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Long-range organization of bacteriochlorophyll in chlorosomes of *Chlorobium tepidum* investigated by cryo-electron microscopy

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Abstract Intact chlorosomes of *Chlorobium tepidum* were embedded in amorphous ice layers and examined by cryo-electron microscopy to study the long-range organization of bacteriochlorophyll (BChl) layers. End-on views reveal that chlorosomes are composed of several multi-layer tubules of variable diameter (20–30 nm) with some locally undulating non-tubular lamellae in between. The multi-layered tubular structures are more regular and larger in a *C. tepidum* mutant that only synthesizes [8-ethyl, 12-methyl]-BChl *d*. Our data show that wild-type *C. tepidum* chlorosomes do not have a highly regular, long-range BChl *c* layer organization and that they contain several multi-layered tubules rather than single-layer tubules or exclusively undulating lamellae as previously proposed.
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1. Introduction

Chlorosomes are light-harvesting organelles found in all green sulfur bacteria (*Chlorobi*), some filamentous anoxygenic phototrophs of the phylum *Chloroflexi*, and the newly discovered aerobic phototroph, *Candidatus* Chloracidobacterium thermophilum [1,2]. They mostly contain special bacteriochlorophylls (BChls *c*, *d* or *e*) that absorb in the near infrared between 720 and 750 nm. Chlorosomes are the largest known antenna structures, and organisms that use chlorosomes for light-harvesting have been found at extraordinarily low light intensities under which no other phototrophs can grow [3,4].

The chlorosomes of the model green sulfur bacterium *Chlorobium tepidum* have been extensively characterized biochemically. These chlorosomes are literally sacs of BChl approximately 100–200 nm in length and 40–60 nm in diameter. A single chlorosome contains about 200 000–250 000 BChl *c* molecules, 2500 BChl *a* molecules, 20 000 carotenoid molecules, 15 000 chlorobiumquinone molecules, 3000 menaquinone-7 molecules, 5000 protein molecules of 10 different types, and about 20 000 lipid molecules [1]. Results from biochemical and genetic studies have established that the chlorosome envelope contains all 10 proteins [1,5] that CsmA binds BChl *a* and forms the chlorosome baseplate and interacts with the Fenna–Matthews–Olson protein [5–9], and that the BChl *c* molecules form large aggregates in the absence of proteins in the hydrophobic interior of the chlorosome [6,10].

Detailed models for the organization of the BChls in chlorosomes date back to the pioneering freeze-fracture electron microscopic studies of Staehelin and coworkers [11,12]. These studies suggested that the BChls are arranged in tubular structures with a diameter of about 10 nm in *Chlorobium limicola* and about 5 nm in *Chloroflexus aurantiacus*. Many advances in electron microscopy have been achieved in the 25 years since the studies of Staehelin and coworkers. However, in spite of much research, rod-based models for BChl organization in chlorosomes have continued to dominate the thinking of researchers until very recently. Pscencik and coworkers were the first to apply cryo-electron microscopy and low-angle X-ray diffraction approaches to re-evaluate the structure of the BChls in chlorosomes. These workers concluded that the BChls are arranged in undulating lamellae rather than tubular structures [13–15]. Based on solid state NMR and small-angle X-ray diffraction, models for the short-range packing have been proposed [15,16].

In the present study cryo-electron microscopy has been used to study chlorosomes from wild-type *C. tepidum* and from a mutant that produces a single homolog of BChl *d* (8-ethyl, 12-methyl, BChl *d*). In contrast to previous models, our results clearly demonstrate that chlorosomes contain one to several multi-layered tubules rather than single-layer tubules or exclusively undulating lamellae.

2. Materials and methods

WT *C. tepidum* and the *bchQ bchR bchU* mutant [17] were grown as described in [18]. Chlorosomes were purified essentially according to Griebenow and Holzwarth [19].

Electron microscopy was performed with a Tecnai G2 Polara electron microscope (FEI, Eindhoven, The Netherlands) equipped with a Gatan energy filter at 78 200× magnification. Images were recorded in the zero-loss imaging mode with a slow-scan CCD camera at 1 μm underfocus, to have optimal phase contrast transfer at 300 kV for details with a periodicity of about 2 nm.

