Regulation and Inhibition of Excitation Energy Transfer in the Light Harvesting Apparatus of Green Photosynthetic Bacteria

Seiji Tokita
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著者 時田誠二

審査担当者
主査 松浦克美
委員 鳩田敬三
委員 平田州雄

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AUTHOR: Seiji Tokita

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Examiner in chief

Examiner

Examiner

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REGULATION AND INHIBITION OF EXCITATION ENERGY TRANSFER IN THE LIGHT HARVESTING APPARATUS OF GREEN PHOTOSYNTHETIC BACTERIA

A doctoral dissertation

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Seiji Tokita

Department of Biology, Graduate School of Science

Tokyo Metropolitan University
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Summary

Normal growth of photosynthetic green sulfur bacteria is influenced by pheophytinization of bacteriochlorophyll (BChl) c and quenching of energy due to oxygen in chlorosomes. Pheophytinization of BChl c and energy transfer in chlorosomes in cells of *Chlorobium (Chl.) tepidum* were studied with the method of absorption spectroscopy, fluorescence measurements and HPLC.

When a small amount of *Chl. tepidum* cells in which BChl c had been completely pheophytinized were transferred to a new culture medium, normal growth was observed after a short lag phase. This result indicates that *Chl. tepidum* can survive even when BChl c has been completely pheophytinized. Upon excitation of bacteriopheophytin (BPhe) c at 550 nm the fluorescence emission spectrum showed maxima at 775 and 810 nm, which correspond to emissions from BChl c and BChl a, respectively. This indicates energy transfer from BPhe c to BChl c and BChl a. In cells where BChl c was completely pheophytinized, fluorescence measurements were indicative of direct energy transfer from BPhe c to baseplate BChl a.

Quenching of BChl c fluorescence was examined in chlorosomes isolated from *Chloroflexus (Cfl.) aurantiacus* by many substituted quinones. The quenching of energy in *Cfl. aurantiacus* is similar to that in *Chl. tepidum* exhibiting sensitivity to O₂. All quinones examined showed quenching and I determined the half-quenching concentrations of the quinones. The ratio of estimated bound naphthoquinone molecules per BChl c molecule at a half-quenching concentration was rather constant among very effective naphthoquinones, gradually increasing with increasing the half-quenching concentration in less-effective quinones. In isolated chlorosomes from *Cfl. aurantiacus*, the hydroxy (or oxo) group in the alpha
position to the quinone carbonyl seems to be very important for the high quenching ability, probably together with a high redox potential and high hydrophobicity of the quinone molecule.

In *Cfl. aurantiacus* cells, the effects of several naphthoquinones on BChl *c* fluorescence and on flash-induced cytochrome *c* oxidation indicated that these naphthoquinones quenched BChl *c* fluorescence in a cell suspension. It was concluded that the added quinones inhibited flash-induced cytochrome *c* oxidation in *Cfl. aurantiacus* by specifically quenching the excited BChl *c* antenna.

Chlorobiumquinones (CK) has been suggested to function as quenchers of chlorosome fluorescence in *Chl. tepidum* under oxic conditions. I suggest that the oxo group neighboring on dicarbonyl group of the CK caused the high quenching effect of CK in chlorosomes from *Chl. tepidum*. 
Abbreviations

AcMNQ, 2-acetyl-3-methyl-1,4-naphthoquinone; AQ, anthraquinone; BChl, bacteriochlorophyll; BPhe, bacteriopheophytin; BQ, benzoquinone; CK, chlorobiumquinone; FMO protein, Fenna-Matthews-Olson protein; Fo, the intensity of fluorescence in the absence of any quencher; Fc, the intensity of fluorescence after the addition of quencher; 5HNQ, 5-hydroxy-1,4-naphthoquinone; HPLC, high-performance liquid chromatography; MK, menaquinone; MOPS, 3-Morpholinopropanesulfonic acid; NQ, naphthoquinone; RC, reaction center; Shikonin, 5,8-dihydroxy-2-(1-hydroxy-4-methyl-3-pentenyl)-1,4-naphthoquinone; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol.
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General introduction

The process of photosynthesis has two parts. First, light energy is captured in antenna pigments and transferred to the photochemical reaction center (RC) complexes. Next is the conversion of light energy to chemical energy as high-energy electrons in the RC. While these electrons are transferred to other electron transfer components, protons are translocated across the membrane (1). ATP and NAD(P)H are synthesized by using the proton gradient.

Green photosynthetic bacteria consist of two families, Chlorobiaceae and Chloroflexaceae. A large evolutionary distance separates these two families. The families contain different types of RC [PS I type in Chlorobiaceae (2) and PS II type in Chloroflexaceae (3)]. Chlorosomes, the light-harvesting apparatus, are almost the same in the two families (4-6).

The chlorosomes of green sulfur bacteria and green filamentous bacteria are ovoid bodies attached to the inner side of the cytoplasmic membrane (7, 8). Chlorosomes contain a large amount of bacteriochlorophyll (BChl) c, d, or e as the main antenna pigment and a small amount of BChl a in the “baseplate”, in addition to carotenoids, isoprenoid quinones, amphipathic lipids, and proteins (9, 10). The baseplate is thought to be located at the cytoplasmic membrane side of the chlorosomes (11, 12). Rod-like structures directed along the axis have been observed in the chlorosomes (5, 6). Olson et al. and Hirota et al. (13, 14) suggested that these rod structures were formed from many aggregated BChl c molecules without any involvement of proteins.

The Fenna-Matthews-Olson (FMO) protein, a soluble BChl a-containing protein in green sulfur bacteria, is located between the chlorosomes and the membrane. It mediates energy transfer from chlorosomes to the RC in the membrane. This
protein complex is not found in the green filamentous bacteria.

The energy transfer from BChl c in chlorosomes to the RC in green sulfur bacteria is thought to occur as follows (11, 15-19):

\[ \text{BChl c (chlorosomes)} \rightarrow \text{BChl a (baseplate)} \rightarrow \text{BChl a (FMO protein)} \rightarrow \text{RC} \]

The green sulfur bacterium, *Chlorobium (Chl.) tepidum*, grown at high light intensity at 46 °C changed color from green to dark brown within 2 weeks after growth initiation. Analyses of this color-change process is described in Chapter 1. Effects of various environmental factors on the color-change process are presented in Chapter 2.

Green sulfur bacteria are strictly anaerobic bacteria. Under reducing conditions, which is the natural state for growth of the bacteria, the chlorosomes are highly fluorescent. Under oxidizing conditions, this fluorescence is quenched and the intrachlorosomal energy transfer from BChl c to BChl a in the baseplate is significantly decreased. It has been proposed that this is caused by the presence of a quenching mechanism inside the chlorosomes that is activated by a high redox potential (18-21). The redox modulation effect is not observed in the green filamentous bacterium *Chloroflexus (Cfl.) aurantiacus* which contains chlorosomes but whose reaction center does not contain low-potential electron acceptors.

Frigaard et al. (10, 22) suggested that chlorobiumquinone (CK) in chlorosomes in *Chl. tepidum* plays a significant, but not exclusive, role in controlling the fluorescence and inhibiting energy transfer under oxic conditions.

By adding different quinones to chlorosomes isolated from *Cfl. aurantiacus*, I observed that the BChl c fluorescence was quenched to different extents depending on the quinones. The results are described in Chapter 3. I suggest that the natural quenching effect of CK is higher than that of MK-7 due to the 1'-oxo group neighboring the quinone group of the CK molecule.
A membrane-bound tetraheme cytochrome $c_{554}$ is the immediate electron donor to the photo-oxidized special pair in the reaction center of *Cfl. aurantiacus*. The two MK-10 molecules in the reaction center complex function as electron acceptors (23, 24). By flash-induced cytochrome $c$ oxidation measurement, the energy transfer in *Cfl. aurantiacus* from BChl $c$ to the RC is not sensitive to O$_2$ in untreated cells (25). I show in Chapter 4 that addition of low concentrations of certain quinones to cells under aerobic conditions induces a quenching of the BChl $c$ antenna and thereby inhibits photosynthetic electron transfer when BChl $c$ is specifically excited.
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Chapter 1

Pheophytinization of bacteriochlorophyll $c$ and energy transfer in cells of *Chlorobium tepidum*
Abstract  Bacteriochlorophyll (BChl) c in whole cells of *Chlorobium tepidum* grown at 46 °C changed into bacteriopheophytin (BPhe) c within 10 days after reaching full growth. When a small amount of *Chl. tepidum* cells in which BChl c had been completely pheophytinized were transferred to a new culture medium, normal growth was observed after a short lag phase, and the absorption spectrum of the growing cells showed the presence of a normal amount of BChl c. During the growth of *Chl. tepidum* in the new culture, the BChl c concentration was nearly proportional to the cell density measured by turbidity (OD₆₄₀). These results indicate that *Chl. tepidum* can survive even when BChl c has been completely pheophytinized and that BChl c is newly synthesized in such cells when transferred to a new culture medium. In partly pheophytinized cells, upon excitation of BPhe c at 550 nm the fluorescence emission spectrum showed maxima at 775 and 810 nm, which correspond to emissions from BChl c and BChl a, respectively. This indicates energy transfer from BPhe c to BChl c and BChl a. In cells in which BChl c was completely pheophytinized, fluorescence measurement were indicative of direct energy transfer from BPhe c to baseplate BChl a. These findings suggest that when BChl c in *C. tepidum* cells is pheophytinized, the product (BPhe c) remains in the chlorosomes and continues to work as a light-harvesting pigment.
Introduction

The light-harvesting apparatus of green sulfur bacteria and green filamentous bacteria, the chlorosomes, are ovoid bodies attached to the inner side of the cytoplasmic membrane (1, 2). Chlorosomes contain a large amount of bacteriochlorophyll (BChl) c, d, or e as the main antenna pigment and a small amount of BChl a in the "baseplate", in addition to carotenoids, isoprenoid quinones, amphipathic lipids, and proteins (3, 4). The baseplate is thought to be located at the cytoplasmic membrane side of the chlorosomes (5, 6). Rod-like structures directed along the longer axis have been observed in the chlorosomes (7, 8). It has been suggested that these rod structures are formed from many aggregated BChl c molecules without any involvement of proteins (9-13).

