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

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Received 28 September 2007; accepted 25 October 2007

Available online 5 November 2007

Edited by Richard Cogdell

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.

Keywords: Chlorosome; Bacteriochlorophyll c; Bacteriochlorophyll d; Cryo-electron microscopy; *Chlorobium tepidum*

1. Introduction

Chlorosomes are light-harvesting organelles found in all green sulfur bacteria (*Chlorobium*), 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 200000–2500000 BChl c molecules, 2500 BChl a molecules, 20000 carotenoid molecules, 15000 chlorobiumquinone molecules, 3000 menaquinone-17 molecules, 5000 protein molecules of 10 different types, and about 20000 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–Olsen 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. Psencik 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 Griebnow 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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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 and C-12 positions are exclusively replaced by [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...
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 tetrapyrole moiety is more planar. An important consequence of eliminating the methylation of the C-8' and C-12' positions is that virtually all of the BChl c is shown in Fig. 5. BChl layers are or-dum solid state NMR might describe packings that are close to question, although current models based on scattering profiles in the absence of such data, the 3D packing of BChl remains an open erentially to about 0.3-nm resolution, is required. In the layers, the diffraction shows a single spacing and to determine the precise 3D packing of BChl molecules a complete set of spacings in 3D, pref-erentially 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. tepi-dum* chlorosomes, based on the observations on both the wild-type and triple mutant, is shown in Fig. 5. BChl layers are organ-ized 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 distin-guish between coaxial cylinders and rolled-up sheets.

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 reg-ular 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 chloro-somes 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 speci-mens. Our model is fully consistent with results of the tilt experiments by these workers, but not with their interpreta-tion, because our additional end-on EM data directly demon-strate 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 sug-gested by [23]. Because the latter contribution not only pre-sents a model for the long-range organization of BChl c but also for the short-range associations between individual mole-cules, it is obvious that further structural data by cryo-electron microscopy, selected area electron diffraction and 13C solid-state NMR will be necessary to obtain a consistent, high-reso-lution model that can explain the long-range and short-range ordering and its resulting spectroscopic signals (e.g., CD) in intact chlorosomes.

**Acknowledgements:** We thank Mrs. Wilma Bergsma-Schutter for skill-ful technical assistance and Prof. Huub de Groot for discussions. E.J.B. thanks the Netherlands Organisation for Scientific Research NWO for funding of a Tecnai G2 Polara electron microscope. This work was supported by Grant DE-FG02-94ER20137 from the US Department of Energy to D.A.B.

**References**