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Aliquots of purified chlorosomes, submitted to holey carbon grids with a thin layer of carbon, were plunge-frozen in liquid ethane with a Vitrobot vitrification system (FEI).

3. Results

Cryo-electron microscopy was performed without any contrasting agent on intact chlorosomes embedded in vitreous ice layers about 100 nm thick. Wild-type *C. tepidum* chlorosomes were mostly observed side-on with their long axis parallel to the plane of the ice layer (Fig. 1, upper frame). In this orientation many chlorosomes show an internal pattern of closely spaced, thin lines, which represent the densely packed BChl layers. To evaluate the periodicity of the layers, Fourier transforms were calculated from a large number of selected arrays of side-on views, of which the best show that the spacing is about 2.1 nm (Fig. 2). The diffraction peaks are not extremely sharp and a second-order of diffraction is not visible, which is a direct indication of some variability in the packing of the BChl layers. Interestingly, some chlorosomes are fixed in a perpendicular orientation in the ice layer (Fig. 1, lower row). These end-on views reveal that individual chlorosomes are formed from multiple tubular structures with a variable diameter between about 10 and 15 nm. The tubules are multi-layered, and the layers have the same spacing (~2.1 nm) as observed in the side-on views. As can be seen in the third frame from the left in Fig. 1, where both orientations are next to each other, the tubules generally scatter more strongly in the end-on position than in the side-on position. This indicates that the tubules have substantial length.

In addition, electron microscopy was performed on chlorosomes isolated from a *bchQ bchR bchU* triple mutant of *C. tepidum*, in which the heterogeneous BChl *c* molecules methylated at the C-8^2 and C-12^1 positions are exclusively replaced by

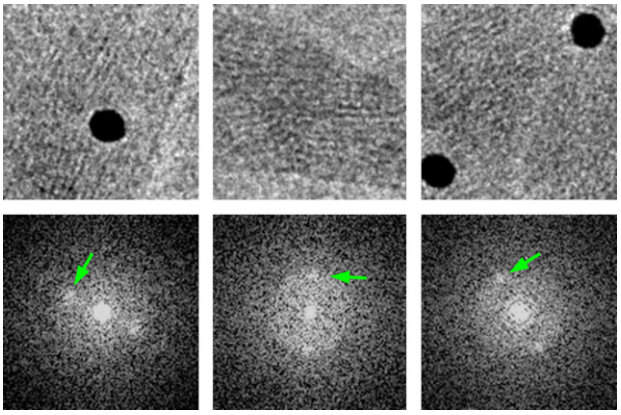

Fig. 2. A gallery of selected areas of side-on views of chlorosomes with the strongest regular spacing (upper row) and their corresponding Fourier transforms (lower row). The green arrows indicate a 2.1-nm spacing between BChl layers.

[8-ethyl, 12-methyl]-BChl *d* [20]. BChl *d* is very similar to BChl *c* but has a hydrogen at the C-20 methine carbon of the tetrapyrrole ring instead of methyl group [20]. The mutant produced chlorosomes of similar length and diameter, but with a slightly different overall shape. In the side-on views, the chlorosomes appeared wedge-shaped or nearly rectangular (Fig 3). A more striking difference was observed in the packing of the layers. The multi-layered tubular structures were more regular and had a larger number of layers than in chlorosomes of wild-type *C. tepidum*. Fourier transformation of side-on views from the mutant indicated, however, that the spacing between the layers was the same as those of the wild-type. In addition to the 2.1 nm spacing, a second-order of reflection at 1.0 nm was visible (Fig. 4, upper green arrow in second frame from left). Furthermore, an 0.8-nm spacing is apparent in two arrays (red arrows). This reflection is not a sharp spot but appears as

