Triplet Exciton Formation as a Novel Photoprotection Mechanism in Chlorosomes of *Chlorobium tepidum*

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ABSTRACT Chlorosomes comprise thousands of bacteriochlorophylls (BChl *c, d*, or *e*) in a closely packed structure surrounded by a lipid-protein envelope and additionally contain considerable amounts of carotenoids, quinones, and BChl *a*. It has been suggested that carotenoids in chlorosomes provide photoprotection by rapidly quenching triplet excited states of BChl via a triplet-triplet energy transfer mechanism that prevents energy transfer to oxygen and the formation of harmful singlet oxygen. In this work we studied triplet energy transfer kinetics and photodegradation of chlorosomes isolated from wild-type *Chlorobium tepidum* and from genetically modified species with different types of carotenoids and from a carotenoid-free mutant. Supporting a photoprotective function of carotenoids, carotenoid-free chlorosomes photodegrade ~3 times faster than wild-type chlorosomes. However, a significant fraction of the BChls forms a long-lived, triplet-like state that does not interact with carotenoids or with oxygen. We propose that these states are triplet excitons that form due to triplet-triplet interaction between the closely packed BChls. Numerical exciton simulations predict that the energy of these triplet excitons may fall below that of singlet oxygen and triplet carotenoids; this would prevent energy transfer from triplet BChl. Thus, the formation of triplet excitons in chlorosomes serves as an alternative photoprotection mechanism.

INTRODUCTION

Chlorosomes, the largest known light-harvesting antenna structures, are found in the photosynthetic green sulfur bacteria and green filamentous bacteria (1–3). The main light-harvesting pigment in chlorosomes is bacteriochlorophyll (BChl) *c* or the closely related molecules BChl *d* or *e*. Each chlorosome of the model green sulfur bacterium *Chlorobium tepidum* was shown to contain as many as 215,000 ± 80,000 BChl *c* molecules (4). Unlike other known pigment-protein complexes in which the pigments are arranged by the surrounding protein (5,6), the spatial organization of the pigments in chlorosomes is governed primarily by the pigment-pigment interactions (7–9). As a consequence, no fixed stoichiometric ratio of the major pigment to protein has been found in chlorosomes, and they exhibit considerable variation in size and shape (10,11). Although the exact structure of chlorosomes is not yet known, several models have been proposed in which the BChl *c* molecules are closely packed into rod-like (tubular) or sheet-like (lamellar) aggregates enclosed into a lipid membrane (12–14).

Along with the highly aggregated BChl *c*, chlorosomes also contain carotenoids, isoprenoid quinones, and a small amount of BChl *a* (3,15). The BChl *a* in chlorosomes is associated with the most abundant chlorosome protein, CsmA, which mediates the transfer of the excitation energy from chlorosomes to the reaction center via the BChl *a* binding Fenna-Matthews-Olson protein (2,15–17). In addition, nine other proteins have been identified in the chlorosomes from *Chlorobium tepidum,* but very little is known about their functions. It has been shown that only CsmA is essential and that none of the other nine proteins are required for the biogenesis, light harvesting, or structural organization of BChl *c* and BChl *a* within chlorosomes (11,18). The quinones in chlorosomes were suggested to act as quenchers of BChl *c* fluorescence depending on the redox state of the cellular medium, which regulates the energy transfer to reaction center to protect the green bacteria from potentially lethal reactions (e.g., superoxide formation) resulting from the reduction of ferredoxin in the presence of oxygen (19,20).

The molar ratio of carotenoids to BChl *c* in chlorosomes is typically around 1:10 in *Chlorobium* species, depending on the species and light conditions (1). Suggested functions for the carotenoids in chlorosomes include light harvesting, photoprotection, and structural stabilization of the BChl aggregates. The light-harvesting function, that includes light energy absorption by carotenoids and subsequent energy transfer to BChl, was confirmed by steady-state and ultrafast time-resolved experiments (17,21–23). The observed efficient energy transfer between carotenoids and BChl *c* implies that a significant fraction of carotenoids are embedded in the BChl *c* antenna structure, since energy transfer depends strongly on the distance between the donor and acceptor molecules (24–26). This is also consistent with the recent x-ray scattering and electron cryomicroscopy data (27) that suggest that carotenoids are localized between the planes of BChl *c* and drive assembly of chlorosomal antenna by augmenting hydrophobic interactions. The photoprotection realized by the quenching of the BChl triplet states (^{3}BChl*) in

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chlorosomes or scavenging singlet oxygen has also been tested, and triplet energy transfer to carotenoids was detected using different experimental techniques (23,28–30). The structural role played by carotenoids in chlorosomes is also manifested by decreased levels of the BChl *a* when carotenoid levels in chlorosomes are reduced (31,32), although this could also be a consequence of lessened photoprotection.

