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Structural Variations in Chlorosomes from Wild-Type and a *bchQR* Mutant of *Chlorobaculum tepidum* Revealed by Single-Molecule Spectroscopy

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Supporting Information

ABSTRACT: Green sulfur bacteria can grow photosynthetically by absorbing only a few photons per bacteriochlorophyll molecule per day. They contain chlorosomes, perhaps the most efficient light-harvesting antenna system found in photosynthetic organisms. Chlorosomes contain supramolecular structures comprising hundreds of thousands of bacteriochlorophyll molecules, which are properly positioned with respect to one another solely by self-assembly and not by using a protein scaffold as a template for directing the mutual arrangement of the monomers. These two features—high efficiency and self-assembly—have attracted considerable attention for developing light-harvesting systems for artificial photosynthesis. However, reflecting the heterogeneity of the natural system, detailed structural information at atomic resolution of the molecular aggregates is not yet available. Here, we compare the results for chlorosomes from the wild type and two mutants of *Chlorobaculum tepidum* obtained by polarization-resolved, single-particle fluorescence-excitation spectroscopy and theoretical modeling with results previously obtained from nuclear-magnetic resonance spectroscopy and cryo-electron microscopy. Only the combination of information obtained from all of these techniques allows for an unambiguous description of the molecular packing of bacteriochlorophylls within chlorosomes. In contrast to some suggestions in the literature, we find that, for the chlorosomes from the wild type as well as for those from mutants, the dominant secondary structural element features tubular symmetry following a very similar construction principle. Moreover, the results suggest that the various options for methylation of the bacteriochlorophyll molecules, which are a primary source of the structural (and spectral) heterogeneity of wild-type chlorosome samples, are exploited by nature to achieve improved spectral coverage at the level of individual chlorosomes.

INTRODUCTION

In photosynthesis, solar energy is absorbed by antenna complexes and efficiently transferred to a photochemical reaction center, where it is used to produce a stable charge-separated state across the membrane.^{1,2} One of the most efficient light-harvesting systems in nature is the chlorosome found in green bacteria.³ It has been reported that a photon flux of only 10^9 photons/(cm²·s) at 750 nm is sufficient to enable photosynthetic growth of some green-sulfur bacteria.^{4–6} For comparison, this is equivalent to illumination conditions that correspond to 1% of light from a full moon!

Chlorosomes are organelles enclosed by a monolipid envelope with dimensions of roughly 40–60 nm in width, 10–40 nm in height, and 100–200 nm in length, which are

filled with hundreds of thousands of bacteriochlorophyll (BChl) molecules.^{7–10} The BChl molecules are organized in self-assembling aggregates that are stabilized by hydroxyl-chelation of the central Mg atom, hydrogen bonds, and van der Waals forces.¹¹ This is in contrast to most of the other antenna systems that are found in chlorophototrophy, for which the cofactors are properly positioned with respect to each other by interactions with a protein scaffold.^{3,12,13} These two features—high light-harvesting efficiency and self-assembly in the absence of a protein scaffold—have inspired many researchers

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to consider chlorosomes as templates for novel organic solar cells.^{14–16} Because the operational mode of antenna structures is determined by the properties of the electronic excited states of the chromophores, which in turn are imposed by the detailed supramolecular organization of the molecular building blocks, the elucidation of high-resolution antenna structures has significantly contributed to our current understanding of the structure–function relationships of the primary reactions in photosynthesis.^{17,18} For chlorosomes, however, details about their structural organization are the subject of an ongoing debate, and several models for their secondary structures, including tubular and lamellar aggregates, have been proposed.^{11,19–33} This discussion in part reflects the structural heterogeneity of the chlorosomes, which can vary with respect to the size of the assemblies, their detailed organisation, and the type and chemical modifications of BChl molecules,³⁴ preventing structure determination by high-resolution X-ray crystallography. In particular, natural chlorosomes contain mixtures of BChl molecules with modifications of the side chains R_1 – R_3 , giving rise to a variety of the possible building blocks for the molecular aggregates within the chlorosomes.^{34,35} In Figure 1, those variations are listed that are relevant for *Chlorobaculum* (*Cba.*) *tepidum*.

Figure 1. Schematic representation of a BChl molecule. For clarity, the farnesyl tail has been omitted. The table summarizes the possible substituents for BChl c, BChl d, and for three mutants in which the substituents of BChl c are controlled (shaded) for the side groups R_1 , R_2 , and R_3 at carbon positions C-20, C-8, and C-12, respectively.

In the past, Bryant and co-workers have deciphered the biosynthesis of BChl c in *Cba. tepidum*, which has led to the preparation of a series of mutants for which the substituents of the side chains could be controlled, resulting in a diminished heterogeneity.^{3,35–37} For the triple mutant *bchQRU*, which contains sole BChl d because the composition of all three side chains can be defined, as shown in Figure 1, the more uniform pigment composition allowed the development of a detailed structural model by combining information from cryo-electron microscopy (EM), nuclear magnetic resonance (NMR), and molecular modeling.²¹ In turn, this model served as a template for a structural model for the wild-type (WT) chlorosomes. Briefly, according to the NMR data, the short-range order of the pigment arrangement corresponds to syn–anti-stacked BChl dimers on a Bravais lattice as shown in Figure 2a. Given the hydrophobic tails of the BChl molecules, the idea is that the concentric tubular superstructures that have been observed by cryo-EM, and which feature a spacing of about 2.1 nm between the tubes (see Figure S1), are obtained by wrapping multiple layers of this grid onto a cylindrical surface along a rolling vector $\vec{C}$. Accordingly, the structures proposed for the chlorosomes of the *bchQRU* triple mutant and the chlorosomes of the WT differ from each other only by the angle δ that is

Figure 2. (a) Bravais lattice obtained from NMR for the short-range order of the BChl molecules according to ref 21. Each arrow represents the $\vec{Q}_i$ transition-dipole moment of a BChl molecule. The molecules in red (green) color are inclined by 4° (-4°) with respect to the plane of the grid and distinguish between the syn and anti orientation of the monomers. The gray shaded area highlights one unit cell with lattice vectors $a = 1.25$ nm, $b = 0.98$ nm that enclose an angle of $\gamma = 122^\circ$. (b) Wrapping the grid onto a cylindrical surface along a rolling vector $\vec{C}$ [black arrow in part (a)] enclosing an angle of $\delta = 70^\circ$ with the a -axis of the grid yields the model structure proposed in ref 49 for the *bchR* mutant.

