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Alternating syn-anti bacteriochlorophylls form concentric helical nanotubes in chlorosomes

Swapna Ganapathy\textsuperscript{a}, Gert T. Oostergetel\textsuperscript{b}, Piotr K. Wawrzyniak\textsuperscript{a}, Michael Reus\textsuperscript{c}, Aline Gomez Maqueo Chew\textsuperscript{d,1}, Francesco Buda\textsuperscript{a}, Egbert J. Boekema\textsuperscript{b}, Donald A. Bryant\textsuperscript{d}, Alfred R. Holzwarth\textsuperscript{c}, and Huub J. M. de Groot\textsuperscript{a,2}

\textsuperscript{a}Leiden Institute of Chemistry, P. O. Box 9502, 2300 RA Leiden, The Netherlands; \textsuperscript{b}Groningen Biomolecular Sciences and Biotechnology Institute, 9747 AG Groningen, The Netherlands; \textsuperscript{c}Max-Planck-Institut für Bioanorganische Chemie, D-45470 Mülheim an der Ruhr, Germany; and \textsuperscript{d}Department of Biochemistry and Molecular Biology, The Pennsylvania State University, University Park, PA 16802

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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) \textit{c, d}, or \textit{e} pigments. Because they form very large and compositionally heterogeneous organelles, they had 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 \textit{c} pigment composition of chlorosomes of the green sulfur bacterium \textit{Chlorobaculum tepidum} was simplified to nearly homogeneous BChl \textit{d}. Computational integration of two different bioimaging techniques, solid-state NMR and cryoEM, revealed an undescribed \textit{syn-anti} stacking mode and showed how ligated BChl \textit{c} and \textit{d} self-assemble into coaxial cylinders to form tubular-shaped elements. A close packing of BChls via \textit{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, \textit{syn-anti} BChl stacks form sheets that allow for strong exciton overlap in two dimensions enabling triplet exciton formation for efficient photoprotection.

\textbf{Fig. 1.} BChl homologues for \textit{C. tepidum}. In the WT, \textit{R}_{1} = \textit{Me}; \textit{R}_{2} = \textit{Et}, \textit{n-Pr}, or \textit{i-Bu}; \textit{R}_{3} = \textit{Me}, or \textit{Et}. In the \textit{bchQRU} mutant \textit{R}_{1} = \textit{H}; \textit{R}_{2} = \textit{Et}; \textit{R}_{3} = \textit{Me}.

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\textit{Chlorobaculum} \textit{tepidum} contain a complex mixture of BChl \textit{c} molecules with variable degrees of methylation at carbons C-8\textsuperscript{2} and C-12\textsuperscript{1} (Fig. 1), variations in the esterifying alcohol side chain of the C-17 carboxyl group, and both \textit{R}- and \textit{S}-chirality at the C-3\textsuperscript{1} 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 \textit{c} have led to a well-characterized \textit{bchQ bchR bchU} (hereafter \textit{bchQRU}) \textit{C. tepidum} triple mutant (12). This mutant synthesizes well-defined, extended chlorosomes that contain >95\% 17\textsuperscript{2}-farnesyl-R-[8-ethyl,12-methyl]BChl \textit{d} (Fig. 1) and form regularly packed, tubular-shaped elements with a diameter >10 nm that are interconnected via curved sheets (16, 18). The


The authors declare no conflict of interest.

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\textsuperscript{1}Present address: Department of Microbiology, Ohio State University, Columbus, OH 43210.

\textsuperscript{2}To whom correspondence should be addressed. E-mail: groot.h@ lic. leidenuniv.nl.