The Fenna-Matthews-Olson (FMO) protein, which is a soluble BChl a-containing protein in green sulfur bacteria, is located between the chlorosomes and the membrane and mediates energy transfer from chlorosomes to the reaction center (RC) in the membrane. In green sulfur bacteria, the energy transfer from BChl c in chlorosomes to the RC is thought to occur as follows (5, 14-18):

\[ \text{BChl c} \rightarrow \text{BChl a (baseplate)} \rightarrow \text{BChl a (FMO protein)} \rightarrow \text{RC} \]

The green sulfur bacterium, *Chlorobium tepidum*, is a moderately thermophilic anoxygenic phototrophic bacterium which has a generation time of approximately 2 h at the optimum growth temperature of 48°C (19).

Here I report that *Chl. tepidum* grown at high light intensity at 46 °C changed in color from green to dark brown within about 2 weeks after growth initiation. In the absorption spectra, the Qy band of chlorosomes was blue-shifted from 740 to 670 nm. The color change of the cells after the stationary phase of growth was caused by loss of the central magnesium atom (pheophytinization) of BChl c in
chlorosomes. Fluorescence spectroscopy suggested that excitation energy was transferred from bacteriopheophytin (BPhe) \( c \) to BChl \( a \) in the cells after the color change.

**Materials and methods**

**Growth conditions**

*Chlorobium tepidum* ATCC49652 cells were grown at 46 °C under long-wavelength fluorescent lamps (Toshiba FL-20S 20W, emission maximum at 740 nm) with an irradiance of 10 Wm\(^{-2}\). The irradiance was measured at a distance of about 15 cm from the lamps using a LI-1000 data logger with a LI-200S sensor (LI-COR, Lincoln, Nebraska, USA). The wavelength range of sensitivity of the sensor was from 400 to 1100 nm. The culture medium, prepared and modified according Wahlund et al. (19), contained per liter: 2.2 g \( \text{KH}_2\text{PO}_4 \); 0.5 g \( \text{K}_2\text{HPO}_4 \); 0.05 g ethylenediamine-\( N,N,N',N' \)-tetraacetic acid, trisodium salt, trihydrate; 0.4 g \( (\text{NH}_4)_2\text{SO}_4 \); 0.4 g \( \text{MgSO}_4 \cdot 7\text{H}_2\text{O} \); 0.4 g \( \text{NaCl} \); 2.0 g \( \text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O} \); 0.1 g \( \text{CaCl}_2 \cdot 2\text{H}_2\text{O} \); 0.005 g ethylenediamine-\( N,N,N',N' \)-tetraacetic acid, Fe (III), sodium salt, trihydrate; 100 \( \mu \)l trace element solution [1.55 g \( \text{H}_3\text{BO}_3 \), 5.58 g \( \text{MnSO}_4 \cdot 4\text{H}_2\text{O} \), 1.46 g \( \text{Co(NO}_3)_2 \cdot 6\text{H}_2\text{O} \), 1.21 g \( \text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O} \), 1.26 g \( \text{CuSO}_4 \cdot 5\text{H}_2\text{O} \), 1.44 g \( \text{ZnSO}_4 \cdot 7\text{H}_2\text{O} \), deionized water to 500 ml]; 500 \( \mu \)l vitamin B\(_{12}\) solution (0.004 % w/v); 0.5 g ammonium acetate. Fifty millilitres of a solution containing 0.6 g \( \text{Na}_2\text{S} \cdot 9\text{H}_2\text{O} \) and 2.0 g \( \text{NaHCO}_3 \) was filtered through a Millex-GS Millipore filter and added to 1 l of the sterilized medium. The final pH of the medium was 6.9.

Ten millilitres of cell culture was added to 1 l of fresh medium. To monitor the growth and pigment content, a small portion of *Chl. tepidum* culture was
withdrawn anoxically for absorption and fluorescence measurements.

Spectroscopic analyses
Absorption spectra were measured with a Shimadzu UV-160 spectrophotometer with opal glasses in the light pathways of the sample and reference beams to reduce the contribution of light scattering to the measured spectra. Sample cuvettes were located 5.5 cm from the detector. Samples for spectroscopy were quantitatively diluted in 10 mM Tris-HCl (pH 8.0) to give an absorbance of 0.2 at 740 or 670 nm before measurements. The absorbance peaks attributed to BChl c and BPhe c in cultures were estimated from second-order differential spectra in order to exclude turbidity effects. All spectra were measured at room temperature. Samples for fluorescence spectroscopy were diluted as described above and supplemented with a small amount of sodium dithionite to avoid fluorescence quenching due to oxidation of chlorosomes. The fluorometer (Ritsu Oyo Kogaku, Japan) was equipped with a Hamamatsu 7102 photomultiplier cooled at 5°C and with a Ritsu MC-20L monochromator on both the excitation and detection sides. The excitation and emission bandwidths were 10 nm.

HPLC measurement
Cells were extracted and analyzed by high-performance liquid chromatography (HPLC) as previously described (4), except that BChl c and BPhe c were determined at 669 nm. BPhe c bands corresponding to the major BChl c homologs were eluted about 12 min later than the corresponding BChl c bands and were well separated from other major peaks (20).

Results and discussion
Changes in BChl c (A\textsubscript{740}) and BPhe c (A\textsubscript{670}) concentration and turbidity (OD\textsubscript{640}) in the growth medium of Chl. tepidum are shown in Fig. 1-1. After 10 ml of Chl. tepidum culture had been transferred to 1 l of fresh medium, the cell density increased exponentially during the first day and the culture reached the stationary phase after the second; the stationary phase continued for about 1 week. After the early stationary phase, the turbidity (OD\textsubscript{640}) decreased slightly for 2\textasciitilde3 days and the culture finally attained a late stationary phase (Fig. 1-1).

A high absorption at 740 nm in the early stationary phase shows that the chlorosomes contained aggregated BChl c (Fig.1-2A). The absorption spectrum for the transition from early stationary phase to late stationary phase has a maximum at 670 nm in addition to the Qy maximum of BChl c at 740 nm (Fig. 1-2B), suggesting a transient phase in which BChl c in the chlorosomes degraded to BPhe c (see below). The absorption spectrum for the late stationary phase had no absorption band at 740 nm, but instead a main maximum at 670 nm and three smaller maxima at 620, 560 and 520 nm (Fig. 1-2C). These maxima are consistent with those of BPhe c (21) and, therefore, the peak at 670 nm was concluded to be due to BPhe c and not monomeric BChl c. Thus, in the late stationary state, BChl c was completely pheophytinized. In this paper, we call the three states (Fig. 1-2A\textendash C) the "chlorophyll" state, the "mixed" state and the "pheophytin" state, respectively.

To confirm the conversion of BChl c to BPhe c in intact cells, we extracted pigments from cells and analyzed them by HPLC (Fig. 1-3). The BChl c content as determined by absorption spectroscopy of intact cells and by HPLC were proportional throughout the experiments. A similar relation was also observed for BPhe c, although some BPhe c was further converted into bacteriopheophorbide c (BPhe c without farnesol), which eluted within 6 min after injection in the HPLC.
During the exponential growth and the early stationary phase, the BChl c concentration ($A_{740}$) was nearly proportional to the cell density measured by turbidity ($OD_{640}$). When the BChl c concentration decreased after the early stationary phase, a concomitant increase of BPhe c was observed (Fig. 1-1). This observation confirmed the conversion of BChl c to BPhe c after the early stationary phase. To examine the viability of "pheophytinized" cells, 10 ml cells was added to 1 l fresh medium under anoxic conditions. Growth occurred after a short lag phase (Fig. 1-4) and 3 days after the inoculation, the absorption spectrum of the new culture corresponded to that of the chlorophyll state shown in Fig. 1-2A. Changes in BChl c and BPhe c concentration after inoculation are also shown in Fig. 1-4. A rapid rise in the BChl c concentration was observed, while BPhe c concentration did not change during the exponential growth phase. This means that BChl c was newly synthesized, and not converted from existing BPhe c by binding Mg$^{2+}$. These observations indicate that Chl. tepidum was still viable in the pheophytin state in which no detectable BChl c was found in the cells.

Fig. 1-5 shows fluorescence emission spectra of Chl. tepidum in the chlorophyll state (Fig. 1-5A), the mixed state (Fig. 1-5B) and the pheophytin state (Fig. 1-5C) excited at 450 nm, where BChl c and carotenoids predominantly absorb. In Fig. 1-5A and B, fluorescence maxima occured at 775 and 810 nm due to BChl c and BChl a, respectively, indicating energy transfer from BChl c to BChl a. No fluorescence band in the 775-nm region was observed in the spectrum of Fig. 1-5C, confirming that aggregated BChl c was totally absent in the chlorosomes. Fig. 1-6 shows the fluorescence emission spectra of cells in the mixed and pheophytin states excited at 550 nm, where mainly BPhe c absorbs. In the mixed state (Fig. 1-6A), emission maxima around 775 and 810 nm were observed. This suggests
energy transfer from BPhe c to BChl c and BChl a. The fluorescence spectrum of cells in the pheophytin state has peaks only at 820 nm and 680 nm, which are emitted from BChl a and BPhe c, respectively (Fig. 1-6B). Since BChl a and carotenoids absorb little at 550 nm, the emission band at 820 nm suggests that energy was transferred from BPhe c to BChl a in pheophytin-state cells.

In order to further examine the possibility of energy transfer from BPhe c to BChl c, a fluorescence excitation spectrum for BChl c monitored at 775 nm was measured in mixed-state cells. A small fluorescence excitation band around 560 nm was observed (Fig. 1-7B). (The presence of this peak is clearer when the spectrum is compared with that of the chlorophyll-state cells; Fig. 1-7A.) This observation indicates that energy was transferred from BPhe c to BChl c, since the excitation bands around 560 and 670 nm coincided with BPhe c absorption band. Direct excitation transfer from BPhe c to BChl a was also observed by recording a fluorescence excitation spectrum monitored at 810 nm (Fig. 1-8). Direct excitation of BChl a was indicated by the band around 610 nm. The excitation band at 560 nm of the pheophytin-state cells (Fig. 1-8B) shows that energy was transferred from BPhe c to BChl a. In the corresponding spectrum for the mixed state, peaks due to BPhe c were also observed although in this case both direct and indirect (via BChl c) energy transfer to BChl a may have occurred (Fig. 1-8A).