Recently, the biosynthetic pathway leading to the production of γ-carotene (β,ψ-carotene) and chlorobactene (φ,ψ-carotene), the major carotenoids of the green sulfur bacterium *Chlorobium tepidum*, has been elucidated by using a combination of genome analysis and gene inactivation (32,33). In this work, the chlorosomes isolated from these carotenoid mutants were used to characterize the photoprotection function of carotenoids using both steady-state and time-resolved optical techniques. Even though carotenoid-depleted chlorosomes have been previously prepared by other groups by applying a carotenoid biosynthesis inhibitor directly to cell cultures (31), the limitation of this approach is that only one sample (carotenoid-depleted chlorosomes) could be produced and studied. Moreover, inhibitors do not completely block carotenoid biosynthesis. The genetic methods employed in this work not only produce chlorosomes that are completely free of carotenoids, but they also produce chlorosomes containing carotenoids with defined but variable numbers of double bonds that could modify the efficiency of their protective function. Based on the experimental results obtained, we propose that BChl aggregates are naturally protected due to the formation of triplet excitons and that carotenoids in chlorosomes are not as essential as they are in other BChl/Chl containing photosynthetic complexes.

MATERIALS AND METHODS

Sample preparation

The construction and characterization of mutant strains of *Chlorobium tepidum* lacking specific enzymes of carotenoid biosynthesis have been described in detail elsewhere (32,33). The isolation and purification of chlorosomes has also been described (11,18,34). Chlorosomes from the wild-type strain contain 57 mg of carotenoids (mainly chlorobactene) per gram of BChl *c*. The chlorosomes of the *crtB* mutant contained no carotenoids; the *crtQ* mutant contained 65 mg of carotenoids (mainly ζ-carotene) and the *crtU* mutant contained 55 mg of carotenoids (mainly γ-carotene) per gram of BChl *c* (32).

For the alkaline treatment, the method of van Walree et al. (35) was followed without any further modification. Briefly, NaOH was added to chlorosomes that were incubated at 40°C for 0.5–1 h, and potassium phosphate buffer pH 6.0 was added to restore the sample to pH 7.2 before the measurement. The control samples were treated in a similar manner, but only 10 mM potassium phosphate buffer at pH 7.2 was added. To resolve the absorption band of BChl *a* around 795 nm, low-temperature or room-temperature absorption spectra were taken to confirm the selective degradation of BChl *a* as required.

For the oxygen tolerance experiment, cells were grown to late exponential phase, diluted 100-fold in aerobic phosphate buffer, and exposed to an air-saturated atmosphere under dark conditions or variable low light-intensity conditions (20, 30, and 40 μmol m^{-2} s^{-1}) for two days. Cells were later diluted 10,000-fold and plated to allow single colonies to develop. The number of colonies was counted after the treatment, and the survival rate was determined by comparison with the number of colonies obtained at time zero.

Spectroscopic measurements

Steady-state absorption spectra were measured using a Perkin Elmer Lambda 3B spectrometer (Wellesley, MA). In time-resolved experiments, 20 ns full width at half maximum pump pulses were generated by a dye laser pumped by an excimer laser (Lambda Physik, Scanmate 2 and EMG102 (Santa Clara, CA)). Probe beams were provided by a broadband xenon arc lamp driven by an ultrastable, pulsed power supply (Applied Photophysics, LKS .60 (Leatherhead, UK)). The latter was capable of producing ~2 ms square probe pulses every 2 s with a 250-μs plateau region with intensity fluctuation level ±0.5%. A set of glass filters transmitting only in the probed spectral region were inserted between the xenon lamp and the sample to reduce the actinic effect. The light from the xenon arc lamp was split into probe and reference beams. The reference beam bypassed the sample, whereas the probe beam was crossed with the pump beam in the sample housed in a 1- to 2-mm thick optical cell and spectrally filtered by a monochromator (Spectra-Physics, 77250 (Stratford, CT)) operated at 10–15 nm bandpass. Both beam intensities were measured by two identical fast low-noise amplified photodiodes (homebuilt), recorded by a digital oscilloscope, and processed in a computer resulting in time-dependent absorption difference (ΔA) profiles. Time resolution of the system was determined by the pump pulse duration (20 ns). The system was optimized for the detection of weak absorption changes $<10^{-3}$ expected in the experiments.

For the photodegradation experiment, ~120 μl of a sample contained in a 1-mm thick glass cell were uniformly irradiated by homebuilt continuum wave Ti:Sapphire laser, which was tuned to the peak of the Q_y absorption band of the major antenna pigments (~750 nm). The initial optical density of the sample was ~1.5 at its maximum absorption, and the absorption spectra were recorded as a function of irradiation time under air-saturated condition. The photodegradation of BChl was assessed as the integrated area of Q_y absorption band. The power density of irradiation was 0.23 W/cm^2, which corresponds to excitation of each BChl *c* ~70 times per second.

For the low temperature absorption measurements, a closed cycle cryostat (DE 202, Air Products (Allentown, PA)) was used with the 1-mm pathlength optical sample cell, providing controlled temperatures down to 10 K. Glycerol was added to the sample at 0.6/0.4 (v/v) ratio to enhance the optical properties of the frozen sample.

