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ANTENNA ORGANIZATION AND EVIDENCE FOR THE FUNCTION OF A NEW ANTENNA PIGMENT SPECIES IN THE GREEN PHOTOSYNTHETIC BACTERIUM *CHLOROFLEXUS AURANTIACUS*

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Whole cells and isolated chlorosomes (antenna complex) of the green photosynthetic bacterium *Chloroflexus aurantiacus* have been studied by absorption spectroscopy (77 K and room temperature), fluorescence spectroscopy, circular dichroism, linear dichroism and electron spin resonance spectroscopy. The chlorosome absorption spectrum has maxima at 450 (contributed by carotenoids and bacteriochlorophyll (BChl) *a* Soret), 742 (BChl *c*) and 792 nm (BChl *a*) with intensity ratios of 20:25. The fluorescence emission spectrum has peaks at 748 and 802 nm when excitation is into either the 742 or 450 nm absorption bands, respectively. Whole cells have fluorescence peaks identical to those in chlorosomes with the addition of a major peak observed at 867 nm. The CD spectrum of isolated chlorosomes has an asymmetric-derivative-shaped CD centered at 739 nm suggestive of exciton interaction at least on the level of dimers. Linear dichroism of oriented chlorosomes shows preferential absorption at 742 nm of light polarized parallel to the long axis of the chlorosome. This implies that the transition dipoles are also oriented more or less parallel to the long axis of the chlorosome. Treatment with ferricyanide results in the appearance of a 2.3 G wide ESR spectrum at *g* 2.002. Whole cells grown under different light conditions exhibit different fluorescence behavior when absorption is normalized at 742 nm. Cells grown under low light conditions have higher fluorescence intensity at 748 nm and lower intensity at 802 nm than cells grown under high light conditions. These results indicate that the BChl *c* in chlorosomes is highly organized, and transfers energy from BChl *c* (742 nm) to a connector of baseplate BChl B792 (BChl *a*) presumably located in the chlorosome baseplate adjacent to the cytoplasmic membrane.

Introduction

The gliding filamentous bacterium *Chloroflexus aurantiacus* is the only member of the green photosynthetic bacteria that grows both anaerobically in the light and aerobically in the dark. As with all green bacteria, *Chloroflexus* contains light-harvesting structures called chlorosomes (formerly called chlorobium vesicles) [1–4]. Chlorosomes are oblong bodies located in close association with the inner surface of the cytoplasmic membrane [5,6] and contain all of the BChl *c* within the cell. *Chloroflexus* also contains BChl *a* which functions both as antenna bacteriochlorophyll and reaction

Abbreviations: BChl, bacteriochlorophyll; BChl B792, bacteriochlorophyll absorbing at 792 nm (probably a new BChl *a* absorbing species).

center. All BChl *a* is localized in the cytoplasmic membrane except for a minor but distinct amount of BChl B792 (presumably BChl *a*) that copurifies with isolated chlorosomes [7–10]. Thus far, this minor BChl *a* species has not been detected by absorption spectroscopy of whole cells due to competing absorption from BChl *a* in the membrane. This 792 nm absorption species has been postulated to be the channel or baseplate bacteriochlorophyll through which the chlorosome funnels energy to the membrane [7–10]. In other green bacteria (*Chlorobium limicola, Prosthecochloris aestuarii*), a two-dimensional crystalline array of a BChl *a* protein [5,12] has been postulated to serve as the chlorosome baseplate or attachment site to the cytoplasmic membrane.

The ultrastructure of chlorosomes in *Ch. limicola* [5] and *C. aurantiacus* [6] has been described by freeze-fracture electron microscopy. The chlorosomes consist of an envelope layer (not bilayer membrane) surrounding a varying number of rod-like structures that are oriented parallel to the major axis of the chlorosome. An electron-dense area between the chlorosome and membrane has been observed [6] and is interpreted to be the baseplate which anchors the chlorosome to the membrane. Theoretically, the baseplate connects the chlorosome energetically to the membrane-bound BChl *a*. The chlorosome and baseplate of *Chloroflexus* have about one-half the dimensions of *Chlorobium* [6].

