduced and CsmC, CsmH, and CsmJ were missing. An apparent splitting of the CsmD band into two bands observed in some analyses of the carotenosomes (Figure 2, lane 7) was apparently due to an artifact that was not observed in other similar SDS-PAGE analyses (Figure 2, lane 10). When samples were compared on the basis of either carotenoid or

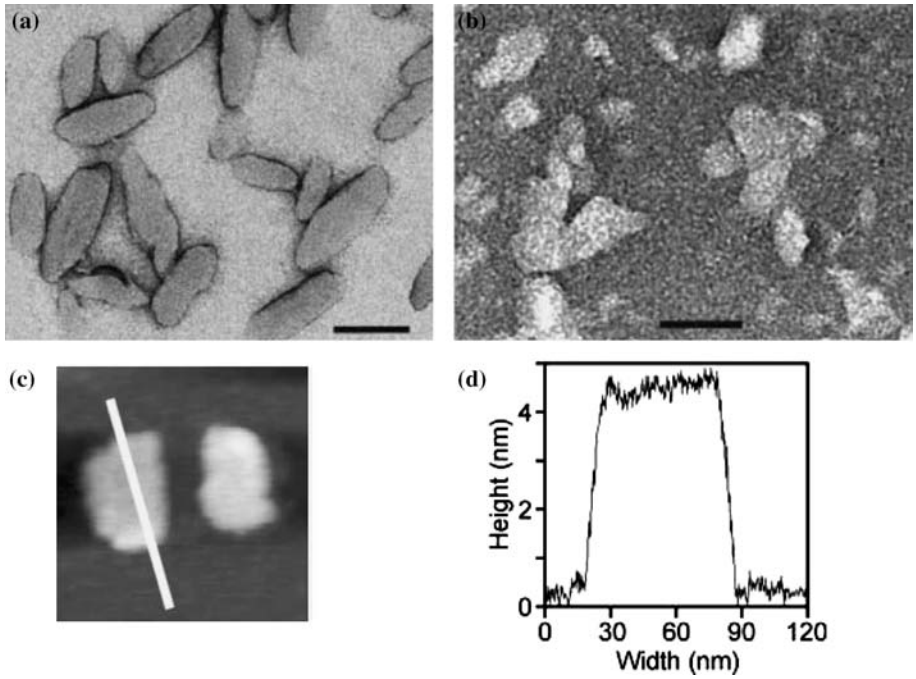

Figure 3. Visualization of wild-type chlorosomes (a) and carotenosomes (b–d) by electron microscopy and negative staining (a, b) or by atomic force microscopy on mica (c, d). The graph in (d) shows the height along the white bar in (c). The black bars in (a) and (b) are 100 nm; the white bar in (c) is 119 nm.

Table 1. Composition of chlorosomes isolated from wild-type and carotenosomes isolated from the *bchK* mutant of *Chlorobium tepidum*[a]

Component	Chlorosomes	Carotenosomes
BChl *c*	17	0
BChl *a*	0.23	0.14
Chlorobiumquinones	0.75	0.25
Menaquinone	0.32	0.13
Bacteriopheophytins *c*	0	< 0.04
Protein	4.0	1.35

[a] On a carotenoid basis (w/w). The values represent the average of at least two experiments in which the standard deviation did not exceed 15% of the average.

BChl *a* content immunoblotting of SDS-PAGE gels of carotenosomes and wild-type chlorosomes similar to the preparations analyzed in Figure 2 (lanes 7 and 8) confirmed that CsmA and CsmD were present in similar amounts in the carotenosomes and chlorosomes. Immunoblotting of carotenosomes also showed that CsmB, CsmE, CsmF, and CsmI were significantly reduced, and that CsmC, CsmH, CsmJ, and CsmX were not detectable (data not shown). N-terminal sequencing failed to detect CsmE, but small amounts of pre-CsmA, CsmB, CsmF, and CsmI were detected by amino acid sequence analysis. N-terminal sequencing also confirmed that both of the proteins from carotenosomes migrating similarly to CsmD in wild-type chlorosomes (Figure 2, lane 7) had identical N-terminal sequences corresponding to CsmD.