Exciton spectrum simulation

Triplet exciton energy levels were computed using the approach described (36–38) for calculating singlet exciton spectra. The total electronic Hamiltonian of *N* coupled BChl *c* molecules in an aggregate can be written as:

$$\hat{H} = \sum_{p=1}^{N} \hat{H}_p + \sum_{p<q}^{N} V_{pq}, \quad (1)$$

where $\hat{H}_p$ is the electronic Hamiltonian for a noninteracting pigment *p* and V_{pq} represents the triplet-triplet interaction between pigments *p* and *q*. The *N* triplet exciton states may be expanded in the basis of the *N* states $|l\rangle$ that localize triplet excitation on single pigments *l*. The triplet exciton energy levels $E_{exc,i}$ are the eigenvalues obtained by diagonalizing the $N \times N$ Hamiltonian matrix with elements

$$H_{l,m} = \left\langle l \middle| \sum_{p=1}^{N} \hat{H}_p + \sum_{p<q}^{N} V_{pq} \middle| m \right\rangle. \quad (2)$$

The diagonal and off-diagonal elements of this matrix are $H_{l,l} = E_l$ and $H_{l,m\neq l} = V_{lm}$, respectively, since all other contributions in Eq. 2 vanish through orthogonality. Here E_l represents the triplet excited state energy of

the pigment *l* in the absence of interaction. Matrix diagonalization was performed on a personal computer using Jacobi transformation (39).

RESULTS

Absorption spectra of chlorosomes

The solid curve of Fig. 1 shows the typical room temperature absorption spectrum of chlorosomes isolated from the wild-type *Chlorobium tepidum* and exhibits the strong Q_y absorption band of BChl *c* centered at 751 nm. The monomeric form of BChl *c* in organic solution (e.g., methanol) has its Q_y absorption band at around 670 nm (7,9,15,40), and the ~80-nm red shift observed in chlorosomes is caused by strong excitonic interactions between the closely packed BChl *c* molecules (1). The absorption band of BChl *a* is not readily resolved in the spectrum shown in Fig. 1 because its molar ratio is ~1:100 relative to BChl *c* (19) and the red tail of the strong BChl *c* absorption band overlaps its absorption band at 795 nm. However, the BChl *a* band can be resolved at low temperature as shown in the inset of Fig. 1.

The presence of carotenoids in chlorosomes is visualized as a shoulder near 500 nm on the Soret absorption band. The dashed curve in Fig. 1 shows the absorption spectrum of the carotenoid-free *crtB* chlorosomes. The carotenoid band is completely missing in *crtB* chlorosomes, which is consistent with the earlier high-performance liquid chromatography analysis of these samples (32). Except for the ~30-nm red shift, the absorption difference spectrum between wild-type and mutant around 500 nm (Fig. 1, *bottom panel*) roughly matches the absorption spectrum in petroleum ether of chlorobactene (41), which is the major carotenoid of the green sulfur bacteria (41–43). In addition, the *crtB* chlorosome sample shows a 9-nm blue shift of the entire Q_y band of the BChl *c* aggregates with respect to wild-type chlorosomes. This indicates that the absence of carotenoids influences the absorption properties of BChl *c* aggregates in chlorosomes, even though their absence does not disrupt the structure drastically. A similar blue shift of 10 nm was also reported for carotenoid-depleted chlorosomes from *Chlorobium phaeobacteroides*, which produces BChl *e* as the major antenna pigment (31).

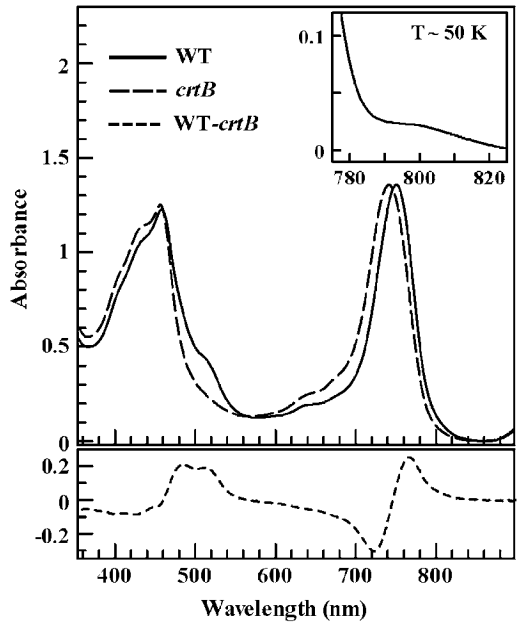

FIGURE 1 The absorption spectra of chlorosomes isolated from wild-type and carotenoid-free *crtB* mutant normalized by the BChl *c* Q_y band. The inset shows the absorption spectrum of wild-type chlorosomes at ~50 K, which resolves the absorption band of BChl *a*. The lower panel shows the difference spectrum for wild-type chlorosomes minus *crtB* mutant chlorosomes.

Photodegradation experiment and the photoprotective function of carotenoids

We performed photodegradation experiments in the presence of atmospheric oxygen levels on chlorosomes isolated from wild-type and several carotenoid mutants to verify the proposed photoprotective function of carotenoids (28,29,44) and to estimate its extent. The irradiation light source was tuned to the maximum absorption wavelength of each sample in the red region and its intensity was kept constant throughout the experiment. The photodegradation of the BChls as a function of irradiation time was measured as the integrated area of the Q_y absorption band, reflecting the amount of undamaged pigments after the irradiation as shown in Fig. 2. As expected, wild-type chlorosomes were more resistant to photodegradation than carotenoid-free chlorosomes from the *crtB* mutant, whereas chlorosomes from the *crtU* mutant, which have slightly lower total carotenoid content than wild-type chlorosomes (32), show an intermediate level of resistance. Quantitatively, the half-times of photodegradation, i.e., the time when the undamaged BChls reached half