enclosed between the a -axis of the grid and the rolling vector $\vec{C}$, that is, 90° for the WT and 0° for the *bchQRU* mutant.²¹

Further structural information can be obtained from optical spectroscopy. In molecular aggregates, the details of the photo-physical properties of the electronically excited states, such as the transition energies, the oscillator strengths, and the polarization behavior, depend crucially on the mutual distances and orientations of the molecular building blocks, that is, on the molecular packing.^{38–40} This allows one to test whether a proposed structural model is compatible with the experimentally observed spectra and to discriminate between different structures. In this context, it is important to note that, given the spacing between the concentric cylinders of about 2.1 nm, the multiple cylinders can be treated as uncoupled systems for modeling the optical spectra, as has been shown previously in ref 40. Still, distinctive spectral signatures that are characteristic of specific structural features might be lost in ensemble-averaged spectra because of the intrinsic heterogeneity of biological samples. This problem can be mitigated by applying single-molecule techniques.

The first studies on single chlorosomes provided information about the ground-state properties and/or about the polarization properties of the excited states for a few fixed excitation wavelengths.^{20,32,41–46} In a previous study, individual WT chlorosomes from *Cba. tepidum* were investigated using polarization-resolved fluorescence excitation spectroscopy.^{47,48} This revealed a strong modulation of the entire fluorescence-excitation spectrum as a function of the polarization of the excitation light, indicating a high degree of spatial organization of the individual BChl molecules within a chlorosome. However, these studies also revealed the large degree of structural disorder in the individual WT chlorosomes from *Cba. tepidum*, which unfortunately prevented the development of a detailed proposal for a structural model.

The same technique was recently applied to individual chlorosomes from the *bchR* mutant of *Cba. tepidum*.⁴⁹ For this strain, one of the BChl c side chains is fixed as a methyl group³⁷ (see Figure 1), resulting in more homogeneous structures and allowing for a detailed theoretical modeling of the spectra.⁴⁹ This yielded a packing of the BChl molecules in tubular symmetry such that the angle β between the monomer

transition-dipole moments and the symmetry axis of the cylinder amounted to about 55° . This was in close agreement with the proposed structure of the *bchQRU* triple mutant.²¹ From cryo-EM experiments, a value of 1.24 nm was obtained for the distance between adjacent, symmetry-equivalent BChls in the grid, which, to our great surprise, was in good agreement with the distances reported for the model structure of the aggregates in the WT chlorosomes. Apparently, the structural models either based on NMR and single-molecule spectroscopy or based on NMR and cryo-EM were not in mutual agreement, and an unambiguous assignment of the BChl assembly in the *bchR* chlorosomes was only possible by combining the information obtained from all three techniques. This yielded a model for the basic structural element that can be envisaged to result from wrapping the lattice obtained from NMR onto a cylindrical surface along a rolling vector that is inclined with respect to the *a*-axis of the grid under an angle δ of about 70° ;⁴⁹ see Figure 2b. The secondary structural element of tubular symmetry that results from this construction principle is compatible with both the mutual orientations of the transition-dipole moments of the optical bands observed in single-chlorosome spectroscopy and the distance between adjacent, symmetry-equivalent BChls observed in cryo-EM. This raises the question whether the model structures proposed for the chlorosomes from the WT and the *bchQRU* mutant that were based solely on NMR and cryo-EM, and in particular the reported rolling angles of 90° and 0° for wrapping the grid onto a tubular surface, might also need some modification after taking into account spectroscopic data from single chlorosomes of these strains.

The *bchU* mutation included in the triple mutant converts BChl *c* into BChl *d*; see Figure 1. Unfortunately, this shifts the monomer absorption from 666 to 655 nm in organic solvents,⁵⁰ and correspondingly, the absorption peak for BChl *d* containing chlorosomes shifts to about 729 nm,⁵¹ which is out of the spectral range accessible for fluorescence-excitation spectroscopy in our experimental setup. Therefore, in this study, we compared chlorosomes from the WT to those from the *bchQR* double mutant. In this mutant strain, the composition of all three side groups of the BChl molecules is defined (see Figure 1), yet the spectral positions of the absorption bands are still within the experimentally accessible range because this mutant still produces BChl *c*^{22,37} (see Figure S2 in the Supporting Information for the ensemble absorption spectra of the mutants). Here, we report the results from low-temperature polarization-resolved fluorescence-excitation spectroscopy on individual chlorosomes of the WT and the *bchQR* mutant strains of *Cba. tepidum*. As expected, our study confirms that the degree of spectral heterogeneity decreases in the order WT, *bchR*, and *bchQR*, suggesting that the spectral heterogeneity is closely connected to the degree of structural disorder. However, more interesting is the finding that the spectra of the chlorosomes of the WT and the *bchQR* mutant can be reproduced using the same arrangement for the monomer transition-dipole moments, as reported previously for the *bchR* mutant. This result, indeed, calls for a modification of the structural model provided in ref 21.

METHODS

Chlorosome Preparations. Chlorosome preparations were made as previously described (refs 49 and 52).

Preparation of the Single-Particle Sample. The stock solutions ($\text{OD}_{733} = 58$ for a sample of 1 cm thickness) of

chlorosomes from the WT and the *bchQR* mutant of *Cba. tepidum* were stored in the dark at -20°C in a buffer [5 mM dipotassium phosphate (K_2HPO_4), 5 mM monopotassium phosphate (KH_2PO_4), 150 mM sodium chloride (NaCl), pH 7.2 at room temperature]. For single-particle experiments, these solutions were diluted in the same buffer to 10^{-9} M, and about 10 μL of the sample solutions was adsorbed onto a SiO_2 glass substrate under nitrogen atmosphere for 30 min and mounted in a helium cryostat.

At room temperature, we found a significant increase in the fluorescence emission upon prereducing the samples. However, at cryogenic temperatures, the fluorescence intensity did not depend on the treatment of the samples, and we therefore refrained from reducing the samples prior to the optical experiment.