Pheophytinization of BChl c has been thought to be merely a process which decomposes bacteriochlorophyll pigments. However, my results show that BPhe c in the chlorosome can still act as a light-harvesting and energy-transferring pigment in photosynthesis. Even when all BChl c was changed to BPhe c, energy transfer from BPhe c to the reaction center occurred. Pheophytinization of BChl c in Chl. tepidum, cultured in high light intensity at 46 °C, occurred about 1 week after inoculation in the mixed state (Fig. 1-1). By the method of inductive coupled
plasma atomic emission, I observed that Mg$^{2+}$ liberated from BChl c in the chlorosomes moved out to the culture medium (data shown in Chapter 2). It is suggested that the pheophytinization of BChl c in the chlorosomes is stimulated when the concentration of Mg$^{2+}$ in the cytoplasm is lowered. The ability of BPhe c to harvest light may enable Chl. tepidum to prolong its viability under natural conditions when growth was ceased due to a shortage of electron donors. Chl. tepidum has been isolated from shallow hot springs exposed to strong sun light (19) where temperature and light conditions seem similar to those used in this study and result in pheophytin formation.

In Chl. tepidum, BChl c was pheophytinized during the stationary phase at 46°C. In the mesophilic green sulfur bacterium, Chlorobium limicola, pheophytinization of BChl c was also observed at 28°C within about 5 months after inoculation (data not shown). Pheophytinization of BChl c may also occur in other green sulfur bacteria.
References


Fig. 1-1 Changes in BChl c (A_{740}, □) and BPhe c (A_{670}, ○) concentration, and turbidity (OD_{640}, △) of a C. tepidum culture at 46 °C. Absorbance values due to BChl c and BPhe c were obtained from second-order differential spectra of cells. Samples of high absorbance were measured after dilution.
Fig. 1-2 Absorption spectra of *C. tepidum* cells in the chlorophyll state (A), the mixed state (B), and pheophytin state (C). Samples A, B, C were taken 3, 10, and 12 days after inoculation, respectively. The spectra were measured after dilution. Peaks around 740 and 670 nm are due to BChl c and BPhe c, respectively (see text). The small peak around 810 nm is due to BChl a.
Fig. 1-3. Changes in BChl c (A740, ●) and BPhe c (A670, ▲) concentration, and changes of HPLC in BChl c (○), BPhe c (△), BPhe c (with tail) (×) and BPhe c (with no tail) (□) concentration. Absorbance value due to BChl c and BPhe c were obtained from second-order differential spectra of cells.
Fig. 1-4. Time course of the changes in BCHl c ($A_{740}$, □) and BPhe c ($A_{670}$, ○) concentration, and turbidity ($OD_{640}$, △) in fresh culture medium inoculated with a small volume of pheophytinized *C. tepidum* culture.
Fig. 1-5 Fluorescence emission spectra of chlorophyll-state (A), mixed-state (B) and pheophytin-state (C) cultures excited at 450 nm, where BChl c and carotenoids predominantly absorb. Samples A, B and C were taken 3, 10, and 12 days after inoculation, respectively. Spectra were measured after addition of a small amount of sodium dithionite to each sample.
Fig. 1-6. Fluorescence emission spectra of *C. tepidum* cells in the mixed state (A) and pheophytin state (B) using an excitation wavelength of 550 nm, where mainly BPhe c absorbs. The samples were prepared and the spectra measured as in Fig. 1-5.
Fig. 1-7. Excitation spectra for BChl c fluorescence in culture of chlorophyll state (A) and mixed state (B). Samples A and B were taken 3 and 10 days, respectively, after inoculation. The spectra were measured with a monitoring wavelength of 775 nm.
Fig. 1-8 Excitation spectra for BChl a fluorescence in cultures of mixed state (A) and pheophytin state (B). Samples A and B were taken 10 and 12 days, respective after inoculation. The spectra were measured with a monitoring wavelength of 810 nm.
Chapter 2

Effects of light intensity, temperature, and magnesium concentration in culture medium on the pheophytinization of bacteriochlorophyll c in *Chlorobium tepidum*
Abstract Phototrophic green sulfur bacterium, *Chl. tepidum*, which has the optimum growth temperature of 47-48 °C, was grown at 46 and 22 °C under various light conditions. BChl c in the chlorosomes were changed to BPhe c at 46 °C after 14 days under high-light intensity and after 32 days under low-light intensity. When *Chl. tepidum* was cultured under high- and low-light conditions at 22 °C, BChl c in the chlorosomes was not pheophytinized. It was suggested that the pheophytinization of BChl c in the chlorosomes was more depended on the temperature rather than the light intensity in the culture medium.

The pH in the cultures and the concentration of magnesium ions (Mg$^{2+}$) in the culture medium, cytoplasm, and chlorosomes were measured. The change of pH in the cultures indicated that the pheophytinization of BChl c in *Chl. tepidum* was independent of the acidification of the culture medium. The change of Mg$^{2+}$ concentrations in the culture medium, cytoplasm and chlorosomes indicated that the Mg$^{2+}$ concentration in the culture medium was decreased during the growth of *Chl. tepidum* cells and increased accompanying the pheophytinization of BChl c in the stationary-phase cells of *Chl. tepidum*. The Mg$^{2+}$ ions liberated from BChl c in the chlorosomes were suggested to move out to the culture medium via the cytoplasm. It was suggested that the pheophytinization of BChl c in chlorosomes of whole cells was promoted by the decrease of the Mg$^{2+}$ concentration in the cytoplasm.
Introduction

*Chl. tepidum*, a moderately thermophilic phototrophic bacterium, has a unique light-harvesting system, chlorosomes, which are ovoid bodies attached to the inside of the cytoplasmic membrane, and contain a large amount of BChl c as the main antenna pigment (1, 2). When *Chl. tepidum* was cultured under high-light intensity at 46 °C, the culture was changed in color from green to dark brown in about two weeks. This was caused by the pheophytinization of BChl c in chlorosomes (Chapter 1). It has been known that decreasing light intensity causes an increase in the number of chlorosomes per cell, while their length or width are not affected (3). On the other hand, Broch-Due et al. (4) reported that thin sections of *Chlorobium limicola f. thiosulfatophilum* revealed that the chlorosomes are larger at low-light than at high-light conditions. To examine the factors that affect the pheophytinization of BChl c in whole cells, *Chl. tepidum* was cultured under high- and low-light conditions at 46 °C and 22 °C. The influence of light intensity and temperature on the growth and the pheophytinization of BChl c in the chlorosomes in *Chl. tepidum* is described.

The pH of the cultures and the concentration of Mg$^{2+}$ in the culture medium, cytoplasm and chlorosomes in the growth of *Chl. tepidum* were measured. It is shown that the pheophytinization of BChl c in *Chl. tepidum* was independent on the acidification of the culture medium. It is also shown that the Mg$^{2+}$ ions liberated from BChl c in the chlorosomes moved out to the culture medium via the cytoplasm.

Materials and methods
Light intensity, temperature and growth conditions

*Chl. tepidum* was grown as described in Chapter 1 (Materials and methods) with an irradiance of 10 Wm\(^{-2}\). The low-light condition was made by wrapping a 1 l culture bottle with a sheet of Kimberly-clark Kimtowel paper and light intensity in the culture was measured with an irradiance of 0.5 Wm\(^{-2}\). Ten milliliter of cell culture was transferred to 1 l of fresh medium. The culture bottles were set under high- and low-light conditions at 46 and 22 °C. A small amount of the medium with grown cells was withdrawn anaerobically for absorption measurements. Absorption spectra were measured with Shimadzu UV-160 spectrophotometer.

Concentration of magnesium ions (Mg\(^{2+}\)) and growth conditions

Two kinds of culture medium described as the proceeding clause except the concentration of magnesium sulfate were made. The concentrations of magnesium sulfate contained in each 1 l of culture medium was 1600 µM (normal concentration) and 160 µM.

Inductive coupled plasma atomic emission

Fifteen milliliter sample of culture was centrifuged at 1000 × g, for 15 min and at 4 °C. The supernatant was determined for the concentration of Mg\(^{2+}\) ions in the culture medium by the method of inductively coupled plasma spectroscopy (ICPS). For separation of the cytoplasm from membranous fraction, 15 ml of distilled water was added to the packed cell. The suspension (5 ml) of cells was fragmented by sonication (twice for 7 min) in ice bath. After sonication, the suspension was centrifuged at 2000 × g, for 15 min and at 4 °C. The precipitate was added with 5 ml of distilled water and the absorption spectrum (spectrum (a)) of the suspension was measured. Spectrum (a) was used to estimate the concentration of Mg\(^{2+}\) ions in
the chlorosomes and the cytoplasmic membrane. The supernatant was centrifuged at 50 000 × g, for 20 min and at 4 °C. After centrifugation, the uncolored supernatant was determined for the concentration of Mg$^{2+}$ ions in the cytoplasm by inductively coupled plasma spectroscopy. The precipitate was washed with small volume of distilled water, including 1 mM EDTA and 100 mM KCl, and was recentrifuged at 50 000 × g, for 20 min and at 4 °C. The precipitate was added with 15 ml of distilled water and the absorption spectrum (spectrum (b)) of the suspension was measured. Spectrum (b) was used to estimate the concentration of Mg$^{2+}$ ions in chlorosomes. This concentration of Mg$^{2+}$ ions was corrected using spectrum (a) for the recovery of chlorosomes. After spectrum (b) was measured, hydrochloric acid was added to change the pH to 1 (or pH 2), and the suspension was centrifuged at 2000 × g, for 15 min and at 4 °C. The supernatant was determined for the concentration of Mg$^{2+}$ ions in the chlorosomes by inductively coupled plasma spectroscopy. Inductively coupled plasma-atomic emission was measured with a Shimadzu ICPS-5000 spectrometer.

Isolation of chlorosomes

One liter of Chl. tepidum cell culture was centrifuged at 600 × g, for 20 min and at 4°C, and the precipitate was suspended in the buffer of 2 M sodium thiocyanate, 10 mM sodium phosphate and 10 mM sodium ascorbate, pH 7.2. After the suspension was centrifuged at 1000 × g, for 10 min, the precipitate was resuspended in 30 ml of the buffer. The suspension (5 ml) of cells was treated with sonication (twice for 7 min) in ice bath. The suspension of the fragmented cells was then loaded onto 13, 23, 33 and 43 % (w/w) sucrose gradients. These gradients were centrifuged approximately 16 h at 4 °C in Hitachi CP70G at 19 000 × g. The main chlorosome band was seen in the part of 23 % of gradients.
Results

Changes in BChl c ($A_{740}$) and BPhe c ($A_{670}$) concentration, and turbidity ($OD_{640}$) of the growing cell suspension of *Chl. tepidum* under high- and low-light intensity at 46 °C are shown in Fig. 2-1. Under high-light intensity, the pheophytinization of BChl c started at 6 days after the inoculation and BChl c disappeared after two weeks. Under low-light intensity, the pheophytinization of BChl c started after 8 days and BChl c disappeared after 32 days. The maximum turbidity ($OD_{640}$) under low-light intensity was lower than that under high-light intensity.