Two additional minor protein bands, with apparent molecular masses of about 35 and 40 kDa, appeared in the SDS-PAGE protein analyses of carotenosomes (Figure 2, lanes 5–7, 10). N-terminal sequencing identified the faster migrating polypeptide as CT0105 (predicted mass 35 kDa) and the more slowly migrating polypeptide as CT0104 (predicted mass 58 kDa). In contrast to wild-type chlorosomes, no detectable FMO protein was found to copurify with the carotenosomes.

Chemical cross-linking of isolated carotenosomes with *N*-ethyl-*N*′-(3-dimethylaminopropyl) carbodiimide (EDC) and subsequent SDS-PAGE protein analysis revealed that the proteins present form oligomeric complexes (Figure 4a). Immunoblotting confirmed that the cross-linked protein complexes are predominantly oligomers of CsmA

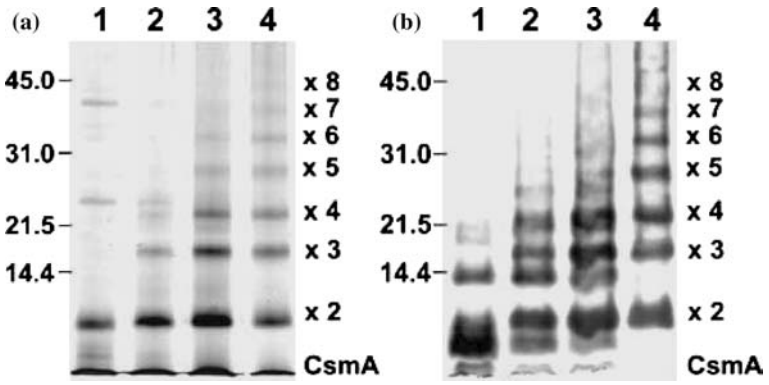

Figure 4. SDS-PAGE protein analysis of isolated carotenosomes treated with the protein cross-linker EDC. (a) Silver-stained gel, (b) immunoblot probed with anti-CsmA. Time of exposure to EDC: lane 1, no EDC; lane 2, 5 min; lane 3, 30 min; lane 4, 3 h.

(Figure 4b). The apparent sizes of the visible protein bands are consistent with the expected sizes for homo-oligomers of CsmA containing up to 8 subunits. Oligomers with higher numbers of subunits are probably formed but cannot easily be resolved under the conditions employed for electrophoresis.

Pigment composition of carotenosomes

Table 1 shows the pigment composition of carotenosomes and chlorosomes. Small amounts of bacteriopheophytins *c* form in the *bchK* mutant, probably due to non-specific esterification of intermediates from the incomplete BChl *c* biosynthetic pathway by BChl *a* synthase and/or Chl *a* synthase (Frigaard et al. 2002). The values in Table 1 correspond to about 12 carotenoid molecules and 4 quinone molecules per molecule of BChl *a* in the carotenosomes and about 80 BChl *c* molecules, 8 carotenoid molecules, and 6 quinones molecules per molecule of BChl *a* in the wild-type chlorosomes.

Optical and energy transfer properties of pigments in carotenosomes

The absorption spectrum of purified carotenosomes in aqueous buffer showed a broad band around 400–530 nm due to carotenoids, a small peak around 675 nm due to bacteriopheophytins *c*, and peaks at around 600 nm and at 797 nm due to BChl *a* (Figure 5). By injecting the same amount of carotenosomes into aqueous buffer and methanol, the absorption coefficients (ε) of the pigments in the carotenosomes was determined as follows: carotenoids at 444 nm had an ε of approximately 165 l g^{-1} cm^{-1} or 88 mM^{-1} cm^{-1} (based on an ε of 265 l g^{-1} cm^{-1} at 490 nm of chlorobactene in methanol; Frigaard et al. 2002); the BChl *a* species had an ε of 88 l g^{-1} cm^{-1} or 80 mM^{-1} cm^{-1} at the 797 nm peak maximum (based on an ε of 60 l g^{-1} cm^{-1} at 770 nm in methanol; Frigaard et al. 2002).