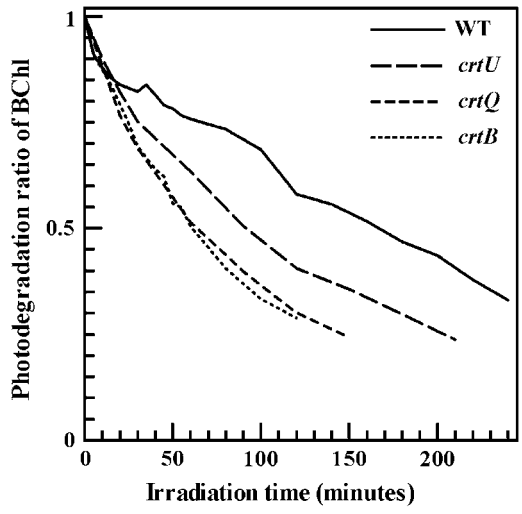

FIGURE 2 The photodegradation kinetics of BChls in chlorosomes as a function of irradiation time. Irradiation was tuned to the maximum absorption wavelength of each sample (730–750 nm).

of the initial contents, were 170, 95, 65, and 60 min for chlorosomes from the wild-type and *crtU*, *crtQ*, and *crtB* mutant strains, respectively. Wild-type chlorosomes thus exhibit ~3 times better photoprotection than carotenoid-free *crtB* chlorosomes. *CrtQ* chlorosomes, which contain primarily ζ-carotene, are no better photoprotected than carotenoid-free *crtB* chlorosomes. On the other hand, under similar irradiation conditions, monomeric BChl *c* dissolved in methanol photodegrades with half-time of ~2 s (not shown), i.e., >3 orders of magnitude faster than even carotenoid-free chlorosomes. The short excited state lifetime of the aggregated BChl *c* in chlorosomes (~100 ps (2,45,46)) in respect to that of monomeric BChl *c* (2.7 ns in methanol (47)) would lead only to ~30 times lower singlet oxygen formation rate due to the lowered triplet state yield, and cannot alone account for the observed photoprotection level. This implies that there is an additional unconventional photoprotection mechanism occurring in chlorosomes that does not involve carotenoids.

Triplet energy transfer from BChl to carotenoid as a photoprotection mechanism

The conventional photoprotection mechanism of carotenoids, i.e., triplet energy transfer from BChl to carotenoid (48), which can effectively compete with energy transfer to oxygen and prevent singlet oxygen formation, was tested by monitoring the transient absorption difference band of carotenoids upon optical excitation into the Q_y band of BChl *c*. Fig. 3 shows the time-dependent ΔA kinetic profile probed at 545 nm (carotenoid band (49,50)) after exciting wild-type chlorosomes under anoxic conditions at 680 nm (the *blue* side of the BChl *c* absorption band). A single decay component of 3.4 ± 0.6 μs was sufficient to fit this profile, and similar kinetic profiles with the same kinetics were recorded with probe wavelengths ranging from 520 to 570 nm. The inset of Fig. 3 shows the decay associated spectrum (DAS) that represents the amplitude of the 3.4 μs component as a function of the probe wavelength. The shape of DAS and the decay lifetime are consistent with the characteristics of triplet excited carotenoids (^{3}Car*) (49–51). Similar results were obtained at low temperature (~50 K), but with somewhat longer ΔA decay times (~10 μs, not shown), which can be explained by the impeded oxygen access to the carotenoids (50,51). A comparable transient ΔA band ascribed to ^{3}Car* was reported for chlorosomes from *Chlorobium phaeobacteroides* and from *Chloroflexus aurantiacus* (23).

As expected, no ^{3}Car* signal was detected around 540 nm upon excitation into the BChl *c* band of chlorosomes from the *crtB* mutant that lacks carotenoids (Fig. 4). However, as will be discussed later, in this case we detected a weak, long-lived (lifetime >100 μs), photobleaching signal at 730 nm (BChl absorption band), which we ascribe to the formation of an unquenched triplet state of bacteriochlorophyll.

BChl *a* free chlorosomes and triplet energy transfer

To ensure that triplet energy transfer to carotenoids occurs from BChl *c* pigments of chlorosomes, BChl *a* was selectively degraded using the alkaline treatment developed by van Walree et al. (35). The inset of Fig. 5 shows the low-temperature absorption spectra taken before and after the treatment of wild-type chlorosomes. The data confirm that this treatment causes selective degradation of the BChl *a* band at ~800 nm but does not cause degradation of the main

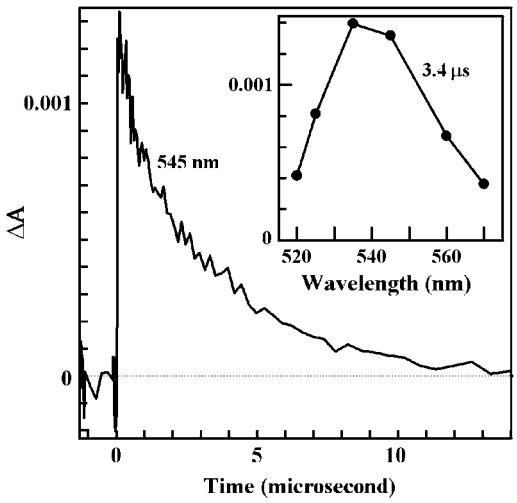

FIGURE 3 The transient absorption difference profile at 545 nm upon 680 nm excitation into the BChl *c* absorption band in wild-type chlorosomes. The inset shows the decay-associated spectrum of the ~3 μs decay component obtained from global fits to all profiles probed between 520 and 570 nm with the pump laser wavelength fixed at 680 nm.