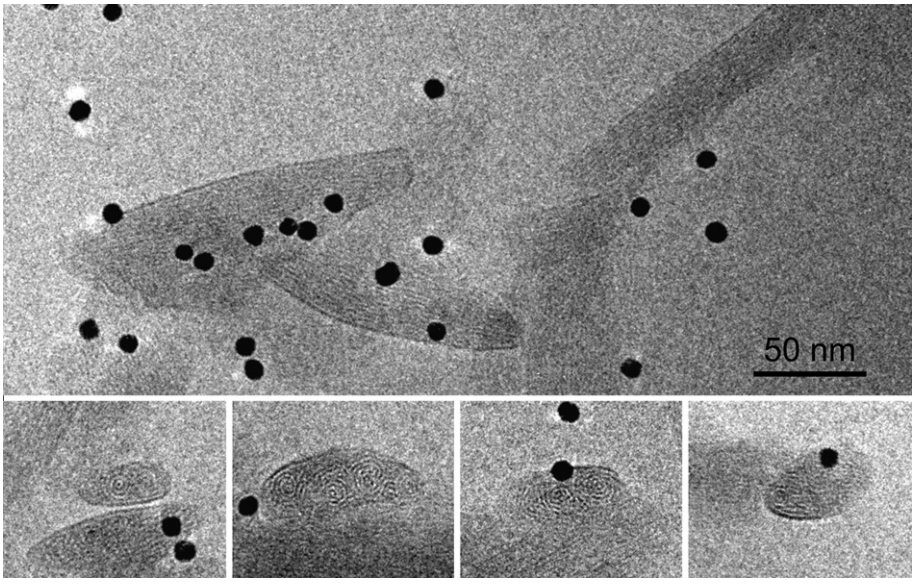

Fig. 1. Cryo-EM images of isolated chlorosomes from *C. tepidum* embedded in amorphous ice. In the upper frame chlorosomes are seen in side-on position; the lower row presents end-on views. The black spheres are 10 nm gold clusters, which were added to perform electron tomography reconstructions. In some images the chlorosome envelope appears to have two thicknesses, and the thicker part might represent the BChl *a*-CsmA complexes of the baseplate.

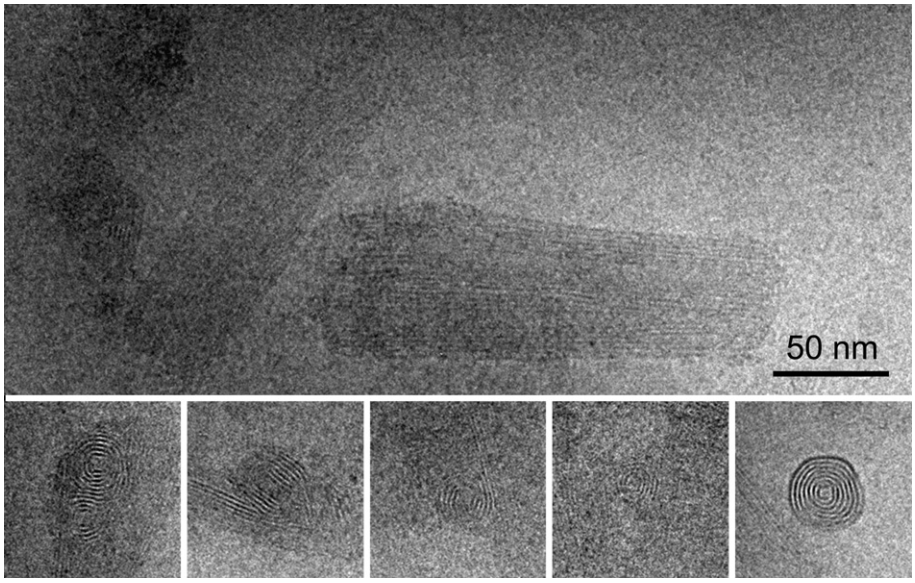

Fig. 3. Cryo-EM images of isolated chlorosomes from the *bchQ bchR bchU* triple mutant of *C. tepidum* embedded in amorphous ice. In the upper frame chlorosomes are seen in side-on position; the lower row presents end-on views.