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Results

The NMR assignments of the $^1$C and $^1$H signals of BChl $d$ molecules in chlorosomes from the *bchQRU* mutant were obtained from 2D $^1$C-$^1$C and $^1$H-$^1$C magic-angle spinning (MAS) solid-state NMR dipolar correlation spectra [supporting information (SI) Figs. S1 and S2 and Tables S1 and S2] collected from $^{13}$C-enriched chlorosome preparations. The lines observed in $^1$C-$^1$C correlation datasets were very narrow, of the order of 1 ppm (Figs. S1 and S2). A $^{13}$C-$^{13}$C dataset recorded at a longer mixing time of 5.1 ms was needed to assign the farnesyl tail of BChl $d$, which meant that the tail is subject to restricted mobility. The assignments were made following previous NMR work on *C. tepidum* chlorosomes, recorded at 17.6 T with 13 kHz short mixing time of 250 $\mu$s was used (Fig. 2A) (19). Intermolecular correlations between BChl $d$ molecules within a chlorosome were detected for $^1$H-$^1$H transfer events of $<3 \text{ Å}$ (Fig. 2A and Table 1). Because no intramolecular pathway that could serve to correlate the resonances over a 250-$\mu$s mixing time exists in the proton network, these correlations could be assigned unambiguously. The C-71/C-18, and C-71/C-18$'$ intermolecular contacts (Fig. 2B) observed were especially significant because very few possibilities for molecular assembly exist in which these atoms can be in close proximity. The data revealed the presence of alternating monomers that form stacks assembled into layers with the farnesyl tails alternately extending on both sides. The two possible intermolecular arrangements that could satisfy these constraints are the new stacking model based on alternating $\text{syn}$ and $\text{anti}$ 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 II of adjacent BChl $d$ molecules. When $\text{syn}$-$\text{anti}$ 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 $^1$H-$^1$H 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 $\mu$s produces $^1$H transfer over short distances $<3$ Å, the interstack correlations provide convincing evidence that $\text{syn}$-$\text{anti}$ monomer stacking is the basic building block in the *bchQRU* chlorosome structure.

In chlorophyll aggregates, the $^1$H signals shift upfield by ring current effects from neighboring molecules (20). Density functional theory calculations were performed to estimate the ring currents for the $\text{syn}$-$\text{anti}$ 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 $\text{syn}$-$\text{anti}$ monomer stack has alter-
nating molecular conformations of the C-3\textsuperscript{\textit{I}} side chain, and \textit{syn} or \textit{anti} refers to the orientation of the OH ligation of adjacent BCHls with respect to the farnesy1 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 \textit{syn-anti} monomer stack and the parallel stack can reproduce the experimental ring current shifts that are observed. In these two arrangements, each BCHl \textsuperscript{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{\textit{I}}–H, and C-3\textsuperscript{\textit{II}}–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 \textit{bchQRU} mutant obtained with cryoEM, the BCHls can be observed to form coaxial cylinders (Fig. 4\textit{B}). In side views the concentric layers produce a regular pattern with a spacing of 2.10 ± 0.12 nm (Fig. 4\textit{A} and \textit{D}) (16, 18). Additionally, a distinct, striped pattern with a spacing of 0.83 ± 0.01 nm was observed at a 90° angle to the layers (Fig. 4\textit{C} and \textit{D}). After Fourier transformation, the layers translated into a single pair of equatorial reflections at 1/(2.1 nm) where 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 \textsuperscript{d} molecules in the multilayer tubular structures of chlorosomes of the \textit{bchQRU} mutant, supramolecular models were built for different orientations of the stacks relative to the tube axis (Figs. 4\textit{ and 5 and 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 \textit{bchQRU} mutant (compare Fig. 4\textit{C} and \textit{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\textit{E} and \textit{F}). The high contrast of the structure in the chlorosomes of the \textit{bchQRU} mutant arises from the orientation of the BCHl molecular planes and the rotational symmetry (Fig. 5\textit{B}). 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 \textit{C. tepidum} WT chlorosomes, the spacing between layers matches the separation of 2.1 nm observed in the chlorosomes of the \textit{bchQRU} mutant (Fig. S5) (18). No other distinct spacing, similar to that in \textit{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 ± 0.03 nm\textsuperscript{-1} (Fig. S5). This periodicity corresponds to the distance between repeating \textit{syn-anti} pair units in the direction of the stacks (Fig. 5\textit{A} and \textit{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 \textit{bchQRU} mutant or the WT.

In models in which the direction of the BCHl stacks deviates slightly from the two orientations for WT and \textit{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 \textit{bchQRU} chlorosomes and stacks forming steeper helices in the WT chlorosomes are consistent with the EM data. In Fig. 5\textit{A} the unit cell of the sheet is indicated, where \(a = 1.25 \text{ nm}\) is in the direction of the stack, and \(b = 0.98 \text{ nm}\) is at an angle of \(y = 122°\) to \(a\). In Fig. 5\textit{B} 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. 5\textit{B}). In contrast, the stacks in Fig. 5\textit{C} do not form rings but lie along helices without rotational symmetry.

**Discussion**

WT chlorosomes are much more heterogeneous than chlorosomes from the \textit{bchQRU} mutant and contain BCHl \textsuperscript{c} molecules with a greater proportion of \(S\)-chirality at carbon C-3\textsuperscript{\textit{I}} and variable degrees of methylation at carbons C-8\textsuperscript{\textit{I}} 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 under-
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 minima in the energy landscape of the suprastructure that permit 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 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 minima in the energy landscape of the suprastructure that permit chemical heterogeneity to be embedded within the structure.

