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Exogenous quinones inhibit photosynthetic electron transfer in *Chloroflexus aurantiacus* by specific quenching of the excited bacteriochlorophyll *c* antenna

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Abstract

In the photosynthetic green filamentous bacterium *Chloroflexus aurantiacus*, 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* and BChl *a* 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 ((*R*)-2-(1-hydroxy-4-methyl-3-pentenyl)-5,8-dihydroxy-1,4-naphthoquinone), 0.9 μM 5-hydroxy-1,4-naphthoquinone, or 4 μM 2-acetyl-3-methyl-1,4-naphthoquinone. Between 25 and 100 times higher quinone concentrations were needed to quench BChl *a* fluorescence to a similar extent. These quinones also efficiently inhibited flash-induced cytochrome *c* oxidation when BChl *c* was excited, but not when BChl *a* was excited. The quenching of BChl *c* fluorescence induced by these quinones correlated with the inhibition of flash-induced cytochrome *c* oxidation. We concluded that the quinones inhibited electron transfer in the reaction center by specifically quenching the excitation energy in the BChl *c* antenna. Our results provide a model system for studying the redox-dependent antenna quenching in green sulfur bacteria because the antennas in these bacteria inherently exhibit a sensitivity to O_2 similar to the quinone-supplemented cells of *Cfx. aurantiacus*.

Keywords: Light-harvesting complex; Chlorosome; Fluorescence quenching; Non-photochemical quenching; Cytochrome *c* photooxidation; Green bacteria

1. Introduction

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 other known light-harvesting complexes. Chlorosomes

Abbreviations: 2HNQ, 2-hydroxy-1,4-naphthoquinone; 5HNQ, 5-hydroxy-1,4-naphthoquinone; AcMNQ, 2-acetyl-3-methyl-1,4-naphthoquinone; BChl, bacteriochlorophyll; HEMNQ, 2-(1-hydroxyethyl)-3-methyl-1,4-naphthoquinone

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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. *Cfx. aurantiacus* also contains the membrane-bound BChl *a*-containing B808–866 light-harvesting complex which resembles the light-harvesting complexes 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 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_2 [1,6]. The quenching mechanism is not clear, but it probably involves chlorobiumquinone (Fig. 1) which seems to be present only in green sulfur bacteria [8–10]. We recently found that although *Cfx. 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 ([11] and S. Tokita, N.-U. Frigaard, M. Hirota, K. Shimada, K. Matsuura, unpublished data).

A membrane-bound tetraheme cytochrome c_{554} is the immediate electron donor to the photooxidized special pair in the reaction center of *Cfx. aurantiacus* and two menaquinone-10 molecules in the reaction center complex function as electron acceptors [12,13]. In the present work, we have used flash-induced cytochrome *c* oxidation to measure the energy transfer in *Cfx. aurantiacus* from either BChl *c* or BChl *a* to the reaction center. This energy transfer is not sensitive to O_2 in untreated cells [7]. However, we show 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. Addition of quinones thus causes the chlorosomal energy transfer in *Cfx. aurantiacus* to exhibit a sensitivity to O_2 similar to that observed in green sulfur bacteria.

2. Materials and methods

Cfx. aurantiacus J-10-fl was grown and spectroscopic measurements carried out as previously [7]. Cells were suspended in 40 mM MOPS buffer pH 7.0 to an A_{745} of 0.5 (this corresponded to 5.4 μM BChl *c* and 0.7 μM BChl *a*). The extent of flash-induced cytochrome *c* oxidation was measured as the absorption change $\Delta A_{554-542}$ induced by a single 5-μs flash from a xenon lamp. The flash filters used for specific BChl *a* and BChl *c* excitation were 810–880- and 700–760-nm broad-band interference filters, respectively (see transmission spectra in [7]). Steady-state 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 previously [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 ((*R*)-2-(1-hydroxy-4-methyl-3-pentenyl)-5,8-dihydroxy-1,4-naphthoquinone) was obtained from Tokyo Chemical Industry, Japan. 2-Acetyl-3-methyl-1,4-naphthoquinone (AcMNQ) and 2-(1-hydroxyethyl)-3-methyl-1,4-naphthoquinone (HEMNQ) were synthesized from 2-methyl-1,4-naphthoquinone as described previously [14,15]. Chlorobiumquinone was purified from cell extracts of *Cb. tepidum* as described previously [9]. Stock solutions of quinones were prepared in 99.5% ethanol. The molecular structures of some quinones are shown in Fig. 1.