Single-Particle Spectroscopy. For polarization-resolved fluorescence-excitation spectroscopy of the WT and the *bchQR* mutant, two independent homebuilt wide-field/confocal microscopes were used. In the following, the specific components are listed that were used in the setup for the experiments on the WT samples, whereas those used in the other setup were given in square brackets. Excitation light was provided by a tuneable titanium:sapphire laser (3900 S, Spectra Physics; [899-01, Coherent]) that was pumped by a frequency doubled continuous-wave neodymium-yttrium-vanadate (Nd:YVO_4) laser (Millenia Vs, Spectra Physics; [Verdi V10, Coherent]). To suppress nonamplified fluorescence from the laser crystal, the output from the laser passed a band-pass filter (SP 785 RazorEdge, AHF Analysentechnik; [BP 736/236 128, AHF Analysentechnik]). Focusing of the laser beam and collection of the emission from the sample were accomplished by an objective (Mikrothek, NA = 0.85; [Edmund Optics, NA 239 = 0.85]) that was mounted in the cryostat. For localizing single chlorosomes, a wide-field fluorescence image was recorded by defocusing the excitation with an additional lens in the excitation path to a spot size of about 20 μm . The resulting wide-field fluorescence image was then registered with a charge-coupled device camera (iKon, Andor; [LUCAEM R 245 604, Andor Technology Ltd.]). From this image, chlorosomes that were well-isolated spatially were selected, the defocusing lens was removed, and the excitation light was focused to a diffraction-limited spot.

For WT samples, the excitation laser wavelength was scanned between 718 and 775 nm (698–795 nm for *bchQR*), with a rate of 3 nm/s (2.8 nm/s) by rotating a birefringent filter via a motorized micrometer screw (Actuator 253 850 F, Motion Controller MM4005, Newport; [Nanomover, 254 Melles Griot]). The accuracy and the reproducibility of the wavelength variation was 1 cm^{-1} , as verified with a wavemeter (WaveMaster, Coherent; both setups). Between two successive scans, the polarization of the excitation light was rotated in steps of 6.2° (3°) by a waveplate (Thorlabs Inc.; both setups) operated by a stepper motor (OWIS GmbH; both setups). The excitation intensity was adjusted to 25 W/ cm^2 (5 W/ cm^2). After passing three band-pass or two long-pass filters (LP 262 780, AHF Analysentechnik; [BP 850/80, AHF Analysentechnik-263 nik]) to block residual laser light, the emission from a single chlorosome was focused onto a single-photon counting avalanche photodiode (PCM-AQR-16, PerkinElmer; [SPCM-266 AQR-15, PerkinElmer]). The profile of the fluorescence excitation spectra did not depend on whether the detection window of the emission filters was sensitive to the spectral range of the emission from the

Figure 3. Examples of fluorescence-excitation spectra from individual chlorosomes in a two-dimensional representation as a function of the polarization of the excitation light. All spectra have been recorded at 1.2 K. (Top row) WT, stack of 97 spectra, recorded with an excitation intensity of 25 W/cm². (Bottom row) *bchQR* mutant stack of 200 spectra recorded with an excitation intensity of 5 W/cm². The relative normalized intensity is given by the color bar.

baseplate or the entire chlorosome. In parallel to the data acquisition, the intensity of the excitation light was recorded as a function of the wavelength using a powermeter (LaserMate-Q, Coherent; both setups), and all spectra are corrected for variations of the laser intensity across the spectra.

RESULTS

Examples of polarization-resolved, fluorescence-excitation spectra from single chlorosomes from *Cba. tepidum* are shown in Figure 3. The spectra in the top row have been taken from single chlorosomes from the WT samples, whereas the spectra in the bottom row have been taken from single chlorosomes from the *bchQR* mutant. Each pattern corresponds to a sequence of 97 (WT) or 200 (*bchQR* mutant) consecutively recorded fluorescence-excitation spectra, for which the horizontal axis corresponds to photon energy, and the vertical axis corresponds to the polarization of the incident radiation. Between the recordings of two subsequent spectra, the polarization was rotated by a few degrees. The resulting fluorescence intensity is given by the color code.

Between individual chlorosomes, the spectral patterns show variations in their appearance. Yet, despite these variations, two observations are striking. First, for the chlorosomes from the *bchQR* mutant (Figure 3 bottom), the widths of spectral features are significantly reduced with respect to those from the WT chlorosomes (Figure 3 top). This clearly reflects the reduction in heterogeneity owing to the definition of the BChl side groups and probably C-3¹ chirality by mutagenesis. Second, for all spectra for both the WT and *bchQR* mutant, the intensity is clearly modulated with a periodicity of 180° as a function of the polarization of the excitation light. This reflects a high degree of organization of the hundreds of thousands of BChl molecules that contribute to a single chlorosome spectrum. Similar to previous work,⁴⁹ the stacks of spectra can be analyzed by a global fit routine that decomposes the spectrum into four spectral bands of the Gaussian form

$$F(E, \Theta) = B + \sum_{i=1}^4 A_i(E) \cdot [\cos(\Theta - \varphi_i)]^2 \cdot \exp\left(-\frac{(E - E_i)^2}{W_i^2/4 \ln 2}\right) \quad (1)$$

Here, “*i*” counts the spectral contributions, *B* denotes an offset that accounts for background signals, *A_i*(*E*) denotes the

amplitude of the respective contribution, Θ corresponds to the polarization angle of the incident light with respect to a laboratory frame, φ_i refers to the angle of the projection of the transition-dipole moment of the respective spectral component on to the sample plane, and the lineshape is modeled as a Gaussian centered at an energy *E_i* with a width [full width at half-maximum (fwhm)] of *W_i*. For the total of about 100–250 spectra from each individual chlorosome, this yields 17 fit parameters where the amplitudes *A_i*(*E*) and the phase angles φ_i were treated as global parameters. For the majority of chlorosomes studied (72 out of 75 for the WT and all the 29 from the *bchQR* mutant) including 4 Gaussians for the fitting sufficed to achieve good agreement between the fit and the experiment, while with less than 4 Gaussians, the fit quality seriously deteriorated.