Chloroflexus can be grown under a wide variety of conditions [3,11,13,14]. During anaerobic photosynthetic growth, the BChl *c*/BChl *a* ratio varies inversely with the growth light intensity [11]. During chlorosome development, it has been postulated that chlorosome length, width and baseplate size are not dependent on the light intensity [11]. The increase in the BChl *c*/BChl *a* ratio as a function of light intensity during growth is a result of an increasing number of chlorosomes per cell and possibly an increase in the thickness of the chlorosomes [11].

The data in this paper confirm the in vivo existence of the 792 nm absorption species BChl B792 (BChl *a*) and that the function of this pigment is to act as an intermediate in energy transfer between the chlorosome and the cytoplasmic membrane. Evidence is also presented suggesting possible BChl *c* interactions within the chlorosome, ordering of pigments within the chlorosome, and for thickening of chlorosomes related inversely to light intensity during growth.

Materials and Methods

Organism and growth conditions. C. aurantiacus strain J-10-F1 was isolated by Pierson and Castenholz [3] and obtained in pure culture from S.C. Holt, Amherst. Cells were grown in suspension at 53°C in medium D [3] supplemented with (w/v) 0.1% yeast extract, 0.25% casamino acids and 0.08% glycylglycine at pH 8.3. Cells grown under high light intensity were cultivated in 900-ml Roux bottles with illumination from four 60 W Lumiline bulbs. High light cells were harvested 18–24 h after inoculation. Cells grown under low light intenisty were grown in 14-l fermentors that were stirred at 200 rpm and illuminated with one 10 W bulb.

Cell harvesting and fractionation. Chloroflexus cells were harvested by centrifugation at $15000 \times g$ for 15 min at 40°C and washed twice with 0.01 M Tris-HCl, pH 8.0. Trace amounts of RNAase A and DNAase were added to the cell suspensions 10–15 min before disruption of the cells by three passes through a French pressure cell at $1 \cdot 10^8$ Pa. Unbroken cells and large debris were removed from the French pressure effluent by centrifugation at $15000 \times g$ for 15 min at 40°C. Particulate material (membranes and chlorosomes) was collected from the $15000 \times g$ supernatant by centrifugation at $184000 \times g$ (45000 rpm, Beckman 50 Ti rotor) for 90 min at 25–35°C, and resuspended in the same buffer.

Chlorosome isolation. Chlorosome and membrane fragments were collected as described above. Chlorosome and membrane fragments were separated by centrifugation through two continuous sucrose density gradients [10]. The BChl *c*-containing band from the first gradient (10–40% (w/v) sucrose over a 60% (w/v) pad, 40000 rpm for 10 h in Beckman 50 Ti rotor at 4°C) was collected and assayed for membrane contamination by looking for absorption at 865 nm. If there was contamination the fraction was dialyzed against four changes of 0.05 M Tris-HCl, pH 8.0, and layered over a second gradient (20–50% (w/v)

sucrose over 60% (w/v) pad, 27000 rpm for 24 h in Beckman SW 27 rotor at 4°C). The BChl *c*-containing band was collected and assayed for purity by comparing absorption at 865 nm and 740 nm.

Absorption spectroscopy. Absorption spectra were taken using a Cary 14 spectrophotometer. Low-temperature absorption spectroscopy was done on the same instrument using an optical Dewar. Liquid nitrogen was used to lower the temperature of samples that were 50–75% (w/v) in glycerol. Cells grown at high and low light intensities were normalized in absorbance by taking absorption spectra and making the proper dilution or by organic extraction into organic solvent (5 : 2 : 1 mixture of methanol/diethyl ether/Skelly B).

Fluorescence spectroscopy. Fluorescence spectra were taken using a Perkin Elmer MPF44 fluorimeter equipped with a Hammamatsu R928 photomultiplier tube. To avoid self-absorption distortion all samples were diluted to the point where the ratios of the fluorescent intensity peaks remained constant.

Electron spin resonance. ESR was done on isolated chlorosomes treated with 0.01 M ferricyanide using a Varian ESR spectrometer.

Circular dichroism. CD spectra were taken of isolated chlorosomes using a Jasco J-40 spectrophotometer.