Figure 2-2 shows changes in BChl c and BPhe c concentration, and the turbidity ($OD_{640}$) of *Chl. tepidum* under high- and low-light intensity at 22 °C. The pheophytinization of BChl c was not observed at the temperature of 22 °C. The experiment under low-light intensity at 22 °C (open symbols, dashed lines) also showed similar changes to those under high-light intensity. The maximum turbidity ($OD_{640}$) and the concentration of BChl c under low-light intensity at 22 °C kept higher levels than those under high-light intensity at 22 °C. The maximum turbidity ($OD_{640}$) under low-light intensity at 22 °C was almost the same as that under high-light intensity at 46 °C (Fig. 2-1). When the temperature was changed from 22 °C to 46 °C after 42 days, both BChl c under high- and low-light intensity were pheophytinized rapidly (Fig. 2-2). After the temperature changed, it took 4 days under low-light intensity and 7 days under high-light intensity for complete pheophytinization of BChl c.

To examine the effect of temperature on the isolated chlorosomes, chlorosomes were suspended in the culture medium in a 200 ml bottle, and heated at various temperature. Even after an autoclave treatment at 105 °C for 5 min, BChl c in chlorosomes was not destroyed or pheophytinized (Fig. 2-3A and B). When *Chl.
Tepidum cells cultured in a 200 ml bottle were also heated in an autoclave at 105 °C for 5 min, only a little BChl c in the chlorosomes were pheophytinized (Fig. 2-4A and B). These results indicate that BChl c in chlorosomes can stand against intense heat.

Two kinds of culture medium were made that were different in the Mg²⁺ concentration of 1600 ìM and 160 ìM. Ten milliliter of cell culture was transferred to each of the two 1 l culture media. Such two kinds of cultures are called as the “A” culture (Mg²⁺, 1600 ìM) and the “B” culture (Mg²⁺, 160 ìM) in this chapter. In the A culture, the pheophytinization of BChl c started at 7 days after the inoculation and BChl c disappeared after 15 days (Fig. 2-5A). The pH of the culture slightly increased to pH 7.15 after 1 day and then decreased. The minimum pH was pH 6.7 that was recorded after 9 days and then increased to pH 6.75. In the B culture, the pheophytinization of BChl c started at 3 days after the inoculation and BChl c disappeared after 10 days (Fig. 2-5B). The pH of the culture decreased gradually. The minimum pH was pH 6.55 at the 7th day and then increased to pH 6.6. The increase of pH after 1 day in the A culture and the almost same level of pH after 1 day in the B culture may be due to the alkalization by sodium sulfide which was added to reduce dissolved oxygen in the culture medium. The decrease of pH after 1 day in these cultures may be due to the acidification by sulfuric acid, which was made from sulfide in the culture medium.

To examine the correlation of the small pH changes of the culture medium to the pheophytinization, 1 l of another culture medium which contained in 1600 ìM of Mg²⁺ was made and 10 ml of cell culture was transferred in it (Fig. 2-6). When the pH of this culture decreased to pH 6.78 from the initial pH 6.95 after 2 days, pH was increased to pH 6.98 by adding the solution of sodium hydroxide. The cells continued to grow as usual grow, and during the growth the pH changes were
within pH unit of 0.1. However, the pheophytinization of BChl c was observed after 5 days almost the same time course as the A culture (Fig. 2-5A) in which pH was decreased to pH 6.7. This suggests that the pheophytinization of BChl c in Chl. tepidum proceeds independently to the acidification of cultures.

The Mg$^{2+}$ concentrations of the culture medium, cytoplasm and chlorosomes in growth were measured and shown in Fig. 2-7. In the A culture, the Mg$^{2+}$ concentration in the culture medium showed a decrease for initial 6 days and then an increase (Fig. 2-7A). The minimum concentration of Mg$^{2+}$ was observed at the 7th-8th day. When BChl c was completely pheophytinized after 14 days, the Mg$^{2+}$ concentration in the medium returned almost to the initial concentration. The Mg$^{2+}$ concentrations in the cytoplasm and chlorosomes showed smaller changes than that in the culture medium; initially increased and then decreased. The maximum concentrations of Mg$^{2+}$ in the cytoplasm were observed at 8 days after inoculation and those in chlorosomes also at 8 days after. When BChl c was completely pheophytinized, both Mg$^{2+}$ concentrations decreased.

In the B culture, the Mg$^{2+}$ concentration in the culture medium showed a similar but somewhat larger change than that in the A culture probably because of the lower initial concentration of Mg$^{2+}$ (Fig. 2-7B). The minimum concentration of Mg$^{2+}$ in the culture medium was observed at the 4th-6th days. When BChl c was completely pheophytinized after 9 days, the Mg$^{2+}$ concentration returned almost to the initial concentration. While, the Mg$^{2+}$ concentrations in the cytoplasm and chlorosomes showed an initial increase followed by a decrease. Maximum Mg$^{2+}$ concentrations were observed on the 2nd day. When BChl c was completely pheophytinized, Mg$^{2+}$ concentrations decreased.

In the A and B cultures, the rapid decrease of the Mg$^{2+}$ concentration in the culture medium was probably caused by the rapid growth of the cells (the
logarithm phase). The rapid increase of that seemed to closely correlate to the pheophytinization of BChl c because the increased concentration of Mg$^{2+}$ in the culture medium seemed to correspond to the increased concentration of BPhe c. In the B culture (Fig. 2-7B), the maximum turbidity (OD$_{640}$) was as high as that in the A culture. However, the pheophytinization of BChl c in the B culture was faster than that in the A culture (Fig. 2-7A). From these results, it was suggested that the pheophytinization of BChl c in *Chl. tepidum* was promoted by the low Mg$^{2+}$ concentration in the culture medium. In the A and B cultures, the decrease and increase concentration of Mg$^{2+}$ in the culture medium corresponded respectively to the increase and decrease of the concentration of Mg$^{2+}$ in the cytoplasm and chlorosomes. Moreover, while the Mg$^{2+}$ concentration in the chlorosomes decreased, the Mg$^{2+}$ concentration in the cytoplasm also decreased. This suggests that the Mg$^{2+}$ ions liberated from BChl c in the chlorosomes moved out to the culture medium via the cytoplasm.

Discussion

The pheophytinization of BChl c in the cultures under high- and low-light intensity at 22 °C was not observed until 42 days after inoculation, when the temperature was changed from 22 °C to 46 °C (Fig. 2-2). This suggests that the pheophytinization of BChl c in *Chl. tepidum* is affected by the temperature of the culture rather than the light intensity. The concentration of BChl c at 22 °C under low-light intensity was kept at a higher level than that under high-light intensity. This was probably caused by the increase in the number of chlorosomes per cell and/or in the size of chlorosomes that was reported to become larger under the low-light intensity in *Chl. limicola* (3, 4). When the temperature was changed from
22 °C to 46 °C, BChl c in the cultures under both high- and low-light intensity was pheophytinized rapidly. Since BChl c in Chl. tepidum itself can stand intense heat, it was suggested that the pheophytinization of BChl c in the culture was not caused by the temperature rise directly.

The increasing concentration of Mg$^{2+}$ in the culture media caused the decrease of Mg$^{2+}$ concentrations in the cytoplasm and chlorosomes (Fig. 2-7A and B). When Mg$^{2+}$ in the cytoplasm fraction decreased, that in the chlorosome also decreased. The ratio of the Mg$^{2+}$ concentration in the cytoplasm to that in the chlorosomes was nearly constant throughout the growth of Chl. tepidum. These suggest that Mg$^{2+}$ ions in the cytoplasm flew out to the culture medium, and Mg$^{2+}$ ions in BChl c moved to the cytoplasm. The Mg$^{2+}$ concentration of the cytoplasm in Chl. tepidum was always higher than that of the chlorosomes. In the A culture, the ratio of the Mg$^{2+}$ concentration in the cytoplasm to that of chlorosomes was nearly 3:1 (Fig. 2-7A). It was suggested that Chl. tepidum required more Mg$^{2+}$ ions for the metabolism in the cytoplasm as the cofactor than those for the photosynthesis as the central metals in bacteriochlorophylls. In the B culture, the ratio of the Mg$^{2+}$ concentration in the cytoplasm to that in the chlorosomes was nearly 1:1 (Fig. 2-7B). Accordingly, it was suggested that the pheophytinization of BChl c in the chlorosomes was stimulated when the Mg$^{2+}$ concentration in the cytoplasm was lowered. In the A and B cultures, the time required to achieve the minimum pH and Mg$^{2+}$ concentration of the culture media is similar. However, when the Mg$^{2+}$ concentration in the culture returned to the initial concentration, the pH in the cultures did not return to the initial pH. It seems that there is no relation between the Mg$^{2+}$ concentration and pH.

It has been reported that in cells of eucaryote, such as ascites cells and thymocyte, exogenous ATP activated Na$^+\cdot$Mg$^{2+}$ antiporter in the membrane of cells
and stimulated magnesium efflux from cells (5-7). Furthermore, it was reported that free cytosolic magnesium concentration was unaffected by Mg$^{2+}$ efflux suggesting that cell signaling involved changes and redistribution of bound Mg$^{2+}$ (5-7). A system of magnesium efflux from the cytoplasm in Chl. tepidum cells similar to eucaryote may exist. The pheophytinization of BChl c in the chlorosomes of Chl. tepidum may occur to control the concentration of Mg$^{2+}$ ions in the cytoplasm.
References


Fig. 2-1 Changes of concentration of BChl c (squares) and BPhe c (circles), and turbidity (OD640) (triangles) in *C. tepidum* cultures under high-light (closed symbols) and low-light (open symbols) intensity at 46 °C.
Fig. 2-2 Changes of concentration of BChl c (squares) and BPhe c (circles), and turbidity (OD \textsubscript{640}) (triangles) in \textit{C. tepidum} cultures under high-light (closed symbols) and low-light (open symbols) intensity at 22 °C. After 42 days temperature was shifted to 46 °C.
Fig. 2-3 Absorption spectra of isolated chlorosomes from *C. tepidum* suspended in the culture medium (in a 200 ml bottle); (A) before heating (B) after an autoclave treatment at 105 °C for 5 min.
Fig. 2-4 Absorption spectra of *C. tepidum* cells cultured in a 200 ml bottle; (A) before heating (B) after an autoclave at 105 °C for 5 min.
Fig. 2-5 Changes in concentration of BChl c (closed squares) and BPhe c (closed circles) in *C. tepidum* cultures during growth under different concentrations of Mg$^{2+}$ in the culture media. Turbidity (OD$_{640}$) (closed triangles), and pH (open circles) are also shown. (A) 1600 μM of Mg$^{2+}$ (B) 160 μM of Mg$^{2+}$. 