The following is based on this majority of chlorosomes, and the details of the analysis will be illustrated by considering the polarization-resolved spectrum from a single WT chlorosome shown in Figure 4. The black line in Figure 4b shows the experimental fluorescence-excitation spectrum averaged over all polarizations of the excitation light. It is centered at 13 274 cm^{−1} and features a width of 740 cm^{−1} (fwhm). The colored lines correspond to the four spectral contributions from the fit routine, and the gray line represents their sum, which nicely reproduces the overall experimental spectrum.

In the following, the four individual bands are labeled 1–4 in order of increasing energy of their center position for further reference. In the example shown in Figure 4, the first band (red) occurs at 13 166 cm^{−1} with a width (fwhm) of *W*₁ = 451 cm^{−1}. The second band (orange) has its peak position at *E*₂ = 13 250 cm^{−1} and a width of *W*₂ = 527 cm^{−1}. The third (cyan) and fourth (dark blue) bands are centered at *E*₃ = 13 527 cm^{−1} and *E*₄ = 13 757 cm^{−1} with widths of *W*₃ = 752 cm^{−1} and *W*₄ = 764 cm^{−1}, respectively. The phase differences, $\Delta\Phi_{ij}$ between the bands were calculated according to $\Delta\Phi_{ij} = |\varphi_i - \varphi_j|$ if $|\varphi_i - \varphi_j| < 90^\circ$, and $\Delta\Phi_{ij} = |180^\circ - |\varphi_i - \varphi_j||$ otherwise. For the example shown in Figure 4, this yields $\Delta\Phi_{12} = 81.2^\circ$, $\Delta\Phi_{13} = 0.5^\circ$, $\Delta\Phi_{14} = 79.5^\circ$, $\Delta\Phi_{23} = 80.7^\circ$, $\Delta\Phi_{24} = 1.7^\circ$, and $\Delta\Phi_{34} = 79.0^\circ$. This procedure was employed to analyze the polarization-resolved, fluorescence-excitation spectra from 72 individual chlorosomes of the WT and 29 individual chlorosomes from the *bchQR* mutant from *Cba. tepidum*.

Table 1. Mean Values of the Spectral Positions E_i and Linewidths W_i (fwhm) of the Gaussians Obtained from Sets of 72, 58, and 29 Individual Chlorosomes of the WT, *bchR*, and *bchQR* Strains^a

Strain	WT (72)	<i>bchR</i> (58)	<i>bchQR</i> (29)
$E_{\text{peak, overall}}$ (sdev)	13366 (97)	13609 (33)	13492 (21)
W_{overall} (sdev)	783 (91)	437 (54)	441 (70)
E_1 (sdev)	13170 (56)	13468 (26)	13452 (18)
W_1 (sdev)	310 (86)	233 (35)	198 (25)
E_2 (sdev)	13274 (59)	13502 (41)	13471 (21)
W_2 (sdev)	432 (76)	270 (41)	230 (28)
E_3 (sdev)	13517 (80)	13685 (39)	13656 (40)
W_3 (sdev)	685 (109)	495 (66)	404 (31)
E_4 (sdev)	13649 (91)	13747 (57)	13695 (45)
W_4 (sdev)	701 (98)	501 (63)	408 (27)

^aThe data for the *bchR* mutant are not shown in Figure 5 and have been taken from ref 49. The colors in the table refer to the color code used for distinguishing the four bands in the figures. The main entry in each cell gives the average value of the quantity considered for the set of chlorosomes studied, while the entry in parentheses gives the corresponding standard deviation. All values are in cm^{-1} .

The histograms in Figure 5 display the statistics of the energetic positions and the widths of the four bands for these chlorosomes. It is important to note that a single chlorosome corresponds to a small subensemble of molecular aggregates. Hence, each entry in the histograms represents an average over such a subensemble, whereas the whole histogram represents the distribution of these averages within the macroscopic ensemble of chlorosomes.

The histograms presented in Figure 5 can be characterized by the statistical parameters summarized in Table 1. For better

Figure 4. (a) Stack of 133 fluorescence-excitation spectra from an individual chlorosome of the WT as a function of the polarization of the excitation light. (b) Fluorescence-excitation spectrum of the pattern averaged over all polarizations (black line) together with its decomposition into four Gaussians (colored) and the sum of the Gaussians (gray). The relative normalized intensity is given by the color bar. The spectra were recorded at 1.2 K using an excitation intensity of 25 W/cm^2 .

Figure 5. Top: WT Bottom: *bchQR* mutant. (a,c) Distributions of the spectral peak positions of the fitted Gaussians and (b,d) distributions of the widths (fwhm) of the fitted Gaussians.

Figure 6. Distributions of the relative phase angles Φ between the bands 1 and 2 and between the bands 3 and 4. The black entries correspond to the WT chlorosomes and the gray entries correspond to the *bchQR* mutant.

comparison, the table lists also the statistical data obtained from the *bchR* mutant that was the subject of a previous study.⁴⁹

For the WT chlorosomes, the widths of 310 and 432 cm^{-1} of the two spectral components E_1 and E_2 are significantly narrower than the 685 and 701 cm^{-1} values that have been found for the spectral components E_3 and E_4 . The same holds true for the spectral components of the *bchR* mutant (233 and

270 cm^{-1} vs 495 and 501 cm^{-1}) and for those from the *bchQR* mutant (198 and 230 cm^{-1} vs 404 and 408 cm^{-1}). In the following analysis, we group the four spectral components into a low-energy pair, E_1 and E_2 , and a high-energy pair, E_3 and E_4 , and determine for each individual chlorosome the difference of the polarization angles, $\Delta\Phi_{12}$ and $\Delta\Phi_{34}$, between the transitions within one pair; see Figure 6 and Table 2.

Table 2. Mean Values (Standard Deviations) of the Relative Phase Angles between the Bands 1 and 2 and between the Bands 3 and 4 for the Three Types of Chlorosomes^a

<i>ij</i>	12			34		
	WT	<i>bchR</i>	<i>bchQR</i>	WT	<i>bchR</i>	<i>bchQR</i>
$\Delta\Phi_{ij}$ (deg)	60 (34)	78 (16)	83 (6)	77 (16)	87 (3)	81 (34)

^aThe data for the *bchR* mutant are not shown in Figure 6 and have been taken from ref 49.