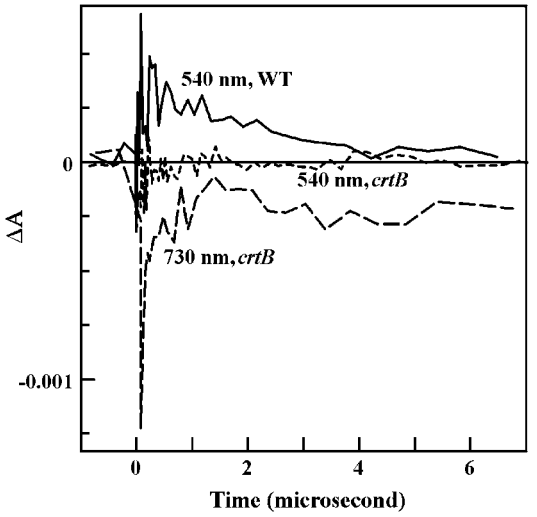

FIGURE 4 The absorption difference profiles of carotenoid-free chlorosomes from the *crtB* mutant upon the BChl excitation (747 nm) along with that of the wild-type at 540 nm as a control. The 730-nm profile of chlorosomes from the *crtB* mutant shows the long-lived photobleaching component whereas the decay time of ^{3}Car* of wild-type chlorosomes is ~3 μs.

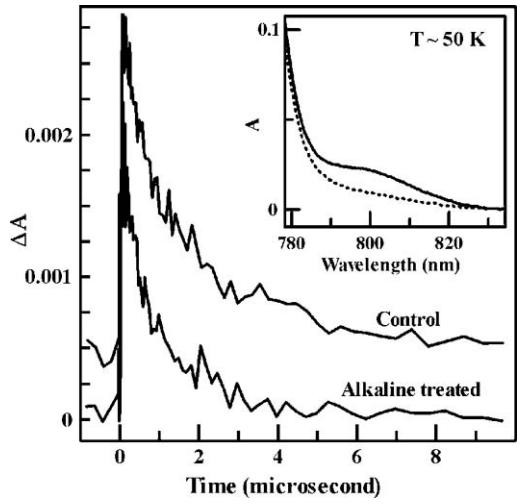

FIGURE 5 The absorption difference profiles at 540 nm before and after the selective degradation of BChl *a* (alkaline treatment) in wild-type chlorosomes. The inset shows the BChl *a* band of untreated sample resolved at 50 K; the dotted line shows the absorption spectrum after the treatment, which confirms the disappearance of the BChl *a* upon the treatment.

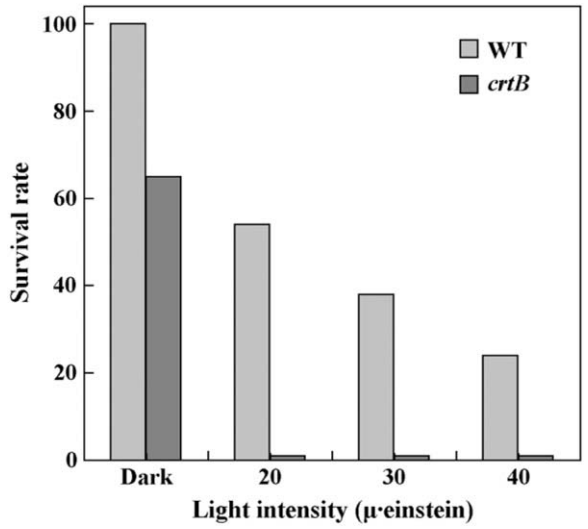

FIGURE 6 The in vivo survival rate of the *Chlorobium tepidum* wild-type and the *crtB* mutant strain, as measured by the number of colony forming units remaining after exposure to the light intensity indicated in the presence of atmospheric levels of oxygen for two days. Although nearly 70% of the *crtB* mutant cells survived a two-day exposure in the dark, no colonies were observed for this mutant in the light.

BChl *c* absorption band centered at ~751 nm. Confirming that the triplet energy transfer occurs from BChl *c* to carotenoids, we observed a ^{3}Car* signal of comparable intensity in both cases (Fig. 5). Because the BChl *a* content of chlorosomes is two orders of magnitude smaller than that of BChl *c*, however, this experiment does not exclude the possibility of additional triplet energy transfer from BChl *a*.