Fig. 2-6 Changes in concentration of BChl c (closed squares) and BPhe c (closed circles), and pH (open circles), in *C. tepidum* cultures including 1600 μM of Mg²⁺ under high light at 46 °C. The pH of the culture was adjusted to pH 6.98 after 2 days from inoculation (arrow).
Fig. 2-7 Changes of Mg\(^{2+}\) concentration in the culture medium (open squares), cytoplasm (open circles) and chlorosomes (open triangles) of *C. tepidum* during culture. Initial concentration of Mg\(^{2+}\) in the medium was (A) 1600 \(\mu\) M and (B) 160 \(\mu\) M. Changes in concentration of BChl c (closed squares) and BPhe c (closed circles), and turbidity (OD\(_{640}\)) (closed triangles) are also shown.
Chapter 3

Quenching of bacteriochlorophyll florescence in chlorosomes from

*Chloroflexus aurantiacus* by added quinones
Abstract  The quenching of bacteriochlorophyll (BChl) c fluorescence in chlorosomes isolated from Chloroflexus aurantiacus was examined by the addition of various benzoquinones, naphthoquinones, and anthraquinones. Many quinones showed strong quenching with half-quenching concentrations in the micromolar or sub-micromolar range. The number of quinone molecules bound to the chlorosomes at the half-quenching concentration was estimated to be as small as 1 quinone molecule per 50 BChl c molecules. It was suggested that quinones which exhibit a high quenching effect have sufficient hydrophobicity and one or more hydroxyl groups in the alpha positions of naphthoquinones and anthraquinones. Chlorobiumquinone has been suggested to be essential for the indigenous quenching of chlorosome fluorescence in Chlorobium tepidum under oxic conditions. It is suggested that the quenching effect of chlorobiumquinone in chlorosomes from Chlorobium tepidum is related to the 1'-oxo group neighboring the dicarbonyl group.
Introduction

Photosynthetic green sulfur and green filamentous bacteria contain chlorosomes as light-harvesting antennae (1, 2). Chlorosomes are ovoid bodies attached on the inner side of the cytoplasmic membrane (3-6). They contain a large amount of bacteriochlorophyll (BChl) c (or d or e) as the main antenna pigment and a small amount of BChl a presumably in the baseplate structure in addition to carotenoids, isoprenoid quinones, amphipathic lipids, and proteins (7, 8). The baseplate is thought to be located at the cytoplasmic membrane side of the chlorosomes (9, 10). The BChl c molecules form large aggregates arranged in rods. Excitation energy is transferred from the bulk BChl c antenna via the baseplate BChl a (and, in green sulfur bacteria, additionally via the BChl a-containing Fenna-Matthews-Olson (FMO) protein) to the reaction centers in the cytoplasmic membrane.

The green sulfur bacteria are strictly anaerobic bacteria. Under reducing conditions, which are natural for the growth of the bacteria, the chlorosomes show considerable fluorescence. Under oxidizing conditions, this fluorescence is significantly quenched and the intrachlorosomal energy transfer from BChl c to BChl a in the baseplate is also decreased. It has been proposed that this is caused by the presence of a quenching mechanism inside the chlorosomes that operates under high-redox-potential conditions (11-13). The large redox effect on the chlorosome fluorescence is not observed in the green filamentous bacterium Chloroflexus (Cfl.) aurantiacus.

It has been shown that the chlorosome antenna of the green sulfur bacterium Chlorobium (Chl.) tepidum contains significant amounts of three isoprenoid quinones, namely, chlorobiumquinone (CK), menaquinone (MK)-7, and an unidentified quinone named “the polar menaquinone” or “Quinone 1” (8).
chlorosome antenna of the green filamentous bacterium *Cfl. aurantiacus* contains MK-10 and only traces of other menaquinones but no CK (8, 14). Frigaard et al. (8, 15) suggested that CK plays a significant, but not exclusive, role in controlling the fluorescence and inhibiting energy transfer in chlorosomes in *Chl. tepidum* under oxic conditions. The absence of CK in *Chloroflexus* chlorosomes may be related to the absence of the redox-dependent fluorescence quenching in green filamentous bacteria. The mechanism of fluorescence quenching of chlorophyll by quinone in solution is thought to be based on an electron transfer from excited chlorophyll to quinone followed by extremely rapid charge recombination to the ground state (16-20).

I recently showed that the bacteriochlorophyll *c* fluorescence from *Chloroflexus* chlorosomes in aqueous suspension could be quenched by the addition of quinones (21). In this study, I examined the effect of externally added quinones in chlorosomes isolated from *Cfl. aurantiacus* and observed that the BChl *c* fluorescence was quenched to different extents depending on the quinone species. I tested 33 substituted benzoquinones (BQ), naphthoquinones (NQ), and anthraquinones (AQ) and determined their half-quenching concentrations. The structural requirements of quinones for obtaining high quenching effects in chlorosomes and the quenching effect of CK (which has a 1′-oxo group) in chlorosomes from *Chl. tepidum* are discussed.

**Materials and methods**

**Growth**

*Cfl. aurantiacus* J-10-fl was grown at 55 °C in 1-l batch cultures using the PE medium of Hanada et al. (22) under a light intensity of 12 Wm⁻² from incandescent
lamps and harvested in the late exponential phase. Chlorosomes were prepared on sucrose gradients containing 2 M NaSCN as described by Gerola and Olson (10).

**Quinones**

Various quinones were purchased from Aldrich, Sigma, Wako (Japan) and Tokyo Kasei Chemical Company (Japan), except for 2-methyl-3-acetyl-1,4-NQ and 2-methyl-3-(1-hydroxyethyl)-1,4-NQ, which were prepared by Drs. Y. Kuwatani and M. Iyoda in the Department of Chemistry at Tokyo Metropolitan University. All the quinones listed in Table 3-1 were dissolved in ethanol, except for 1,5-dihydroxy-9,10-AQ and 9,10-AQ, which were dissolved in ethanol-acetone (1:1 by vol) and dimethyl sulfoxide, respectively. Solvent concentrations of less than 4% (by volume) had no significant effect on the chlorosome fluorescence intensity in aqueous suspension. Since no reductants were present, all quinones could be considered to be in the oxidized form. Figure 3-1 shows the chemical structure of the naphthoquinones extensively used in this study as well as those of CK and MK-7.

**Spectroscopy**

For spectroscopic measurements, chlorosomes were suspended in a 20-mM MOPS buffer (pH 7.0) to give an absorbance of about 0.3 at 740 nm. Absorption spectra were measured with a Shimadzu UV-160 spectrophotometer. Chlorosomes were excited with a 10-nm (half-maximum) band beam peaking at 725 nm, and fluorescence in the range 740-840 nm was measured with a Hamamatsu 7102 photomultiplier, as previously described (23). Absorption and fluorescence emission spectra were measured at room temperature.
**Quinone Binding**

To estimate the amount of chlorosome-bound naphthoquinones, we measured the quinone concentrations in the aqueous phase of the chlorosome suspension supplemented with quinones. To the chlorosome suspensions of $A_{740} = 0.5$, quinones were added to 50 µM and ultracentrifuged (440 000 × g, 30 min). From the difference of the absorption spectra of quinones in the supernatants and those of aqueous solutions of 50 µM without chlorosomes, the amounts of quinones absorbed in the chlorosomes were estimated. The absorption coefficient of BChl c in chlorosomes from *Cfl. aurantiacus* in aqueous suspension was estimated to be 95 mM⁻¹cm⁻¹ at the Qy maximum (approx. 740 nm) by comparing the absorption spectrum of the same amount of chlorosomes in the MOPS buffer and in methanol solution.

**Results**

**Quinone-induced fluorescence quenching**

The emission spectra of BChl c in chlorosomes from *Cfl. aurantiacus* in the presence of various concentrations of 5-hydroxy-1,4-NQ are shown in Fig. 3-2. When quinones were added the BChl c emission maximum at 758 nm was not changed, but the intensity was largely decreased. The BChl a emission maximum at 805 nm after energy transfer from BChl c was similarly, or a little more extensively, decreased. All quinones examined showed quenching of fluorescence, but the concentrations needed for the quenching were very different depending on the quinone species. Table 3-1 summarizes the fluorescence half-quenching concentrations, namely, which is the quinone concentration necessary to induce a decrease in BChl c fluorescence intensity to 50 % of the level in unsupplemented
chlorosomes. Substituted benzoquinones generally exhibited weaker quenching effects than substituted naphthoquinones, as reported by Karukstis et al. (24) and Lee et al. (25), who used other chlorophyll systems. Substituted naphthoquinones generally exhibited weaker quenching effects than substituted anthraquinones.

**Fluorescence quenching by substituted benzoquinones**

Figure 3-3A shows the relative BCHl c fluorescence at 758 nm in *Chloroflexus* chlorosomes as a function of the concentration of added 2,3,5,6-tetramethyl-1,4-BQ (TMBQ). The linearity of the Stern-Volmer plot (20, 24, 25) of the inset indicates that, for the given quinone concentration range, all BCHl c fluorescence is sensitive to TMBQ as in Photosystem (PS) II fluorescence in barley chloroplasts (24).

**Fluorescence quenching by substituted naphthoquinones**

Figure 3-3B shows the chlorosome BCHl c fluorescence in *Chloroflexus* chlorosomes as a function of the concentration of added 1,4-NQ, and the inset shows the linear Stern-Volmer plot. Although 1,4-NQ exhibited a high quenching effect on chlorosome fluorescence, 1,2-NQ exhibited a lower quenching effect (Table 3-1). The hydroxyl groups at certain positions increased the quenching effects considerably (Table 3-1, Fig. 3-1). Naphthoquinones having hydroxy groups at position 5 (5-hydroxy-1,4-NQ) or 5 and 8 (5,8-dihydroxy-1,4-NQ) exhibited a higher effect than that of 1,4-NQ; 5,8-dihydroxy-1,4-NQ exhibited a higher effect than that of 5-hydroxy-1,4-NQ. 5,8-Dihydroxy-2-(1-hydroxy-4-methyl-3-pentenyl)-1,4-NQ (shikonin) exhibited a higher effect than 5,8-dihydroxy-1,4-NQ. On the other hand, naphthoquinone with a substituent of the hydroxy group at position 2 (2-hydroxy-1,4-NQ) exhibited a lower effect that of
1,4-NQ. 2-Hydroxy-1,4-NQ is also reported to be a weak quencher of chloroplast fluorescence (24).