For the WT sample, the differences in phase angles between bands 1 and 2 form a bimodal distribution peaking around 0° and 90°, which is averaged out to the values of $60^\circ \pm 34^\circ$ for the mean and the standard deviation. This illustrates the added value of single-molecule techniques, which allow one to obtain information about the distributions of parameters rather than only about their average values. In contrast, the entries in the histogram for the phase difference between bands 3 and 4, as well as the entries in both histograms for the *bchQR* mutant show pronounced maxima close to a value of 90°.

DISCUSSION

Comparison of the histograms presented in Figure 5 reveals a striking reduction in the widths of the corresponding distributions when comparing WT chlorosomes to those from the *bchQR* mutant. This statement is quantified by the data provided in Table 1. All entries in the table that can be associated with structural disorder, namely, the standard deviations of the distributions of the spectral peak positions E_1 – E_4 , the mean values of the widths of these peaks W_1 – W_4 , and the standard deviations of the distributions of these widths, decrease in the order WT, *bchR* single mutant, *bchQR* double mutant. These observations confirm the idea that the structural heterogeneity of the BChl aggregates within the chlorosomes can be very substantially reduced by decreasing the BChl homolog (sidechain) heterogeneity (Figure 1 and Table 1; also see ref 37). Nevertheless, despite the differences in BChl homolog composition, the spectra from all three strains follow the same general pattern. For the chlorosomes of each strain, the observed spectral features can be separated into a low-energy and a high-energy doublet, and for each chlorosome type, the widths of the bands in the high-energy doublet exceed those in the low-energy doublet by a factor of about 2.0 ± 0.4 . This suggests that the formation of the molecular aggregates within the chlorosomes follows a very similar construction principle. For the chlorosomes from the *bchQR* mutant, the phase differences between the bands within the low-energy pair and the high-energy pair, $\Delta\Phi_{12}$ and $\Delta\Phi_{34}$, mostly occur at 90°; see Figure 6. This is a clear indication of an underlying cylindrical symmetry of the BChl aggregates within the chlorosomes⁵³ (also see below for further explanation and interpretation) and resembles the situation that was found earlier for the *bchR* mutant.⁴⁹ For the chlorosomes from the WT, the observation is not that straightforward. Here, the phase differences between the

Figure 7. Correlation between the phase differences between the bands within the low-energy pair, $\Delta\Phi_{12}$, and the bands 1 and 3, $\Delta\Phi_{13}$, for individual chlorosomes from the WT (crosses) and from the *bchQR* mutant (blue circles). The shaded area indicates those chlorosomes that are referred to as group 1 in the text.

bands in the high-energy pair, $\Delta\Phi_{34}$, also accumulate at 90°, whereas those between the low-energy pair, $\Delta\Phi_{12}$, show a bimodal distribution peaking at 0° and at 90°. To analyze this observation in more detail, Figure 7 displays the correlation between the phase differences $\Delta\Phi_{12}$ and $\Delta\Phi_{13}$ for both the WT and the *bchQR* mutant. The data points can be divided roughly into two groups, namely, those that exhibit hardly any correlation between $\Delta\Phi_{12}$ and $\Delta\Phi_{13}$ (group 1; shaded area in Figure 7) and those that do exhibit strong correlation (group 2). Group 1 includes all chlorosomes of the *bchQR* mutant and 53 chlorosomes from the WT, corresponding to 74% of the WT chlorosomes studied. For the chlorosomes of group 1, the data points cluster around $\Delta\Phi_{12} \approx 70^\circ - 90^\circ$, and the spectra feature approximately two mutually orthogonally polarized bands within each pair (in short: 1 $\perp$ 2 and 3 $\perp$ 4) and in addition two predominantly parallel-polarized pairs of bands 1 $\parallel$ 3 and 2 $\parallel$ 4. Group 2 includes exclusively the remaining 26% of the WT chlorosomes studied. Here, we find $\Delta\Phi_{13} \approx 65^\circ - 90^\circ$ and $\Delta\Phi_{12} \leq 30^\circ$. For this group of chlorosomes, the analysis of the polarization behavior of the spectral bands yields approximately 1 $\parallel$ 2, 3 $\perp$ 4, and 1 $\perp$ 3 (implying 1 $\parallel$ 4, 2 $\perp$ 3, and 2 $\parallel$ 4).

In the following, we refer to the WT chlorosomes in the two groups as WT-group1 and WT-group2, respectively. For the WT-group1 chlorosomes, the distributions for the energetic separations, ΔE_{12} and ΔE_{34} , the ratios of the oscillator strength, O_1/O_2 and O_3/O_4 , and the difference of the polarization angles, $\Delta\Phi_{ij}$, are given in Figure 8, together with the corresponding distributions for the *bchQR* mutant.

For the WT-group1 chlorosomes, the distributions for the energetic separations of the spectral components in the low- and high-energy pairs are rather wide with respect to those from the *bchQR* mutant that generally are separated by less than 100 cm^{-1} . The same holds true for the widths of the distributions of the ratios of the oscillator strengths and the mutual phase differences. The widths of the distributions for the mutant are clearly narrower than those of the corresponding distributions for the WT-group1 chlorosomes. This is clearly reflected by the corresponding standard deviations (see Tables 3 and 4), which decrease in the order

Figure 8. (a,b) Distributions of the energetic separation between the low-energy bands and the high-energy bands for an individual chlorosome. (c,d) Distributions of the ratio of the oscillator strength between bands 1 and 2 and between bands 3 and 4, respectively. (e–j) Distributions of the relative phase angles Φ between the bands 1, 2, 3, and 4. The black entries correspond to the WT-group1 chlorosomes and the gray entries correspond to the *bchQR* mutant.

WT-group1, *bchR* (single mutation), *bchQR* (double mutation).