To couple non-protein-based light harvesting to gene evolution required the optimization of BChls and chlorosomes with respect to the predominant environmental constraint, low light intensity. Unmodified [8-ethyl,12-methyl]BChl d, found in the bchQRU mutant, with absorption similar to Chl d, is the easiest of the compounds to synthesize, requiring only three steps beyond chlorophyll. Likewise, the suprastructure must accommodate the variable amounts of BChls with R- and S-chirality at C-31 that the WT organism normally produces. This provides a window for evolutionary optimization through natural selection by diversification in the BChl side chains by coupling the replication/reproduction machinery (i.e., the growth rate) to the biosynthesis of the chlorophylls.

The final chlorosome suspensions in Tris-HCl (pH 8.0) were isolated and analyzed following the methods described in Steensgaard et al. (25). The final chlorosome suspensions in Tris-HCl (pH 8.0) were isolated and analyzed following the methods described in Steensgaard et al. (25). The final chlorosome suspensions in Tris-HCl (pH 8.0) were isolated and analyzed following the methods described in Steensgaard et al. (25). The final chlorosome suspensions in Tris-HCl (pH 8.0) were isolated and analyzed following the methods described in Steensgaard et al. (25). The final chlorosome suspensions in Tris-HCl (pH 8.0) were isolated and analyzed following the methods described in Steensgaard et al. (25).

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Finally, the scaffolding according to Figs. 4 and 5 confirms that the WT organism normally produces chlorosomes with a negative charge on oxygen interacts electrostatically with the Mg²⁺, which is considered the most important electrostatic contribution to the aggregation (2). Because it does not involve any direct binding of the keto moiety to the metal, it contributes to strengthening the hydrogen bond, in contrast to inferences from chemical modeling studies (5).

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 crystallized or are inherently disordered. The mutant structure provides a template for the evaluation of the more complex structure of the WT. Both chlorosome suprastructure 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.
frequency-driven, dipolar recoupling sequence (20) with phase-sensitive detection in 1H at mixing times of 1.4 ms (Fig. 51), 2.9 ms (Fig. 52), and 5.1 ms. A 1H–2 pulse length of 3.1 µs 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 13C–1H spectra were recorded by using the CHHC/CP sequence (19, 29) for indirect detection of 1H–1H contacts at 1H diffusion times of 250 µs (Fig. 2A) and 325 µs. The initial CP contact time was set to 256 µs. Short CP contact times of 128 µs enclosing the 1H–1H spin diffusion step were used to ensure that the polarization transfer was restricted to directly bonded 1H–13C spin pairs. For each of the 256 steps in the indirect dimension, 128 scans were collected. Two-dimensional 1H–13C heteronuclear correlation data were collected by using the frequency-switched Lee–Goldburg (FSG) experiment (30), with a short CP time of 256 µs and a 1H 90° pulse of 3.1 µs (Fig. 51). The 1H chemical shift scale was calibrated from a FSG spectrum of solid tyrosine-HCl salt and validated with the chemical shift correlation plots (Fig. 56). For each of the 128 steps in the indirect 1H dimension, 256 1H 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 X magnification and a specimen temperature of 80 K. Images were recorded in the zero-loss imaging mode, by using a slit-width of 20 e−/Å2 at 115,000 frequency-driven, dipolar recoupling sequence (28) with phase-sensitive detection in 1H at mixing times of 1.4 ms (Fig. 51), 2.9 ms (Fig. 52), and 5.1 ms. A 1H–2 pulse length of 3.1 µs 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 1H–13C–1H spectral data were recorded by using the CHHC/CP sequence (19, 29) for indirect detection of 1H–1H contacts at 1H diffusion times of 250 µs (Fig. 2A) and 325 µs. The initial CP contact time was set to 256 µs. Short CP contact times of 128 µs enclosing the 1H–1H spin diffusion step were used to ensure that the polarization transfer was restricted to directly bonded 1H–13C spin pairs. For each of the 256 steps in the indirect dimension, 128 scans were collected. Two-dimensional 1H–13C heteronuclear correlation data were collected by using the frequency-switched Lee–Goldburg (FSG) experiment (30), with a short CP time of 256 µs and a 1H 90° pulse of 3.1 µs (Fig. 51). The 1H chemical shift scale was calibrated from a FSG spectrum of solid tyrosine-HCl salt and validated with the chemical shift correlation plots (Fig. 56). For each of the 128 steps in the indirect 1H dimension, 256 1H scans were accumulated.

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