The drastic change in absorption properties of the carotenoids upon dissolution in methanol (Figure 5) suggests that most of the carotenoids are located in a highly apolar environment in the carotenosomes and that these carotenoids strongly interact with each other. On the other hand, the BChl *a* absorption properties in intact carotenosomes suggest that this BChl *a* only forms weak interactions with neighboring BChl *a* molecules. A similar suggestion about the BChl *a* molecules in

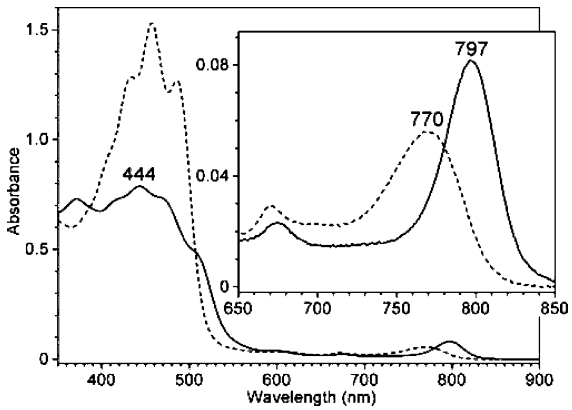

Figure 5. Absorption spectra of isolated carotenosomes diluted into an aqueous buffer (solid line) and into an equivalent amount of methanol (dotted line).

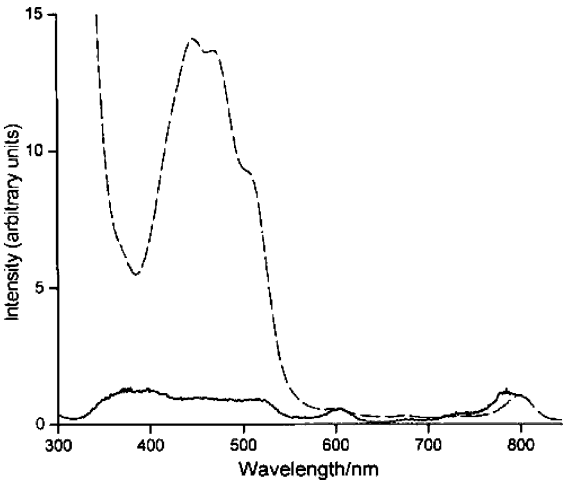

Figure 6. Overlaid absorption spectrum (dashed line) and fluorescence excitation spectrum (solid line, emission wavelength 810 nm) of carotenosomes under reducing conditions.

the CsmA complex from *Cfx. aurantiacus* was made based on circular dichroism spectroscopy (Montaño et al. 2003).

The fluorescence emission of the BChl *a* species in carotenosomes was maximal at 805 nm (data not shown). The BChl *a* emission intensity increased by a factor of about 3 upon reduction with sodium dithionite, and all fluorescence measurements described below were performed under reducing conditions unless otherwise stated. The fluorescence excitation spectrum of carotenosomes, when monitored at 810 nm, showed that the energy transfer from carotenoids to BChl *a* is rather small. As estimated by a comparison of the absorption spectrum and the excitation spectrum, only about 5% or less of the total energy absorbed by carotenoids is transferred to BChl *a* (Figure 6). Energy transfer was neither detected from carotenoids to the pheophytin, nor from the pheophytin to BChl *a*.