The chlorosomes from the *bchQR* mutant as well as those assigned to WT-group1 exhibit the typical polarization behavior that is associated with molecular aggregates with an underlying tubular structure,^{39,53,54} that is, mutually orthogonal transition-dipole moments between exciton states. Briefly, for an ideal and sufficiently long molecular aggregate of cylindrical

Table 4. Mean Values (Standard Deviations) of the Relative Phase Angles between the Bands 1, 2, 3, and 4 for the Three Types of Chlorosomes (WT-Group1, *bchR*, and *bchQR*)^a

$\Delta\Phi_{ij}$ (deg)	12	13	14	23	24	34
WT-group1	79 (10)	15 (16)	63 (24)	76 (16)	19 (22)	76 (17)
<i>bchR</i>	78 (16)	28 (27)	62 (28)	67 (26)	23 (26)	87 (3)
<i>bchQR</i>	83 (6)	17 (27)	69 (24)	68 (22)	19 (25)	81 (34)

^aThe data for the *bchR* mutant are not shown in Figure 8 and have been taken from ref 49.

symmetry, that is, exhibiting neither structural nor energetic disorder, the electronic coupling between the monomers gives rise to only three exciton states with nonzero transition-dipole moment for transitions from and to the electronic ground state. The transition-dipole moment of the lowest exciton state, $\mu_{\parallel}$, is oriented parallel to the symmetry axis of the cylinder, whereas the other two transition-dipole moments result from a pair of degenerate exciton states with mutually orthogonal transition-dipole-moments, $\mu_{\perp x}$ and $\mu_{\perp y}$, both oriented perpendicularly with respect to the symmetry axis of the tube; see Figure 9a. Together with the information that the chlorosomes are oriented with their long axis parallel to the surface of the substrate³² and that for (the vast majority of) the molecular aggregates the long axes of the secondary structures within a chlorosome are oriented parallel with respect to each other,^{5,21,27} this leads us to a clear interpretation of bands 1–4 found in our analyses for the WT-group1 and QR chlorosomes. Bands 1 and 2 result from pairs of exciton states with transition dipoles parallel and perpendicular, respectively, to the axes of the cylindrical aggregates that make up the chlorosomes. Bands 3 and 4 derive from similar pairs with higher energies and larger energy separations ΔE_{34} . Below, we will come back to the possible relation between both pairs.

The abovementioned interpretation yields a direct relationship between the oscillator strength of the parallel and perpendicular-polarized exciton transitions and the angle β between the transition-dipole moments of the monomers and the symmetry axis of the cylinder⁵³

$$\beta = \arctan \sqrt{\frac{2O_{\perp}}{O_{\parallel}}} \quad (2)$$

Here, the factor of 2 accounts for the fact that only half of the oscillator strengths of the $\mu_{\perp}$ transition-dipole moments can be detected in the polarization-resolved experiments, given the orientation of the tubular structures with respect to the optical axis. Denoting the monomer dipole angles thus derived from the low-energy and the high-energy pair as β_{12} and β_{34} , respectively, and using the data for the oscillator strengths shown in Figure 8c,d, we obtain the distributions of these angles as shown in Figure 9b for the WT-group1 and in Figure 9c for the *bchQR* mutant. The mean values and standard

Table 3. Mean Values (Standard Deviations) of the Energetic Separations and the Ratios of the Oscillator Strengths between the Bands 1 and 2 as Well as between the Bands 3 and 4 for the Three Types of Chlorosomes (WT-Group1, *bchR*, and *bchQR*)^a

ij	12			34		
	WT-group1	<i>bchR</i>	<i>bchQR</i>	WT-group1	<i>bchR</i>	<i>bchQR</i>
ΔE_{ij} (cm ^{−1})	71 (66)	34 (31)	19 (13)	101 (107)	62 (53)	38 (32)
O_i/O_j	1.04 (0.95)	0.95 (0.35)	0.95 (0.34)	1.42 (0.77)	1.24 (0.30)	1.13 (0.13)

^aThe data for the *bchR* mutant are not shown in Figure 8 and have been taken from ref 49.

Figure 9. (a) Molecular aggregate with cylindrical symmetry. Electronic coupling yields three exciton states with dipole-allowed transitions to the electronic ground state. The corresponding transition-dipole moments are oriented parallel to the symmetry axis ($\mu_{\parallel}$) and mutually orthogonal (μ_{Lx} , μ_{Ly}) with respect to that axis. (b,c) Distributions of the angles β_{12} (low-energy pair) and β_{34} (high-energy pair) that are enclosed between the transition-dipole moments of the monomers and the symmetry axis of the cylinder for the WT-group1 (b) and the bchQR mutant (c).

Table 5. Mean Values (Standard Deviations) of the Distributions of the Molecular Dipole Angles β_{12} (Low-Energy Doublet) and β_{34} (High-Energy Doublet) Obtained from Our Spectral Analyses and Distances between Adjacent, Symmetry Equivalent BChls, d , of the Supramolecular Structures in Chlorosomes Obtained from Cryo-EM^{21,22a}

	WT-group1	bchR	bchQR
β_{12}	57.3° (9.6°)	56° (4.7°)	56.1° (4.5°)
β_{34}	51.7° (7.4°)	52° (3.4°)	53.1° (1.6°)
d/nm	1.22/1.25 ²¹	1.24 ⁴⁹	0.69 ²²

^aThe data for the bchR mutant are not shown in Figure 9 and have been taken from ref 49.

deviations of these histograms are given in Table 5 and are compared with the data obtained previously from chlorosomes of the bchR mutant. Again, the widths of the distributions decrease in the order WT-group1, bchR, bchQR, which suggests that the structures of the molecular aggregates within the chlorosomes are better defined when the homolog variation due to the BChl side groups is more restricted (i.e., better defined). Yet, within statistical accuracy, all distributions are centered around a similar mean of about 56° for β_{12} and 52° for β_{34} .⁵⁵