Linear dichroism. Chlorosomes were suspended in poly(vinyl alcohol) (Polysciences, Warrington, PA Catalog No. 2815), dried into a film and stretched. LD spectra were recorded on a Cary 14 spectrophotometer equipped with Oriel 2520-3 polarizing prisms in both sample and reference beams. Absorption spectra and baselines were recorded with the polarizers oriented parallel and perpendicular to the stretch axis. The spectra were digitized using a Numonic digitizer and a Hewlett Packard 9825 A computer, baselines substracted, and absorbance adjusted to zero at 900 nm. Data were analyzed using the method of Thurlstrup and Michl [15].

Results

Absorption spectra

The room-temperature absorption spectra of whole cells (high light cells, Fig. 1a; low light cells, Fig. 1b) show peaks at 742 (BChl *c*), 805 (BChl *a*) and 865 nm (BChl *a*) with the BChl *c*/BChl *a* ratio being dependent on light intensity during growth [12]. Low-temperature absorption spectra (77 K, Fig. 1a and b) show sharpening of absorp-

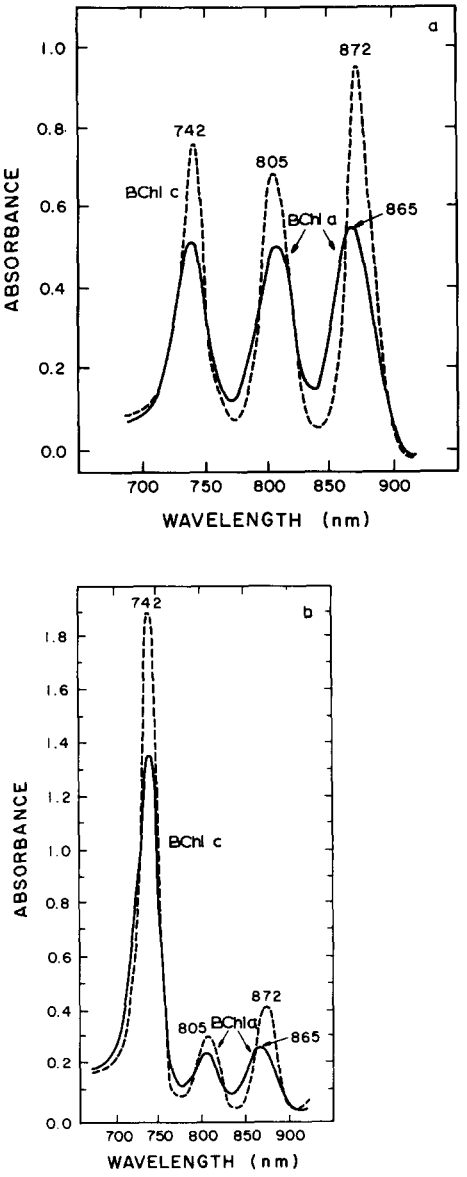

Fig. 1. (a) (———) Room-temperature absorption spectrum of *Chloroflexus* whole cells grown under high light intensities. (- - - - -) Low-temperature (77 K) absorption spectrum of same sample. (b) (———) Room-temperature absorption spectrum of *Chloroflexus* whole cells grown at low light intensity. (- - - - -) Low-temperature (77 K) absorption spectrum of same sample.

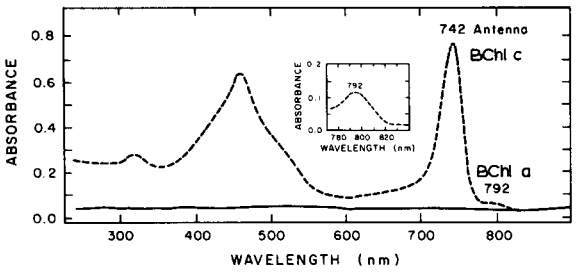

Fig. 2. Room-temperature absorption spectrum of isolated chlorosomes from *Chloroflexus*. (Inset) Low-temperature (77 K) absorption spectrum of 792 nm peak of chlorosomes.

tion peaks and a shift of the 865 nm peak to 872 nm. No fine structure in any peak is observed at 77 K, in contrast to results obtained using *Ch. limicola* and *P. aestuarii* [17].