Naphthoquinones with substituents of methyl groups at position 2 weakened the quenching effect (Table 3-1, Fig. 3-1). 2-Methyl-1,4-NQ and 5-hydroxy-2-methyl-1,4-NQ exhibited lower quenching effects than 1,4-NQ and 5-hydroxy-1,4-NQ, respectively. Although the introduction of a methyl group in the quinone increases the binding affinity to the chlorosomes (see below) probably because of its increased hydrophobicity, no increase of the effect of quenching BChl c fluorescence was observed.

2-Methyl-3-acetyl-1,4-NQ containing an oxo group in the 1' position of the side chain similar to CK exhibited a 5-times higher quenching effect than 2-methyl-3-(1-hydroxyethyl)-1,4-NQ (Table 3-1, Fig. 3-1).

Fluorescence quenching by substituted anthraquinones

1,2,4-Trihydroxy-9,10-AQ added to Chloroflexus chlorosomes showed the quenching of BChl c fluorescence at very low concentrations (Fig. 3-3C). The inset shows the linear Stern-Volmer plot of the same data. These observations indicate that BChl c fluorescence in chlorosomes from Cfl. aurantiacus is highly susceptible to 1,2,4-trihydroxy-9,10-AQ.

Anthraquinones having substituents of two hydroxy group at positions 1 and 4, 1 and 5, or 1 and 8 also exhibited very high quenching effects of BChl c fluorescence. However, those having the substituents at positions 1 and 2 or 2 and 6 exhibited relatively low quenching effects (Table 3-1). Anthraquinones having methyl groups at positions 2 or 2 and 3 weakened the quenching effect similarly as similar to the substituted naphthoquinones.
**Binding of quinones to chlorosomes**

The binding of eleven naphthoquinones to chlorosomes was examined by monitoring the optical absorption of the aqueous phase of chlorosome suspension after the quinone additions. Table 3-2 summarizes the results together with the estimated molar ratio of bound quinones to BChl c. Since only a small fraction of added quinones was bound to chlorosomes in most cases, we used a rather high concentration of quinones, 50 µM, for the binding measurements. We also estimated the molar ratio of bound quinones per BChl c at each half-quenching concentration, assuming that the amount of bound quinones is proportional to the amount of added quinones, which was approximately the case with 5-hydroxy-1,4-NQ (data not shown) (Table 3-2). The examined quinones seem to belong to two groups. One is a group with a low quinone / BChl c ratio of 0.02-0.04 at the half-quenching concentration. The other is a group with ratios higher than 0.1.

**Discussion**

I analyzed the specificity of quinone species in the quenching of BChl c fluorescence using *Cfl. aurantiacus* chlorosomes by the addition of substituted benzoquinones, naphthoquinones, and anthraquinones. Many of these were potent quenchers of BChl fluorescence in the chlorosomes and also inhibited energy transfer from BChl c to BChl a in the baseplate.

I used the half-quenching concentration of BChl c fluorescence to assess the activities of benzoquinones, naphthoquinones, and anthraquinones that govern the quenching process. The ratio of estimated bound naphthoquinone molecule per BChl c molecule at a half-quenching concentration (Table 3-2) was rather constant among very effective naphthoquinones, gradually increasing with increasing the
half-quenching concentration in less-effective quinones.

The mechanism of fluorescence quenching by extrinsic quinones in green algae or higher plants involves the interaction of quinones with light-harvesting chlorophyll-protein complexes of Photosystem I and II and a subsequent alteration of the excitation-transfer process among chlorophyll antennae (24, 26, 27). Higher quinone concentrations are reportedly needed for chlorophyll fluorescence quenching on the order of one quinone molecule per light-harvesting chlorophyll molecule in Photosystem II from barley chloroplasts (24). On the other hand, the ratio of the bound quinone molecules to the BChl c molecules in chlorosomes from *Cfl. aurantiacus*, as shown in this study, is remarkably small (Table 3-2). The strongest quencher of the naphthoquinones examined, shikonin (with a half-quenching concentration of 0.4 µM), showed an estimated ratio of bound quinones at the half-quenching concentration of as small as one quinone molecule per 50 BChl c molecules. This suggests that the added quinones in chlorosomes from *Cfl. aurantiacus* are extremely effective for the quenching of BChl c fluorescence compared with the quinones in Photosystem II from barley chloroplasts. This also supports the suggestion that physiological quinones, especially CK, work very effectively as quenchers of BChl c fluorescence in chlorosomes from *Chl. tepidum*, as discussed below.

Among the naphthoquinones and anthraquinones in Table 3-1, the presence of hydroxy substituents at positions 5 and 8 of naphthoquinones and at positions 1, 4, 5, and 8 of anthraquinones increased the quenching effects of fluorescence. The presence of substituents, except for the chloro group, at positions 1 and 2 of naphthoquinones and at positions 2, 3, 6, and 7 of anthraquinones decreased the quenching effect of the fluorescence. This suggests that quinones which exhibit a high quenching effect have sufficient hydrophobicity and (a) hydroxyl group(s) in
the alpha position to the quinone carbonyl. These effects of hydroxy quinones may be explained by a function of the hydroxy groups as proton donors (28). If the hydroxyl substituent is at the alpha position to the carbonyl, an intramolecular hydrogen bond may stabilize the semiquinone species (28) and then increase the quenching effect. The presence of a chloro group increased the quenching effect as reported by Natarajan and Blankenship (20).

Figure 3-1 summarizes the relation between the substituent structures and the half-quenching concentration (in parentheses) of the naphthoquinones. Shikonin (a) exhibits a higher quenching effect than 5,8-dihydroxy-1,4-NQ (b), and 2-hydroxy-3-(3-methyl-2-butenyl)-1,4-NQ (i) exhibits a higher quenching effect than 2-hydroxy-1,4-NQ (k). The hydrophobic side chain including the dimethyl group may cause the increase of the quenching abilities. There is also a high possibility that the double bond in the side chain contributes to the quenching effect.

In organic solution, the quenching ability of quinones of chlorophyll fluorescence is mainly determined by the redox midpoint potential of the quinone/semiquinone couple, the more oxidizing quinones being better quenchers (20). However, in isolated chlorosomes from *Cfl. aurantiacus*, as shown in this study, the hydroxy (or oxo) group in the alpha position to the quinone carbonyl seems to be very important for the high quenching ability, probably together with a high redox potential and high hydrophobicity of the quinone molecule. These criteria are consistent with our findings that 5-hydroxy-1,4-NQ, 5,8-dihydroxy-NQ, and shikonin were efficient quenchers of flash-induced cytochrome c oxidation in *Cfl. aurantiacus* when BCHl c was excited, whereas 2-hydroxy-NQ was not (29).

Chlorosomes of *Chl. tepidum* contain significant amounts of three quinones, namely CK, MK-7 (Fig. 3-1), and an unidentified quinone named Quinone 1. Chlorobiumquinone is mainly located in the chlorosomes, whereas MK-7 and
Quinone 1 are contained both in the chlorosomes and the cytoplasmic membrane (8). From the fluorescence measurements of chlorosomes, it was suggested that CK is an important component in the fluorescence-quenching mechanism in chlorosomes from green sulfur bacteria (8, 15). *Chloroflexus* cells contain MK-10 and traces of other quinones, but no CK (8, 14). The energy transfer in *Chloroflexus* chlorosomes is either not regulated by the redox potential or regulated to a much lesser extent than in *Chlorobium* chlorosomes. From these observations, we have suggested that the large redox-dependent fluorescence ratio in *Chlorobium* chlorosomes is linked to the presence of CK, though MK-7 can also be of some importance as a quencher in *Chlorobium* (8, 15). To elucidate the quenching mechanism of CK in *Chl. tepidum*, we compared the chemical structures of CK and MK-7 to those of other quinones (Fig. 3-1). Chlorobiumquinone differs from MK-7 in having a 1'-oxo group in the side chain. 2-Methyl-3-acetyl-1,4-NQ (g) also has a 1'-oxo group in the side chain and exhibits a higher quenching effect than 2-methyl-3-(1-hydroxyethyl)-1,4-NQ (j), which has no 1'-oxo group (Fig. 3-1). I suggest that the 1'-oxo group of CK contributes to the high quenching effect of fluorescence in the chlorosomes from *Chl. tepidum*. 
References


Table 3-1. Half-quenching concentrations for BChl c fluorescence quenching in chlorosomes from *C. aurantiacus* by substituted benzoquinones, naphthoquinones and anthraquinones*

