Alternating syn-anti bacteriochlorophylls form concentric helical nanotubes in chlorosomes

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Chlorosomes are the largest and most efficient light-harvesting antennae found in nature, and they are constructed from hundreds of thousands of self-assembled bacteriochlorophyll (BChl) c, d, or e pigments. Because they form very large and compositionally heterogeneous organelles, they have been the only photosynthetic antenna system for which no detailed structural information was available. In our approach, the structure of a member of the chlorosome class was determined and compared with the wild type (WT) to resolve how the biological light-harvesting function of the chlorosome is established. By constructing a triple mutant, the heterogeneous BChl c pigment composition of chlorosomes of the green sulfur bacterium Chlorobaculum tepidum was simplified to nearly homogeneous BChl d. Computational integration of two different bioimaging techniques, solid-state NMR and cryoEM, revealed an undescribed syn-anti stacking mode and showed how ligated BChl c and d self-assemble into coaxial cylinders to form tubular-shaped elements. A close packing of BChls via π–π stacking and helical H-bonding networks present in both the mutant and in the WT forms the basis for ultrafast, long-distance transmission of excitation energy. The structural framework is robust and can accommodate extensive chemical heterogeneity in the BChl side chains for adaptive optimization of the light-harvesting functionality in low-light environments. In addition, syn-anti BChl stacks form sheets that allow for strong exciton overlap in two dimensions enabling triplet exciton formation for efficient photoprotection.

Chlorosomes are assembled from BChl c, d, or e and contain naturally heterogeneous molecular mixtures of BChls with different side chain modifications and stereochemistry (Fig. 1). In particular, chlorosomes of the green sulfur bacterium Chlorobaculum tepidum contain a complex mixture of BChl c molecules with variable degrees of methylation at carbons C-8 and C-121 (Fig. 1), variations in the esterifying alcohol side chain of the C-17 carboxyl group, and both R- and S-chirality at the C-31 carbon (1, 12). Because a heterogeneous structure cannot be determined by crystallographic methods, chlorosomes are the only class of antennae for which no structural information is currently available. Conflicting models have been derived from spectroscopic and electron microscopic assessments (13–16) and from studies with chemical analogs (17).

Recent advances in understanding the biosynthesis of BChl c have led to a well-characterized bchQ bchR bchU (hereafter bchQRU) C. tepidum triple mutant (12). This mutant synthesizes well-defined, extended chlorosomes that contain >95% 172-farnesyl-R-[8-ethyl,12-methyl]BChl d (Fig. 1) and form regularly packed, tubular-shaped elements with a diameter >10 nm that are interconnected via curved sheets (16, 18). The


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Fig. 1. BChl homologues for C. tepidum. In the WT, R1 = Me; R2 = Et, n-Pr, or i-Bu; R3 = Me, or Et. In the bchQRU mutant R1 = H; R2 = Et; R3 = Me.
of alternating supramolecular arrangements that could satisfy these constraints are the new stacking model based on alternating syn-anti monomers (Fig. 3D) or antiparallel monomer stacking (Fig. 3C).

In addition to the intrastack correlations, intermolecular correlations between carbons C-12, C-3, C-12, C-2, and C-12, C-3 were detected between rings I and III of adjacent BChl d molecules. When syn-anti monomer stacks are combined to form a sheet, these distances are very short (Fig. 2C and Table 1), whereas for the antiparallel monomer stacking the shortest 1H-1H distances between C-12/C-3, C-12/C-2, and C-12/C-3 are 3.46, 6.28, and 2.20 Å, respectively. Because the short mixing time of 250 μs produces 1H transfer over short distances <3 Å, the interstack correlations provide convincing evidence that syn-anti monomer stacking is the basic building block in the bchQRU chlorosome structure.