In vivo oxygen tolerance

Fig. 6 shows the normalized survival rate of the wild-type and *crtB* mutant cells after exposure to low light intensities (20–40 μmol photons m^{-2} s^{-1}) in the presence of atmospheric levels of oxygen (air-saturated buffer). The lowest light intensity was sufficient to kill all of the mutant cells in two days, although during the same exposure only ~50% of the wild-type cells were killed. Although this experiment demonstrates the importance of carotenoids in photoprotection, the experiments on whole cells are dependent upon the cumulative photodamage to all parts of the photosynthetic apparatus and not just chlorosomes. Indeed, as evidenced by comparison between Figs. 6 and 2, the photoprotection of chlorosomes by carotenoids is less drastic. This suggests that other parts of photosynthetic apparatus, most likely the photochemical reaction centers, are significantly more vulnerable to photodamage than chlorosomes. This observation implies that there is an additional unconventional photoprotection mechanism occurring in chlorosomes that does not involve carotenoids.

Long-lived states of BChls under excitation by intense light

Fig. 7 *a* shows ΔA profiles probed at 540 nm after exciting chlorosomes into the BChl *c* absorption band with increasing pump pulse energies. Surprisingly, at excitation pulse energies above 3 mJ cm^{-2} the additional absorption expected at 540 nm due to ^{3}Car* formation becomes superseded with photobleaching with the major decay component of longer than 1 ms (the time window of our experimental setup). A similar millisecond photobleaching signal of much greater amplitude was detected at 770 nm (Fig. 7 *b*). Fig. 7 *c* shows the DAS of this millisecond component along with the two somewhat weaker microsecond components required for fitting the measured wavelength-dependent kinetics globally. The spectral shape of the millisecond component is consistent with the bleaching of the BChl *c* ground state, and its lifetime is consistent with the decay of the unquenched ^{3}BChl* state (40). The observed kinetics resemble closely those obtained for the carotenoid-free chlorosomes from the *crtB* mutant even at weaker excitation energy levels (Fig. 4). The observed kinetics were independent of the oxygen concentration in the sample, and similar profiles were obtained in oxic conditions (data not shown).

DISCUSSION

The photoprotection of green sulfur bacteria against singlet oxygen generation may seem to be unnecessary because these bacteria are strictly anaerobic (52). However, most of the *Chlorobium* species grow in water at depths of few meters (53,54), where the ambient oxygen concentration is still $\geq$10 μM. At this concentration diffusion-limited triplet energy transfer from ^{3}BChl* to oxygen (and $^1O_2$ production) occurs with lifetime of $\leq$50 μs (40), which is at least 40 times faster than the natural decay of ~2 ms reported for

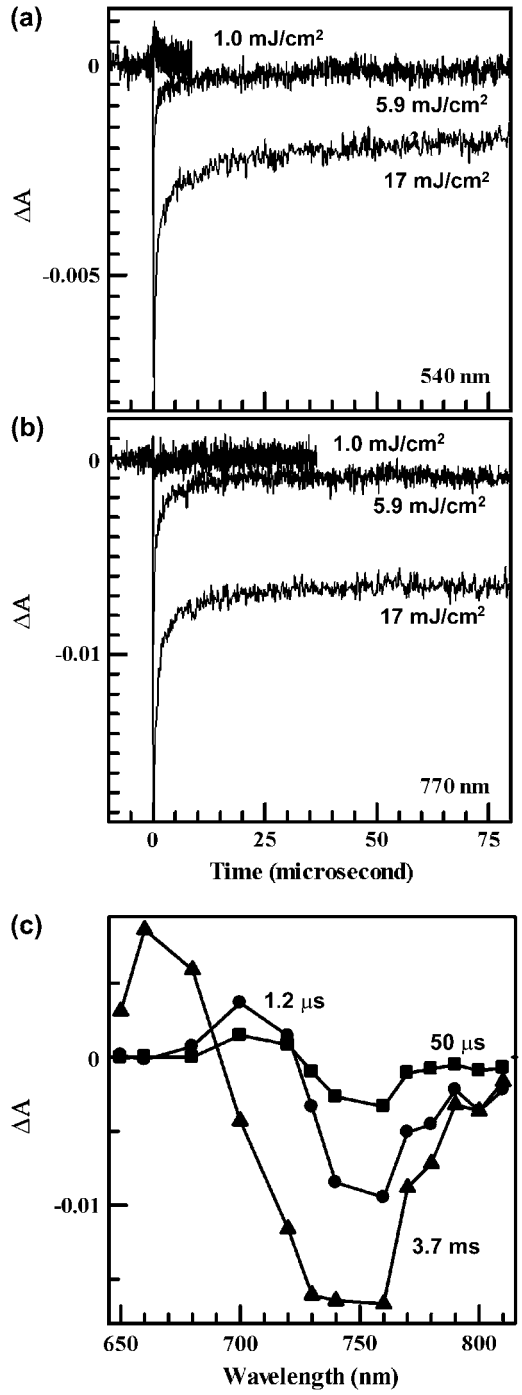

FIGURE 7 The power dependence of the absorption difference profiles of long-lived BChl states around (*a*) carotenoid (probed at 540 nm) and (*b*) Q_y absorption band of wild-type chlorosomes (probed at 770 nm) under strong excitation at 751 nm. (*c*) DAS obtained from the global fitting of the transient profiles measured with 17 mJ cm^{-2} pump pulse energy density with the probe wavelength varying between 650 and 810 nm with three decay components.