To be able to translate the information on the angles β_{12} and β_{34} unambiguously into molecular arrangements on cylindrical surfaces (specifically, the rolling angle δ defined in Figure 2), information from cryo-EM is needed. Therefore, Table 5 provides the distances between adjacent, symmetry-equivalent BChls within the supramolecular structures that were obtained from cryo-EM for the three species. Here, the value of 0.69 nm found for the bchQR mutant²² is significantly smaller than the 1.22 nm/1.24 nm values that have been found for the WT and the bchR mutant,^{21,49} respectively, as well as the 1.25 nm reported for the model structure of the WT.²¹ However, this discrepancy is easy to understand. According to ref 22, the structural changes induced by the bchQR mutation lead to a deformation of the tetrapyrrole backbone of the BChl molecules that induces an arrangement of the BChl molecules in extended domains of alternating stacks of BChl molecules either in syn or anti orientation. In other words, in contrast to the chlorosomes of the WT and bchR strains, for which the molecules are arranged in dimeric syn–anti stacks, the BChl molecules in the bchQR mutant are arranged in monomer stacks. As a consequence of this, the distance between adjacent, symmetry-equivalent BChls of 0.69 nm refers to the monomer

repeat distance and not to the dimer repeat distance as for the other two species. Considering that in the bchQR mutant the assembly in parallel monomers is imposed by structural deformations of the BChl molecules, the value of 0.69 nm is in reasonable agreement with the 0.63 nm that results when the lattice parameter of $a \approx 1.25$ nm of the unit cell shown in Figure 2a is cut in half. At any rate, from the perspective of spectroscopy, it is impossible to distinguish between dimeric syn–anti stacking or extended domains of monomeric (whether syn or anti) BChl arrangements because for syn–anti stacking, the concomitant alternation of the transition-dipole moments by only $\pm 4^\circ$ with respect to the plane of the cylinder is too small to make a detectable impact on the spectra.

Thus, the important conclusion from the above-presented data from NMR, cryo-EM, and single-molecule spectroscopy yields for all three species, WT-group1, bchR, and bchQR, the same basic arrangement of the transition-dipole moments, that can be visualized by wrapping the lattice obtained from NMR onto a cylindrical surface along a chiral vector that includes an angle δ with the a -axis, as proposed in ref 49 and illustrated in Figure 2. The angle δ is found to be the same for these three species, namely, about 70°, a result that contradicts earlier findings, where for the WT, this angle was reported to be 90°. ²¹ For the bchR and bchQR mutants, the fact that the molecular packing basically is the same is in agreement with results from ensemble circular dichroism (CD) spectroscopy (see Figure S3), which yields nearly identical CD spectra for these two strains. While the ensemble CD spectrum from the WT chlorosomes is in qualitative agreement with those for chlorosomes from the bchR and the bchQR mutant, it appears quantitatively slightly different from the other two. We speculate that this difference might originate from the superposition of CD spectra from WT-group1 and WT-group2 chlorosomes.

We now turn to the physical interpretation of the energy differences ΔE_{12} and ΔE_{34} within both pairs of transitions for the WT-group1, bchR, and bchQR species and a speculation about the relation between both pairs. For single-wall aggregates of cylindrical symmetry, the energy splitting between both exciton bands that are polarized perpendicular to each other is directly related to the curvature (i.e., the radius) of the cylindrical aggregate; see Figure S7 in the Supporting Information and ref 39. Explicitly, the splitting scales are roughly inversely proportional to the radius. In Figure 10, the energetic separations between the bands in the high-energy doublet, ΔE_{34} , are correlated with the energetic

Figure 10. Correlation between the energetic separations of the low- and high-energy doublet. The shaded area indicates the range of the most frequently observed energy splittings. The structures on the top and on the right-hand side of the diagram illustrate the radii of tubular structures that are consistent with the respective energy differences. The particular radius scales displayed here have been obtained using exciton model calculations similar to those in ref 49 (see Figure S7).

separations between the bands in the low-energy doublet, ΔE_{12} , for all three species (WT-group1, *bchR*, and *bchQR*). Remarkably, for all three types of chlorosomes, the data points accumulate around a line that roughly satisfies the relation $\Delta E_{34} \approx 1.5 \cdot \Delta E_{12}$ indicated by the gray shaded area in Figure 10 that includes 89% of the total data points (87% for WT-group1, 86% for *bchR*, 97% for *bchQR*). For the chlorosomes from the WT-group1 and the *bchR* mutant, ΔE_{12} (ΔE_{34}) covers the range of 0 to 150 cm^{-1} (250 cm^{-1}), whereas these ranges are restricted for ΔE_{12} (ΔE_{34}) to 0–50 cm^{-1} (150 cm^{-1}), respectively, for the *bchQR* mutant. The reasonable degree of correlation found for all three chlorosome types is another independent and strong indication for close similarities in the molecular arrangement of the secondary structures of the BChls within those chlorosomes. Note that this correlation implies, in particular, that the structure associated with the low-energy doublet (12) has a larger radius than the one associated with the high-energy doublet (34). This would be consistent with interpreting both doublets to derive from concentric double- or multilayer tubular arrangements of the BChl aggregates within the chlorosomes. The reason both doublets have a different average transition energy may originate from different solvent shifts for the molecular transition energies, owing to a different environment within a multilayer structure and (or) to slightly different lattice parameters for aggregates of different radii. The close connection between both doublets suggested here is also consistent with the fact that the higher-energy doublet is characterized by larger spectral linewidths than the lower-

energy pair (Table 1), which could result from relaxation of the excitons in the higher-energy doublet to the ones of the lower-energy one.

While the abovementioned speculation about the physical origin of the correlation between ΔE_{12} and ΔE_{34} in terms of concentric multilayer tubular BChl aggregates will need further investigation, it can safely be stated that this correlation is a strong signature for an underlying structural hierarchy within the overall organization of the BChl assembly. At the same time, it is obvious that for all three species, the average spectral separations, and thus the curvatures of the secondary structural elements within a chlorosome, feature strong variations between individual chlorosomes. Using exciton model calculations similar to those in ref 49 (also see Figure S7), this variation is found to cover a range of about 4 to 20 nm for WT-group1 and *bchR*, whereas this inter-chlorosome variation is less pronounced for the *bchQR* mutant featuring structures with radii in the range of 7–20 nm, that is lacking the structures with the strongest curvature (see right and top axes in Figure 10). Still, on the mesoscale, we find strong similarities for the underlying arrangements of the transition-dipole moments of the BChl molecules within the molecular aggregates in the chlorosomes from WT-group1 and the two mutants *bchR* and *bchQR*.

The observed variations of the curvatures within one species may reflect differences in the chlorosome structure during the course of growth and biogenesis of these structures. This idea is inspired by the existence of some degree of correlation between the energetic splitting within one of the doublets (i.e., $\sim 1/\text{curvature}$) and the corresponding emitted intensity (i.e., $\sim \text{number of monomers in the chlorosome}$) (see Figure S8), which suggests that during growth, the average radius of the tubular aggregates within a chlorosome increases. This might result from an increasing occurrence of multilayer concentric BChl aggregates during growth, which would be favorable, as it leads to a high-density packing of molecules.