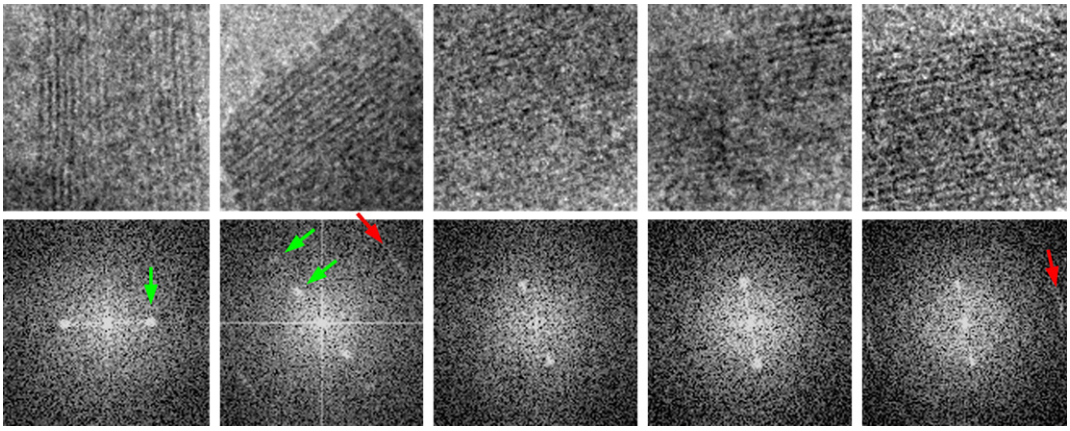

Fig. 4. A gallery of selected side-on arrays with highest regularity in layer spacing and their corresponding Fourier transforms. The green arrows indicate a 2.1-nm spacing between BChl layers, and a 1.0-nm spacing in one particular array; the red arrows indicate a 0.8-nm spacing along the BChl layers.

an arc. Because it occurs at an angle of about 90° relative to the 2.1-nm spacing, it must be produced by a repeating structure along the long axis of the tubules. This spacing reflects a lattice plane or layerline distance (in the case of helical symmetry). Without more knowledge about the packing of the asymmetric units (monomer or dimer) nothing can be concluded about the orientation of the tetrapyrrole ring or the content of the unit cell.

Finally, it was difficult to determine whether the tubules are composed of closed coaxial cylinders or of ''rolled-up'' sheets. However, the left panel from the mutant (Fig. 3) shows an end-on view in which some S-shaped layers seem to extend from one tubule to the other. This suggests that the BChls may form roll-up sheets. Similar rolled-up, supramolecular structures have been observed for several synthetic, amphipathic molecules nearly two decades ago [21].

4. Discussion

The exact supramolecular organization of the BChls within the chlorosomes has been a subject of long-standing discussion. Our original attempt to determine the structure was to perform 3D reconstructions by a method known as electron tomography on single non-stained, intact chlorosomes [22]. Tomography at a resolution of at least 2 nm, which is necessary to resolve the layer spacing of 2.1 nm in a relatively large object such as the chlorosome, is challenging and at the limit of current instrumentation. We recorded optimal tilt series with zero-loss imaging by tilting several chlorosomes along their long axis. Zero-loss imaging is a new but established technique to remove the noise contribution of non-elastically scattered electrons, which may contribute up to ca. 70% of the total signal of biological specimens. However, the obtained electron