An absorption spectrum of isolated chlorosomes (Fig. 2) shows peaks at 450, 742 nm (BChl *c*) and a small shoulder on the long-wavelength side of the 742 nm absorbance that can be resolved at 77 K (Fig. 2, inset) into a 792 nm BChl *a* peak. At 77 K there is sharpening of the 742 nm BChl *c* absorption peak but no fine structure or shift in absorption maxima was observed.

Fluorescence spectroscopy

Isolated chlorosomes excited at 720 nm show emission peaks at 748 nm (from the 742 nm absorbing BChl *c*) and 802 nm (from the 792 nm absorbing BChl *a*) (Fig. 3) with the 748 nm/802 nm emission ratio varying somewhat with each

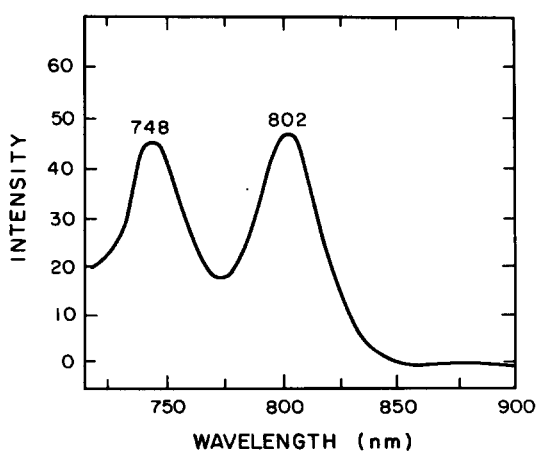

Fig. 3. Fluorescence emission spectrum of isolated chlorosomes excited at 720 nm.

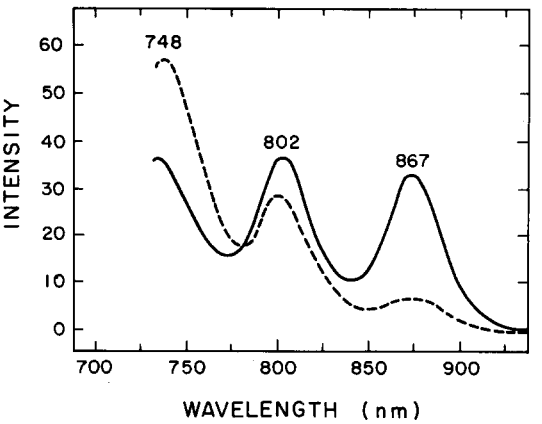

Fig. 4. Fluorescence emission spectrum of whole cells grown at high light intensities (———) or at low light intensities (- - - - -) with excitation into the normalized 742 nm absorbance.

chlorosome preparation. Whole cells excited at the same wavelength have emission peaks also at 748 and 802 nm with an additional peak observed at 867 nm (Fig. 4). The fluorescence at 867 nm is ascribed to be from the 865 nm absorbance (artificially shifted to shorter wavelengths due to photomultiplier tube response). The reaction center fluorescence is probably out of the range of the R 928 photomultiplier. Cells grown at high or low light intensities and normalized in absorbance at 742 nm show the same emission peaks with different intensities when excitation is into the 742 nm absorbance band (Fig. 4). Cells grown at high light intensity show a smaller emission peak at 748 nm and larger peaks at 802 and 867 nm than cells grown at low light intensities.

Circular dichroism

CD done on isolated chlorosomes (Fig. 5) shows an asymmetric-derivative-shaped trace centered at 739 nm and a positive CD in the 400–500 nm

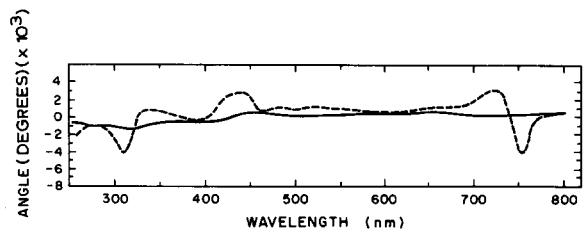

Fig. 5. CD spectrum of isolated chlorosomes.