In chlorophyll aggregates, the 1H signals shift upfield by ring current effects from neighboring molecules (20). Density functional theory calculations were performed to estimate the ring currents for the syn-anti monomer model that was determined from the distance constraints, the antiparallel monomer model, and two earlier structural models that were proposed for BChl c in chlorosomes: the monomer-based, parallel-stack model (21) and the piggy-back dimer model (22) (Fig. 3). The syn-anti monomer stack has alter-
nating molecular conformations of the C-3\textsuperscript{1} side chain, and syn or anti refers to the orientation of the OH ligation of adjacent BChls with respect to the farnesyl side chain. The calculated shifts for the antiparallel monomer stack model and the piggy-back dimer model are much larger than the experimentally observed shifts (see Table S1 and Fig. S3). These calculations showed that only the syn-anti monomer stack and the parallel stack can reproduce the experimental ring current shifts that are observed. In these two arrangements, each BChl d molecule has significant overlap with two adjacent molecules, via rings I and III, at opposite sides of the macroaromatic cycle (Fig. S4). The calculations for the piggy-back dimer model gave anomalously large proton ring current shifts at C-5–H, C-3\textsuperscript{1}–H, and C-3\textsuperscript{3}–H\textsubscript{3} whereas the antiparallel monomer stack gave a mismatch over the entire overlap region of C-1–C-5 and C-15–C-13\textsuperscript{2}. In these two arrangements, there is pronounced overlap with two neighbors at ring I, with correspondingly large ring current shifts, and little overlap at ring III, and this produces strongly asymmetric ring current shift patterns that were not observed experimentally (Fig. S4).

In end-on views of chlorosomes from the bchQRU mutant obtained with cryoEM, the BChls can be observed to form coaxial cylinders (Fig. 4B). In side views the concentric layers produce a regular pattern with a spacing of 2.10 \pm 0.12 nm (Fig. 4A and D) (16, 18). Additionally, a distinct, striped pattern with a spacing of 0.83 \pm 0.01 nm was observed at a 90° angle to the layers (Fig. 4C and D). After Fourier transformation, the layers translated into a single pair of equatorial reflections at 1/(2.1 nm) whereas the stripes gave rise to strong layer lines that revealed a predominant, helical arrangement with an axial repeat of 0.83 nm. In addition to the reflections from the 2.1-nm layer spacing, this spacing can clearly and reproducibly be observed in many images at high magnification.

To determine the arrangement of the BChl d molecules in the multilayer tubular structures of chlorosomes of the bchQRU mutant, supramolecular models were built for different orientations of the stacks relative to the tube axis (Figs. 4 and 5 and Fig. S5). With stacks running perpendicular to the tube axis along the circumference of a cylinder in rings, the simulated image and its Fourier transform reproduced the strong periodicity of 0.83 nm and the distinct striped appearance that is observed in the cryoEM images of the chlorosomes of the bchQRU mutant (compare Fig. 4 C and E). The 2.1-nm spacing is reproduced by combining several coaxial cylinders with an increment in the radius of 2.1 nm (Fig. 4 E and F). The high contrast of the structure in the chlorosomes of the bchQRU mutant arises from the orientation of the BChl molecular planes and the rotational symmetry (Fig. 5B). The plane of the molecules lies along the optical axis, i.e., the projection direction of the microscope, and the direction of the stacks lies along the circumference of the tubes. This organization leads to a strong alternation of high and low projected density along the tube axis direction.

For the C. tepidum WT chlorosomes, the spacing between layers matches the separation of 2.1 nm observed in the chlorosomes of the bchQRU mutant (Fig. S5) (18). No other distinct spacing, similar to that in bchQRU mutant chlorosomes, could be detected visually in the cryoEM images. However, Fourier transforms of the images clearly showed the presence of a weak layerline at 1.22 \pm 0.03 nm\textsuperscript{-1} (Fig. S5). This periodicity corresponds to the distance between repeating syn-anti pair units in the direction of the stacks (Fig. 5 A and C), and the Fourier transform of the projected model structure shows a layer line at 1/1.25 nm\textsuperscript{-1} (Fig. S5). Intermediate orientations of the stacks produced periodicities between 0.83 and 1.25 nm and failed to reproduce the characteristic cryoEM appearance of the chlorosomes of the bchQRU mutant or the WT.

In models in which the direction of the BChl stacks deviates slightly from the two orientations for WT and bchQRU mutant presented in Fig. 5, the expected spacing in the direction of the tube is insensitive to small variations around the 0 and 90° orientation of the stacks. Heterogeneity in the chlorosomes system generally will lead to partial symmetry breaking and a disappearance of the weaker layer lines while only the strongest one remains visible. For instance, in Fig. S5, for a model with stacks forming shallow helices instead of perfectly symmetric rings, only the layer line at 1/0.83 nm appears in the Fourier transform (Fig. S5), which is in agreement with Fourier transforms of the cryoEM images. Thus, stacks forming shallow left or right helices in bchQRU chlorosomes and stacks forming steeper helices in the WT chlorosomes are consistent with the EM data. In Fig. 5A the unit cell of the sheet is indicated, where \( a = 1.25 \) nm is in the direction of the stack, and \( b = 0.98 \) nm is at an angle of \( \gamma = 122^{\circ} \) to \( a \). In Fig. 5B the basic helix is along the \( b \)-direction of the unit cell, with the stack perpendicular to the tube axis. For \( n \) BChl pairs in each ring, the tube comprises a multistart helix with \( n \)-fold rotational symmetry (Fig. 5B). In contrast, the stacks in Fig. 5C do not form rings but lie along helices without rotational symmetry.