^{3}(B)Chl* in anaerobic environments (40,55,56). Moreover, the oxygen concentration within the hydrophobic environment of proteins, membranes, and the hydrophobic interior of their light-harvesting chlorosomes could be higher than that in the surrounding water, and therefore protection against singlet oxygen generation should be vitally important for such organisms. Consistent with this idea, the genome of *Chlorobium tepidum* contains about a dozen genes that are predicted to be directly or indirectly involved in protection against oxygen and reactive oxygen species (57).

It has been shown that under aerobic conditions the excited states of chlorosomes are strongly quenched (21,45, 58–60), and it has been suggested that this is one of the protection mechanisms cells have developed to minimize the yield of ^{3}BChl* formation and, as a result, to decrease singlet oxygen production under accidental exposure to an oxic environment. Our experiments provide strong evidence that chlorobactene molecules incorporated into wild-type chlorosomes in *Chlorobium tepidum* provide additional photoprotection (Fig. 2). However, the photoprotection provided by carotenoids was not as extensive as expected, since the BChl molecules in carotenoid-free chlorosomes from the *crtB* mutant were only ~3 times more sensitive to photodamage than those in wild-type chlorosomes, whereas the monomeric BChl *c* in methanol photodegraded ~1000 times faster. Moreover, the formation of ^{3}Car* upon excitation of BChl *c* was saturated at pump beam intensities that correspond to the accumulation of ~0.5–1.0% of the BChls in the chlorosomes in triplet states (Fig. 7). This indicates that not all of the carotenoids are involved in photoprotection and that a significant number of BChl *c* molecules end up in long-lived states with optical characteristics similar to that of ^{3}BChl*.

The photoprotection of chlorosomes offered by different carotenoids shows significant variations (Fig. 2). The BChls in chlorosomes from the *crtQ* mutant that contain only ζ-carotene photodegrade at the same rate as those in the chlorosomes from the carotenoid-free c*rtB* mutant, whereas the photoprotection provided by γ-carotene molecules in chlorosomes from the *crtU* mutant is intermediate between wild-type chlorosomes containing chlorobactene and the carotenoid-free chlorosomes of the c*rtB* mutant. Assuming that the structural positions of the molecules of chlorobactene, γ-carotene, and ζ-carotene are similar within the wild-type and mutant chlorosomes, these results imply that the properties of the triplet states in these three carotenoids are different. The energies of the singlet oxygen and triplet excited state of monomeric BChl *c* are 0.97 eV (61) and 1.29 eV (40), respectively. To provide photoprotection, the triplet state energy of the carotenoid must be below that of both ^{3}BChl* and $^1O_2$, i.e., <0.97 eV. Because the measurement is technically difficult to perform, the triplet state energies of chlorobactene, γ-carotene, and ζ-carotene have unfortunately not been measured. However, chlorobactene, which is found in wild-type chlorosomes, is structurally similar to the β-carotene (both have 11 conjugated double bonds (62)), for which the triplet state energy of 0.91 ± 0.03 eV (61) was measured. This would explain the highly effective quenching of both ^{3}BChl* and $^1O_2$ in wild-type chlorosomes. We

propose that the triplet energy level of γ-carotene is close to but slightly below the ^{3}BChl* level but higher than that of $^1O_2$; this would lower the energy transfer efficiency from ^{3}BChl* to Car, and/or completely block the quenching of the harmful singlet oxygen. The triplet energy of ζ-carotene must be $>$1.29 eV to explain the photodegradation data presented in Fig. 2. The elevated triplet state energy of ζ-carotene (seven conjugated double bonds (62,63)) with respect to that of chlorobactene is in general agreement with previous reports (63,64), which predict higher triplet energy levels for polyenes with a smaller number of conjugated double bonds.

While the protective roles of carotenoids in isolated chlorosomes in vitro appear to be modest, the in vivo oxygen tolerance experiments on whole cells show a much stronger dependence (compare Figs. 6 and 2). Even the lowest light intensities applied in the presence of oxygen were lethal for the *crtB* mutant. Since the Fenna-Matthews-Olson complex does not contain carotenoids, this observation suggests that the photoprotective role of carotenoids in chlorosomes is significantly less important than in other parts of photosynthetic apparatus, such as the Type 1 photochemical reaction centers (65–67).

The modest protective role of carotenoids in chlorosomes suggests that another photoprotection mechanism may function in these complexes. Indeed, the experiments showed that at higher excitation intensities, when more than 0.5–1% of BChls form triplet states, a long-lived BChl ground state bleaching is clearly observed (Fig. 7 *b*). The spectral shape and the lifetime of the major component ($>$1 ms) are consistent with the characteristics expected for the formation and natural decay of a significant amount of ^{3}BChl* that is not quenched by carotenoids or by oxygen. The proposed structures of chlorosomes (12,13,27), as well as the observed dependence of the optical absorption of chlorosomes in the presence of carotenoids (Fig. 1) and efficient singlet energy transfer between carotenoids and BChl *c* (17,21–23), suggest that carotenoid molecules are distributed rather evenly within the chlorosome and that a single carotenoid could serve as a triplet energy quencher for many surrounding BChl *c* molecules. However, our experiments indicate that only one of 100 BChl *c* molecules readily transfers its triplet energy to a nearby carotenoid. Stated differently, only one of 10 carotenoids receives triplet energy from the adjacent pool of BChl *c* molecules. Moreover, the long-lived (presumably) ^{3}BChl* states are not quenched by oxygen, which is expected to have ready access to the BChl *c* molecules within chlorosomes under oxic conditions. To explain the observed results, we propose that the energy of the triplet state of the aggregated BChl *c* in chlorosomes is dramatically lowered. This causes it to drop below the energies of ^{3}Car* and $^1O_2$ due to the strong excitonic interactions between the triplet states of closely spaced BChl *c* pigments in a way similar to the ~80 nm excitonic red shift of the Q_y band of the aggregated BChl *c* in chlorosomes.