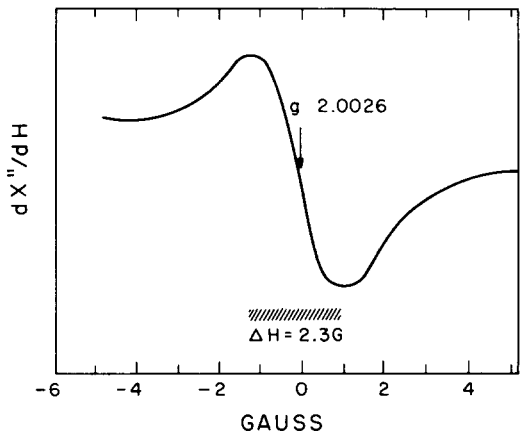

Fig. 6. ESR signal generated by treatment of isolated chlorosomes with 0.01 M ferricyanide.

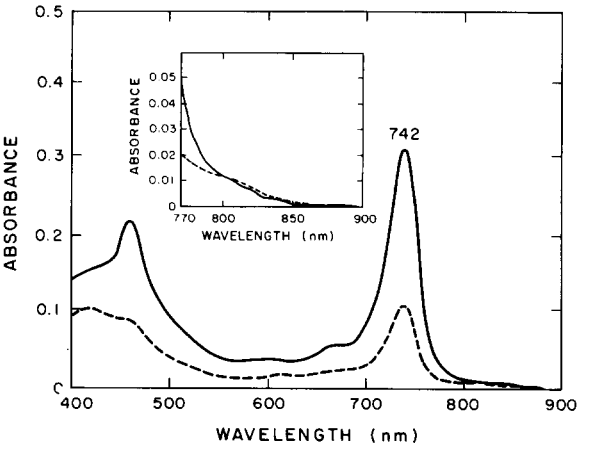

Fig. 7. (———) Absorption spectrum of oriented chlorosoems when polarized light is irradiated parallel to the stretch axis. (- - - - - -) Absorption spectrum of oriented chlorosomes when polarized light is irradiated perpendicular to the stretch axis. (Inset) (———) Enlarged absorption spectrum of parallel irradiation and (- - - - - -) perpendicular irradiation.

region. The internal structure of the chlorosome is entirely unknown except for striations that appear in freeze-fracture electron micrographs [5,6,11]. The CD feature centered at 739 nm suggests that there is BChl *c* exciton interaction within the chlorosome at least at the level of dimers [17].

Electron spin resonance

Chlorosomes treated with ferricyanide show a detergent-sensitive ESR signal with $\Delta H = 2.3$ G and *g* 2.0026 (Fig. 6). The narrowness of the ESR spectrum and *g* value suggest that a chemically generated free electron in a chlorosome can delocalize over a large number of bacteriochlorophyll molecules. The delocalization probably occurs between BChl *c* molecules but it is possible that delocalization does include the 792 nm BChl *a* molecules.

Linear dichroism

Chlorosomes oriented in poly(vinyl alcohol) films exhibit the 742 nm absorbance polarized largely parallel to the stretch axis (Fig. 7). This means that the 742 nm absorbing BChl *c* molecules have their transition dipoles more or less parallel to the long axis, since the molecule (or other assymetric structure) invariably orients itself with its long axis parallel to the stretch direction [18]. The root mean square angle is equal to 40 ± 2°. This angle may have contributions both from imperfectly oriented chlorosomes and from an intrinsic BChl *c* arrangement. It is impossible to determine which of these two effects is the most important. The 792 nm absorbing BChl *a* molecules exhibit no dichroism (Fig. 7). This means either the 792 nm absorbing BChl *a* molecules are randomly oriented with respect to the long axis of the chlorosome, or that they are oriented with their transition dipoles making an angle of nearly the 'magic angle' [15]. As considered in the Discussion the latter possibility seems more likely.