<table>
<thead>
<tr>
<th>Quinone</th>
<th>Half-quenching concentration (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,4-Benzquinone</td>
<td>&gt;500</td>
</tr>
<tr>
<td>2,5-Dimethyl-1,4-benzoquinone</td>
<td>&gt;100</td>
</tr>
<tr>
<td>2,3,5,6-Tetramethyl-1,4-benzoquinone</td>
<td>55</td>
</tr>
<tr>
<td>2,3,5,6-Tetrachloro-1,4-benzoquinone</td>
<td>17</td>
</tr>
<tr>
<td>1,4-Naphthoquinone</td>
<td>7</td>
</tr>
<tr>
<td>1,2-Naphthoquinone</td>
<td>21</td>
</tr>
<tr>
<td>5-Hydroxy-1,4-naphthoquinone</td>
<td>4</td>
</tr>
<tr>
<td>5-Hydroxy-2-methyl-1,4-naphthoquinone</td>
<td>4.7</td>
</tr>
<tr>
<td>5,8-Dihydroxy-1,4-naphthoquinone</td>
<td>1.9</td>
</tr>
<tr>
<td>Shikonin †</td>
<td>0.4</td>
</tr>
<tr>
<td>2,3-Dichloro-5,8-dihydroxy-1,4-naphthoquinone</td>
<td>0.4</td>
</tr>
<tr>
<td>2-Hydroxy-1,4-naphthoquinone</td>
<td>&gt;250</td>
</tr>
<tr>
<td>2-Hydroxy-3-(3-methyl-2-butenyl)-1,4-naphthoquinone</td>
<td>50</td>
</tr>
<tr>
<td>2-Methyl-1,4-naphthoquinone</td>
<td>12</td>
</tr>
<tr>
<td>2-Methyl-3-acetyl-1,4-naphthoquinone</td>
<td>16</td>
</tr>
<tr>
<td>2-Methyl-3-(1-hydroxyethyl)-1,4-naphthoquinone</td>
<td>80</td>
</tr>
<tr>
<td>2,3-Dichloro-1,4-naphthoquinone</td>
<td>1</td>
</tr>
<tr>
<td>2,3-Dichloro-5-nitro-1,4-naphthoquinone</td>
<td>6</td>
</tr>
<tr>
<td>2-amino-3-chloro-1,4-naphthoquinone</td>
<td>27</td>
</tr>
<tr>
<td>9,10-Anthraquinone</td>
<td>3</td>
</tr>
<tr>
<td>1-Hydroxy-9,10-anthraquinone</td>
<td>0.6</td>
</tr>
<tr>
<td>1,5-Dihydroxy-9,10-anthraquinone</td>
<td>0.4</td>
</tr>
<tr>
<td>1,4-Dihydroxy-9,10-anthraquinone</td>
<td>0.4</td>
</tr>
<tr>
<td>1,8-Dihydroxy-9,10-anthraquinone</td>
<td>0.4</td>
</tr>
<tr>
<td>1,2-Dihydroxy-9,10-anthraquinone</td>
<td>11</td>
</tr>
<tr>
<td>2,6-Dihydroxy-9,10-anthraquinone</td>
<td>100</td>
</tr>
<tr>
<td>1,8-Dihydroxy-3-methyl-9,10-anthraquinone</td>
<td>0.6</td>
</tr>
<tr>
<td>1,4-Dihydroxy-2,3-dimethyl-9,10-anthraquinone</td>
<td>0.6</td>
</tr>
<tr>
<td>1,2,4-Trihydroxy-9,10-anthraquinone</td>
<td>0.9</td>
</tr>
<tr>
<td>2-Methyl-9,10-anthraquinone</td>
<td>3</td>
</tr>
<tr>
<td>2,3-Dimethyl-9,10-anthraquinone</td>
<td>100</td>
</tr>
<tr>
<td>1-Chloro-9,10-anthraquinone</td>
<td>0.6</td>
</tr>
<tr>
<td>1,5-Dichloro-9,10-anthraquinone</td>
<td>0.6</td>
</tr>
</tbody>
</table>

*The concentration is obtained by the fluorescence emission spectra of BChl c at 758 nm in chlorosomes from *C. aurantiacus* in the presence of various concentrations of quinones. Fluorescence emission is excited at 725 nm. Chlorosomes were suspended in MOPS buffer (pH 7.0) to give an absorbance of about 0.3 at 740 nm.

†5,8-Dihydroxy-2-(1-hydroxy-4-methyl-3-pentenyl)-1,4-naphthoquinone
Fig. 3-1. The chemical structures of the naphthoquinones listed in Table 3-2, and chlorobiumquinone and menaquinone-7. Naphthoquinones that differ by a single substitution is connected with a line. Values in parentheses show the half-quenching concentration in Table 3-1.
Fig. 3-2. Fluorescence emission spectra of BChl $c$ (a peak at around 758 nm) and BChl $a$ (a peak at around 805 nm) in chlorosomes from *Chloroflexus aurantiacus* in the presence and absence of added 5-hydroxy-1,4-naphthoquinone (5-OH-1,4-NQ), excited at 725 nm. Chlorosomes were suspended in MOPS-buffer (pH 7.0) to give an absorbance of 0.3 at 740 nm. The highest fluorescence emission is the control, and the lower emissions are due to added 5-OH-1,4-NQ with the concentration of 2.5, 5, 10, 25, 50, 100, and 250 μM in order.
Fig. 3-3. Quinone concentration dependency of BChl c fluorescence (at 758 nm) from Chl. aurantiacus chlorosomes. A, 2,3,5,6-tetramethyl-1,4-BQ (TMBQ); B, 1,4-naphthoquinone (NQ); C, 1,2,4-trihydroxy-9,10-anthraquinone (AQ). The insets show the Stern-Volmer plots for each. $F_0$ is the intensity of fluorescence in the absence of quencher, and $F_c$ is the fluorescence intensity in the presence of the indicated concentration of the quencher.
Table 3-2. The binding of various naphthoquinones to chlorosomes from *Cfl. aurantiacus*.

<table>
<thead>
<tr>
<th>Quinone species</th>
<th>Quinone bound ratio (%)</th>
<th>Bound quinone /BClH c (molar ratio) with 50 μM quinones</th>
<th>Half-quenching concentration (μM)</th>
<th>Estimated bound quinone / BChl c (molar ratio) at half-quenching concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shikonin</td>
<td>20.5</td>
<td>2.3</td>
<td>0.4</td>
<td>0.02</td>
</tr>
<tr>
<td>5,8-DiOH-1,4-NQ</td>
<td>9.7</td>
<td>1.0</td>
<td>1.9</td>
<td>0.04</td>
</tr>
<tr>
<td>5-OH-1,4-NQ</td>
<td>4.0</td>
<td>0.4</td>
<td>4</td>
<td>0.03</td>
</tr>
<tr>
<td>5-OH-2-CH3-1,4-NQ</td>
<td>2.1</td>
<td>0.2</td>
<td>4.7</td>
<td>0.02</td>
</tr>
<tr>
<td>1,4-NQ</td>
<td>2.2</td>
<td>0.2</td>
<td>7</td>
<td>0.03</td>
</tr>
<tr>
<td>2-CH3-1,4-NQ</td>
<td>3.7</td>
<td>0.4</td>
<td>12</td>
<td>0.1</td>
</tr>
<tr>
<td>2-CH3-3-acetyl-1,4-NQ</td>
<td>2.6</td>
<td>0.3</td>
<td>16</td>
<td>0.1</td>
</tr>
<tr>
<td>1,2-NQ</td>
<td>4.1</td>
<td>0.4</td>
<td>21</td>
<td>0.2</td>
</tr>
<tr>
<td>2-OH-3-(3-CH3-2-butenyl)-1,4-NQ</td>
<td>3.6</td>
<td>0.4</td>
<td>50</td>
<td>0.4</td>
</tr>
<tr>
<td>2-CH3-3-(1-hydroxyethyl)-1,4-NQ</td>
<td>3.0</td>
<td>0.3</td>
<td>80</td>
<td>0.5</td>
</tr>
<tr>
<td>2-OH-1,4-NQ</td>
<td>1.7</td>
<td>0.2</td>
<td>&gt;250</td>
<td>&gt;1.0</td>
</tr>
</tbody>
</table>

*Quinones (50 μM each) were added to the chlorosome suspensions (A740 = 0.5), and ultracentrifuged (440,000 xg, 30 min). The amounts of quinones absorbed in the chlorosomes were estimated from the difference of the absorption spectra of quinones in the supernatants and those of aqueous solutions of 50 μM without chlorosomes.*
Chapter 4

Inhibition of the photosynthetic electron transfer in *Chloroflexus aurantiacus* by added quinones
Abstract In the photosynthetic green filamentous bacterium *Chloroflexus autantiacus* excitation energy is transferred from a large bacteriochlorophyll (BChl) c antenna via smaller BChl a antennas to the reaction center. The effects of substituted 1,4-naphthoquinones on BChl c fluorescence and on flash-induced cytochrome c oxidation were studied in whole cells under aerobic conditions. BChl c fluorescence in a cell suspension with 5.4 µM BChl c was quenched to 50% by addition of 0.6 µM shikonin, 0.9 µM 5-hydroxy-1,4-naphthoquinone, or 4 µM 2-acetyl-3-methyl-1,4-naphthoquinone. These quinones also efficiently inhibited flash-induced cytochrome c oxidation when BChl c was excited. The quenching of BChl c fluorescence induced by these quinones correlated with the inhibition of flash-induced cytochrome c oxidation. It was concluded that the quinones inhibited electron transfer in the reaction center by specifically quenching the excitation energy in the BChl c antenna.
Photosynthetic green filamentous bacteria (such as *Chloroflexus aurantiacus*) and green sulfur bacteria (such as *Chlorobium tepidum*) both employ a light-harvesting antenna known as the chlorosome (1-3). This organelle contains thousands of bacteriochlorophyll (BChl) c molecules which are organized primarily by pigment-pigment interactions and not by pigment-protein interactions as in most other known light-harvesting complexes. Chlorosomes also contain a small amount of BChl a which absorbs around 795 nm and whose organization probably involves interactions with protein (1, 4). This chlorosomal BChl a is thought to mediate energy transfer from BChl c in the chlorosome to BChl a in the cytoplasmic membrane. *Cfl. aurantiacus* also contains the membrane-bound BChl a-containing B808-866 light-harvesting complex which resembles the light-harvesting complex in purple bacteria in terms of polypeptide composition and spectral properties (1).

The chlorosome structure is similar in green filamentous bacteria and green sulfur bacteria, but a noticeable difference is that chlorosomes from green sulfur bacteria exhibit a redox-dependent quenching. This quenching is activated under oxidizing conditions and thereby inhibits energy transfer to the reaction center (5-7). This is thought to function as a protection mechanism against formation of toxic reactive oxygen species if the bacteria become exposed to O\textsubscript{2} (1, 6). The quenching mechanism is not clear but it probably involves chlorobiumquinone (Fig. 4-1) which seems to be present only in green sulfur bacteria (8-10). I recently found that although *Cfl. aurantiacus* does not inherently possess a similar quenching mechanism, addition of certain quinones to isolated chlorosomes in aerobic suspension causes quenching of the BChl fluorescence (ref. 11 and Chapter 3).
A membrane-bound tetraheme cytochrome $c_{554}$ is the immediate electron donor to the photo-oxidized special pair in the reaction center of *Cfl. aurantiacus* and two menaquinone-10 molecules in the reaction center complex function as electron acceptors (12, 13). In the present work I have used flash-induced cytochrome $c$ oxidation to measure the energy transfer in *Cfl. aurantiacus* from BChl $c$ to the reaction center. This energy transfer is not sensitive to $O_2$ in untreated cells (7). However, addition of low concentrations of certain quinones to cells under aerobic conditions induces a quenching of the BChl $c$ antenna and thereby inhibits photosynthetic electron transfer when BChl $c$ is specifically excited. Addition of quinones thus causes the chlorosomal energy transfer in *Cfl. aurantiacus* to exhibit a sensitivity to $O_2$ similar to that observed in green sulfur bacteria.