**Discussion**

WT chlorosomes are much more heterogeneous than chlorosomes from the bchQRU mutant and contain BChl c molecules with a greater proportion of S-chirality at carbon C-3\textsuperscript{1} and variable degrees of methylation at carbons C-8\textsuperscript{2} and C-12\textsuperscript{2}.
Additionally, end views of WT chlorosomes by cryoEM had shown that the internal organization of BChls was unique in individual chlorosomes (18). This extensive heterogeneity had thus far prevented a precise determination of the structure at the molecular level. However, the chlorosomes of the bchQRU mutant provided the possibility for understanding the underpinning of the chlorosome suprastructure, optical properties, and variability among species.

The two supramolecular arrangements, with stacks running parallel or perpendicular to the tube axis, produce similar O—H—O—C exciton delocalization pathways. Both for syn-anti monomer stacks running perpendicular and for stacks parallel to the tube axis, the monomer transition moments align along the hydrogen bonds. After excitation, the excitons would move through the BChl aggregates along helical trajectories following the BChl aggregates along helical trajectories following the O—H—O—C connection pathway, which reflects the macroscopic chirality of the system (8). When tubes are formed by bending the molecular arrays in the opposite direction, the helicities also change sign. The bending direction will thus affect the circular dichroism sign, which has often been reported to vary in different preparations (23). The inclination of the optical transition dipole moment of the monomers with respect to the long axis of the tube in Fig. 5B is ≈55°. The structural arrangement of the BChls in sheets with overlap between the stacks allows for strong triplet–triplet exciton interactions, which forms the basis for the self-protection of the chlorosome against photodegradation in high light under oxic conditions (6).

Chlorosomes were probably an early invention in biology because they are found in three of the six groups of chlorophototrophs, and two of those three groups have homodimeric type 1 reaction centers (1, 2). The self-organization of the BChls into layers spaced by 2.1 nm is a common property that is preserved across species and allows for the inclusion of carotenoids and quinones by packing between the esterifying alcohols of the chlorins (18). The concentric tube structure of chlorosomes is optimal for very efficient energy transfer in a very large antenna, a prerequisite for photosynthetic growth under very weak light conditions. The suprastructure is stabilized by the underlying principles of the self-assembly process, whereas its size and heterogeneity can be optimized for optimal functionality. As measured by the growth rate of cells, the larger, more complex and heterogeneous WT chlorosomes outperform the chlorosomes of the bchQRU mutant at all light intensities between 5 and 200 μmol of photons m⁻² s⁻¹ (12). Consistent with the idea that BChls c in a larger number of environments leads to better light harvesting, the half-band width of the Qₐ absorption maximum of the BChl c aggregates is largest for WT chlorosomes isolated from cells grown at low light intensity and is also much broader than for chlorosomes of the bchQRU mutant (12). When grown at very low

[Fig. 5. Structural models of chlorosomes in the bchQRU mutant and the WT. (A) 7 × 5 section of the syn-anti array. The stacks are indicated by red lines. The separation perpendicular (⊥) to the stacks is 0.83 nm. The unit cell dimensions are: a = 1.25, b = 0.98, γ = 122°. The repeat in the direction parallel (∥) to the stack is 1.25 nm. (B and C) Multistart helices with the long axis running perpendicular or parallel to the stacks. Periodicities of 1.25 nm and 0.83 nm are indicated. The syn-anti pairs in B form a multistart helix with rotational symmetry. The H-bond helices that connect BChl stacks via the carboxyls are shown in blue and are right-handed in B and left-handed in C.]