Discussion

The ability to isolate pure chlorosomes from *C. aurantiacus* as well as the identification of BChl *c* (742 nm) and a small amount of BChl *a* (B792) in the isolated chlorosome is well established [8–10]. Until now, the in vivo existence of the 792 nm peak as well as a possible function for the pigment has been a matter of speculation [11,12]. The fact that in chlorosomes energy absorbed by the 742 nm peak can fluoresce at 748 and 802 nm (presumably from 792 nm absorbing BChl *a*) and that the same emission peaks are present in whole cell fluorescence spectra suggest that the 792 nm absorbing BChl *a* does exist in vivo and is an active part of the energy-transfer system from antenna BChl *c* to membrane-bound BChl *a*.

Since energy transfer between the 742 and 792 nm absorbing bands occurs even in extremely dilute solution, this indicates that these pigments must be in close proximity. The linear dichroism experiments indicate that the BChl *a* is aligned largely parallel to the membrane plane, in agreement with results reported by Swarthoff et al. [19]. It is also consistent with the highly organized arrangement of BChl *c* as proposed from structural studies of Staehelin et al. [6]. The average angles between 742 nm absorbing BChl *c* and the 792 nm absorbing BChl *a* transition dipoles would allow energy transfer between these pigments. An additional factor in energy transfer from BChl *c* (742 nm) to BChl *a* (B792) is that while the bulk of the BChl *c* may be oriented at a large angle to the BChl *a*, the specific BChl *c* closest to the BChl *a* could have an orientation atypical of the bulk BChl *c*, thus allowing very efficient energy transfer.

Electron microscopic studies have shown a crystalline array structure at the interface between the chlorosome and the cytoplasmic membrane [10,11]. It is attractive to speculate that the 792 nm absorbing BChl *a* is associated with this baseplate and may function in energy transfer between the two structures. In other green bacteria, it has been postulated that the baseplate for the chlorosomes is a BChl *a*-protein complex that exists as a trimer with seven BChl *a* per subunit [20]. Evidence for the existence of a BChl *a* complex first came from low-temperature absorption spectroscopy of membrane vesicles [16]. *Chlorobium* and *Prosthecochloris* vesicles show splitting at low temperature in a BChl *a* peak centered at about 809 nm. In *Chloroflexus*, low-temperature spectroscopy reveals no splitting in its major BChl *a* antenna pigments (805 nm, 865 nm) or in the minor 792 nm absorbing BChl B790. This suggests that a BChl *a*-protein complex with the same spectral properties as other green bacterial BChl *a*-protein complexes does not exist in *Chloroflexus*. In addition, attempts to isolate a BChl *a* protein trimer from *Chloroflexus* by established procedures have failed [21].

The BChl *c*/BChl *a* ratio in *Chloroflexus* is inversely proportional to the light intensity during growth [3,4,11]. The changing ratio can be explained by increasing the number of chlorosomes per cell, increasing the number of BChl *c* molecules per chlorosome or a combination of both processes. It has been previously shown by freeze-fracture electron microscopy that cells increase the number of chlorosomes per cell in reponse to low light growth conditions. The length and width of the chlorosomes as well as the baseplate size do not seem to change but there is some indication that there is a thickening of the chlorosome during its development [10].

Whole cells grown at high and low light intensities, with the absorbance at 742 nm matched, will have the same number of BChl *c* molecules. If these cells are excited into the 742 nm absorbance band, the high light cells show a lower fluorescence intensity at 748 nm and a higher intensity at 802 nm relative to low light cells (Fig. 4). Since each cell type has the same number of absorbing BChl *c* molecules, the only difference in fluorescence intensity at 748 nm should be due to relative efficiencies of energy transfer from the chlorosome BChl *c* (742 nm) to the baseplate BChl *a* (792 nm). If the assumptions are made that the length, width and baseplate size [11] of chlorosomes, as well as that any additional BChl *c* that is added to chlorosomes in cells grown at low light intensities, is packed in the same way as the initial BChl *c*, then these data indicate that the chlorosomes of low light cells are thicker than those of high light cells. Electron micrographs [10] support this conclusion. If the chlorosomes of low light cells are thicker than those of the high light cells, fluorescence at 748 nm should be greater in low light cells because of the greater distance across which energy transfer must occur before encountering the baseplate BChl *a*. If the low light cells have more BChl *c* per chlorosome there will be more chlorosome bodies in a high light cell sample relative to a normalized low light cell mixture. It has been shown [11] that each chlorosome has the same sized baseplate. If in normalized samples of high and low light cells, high light cells have more chlorosomes, they would also have more baseplates or more BChl *a* (792 nm). If these assumptions are correct, the experimental result of a higher fluorescence intensity at 802 nm in high light cells can be attributed to a larger amount of BChl *a* (792 nm) in high light cells relative to low light cells.