**Materials and methods**

*Cfl. aurantiacus* J-10-fl was grown and spectroscopic measurements carried out as Chapter 3. Cells were suspended in 40 mM MOPS buffer pH 7.0 to an $A_{745}$ of 0.5 (this corresponded to 5.4 $\mu$M BChl $c$). The extent flash-induced cytochrome $c$ oxidation was measured as the absorption change $\Delta A_{554-542}$ induced by a single 5-$\mu$s flash from a xenon lamp. The flash filter used for specific BChl $c$ excitation was 700-760-nm broad band interference filter (see transmission spectra in ref. 7). Steady fluorescence was recorded with slit widths of 10 nm on the monochromators. All spectroscopic measurements were carried out at room temperature and under aerobic conditions unless otherwise stated. Cytoplasmic membranes containing chlorosomes were prepared and flash-induced cytochrome $c$ oxidation was measured at a poised redox potential as described in (7), except that the membranes were suspended to an $A_{745}$ of 0.5 in MOPS buffer containing 20
µM each of phenazine methosulfate, 2,3,5,6-tetramethyl-p-phenylenediamine, and $N,N,N',N'$-tetramethyl-p-phenylenediamine, and 50 µM of Fe-EDTA.

Shikonin (5,8-dihydroxy-2-(1-hydroxy-4-methyl-3-pentenyl)-1,4-naphthoquinone), 5-hydroxy-1,4-naphthoquinone (5HNQ) and 2-acetyl-3-methyl-1,4-naphthoquinone (AcMNQ) were obtained as previously (Chapter 3).

Results

Quenching of bacteriochlorophyll fluorescence

When BChl c was excited at 720 nm in a cell suspension of *Cfl. aurantiacus*, steady state fluorescence emission was observed from chlorosomal BChl c at 754 nm. To determine the quenching ability of quinones, cells were washed once and incubated aerobically for at least two hours in the dark; then quinones were added to various concentrations. The quenching of BChl c fluorescence induced by 5HNQ followed a Stern-Volmer relationship (14) with a half-quenching concentration of 0.9 µM 5HNQ (Fig. 4-1). The values of half-quenching concentrations for other quinones are similar to those previously determined on isolated chlorosomes from *Cfl. aurantiacus* (ref. 11 and Chapter 3). When 50 µM 5HNQ was added to an anaerobic cell suspension containing 1 mM sodium sulfide or 5 mM sodium dithionite, no significant quenching of BChl c fluorescence was observed (data not shown), probably because the quinone was reduced to a non-quenching species.

Inhibition of flash-induced cytochrome c oxidation

Cytochrome c oxidation induced by a single flash was measured in cell suspensions made from fresh cell culture diluted 3-6 times with buffer to an
appropriate cell density. Figure 4-2 shows the extent of cytochrome c oxidation at different flash intensities when BChl c was specifically excited. When 50 µM 5HNQ was added to the cell suspension BChl c fluorescence was almost completely quenched (data not shown). Flash-induced cytochrome c oxidation was also almost completely eliminated when BChl c was excited (Fig. 4-2). This predicts that the BChl c antenna is efficiently quenched with 50 µM 5HNQ.

Figure 4-3a shows the flash-induced cytochrome c oxidation at various concentrations of 5HNQ when BChl c was excited. (The flash intensity in these experiments was reduced to approach flash-limiting conditions.) Figure 4-3b shows the fluorescence intensity of BChl c determined on the same samples used for Fig. 4-3a. The results show that the inhibition of BChl c-coupled cytochrome oxidation correlated with the quenching of BChl c fluorescence and that both were almost eliminated at 5HNQ concentrations above 8 µM.

In the experiments for Fig. 4-4, the BChl c fluorescence was quenched to various degrees by addition of various concentrations of three different quinones. In these samples the BChl c-coupled cytochrome oxidation was approximately proportional to the BChl c fluorescence, irrespective of the quinone used.

**Flash-induced cytochrome c oxidation in isolated membranes**

When flash-induced cytochrome c oxidation obtained by BChl c excitation in the membrane preparation (Fig. 4-5, circles) was compared to whole cells (Fig. 4-2, circles), the half-saturation flash intensity was approximately five times larger in the membrane preparation. These results indicate that some of the BChl c antenna in the membrane preparation was functionally disconnected from the membranous antennas. A similar uncoupling of the chlorosomes from the photosynthetic membrane has also been observed in previous work with membrane preparations.
from *Cfl. aurantiacus* (15) and *Chl. tepidum* (7). Due to this uncoupling problem I have focused the experiments on whole cells.

Nevertheless, I observed a similar effect of added quinone in the membrane preparation to that in whole cell. When 100 μM 5HNQ was added to a membrane suspension poised at a redox potential of +100 mV, the intensity of BChl c fluorescence decreased approximately four times and the flash-induced cytochrome c oxidation was significantly inhibited when BChl c was excited (Fig. 4-5).

**Discussion**

Natural and synthetic quinones are potential inhibitors of electron transfer in membrane complexes such as the cytochrome *bc₁* complex (16) and photosystem II-like reaction centers (17). Quinones are also well known quenchers of chlorophyll fluorescence in organic solution (18) and several authors have observed that exogenous quinones are capable of quenching the fluorescence from antenna chlorophyll in chloroplasts and algae (19-25). The latter quenching phenomenon has been explained either by direct interaction between the added quinone and the excited chlorophyll molecules or by displacement of the endogenous quinone from its Q₉ binding site by the added quinone.

In the present work I have shown that when 5HNQ, AcMNQ, or shikonin was added to a suspension of whole cells of *Cfl. aurantiacus*, flash-induced cytochrome c oxidation was inhibited when the BChl c antenna was excited (Figs. 4-2-5). Therefore the inhibition was due to interaction between the added quinone and the BChl c antenna. I have also shown that the quenching of BChl c fluorescence correlated with the inhibition of flash-induced cytochrome c oxidation (Figs. 4-3
and 4). Therefore it was concluded that the added quinones inhibited flash-induced cytochrome c oxidation in *Cfl. aurantiacus* by specifically quenching the excited BChl c antenna.

In experiments with whole cells an additional requirement presumably is that the quinones must be capable of penetrating the cell because chlorosomes are intracellular organelles. Shikonin, 5HNQ and AcMNQ are all water-soluble at low concentrations but they are also sufficiently hydrophobic to concentrate in hydrophobic environments in the cell such as the cytoplasmic membrane and the chlorosomes. The observed abilities of the above quinones to quench BChl c fluorescence are consistent with the above-mentioned criteria: shikonin and 5HNQ have the highest midpoint potential and are very hydrophobic and these quinones are the best quenchers.

In conclusion, these results show that addition of low concentrations of certain quinones to *Cfl. aurantiacus* induced a quenching of the BChl c antenna under aerobic conditions and thereby induced a situation similar to that naturally present in green sulfur bacteria (7). This suggests that the chlorosome structure is similar in these two types of bacteria and that the redox-dependent quenching in green sulfur bacteria could be caused solely by endogenous quinones.
References


Chloroflexus aurantiacus. Biochemistry 26, 8652-8658.


Fig. 4-1. Quenching of BCHl c fluorescence (excitation at 720 nm and emission at 754 nm, circles) in washed and aerated cells by 5HNQ. The data are normalized to the fluorescence intensity when no quinone was added.
Fig. 4-2. Extent of flash-induced cytochrome c oxidation ($\Delta A_{554.542}$) in diluted cell culture measured in the absence (circles) or presence (triangles) of 50 µM SHNQ when BChl c was specifically excited. Each data point represents an average of 40 flashes per wavelength recorded of a 10-ms time scale and separated by 20 s.
Fig. 4-3. Effect of various concentrations of 5HNQ on (a) the extent of flash-induced cytochrome c oxidation ($-\Delta A_{554-542}$) and (b) BChl c fluorescence in diluted cell culture. Cytochrome c oxidation was measured as described in Fig. 4-2, except that BChl c was excited with 10% flash intensity. Steady state fluorescence intensities of BChl c were measured as in Fig. 4-1.
Fig. 4-4. Correlation between the extent of flash-induced cytochrome c oxidation upon excitation of BChl c (\(-\Delta A_{564-542}\)) and BChl c fluorescence in diluted cell culture. Cytochrome c oxidation was measured as in Fig. 4-3 and fluorescence as in Fig. 4-1. Different degree of fluorescence quenching were obtained by adding different concentrations of 5HNQ (triangles), AcMNQ (squares), or shikonin (diamonds); controls are shown with circles. The data are fitted to a straight line through (0, 0).
Fig. 4-5. Extent of flash-induced cytochrome c oxidation ($-\Delta A_{554-542}$) in a membrane suspension poised at a redox potential of +100 mV in the absence (circles) or presence (triangles) of 100 µM 5HNQ and when BChl c was specifically excited. Each data point represents an average of 10 flashes per wavelength recorded on a 256-ms time scale and separated by 30 s.
Conclusion

1. BPhe\textsubscript{c} formed from BChl\textsubscript{c} in the chlorosomes of aged cells of \textit{Chl. tepidum} can still act as a light-harvesting and energy-transferring pigment in photosynthesis. The ability of BPhe\textsubscript{c} to harvest light may enable \textit{Chl. tepidum} to prolong its viability under conditions when growth was ceased due to a shortage of electron donors.

2. I suggest that the pheophytinization of BChl\textsubscript{c} in the chlorosomes from \textit{Chl. tepidum} is stimulated when the concentration of Mg\textsuperscript{2+} in the cytoplasm is lowered.

3. Pheophytinization of BChl\textsubscript{c} in \textit{Chl. tepidum} was independent of the acidification of the culture medium and seemed to be affected by the temperature of the culture rather than the light intensity.

4. Many quinones added to \textit{Cfl. aurantiacus} chlorosomes are potent for quenching of BChl\textsubscript{c} fluorescence in the chlorosomes and also inhibits energy transfer from BChl\textsubscript{c} to BChl\textsubscript{a} in the baseplate.

5. The ratio of estimated bound naphthoquinone molecule per BChl\textsubscript{c} molecule in the chlorosomes from \textit{Cfl. aurantiacus} at a half-quenching concentration was rather constant among very effective naphthoquinones, gradually increasing the increasing the half-quenching concentration in less-effective quinones.

6. In isolated chlorosomes from \textit{Cfl. aurantiacus}, the hydroxy (or oxo) group in the alpha position to the quinone carbonyl seems to be very important for the high quenching ability, probably together with a high redox potential and high hydrophobicity of the quinone molecule.

7. The 1'-oxo group of CK is considered to contribute to the high quenching effect of fluorescence in the chlorosomes from \textit{Chl. tepidum}. 
8. The externally added quinones inhibit electron transfer in the reaction center from *Cfl. aurantiacus* by specifically quenching the excitation energy in the BChl c antenna.