We finally shift attention to the chlorosomes that were assigned to WT-group2, which make up 26% of population observed. For this group, we found above that the polarization-resolved fluorescence-excitation spectra do not follow the “standard” pattern that may be attributed to tubular structures. Instead, their spectra can be decomposed into four contributions whose gross mutual polarization behavior can be expressed as $1||2$, $1\perp3$, and $3\perp4$ (implying $1||4$ and $2||4$). At first sight, the mutually orthogonal transition-dipole moments for the two transitions highest in energy (see Figure S9) seem to suggest that some structural elements of cylindrical symmetry prevail as well for the WT-group2 chlorosomes. However, it is worth noting that except for the deviation of the polarization pattern from that of the WT-group1 chlorosomes, we did not find any other observations that could be used for discriminating the two groups of chlorosomes. The distributions of the spectral peak positions E_1 – E_4 and the widths W_1 – W_4 of the four bands cover a similar range for both groups; see Figure 11. Summing the polarization-averaged spectra from individual chlorosomes for each group separately yields similar fluorescence-excitation spectra, peaking at 13 458 cm^{-1} (743.1 nm) and 13 435 cm^{-1} (744.3 nm) with widths (fwhm) of 785 and 766 cm^{-1} for WT-group1 and WT-group2, respectively. Interestingly, comparing the distributions of the spectral peak positions shown in Figure 11a,c reveals that for the WT-group2 chlorosomes, the peak positions of the four fitted bands are spread evenly across the

Figure 11. Top: WT-group1; Bottom: WT-group2. (a,c) Distributions of the spectral peak positions of the fitted Gaussians and (b,d) distributions of the widths (fwhm) of the fitted Gaussians.

spectrum. In other words, grouping the four bands into a low-energy pair and a high-energy pair, which was an obvious step to take for the chlorosomes from WT-group1, cannot be easily justified for the WT-group2 chlorosomes. Hence, on the basis of the information at hand, it cannot be ruled out that all four transitions come from one superstructure. As has been shown by the Vacha group,²⁰ it is already sufficient to consider only a quarter of a cylinder for the structural elements of the BChl arrangement to obtain similar spectral features as those observed for a closed cylinder. Therefore, we can speculate that the arrangement of the molecular aggregates in the WT-group2 chlorosomes resembles partial cylinders or lamella, possibly in addition to complete cylinders. Such structural elements have been observed also by EM.^{27,29}

CONCLUSIONS

We have studied individual chlorosomes from WT and a *bchQR* double mutant by polarization-resolved, fluorescence-excitation spectroscopy, and we have compared the results with those obtained from a *bchR* single mutant in a previous study. The spectral heterogeneity diminished in the order WT > *bchR* single mutant > *bchQR* double mutant, clearly demonstrating that the higher degree of structural order achieved through the mutations allows better resolution of distinctive spectral features (also see ref 37). Because the *bchR* and *bchQR* mutations correspond to a removal of methyl groups from the sidechains of the BChl *c* molecules, namely, one at C-12 for *bchR*, and additional ones at C-8 for *bchQR* (see also Figure 1), it is tempting to speculate that the methylations effectively substitute for protein interactions for fine tuning the site energies of the monomers to achieve a broadening of the absorption band for better spectral coverage in the natural organisms. If so, the methylations and the induced structural disorder would be of functional importance for optimizing the light-harvesting efficiency of the chlorosomes. From the linewidth distributions of the single chlorosome spectra (see Figure 5b,d), it becomes apparent that this overall broadening is already a property of the individual chlorosomes rather than of the macroscopic population as a whole.

Moreover, we have found two types of spectral patterns for the chlorosomes of the WT. For the vast majority of the WT

chlorosomes, that is about 75%, as well as for the chlorosomes from the two mutants studied, the predominant structural element for the meso-organization of the monomers features tubular symmetry. The molecular packing of these aggregates can be reproduced by wrapping the NMR lattice reported in ref 21 onto a cylindrical surface along a rolling vector that is inclined by about 70° with respect to the *a*-axis of the grid, that is, the same model structure that was obtained for the *bchR* mutant in a previous study.⁴⁹ For the WT chlorosomes, this is in contrast to what has been reported before.²¹ Yet, differences in the details of the monomer arrangement between the strains were found as well, namely, between the secondary structures in the (majority) WT and *bchR* strains on the one hand, and the *bchQR* mutant on the other hand. It appears that in the *bchQR* chlorosomes, the tubular elements with small radii are lacking, which is in contrast to those from the other two strains. A second spectral pattern that was found for about one quarter of the WT chlorosomes showed features that might originate from cylindrical as well as other secondary structural elements, allowing some leeway for speculation, for example, being associated with those (curved) lamellar arrangements that are still the subject of ongoing debates in the literature.^{19,21,22,25,28}

Finally, we have found that the energy splittings observed between the various spectral peaks of individual chlorosomes of the (majority) WT, *bchR*, and *bchQR* strains reveal interesting information. In particular, (i) a strong correlation exists between the energetic separations within the absorption doublets that are observed (i.e., ΔE_{12} vs ΔE_{34}). This provides strong evidence for a structural hierarchy in the molecular packing, possibly because of concentric double- or multiwall cylindrical arrangements. (ii) Within an ensemble of chlorosomes from the same strain, the individual aggregates feature strong variations in the curvature of their secondary structural elements. (iii) Between different strains (here WT, *bchR* vs *bchQR*), the chlorosomes feature strong variations in the radii of their secondary structural elements. Further studies will be needed to provide a more thorough interpretation of these observations.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jpcb.8b02875.

Figure of the model structure as a double layer, ensemble absorption and CD spectra, calculations testing the influence of a misalignment of some secondary structural elements on the spectral properties, the result of simulations concerning the relation between the spectral separation of the two mutually orthogonal polarized exciton transitions and the radius of a tubular structure, experimental data showing the correlation between the energetic splitting and the emitted intensity, and some additional data for chlorosomes of the WT-group2 (PDF)

Visualization of the model structures (MPG)

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Notes

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