Other BChl *a* (805 nm, 865 nm) species exist in the membrane of *Chloroflexus*. The 865 nm ab-

sorbing BChl *a* is 10–14% reversibly photobleachable [21] and is therefore postulated to be partially due to reaction center. The remaining 865 and 805 nm absorbing BChl *a* species are additional antenna pigment. Little or no fluorescence is observed arising from the 805 nm peak. This suggests that energy transfer through the 805 nm pigment is very efficient.

These results suggest a model for energy transfer in *Chloroflexus* (Fig. 8). The energy-transfer sequence is: 742 nm BChl *c* → 792 nm BChl *a* → 805 nm BChl *a* → 865 nm BChl *a* → 865 nm BChl *a* reaction center. There is no clear evidence as to whether only one reaction center is associated with a single chlorosome.

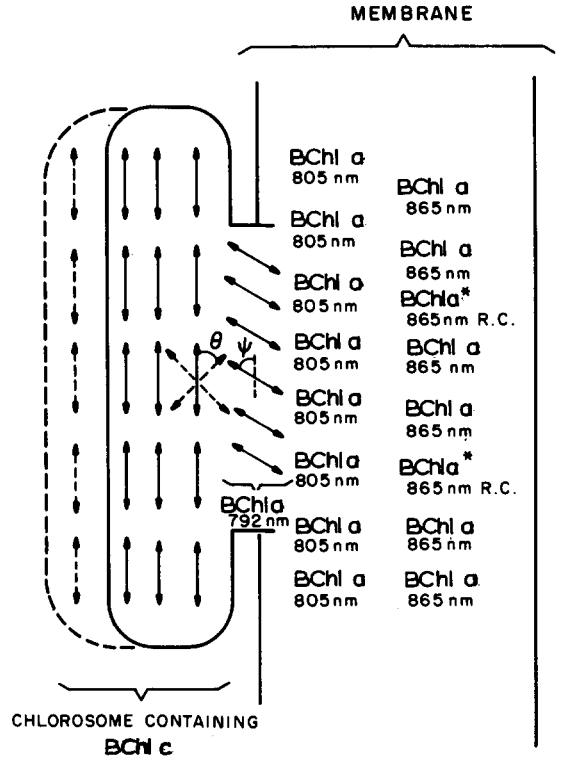

Fig. 8. Model of possible bacteriochlorophyll arrangement both in the chlorosome and membrane. The unbroken lines indicate transition dipole orientations as determined by LD with $\theta = 40 \pm 2°$ and $\psi = 54.7°$. The dashed lines on the outer part of the chlorosome indicate a possible chlorosome width depdendence on growth conditions. The BChl *a* (792 nm) is shown to be a possible baseplate for the chlorosome. The BChl *a* within the membrane is not illustrated in any particular manner except to allow for downhill energy transfer to the reaction center. The number of reaction centers per chlorosome is not known.

The model presented suggest that photosynthetic energy transfer in *C. aurantiacus* is very different from that in other green photosynthetic bacteria. Energy is transferred from the chlorosome to BChl 792 (BChl *a*) baseplate and not to a BChl *a* protein trimer as in *P. aestuarii* or *Ch. limicola* [16]. Reaction center activity is seen at 865 nm and not at 840 nm [22,23]. In addition, chlorosome absorbance is at 745 nm in *P. aestuarii* and shifts to 753 nm [19] at 77 K while in *Chloroflexus* no shift is seen from its 742 nm chlorosome absorbance. Although *Chloroflexus* resembles the Chlorobiaceae with respect to cytological organization and types of bacteriochlorophylls, it is apparent that there are clear and important differences in antenna structure and reaction center function.

Acknowledgements

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