Triplet excitons have been extensively studied in molecular crystals (see, for example, Avakian and Merrifield (68)). To estimate the energies of the triplet excitonic levels, one needs to know triplet-triplet coupling energies between the molecules and compute excitonic levels in the same way as singlet excitonic levels (see, for example, (36–38)). Because the triplet-triplet energy transfer occurs via a Dexter-type electron exchange mechanism, triplet-triplet interactions decay exponentially with the distance between the π-bonds of the interacting molecules (69). According to the high-resolution images of chlorosomes by cryoelectron microscopy (13), the stacking distance between the chlorin rings of BChl *c* is within the range of 3.3–4.2 Å, which is also consistent with NMR measurements (70). Recently, You et al. (69) calculated the triplet-triplet coupling energy between two stacked π-bonds separated by 3.5 Å to be 0.1 eV. To estimate the energies of triplet excitonic levels, we assumed that the BChl *c* molecules are arranged into a flat periodic rectangular net (sheet) and that each of the BChl *c* molecules only interacts with its four nearest neighbors with an exchange energy $V_{lm} = 0.1$ eV. Assuming that the monomeric ^{3}BChl* energy $E_1 = 1.29$ eV (40), an excitonic energy calculation described in the Materials and Methods section yields the lowest triplet excitonic energy level in BChl *c* aggregate at 0.89 eV, i.e., below that of both ^{3}Car* and $^1O_2$. A similar triplet excitonic energy was obtained for the model with a tubular arrangement of the BChl *c*. Based on these results, we propose that only ~1% of weakly coupled BChl *c*, as well as uncoupled BChl *a* molecules, form triplet states with the energies above the energy of ^{3}Car* and are quenched by carotenoids. The majority of the strongly coupled BChl *c* form low-energy triplet excitons that are unable to transfer energy to carotenoids or oxygen molecules. The presence of the microsecond components in the decay of BChl *c* (Fig. 7 *c*) may be explained by some variation in couplings within the chlorosomal structure, which lead to higher triplet excitonic energies that approach those of the ^{3}Car* or $^1O_2$. Thus, we suggest that the aggregation of BChl *c* within the chlorosome not only increases the efficiency of the excitation energy transfer to reaction centers but also serves to protect the chlorosome against photodegradation. This hypothesis is consistent with the earlier suggestion that the dimerization of BChls in several reaction centers lowers the triplet energy below the energy of singlet oxygen (71). The results presented here are also consistent with the work by Krasnovsky et al. (72), in which singlet oxygen formation was not observed in artificial BChl *d* oligomers or in chlorosomes, although these authors suggested that the effect was caused only by the lower yield of ^{3}BChl* formation due to the rapid decay of singlet excited state.

We will now address an alternative scenario that is based upon the possible involvement of quinones in quenching of ^{3}BChl* states. Both carotenoid-containing and carotenoid-free mutant strains incorporate significant amounts of quinones into their chlorosomes, with a molar ratio of quinones

to BChl *c* of ~1:10 (3). Several groups have reported that triplet excited states of Chl *a* in solution can be quenched by quinones (73–75). It was proposed that the quenching occurs as a result of electron transfer from the triplet excited state of the Chl to a nearby quinone with the consequent backward electron transfer from the reduced quinone to the ground state of the Chl (within ~200 μs). In this scenario the long-lived transient spectral component shown in Fig. 7, *b* and *c*, would represent the intermediate oxidized state of the BChl ($BChl^{+}$), whose spectrum is similar to that of ^{3}BChl*. To test this scenario we prereduced quinones within chlorosomes by adding 20 mM sodium dithionite to the sample. The quinone reduction effect of dithionite in chlorosomes is well documented by several groups (19,59,60). The freshness of dithionite solution was ensured by testing its reducing effect on the heme of the cytochrome *f* complex that could be verified optically (76). Prereduction of quinones in chlorosomes should prevent the possible electron transfer from ^{3}BChl* to quinone. However, the transient absorption kinetics shown in Fig. 7 were found to be independent of the redox state of the quinones, and therefore the involvement of quinones in ^{3}BChl* quenching can safely be ruled out.

In conclusion, the protective role of the carotenoids is found to be modest in isolated chlorosomes, and it is proposed that aggregates of BChl *c* molecules are naturally protected from harmful singlet oxygen production by the formation of triplet excitonic states with the energies that lie below the energy of singlet oxygen. This high intrinsic photostability makes artificial oligomers of BChls (77) especially suitable candidates for the production of artificial antennae. A similar protection mechanism may function in other photosynthetic proteins that contain strongly interacting (B)Chl pigments.

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