Properties of the CsmA–BChl a *protein complex*

The BChl *a* protein complex was remarkably stable under a number of harsh chemical or physical treatments. Treatment of isolated carotenosomes with 6 M guanidine hydrochloride, 8 M urea, 2 M sodium chloride, boiling for 3 min, or exposure to pH 6 or 9 did not affect the absorption spectrum of BChl *a* or carotenoids. Nor did these treatments apparently disrupt the physical structure of the carotenosomes, since they were retained on a 100,000 molecular-weight cut-off centrifugal filter device. However, the carotenosomes could be efficiently solubilized by treatment with 1% (w/v) Triton X-100 and 1% (v/v) 1-hexanol in 20 mM Tris–HCl buffer (pH 8), but this treatment also caused monomerization of the BChl *a*.

Discussion

Formation and structure of carotenosomes

Preventing the synthesis of BChl *c* in *Chl. tepidum* by mutagenesis did not prevent the cells from assembling vestigial chlorosome structures, which we have named carotenosomes. However, the carotenosomes are not merely chlorosomes devoid of BChl *c*, since their protein composition is dramatically altered and reduced as well. In contrast, genetic elimination of carotenoids or chlorosome proteins (other than CsmA) has much less, if any, effect on chlorosome formation (Frigaard et al. 2004a, b; H. Li et al. unpublished results).

Another important observation is that the physical, chemical, and optical properties of carotenosomes are direct evidence of the so-called baseplate structure. In the late 1970s, the baseplate was hypothesized to contain BChl *a* and to be a flat, paracrystalline structure attaching the chlorosome to the FMO protein and cytoplasmic membrane (Staehelin et al. 1978, 1980). The microscopy and protein cross-linking results presented here are in agreement with this idea. Our results also indicate that the baseplate in the carotenosomes is very similar to the baseplate in wild-type chlorosomes and that it is formed and functions independently of BChl *c* and chlorosome proteins other than CsmA.

Component interdependence and assembly order of chlorosomes and carotenosomes

Previous results have shown that an 80% reduction in the BChl *c* content of *Chlorobium vibrioforme* cells by inhibition of BChl *c* biosynthesis with acetylene does not significantly affect the cellular content of chlorosome proteins (Vassilieva et al. 2002a). In another experiment, Foidl et al. (1998) transferred *Cfx. aurantiacus* cells from chemotrophic (aerobic) to phototrophic (anaerobic) conditions and observed that the BChl *c*

content increased about 76-fold whereas the chlorosome protein content increased only about 10-fold. Thus, neither *Chl. vibrioforme* nor *Cfx. aurantiacus* has a fixed stoichiometric relationship between BChl *c* and the chlorosome proteins. The authors of these works also suggested that chlorosomes are synthesized by the filling of empty 'chlorosome bags' consisting of lipid and protein with BChl *c*.

However, in both aforementioned cases, varying levels of BChl *c* were present in the cells at all times, and thus the experiments cannot be directly compared to those presented here with the *Chl. tepidum bchK* mutant that completely lacks BChl *c*. In this mutant, the presence of chlorosome proteins, other than CsmA and CsmD, appears to be dependent on the presence of BChl *c*. This can be explained if the synthesis of the chlorosome proteins is affected at the transcriptional level by the presence of BChl *c*. Alternatively, all the chlorosome proteins may be synthesized constitutively. Either the insertion of these proteins into the chlorosome envelope requires the prior presence of BChl *c* or most of these proteins may be unstable and degraded in the absence of BChl *c*. Thus, stable incorporation of most chlorosome proteins into chlorosomes in *Chl. tepidum* seems to follow, or at least occur simultaneously with, the incorporation of BChl *c*. On the other hand, chlorosomes still form that are similar in stability and function to those of the wild-type in mutants unable to synthesize various chlorosome envelope proteins in *Chl. tepidum* (Frigaard et al. 2004a; H. Li et al. unpublished data). These studies imply that there is little or no significant interaction between BChl *c* and these proteins. It is therefore intriguing that the protein composition of the carotenosomes is so dramatically different from that of wild-type chlorosomes, because this change must be directly or indirectly due to the absence of BChl *c*. The 'empty bag' model for chlorosome biogenesis probably still has legitimacy, but if true, it appears that most of the chlorosome proteins are not present at the beginning of chlorosome assembly and are not essential for the biogenesis of this light-harvesting structure. We imagine that an oligomeric, BChl *a*-containing baseplate, which is stabilized with chlorosome-specific glycolipids, is synthesized first from CsmA. Next, other components (carotenoids, isoprenoid quinones, BChl *c*) accumulate in the chlorosome and only as BChl *c* accumulates, are the remaining chlorosome proteins incorporated.