[Fig. 4. Integration of cryoEM and NMR data to resolve the bchQRU mutant chlorosome structure. (A and B) CryoEM images of isolated chlorosomes from the bchQRU mutant of C. tepidum embedded in amorphous ice. A chlorosome is seen in side-on view (A) and in end-on view, showing the involuted layering of the BChls (B). (C) Enlarged region of the side-on view, indicated by the red box in A. (D) Fourier transform of the red-boxed region in A. The red arrows indicate reflections from a 2.1-nm spacing between BChl layers and a 0.83-nm spacing along the layers. (E and F) Simulated EM image and its Fourier transform, from a 172-farnesyl-R-[8-ethyl,12-methyl]BChl d helical rod model comprising 4 tubes built from NMR-derived and geometry-optimized syn-anti monomer stacks that run along the circumference of the tubes, perpendicular to their long axis. The 1/0.83-nm periodicity corresponds to the spacing between adjacent BChl stacks running perpendicular to the tube axis. As explained in the text, there are no additional layer lines for shallow helices, and the blurring of the 2.1-nm reflections on the equator is because of disorder. (G) Enlarged view of the boxed area in E. (H) Top view, i.e., along the tube axis.]
light intensity (~5 μmol of photons m⁻² s⁻¹), C. tepidum cells produce approximately three times more BChl c than cells grown at high light intensity (~100 μmol of photons m⁻² s⁻¹). Because the cell size does not change and the number of chlorosomes per cell does not change dramatically, the number of BChls per chlorosome must increase by ~3-fold (12).

Because the electronic properties of the supramolecular system and the absorption cross-section of the antenna can be modified by introducing disorder at the molecular level, functional advantages are achieved by adopting a suprastructure that is sufficiently plastic and flexible to accommodate considerable chemical heterogeneity. In the modeling, the two configurations having stacks running parallel or perpendicular to the tube axis correspond to local minima in the energy landscape of the suprastructure that permit chemical heterogeneity to be embedded within the structure. The layer lines observed in the Fourier transforms of the cryoEM data from the WT reveal extended periodic regions of stacks built from BChl pairs that run parallel to the tube axis in the suprastructure layer lines observed in the Fourier transforms of the cryoEM data.

In conclusion, researchers have debated for decades about the structure of the chlorosome, which consists of hundreds of thousands of BChls self-assembled into the most efficient light-harvesting antennae in nature. By genetic modification of the BChl biosynthesis pathway, the inherent disorder in the chlorosomes of C. tepidum was reduced without functionally disturbing the self-assembly process. Computational integration of cryoEM and solid-state NMR was used in this work to extend and connect the structure information from the molecular to the supramolecular level for the bchQRU mutant to obtain very detailed structure information. This method may well prove useful in the future for the solution of other biological structures that cannot be crystalized or are inherently disordered. The mutant structure provides a template for the evaluation of the more complex structure of the WT. Both chlorosome suprastructures are built from syn-anti monomer stacks self-assembled into nanotubes. The basis for the efficient and ultrafast light harvesting is a helical exciton delocalization pathway that is realized with stacks running parallel or perpendicular to the tube axis in WT and mutant, respectively. The suprastructure can accommodate heterogeneity in the side chains for evolutionary optimization of light harvesting without the direct intervention of proteins to scaffold the pigment molecules. This latter feature must have been critical to the evolution of these structures in natural environments that are severely energy limited.