tomograms did not resolve the internal 2.1 nm pattern, which is visible in projection in individual cryo-EM pictures. Nevertheless, zero-loss imaging, as carried out in this investigation, made it possible to produce high-contrast images of chlorosomes in relatively thick layers of ice. In thick layers some chlorosomes are oriented in a vertical position, yielding end-on views. The end-on views of *C. tepidum* chlorosomes of the wild-type and a mutant producing only [8-ethyl, 12-methyl]-BChl *d* have provided for the first time direct clues to the packing of BChl within the chlorosome interior. They show the presence of multi-layer BChl *c* or BChl *d* tubules of variable size with some non-tubular, locally undulating layers in between in chlorosomes from wild-type *C. tepidum*. In the *bchQ bchR bchU* mutant containing BChl *d*, the multi-layered tubules are much more regular. The methyl side chain of the C-20 methine carbon of BChl *c* is replaced by a hydrogen in BChl *d* [17]. Since the methyl group packs next to other side chains, steric hindrance causes a bend in the overall conformation of plane of the tetrapyrrole group in BChl *c*. In BChl *d*, however, the entire tetrapyrrole moiety is more planar. An important consequence of eliminating the methylation of the C-8^2 and C-12^1 positions is that virtually all of the BChl *c* should have *R* stereochemistry at the C-3^1 chiral carbon [17]. This may significantly reduce the tendency towards disorder observed in the wild-type chlorosomes and lead to the much more highly ordered structure that is observed. However, our electron microscopy data do not give strong clues to the atomic-level, short-range packing of the BChl molecules within the layers, and therefore it is not easy to reconcile these new images with the existing models for packing BChl *c* based upon small-angle X-ray and solid state NMR [13–16]. The only exception is that there must be a 0.8-nm spacing in a structural motif perpendicular to the spacing of the layers. However, the diffraction shows a single spacing and to determine the precise 3D packing of BChl molecules a complete set of spacings in 3D, preferentially to about 0.3-nm resolution, is required. In the absence of such data, the 3D packing of BChl remains an open question, although current models based on scattering profiles by small-angle X-ray diffraction and distances measured by solid state NMR might describe packings that are close to the actual situation [15,16].

A model for the long-range organization of BChl in *C. tepidum* chlorosomes, based on the observations on both the wild-type and triple mutant, is shown in Fig. 5. BChl layers are organized as coaxial cylinders or rolled-up sheets, embedded in a matrix of structurally less well defined BChl assemblies. In the *bchQ bchR bchU* mutant most of the BChl is located in the cylindrical structures with a diameter of 20–30 nm, whereas in the wild-type chlorosomes the BChl is less well ordered and the diameter of the cylindrical structures is limited to 10–15 nm. Based on our current EM results we cannot distinguish between coaxial cylinders and rolled-up sheets.

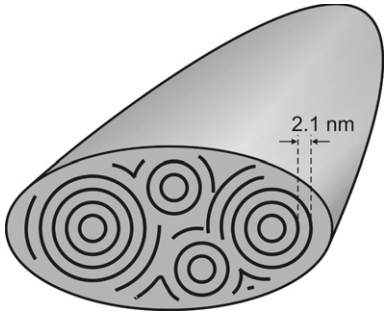

Fig. 5. Schematic model of the BChl aggregates in the chlorosomes of *C. tepidum*. Multi-layered tubular or rolled-up sheets of variable diameter are embedded in a structurally less well-defined matrix. The spacing between BChl layers is 2.1 nm.

From the side-on views of chlorosomes from wild-type *C. tepidum*, we can deduce that the tubules have a length of up to 200 nm. It is likely that they extend through the entire length of the chlorosome. This was also previously suggested by freeze-fracturing electron microscopy [11]. The overall impression is that the chlorosome does not have a highly regular supramolecular organization of its Bchl *c* molecules. The tubules do not have a highly defined diameter and packing, which is consistent with the original EM images from freeze-fracturing [11].

Recently, an undulating lamellar model was proposed for the long-range arrangement of BChl aggregates in chlorosomes of three different *Chlorobium* species, which contain BChl *c* or BChl *e* as the main pigments [13,14]. The model was obtained by electron microscopy, based on tilted specimens. Our model is fully consistent with results of the tilt experiments by these workers, but not with their interpretation, because our additional end-on EM data directly demonstrate that at least the majority of the BChls in the chlorosomes in *C. tepidum* form multi-layered cylinders. Moreover, the tubular structures are not arranged in single layers as suggested by [23]. Because the latter contribution not only presents a model for the long-range organization of BChl *c* but also for the short-range associations between individual molecules, it is obvious that further structural data by cryo-electron microscopy, selected area electron diffraction and ^{13}C solid-state NMR will be necessary to obtain a consistent, high-resolution model that can explain the long-range and short-range ordering and its resulting spectroscopic signals (e.g., CD) in intact chlorosomes.

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