The BChl a*–CsmA antenna complex*

Approximately 90% of the protein in carotenosomes was CsmA as estimated from the band intensities in SDS-PAGE analysis (Figure 2), and thus the content of CsmA was about 1.2 w/w on a carotenoid basis. The BChl *a* content was measured to about 0.14 w/w on a carotenoid basis, which gives an estimated ratio of about 0.8 BChl *a* molecules per CsmA molecule. This is very close to the suggested ratio of 1.0 (Sakuragi et al. 1999; Bryant et al. 2002; Montaño et al. 2003; Frigaard et al. 2004a) and also suggests that all BChl *a* in the carotenosomes is bound to CsmA.

The carotenosome-bound BChl *a* has an absorption maximum (797 nm) and an absorption coefficient (80 mM^{-1} cm^{-1}) that are significantly lower than the corresponding values in other BChl *a*-containing proteins such as the FMO protein and the LH1 and LH2 complexes in purple bacteria. This suggests a significantly lower degree of interaction among the BChl *a* molecules in the CsmA multimers than in the other antenna proteins mentioned. These observations are also consistent with the idea that each CsmA protein subunit binds a single BChl *a* molecule.

Each CsmA protein complex probably contains one or possibly two carotenoid molecules (Montaño et al. 2003). However, the carotenosomes contained about 12 carotenoid molecules per BChl *a* molecule. The excess carotenoids are probably non-specifically aggregated in the hydrophobic interior of the carotenosomes. This suggestion is supported by the observation that the absorption spectrum of the carotenoids drastically changes upon their dissolution in methanol. As expected for carotenoid molecules in such a physical arrangement, the excitation energy transfer efficiency from carotenoids to BChl *a* was very low (about 5%). If each CsmA complex was to contain one carotenoid molecule, this value could correspond to an energy transfer efficiency of roughly 60% from the carotenoid molecule to the BChl *a* molecule. Montaño et al. (2003) found an energy transfer efficiency of 30% in a

chlorosome baseplate preparation from *Cfx. aurantiacus* with a content of 2.6 mol carotenoids per mol BChl *a*; this corresponds to about 80% efficiency if each CsmA complex contained one carotenoid molecule. These values are similar to the efficiencies found in the LH1 and LH2 complexes of purple bacteria (about 40–70%; Frank and Cogdell 1996).

The BChl *a*–CsmA complex is interesting from several points of view. Its evolutionary origin is completely obscure but it seems likely to be linked to the origin and evolution of chlorosomes. In addition, it is as small as the α and β subunits of the LH1 and LH2 complexes of purple bacteria and filamentous anoxygenic phototrophic bacteria, yet it appears to be organized in a completely different manner at the tertiary and quaternary levels. Structural information would thus be of great interest. However, based on the changes in BChl *a* absorption upon monomerization (Montaño et al. 2004; this work), it apparently will be difficult, and may even be impossible, to isolate the BChl *a*–CsmA complex in its monomeric form with the BChl *a* organization intact. The simplicity of the protein composition in carotenosomes (>90% CsmA) and the observation that the CsmA organization in carotenosomes and wild-type chlorosomes are similar, if not identical, may help in understanding this antenna complex in green bacteria. Efforts are therefore currently underway to obtain structural information on the CsmA protein and the baseplate in isolated carotenosomes by solid-state NMR.

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