Materials and Methods

Sample Preparation. Cells of the C. tepidum bchQRU triple mutant strain described by Gomez Maqueo Chew et al. (12) were grown as described by Balaban et al. (14). Samples of the corresponding WT strain were also prepared for comparison. Cultures were grown in 1.5-L fermentation bottles with continuous slow stirring at 40 °C. Growth light was controlled at the surface of the bottles (25 μEm⁻² s⁻¹ photosynthetically active radiation) provided by two 40-W fluorescent neon tubes. For the bchQRU mutant and the WT, cells uniformly labeled with 13C were obtained in steps. First, a 50-mL inoculum of cells grown in Waulund medium was inoculated into Waulund medium without acetate (1 L). In the next step, a 50-mL cell inoculum obtained from the acetate-free culture was inoculated into Waulund medium containing sodium acetate (40 mL). This provided a carbon source. Cells were grown for 3 days and then harvested by centrifugation. Chlorosomes from the two cell types, with the BChl a-containing baseplate attached, were isolated as described in ref. 14. Membranes were incubated for 1 h with 0.7% (wt/vol) lauryl dimethylamine oxide (LDAO) at an OD₅₀₋₅₄ ~ 20 cm⁻¹ at the wavelength of maximum absorbance in the Q₃ region between 700 and 750 nm. Chlorosomes were isolated by sucrose density gradient centrifugation; the gradient had a bottom layer of 10% (wt/vol) sucrose and a linear gradient from 10 to 40% (wt/vol) sucrose. The collected chlorosome band was diluted in 20 mM Tris-HCl (pH 8.0) to bring the sample below the critical micellar concentration (~1–2 mM) of LDAO. The sample was washed to remove sucrose and LDAO by ultrafiltration in an Amicon concentrator equipped with a YM-100 membrane. The final chlorosome suspensions in Tris-HCl buffer showed the expected visible absorption maxima. Chlorosomes were kept in the dark at ~4 °C before EM and NMR data collection. The pigments from the chlorosomes of the bchQRU mutant were also isolated and analyzed following the methods described in Steensgaard et al. (25), and ~95% of 17-farnesyl-R-[8-ethyl,12-methyl]BChl d was found. For the EM, chlorosomes were also prepared with the NaSCN method (26). To facilitate the freezing process for EM, the 2 M NaSCN was removed by dialysis against buffer. The tendency of chlorosomes to clump together was much less with the NaSCN than for the LDAO preparation, which facilitated the EM measurements.

NMR Measurements. All solid-state, MAS NMR experiments were performed with a Bruker AV-750 spectrometer equipped with a 4-mm triple resonance MAS probe head (Bruker) by using a 13C radio frequency of 188.6 MHz and a sample temperature of 277 K. Spinning frequencies of 11 kHz ± 5 Hz and 13 kHz ± 5 Hz were used for 2D 13C-1H homo- and 1H-13C heteronuclear correlation experiments, respectively. The 1H spins were decoupled during acquisition by using the two-pulse phase modulation scheme (27) in all of the experiments. Two-dimensional 13C-13C dipolar correlation spectra were recorded by using the radio
frequency-driven, dipolar recoupling sequence ($\tilde{\varphi}^2$) with phase-sensitive detection in $\omega_n$ at mixing times of 1.4 ms (Fig. S1), 2.9 ms (Fig. S2), and 3.1 ms. A $\tilde{\varphi}^2$ pulse length of 3.1 $\mu$s was used with cross-polarization periods of 2 ms. For each of the 256 steps in the indirect dimension, 128 scans were collected. Two-dimensional $^{13}C$–$^{13}C$ spectra were recorded using the CHHC/CP sequence (19, 29) for indirect detection of $^{13}C$–$^{13}C$ contacts at $\Delta$ diffusion times of 250 $\mu$s (Fig. 2A) and 325 $\mu$s. The initial CP contact time was set to 256 $\mu$s. Short CP contact times of 128 $\mu$s enclosing the $^{13}C$–$^{13}C$ spin diffusion step were used to ensure that the polarization transfer was restricted to directly bonded $^{13}C$–$^{13}C$ spin pairs. For each of the 256 steps in the indirect dimension, 128 scans were collected. Two-dimensional $^{13}C$–$^{13}C$ heteronuclear correlation data were collected using the frequency-switched Lee–Goldburg (FSLG) experiment (30), with a short CP time of 256 $\mu$s and a $\tilde{\varphi}^1$ 90° pulse of 3.1 $\mu$s (Fig. S1). The $^{13}C$ chemical shift was calibrated from a FSLG spectrum of solid urea with STC and validated with the chemical shift correlation plots (Fig. S6). For each of the 128 steps in the indirect $^{13}C$ dimension, 128 $^{13}C$ scans were accumulated.

**CryoEM Measurements.** Aliquots of purified chlorosomes were applied to holey carbon grids with a thin layer of carbon and were plunge-frozen in liquid ethane at 83 K with a Vitrobot vitrification system (FEI). Electron microscopy was performed with a Tecnai G2 Polara electron microscope (FEI) equipped with a Gatan energy filter at 115,000 eV, with a slow-scan CCD camera at 1 eV. With the combination of cryoEM and NMR, which can be visually compared with the biological specimen (Fig. 4), density functional theory calculations were performed using the Gaussian 03 software package (32) and the Becke, Lee, Yang, and Parr (BLYP) (33, 34) exchange–correlation functional, which has been used before to estimate NMR shifts for Chl systems (35). Ring current shifts were calculated using the gauge-independent atomic orbital method (37–39).

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