3. Results

3.1. Quenching of bacteriochlorophyll fluorescence

When BChl *c* was excited at 720 nm in a cell suspension of *Cfx. aurantiacus*, steady-state fluorescence emission was observed from chlorosomal BChl *c* at 754 nm, from chlorosomal BChl *a* at 803 nm, and

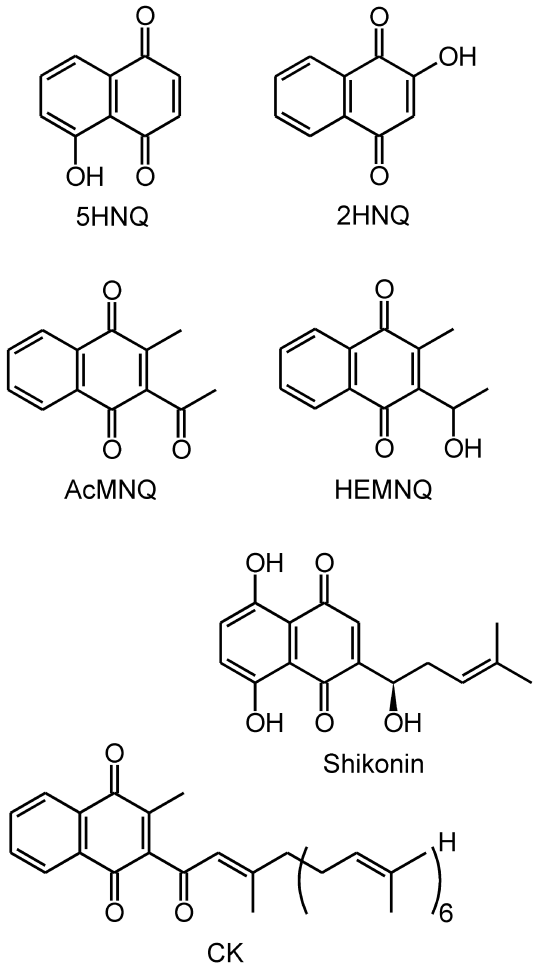

Fig. 1. Molecular structures of quinones used in this study. 2HNQ, 2-hydroxy-1,4-naphthoquinone; 5HNQ, 5-hydroxy-1,4-naphthoquinone; AcMNQ, 2-acetyl-3-methyl-1,4-naphthoquinone; CK, chlorobiumquinone (1′-oxomenaquinone-7); HEMNQ, 2-(1-hydroxyethyl)-3-methyl-1,4-naphthoquinone; shikonin ((*R*)-2-(1-hydroxy-4-methyl-3-pentenyl)-5,8-dihydroxy-1,4-naphthoquinone).

from membranous BChl *a* at 883 nm (data not shown; see e.g. Fig. 2c in [7]). To determine the quenching ability of quinones, cells were washed once and incubated aerobically for at least 2 h in the dark; then quinones were added to various concentrations and the suspensions incubated for at least 1 h in the dark before fluorescence was measured. The quenching of BChl *c* fluorescence induced by 5-hydroxy-1,4-naphthoquinone (5HNQ) followed a Stern–Volmer relationship [16] with a half-quenching concentration of 0.9 μM 5HNQ (Fig. 2, circles). The quenching degree of BChl *a* fluorescence was identical to the quenching degree of BChl *c* fluorescence when the excitation wavelength was 720 nm (data not shown). Half-quenching concentrations determined for other quinones are shown in Table 1. The values for BChl *c* fluorescence quenching are similar to those previously determined on isolated chlorosomes from *Cfx. aurantiacus* ([11] and S. Tokita, N.-U. Frigaard, M. Hirota, K. Shimada, K. Matsuura, unpublished data). No significant quenching was observed when chlorobiumquinone or phylloquinone (2-phytyl-3-methyl-1,4-naphthoquinone) were added in various concentrations up to 200 μM. However, these isoprenoid quinones were difficult to assay because they precipitated in the aqueous phase and probably did not penetrate the interior of the cells.

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.

When BChl *a* was directly excited at 800 nm (both chlorosomal and membranous BChl *a* absorb at this wavelength), we observed fluorescence emission from membranous BChl *a* at 883 nm (data not shown). Significantly higher quinone concentrations were needed to obtain the same degree of quenching of this BChl *a* fluorescence when compared to BChl *c* fluorescence (Fig. 2, triangles and Table 1). Thus, the BChl *a* antennas were much less sensitive to quenching by added quinone than the BChl *c* antenna.

When BChl *a* was excited at 815 nm (mainly membranous BChl *a* absorbs at this wavelength), the half-

Table 1
Concentrations of added quinone that quenched half of the BChl fluorescence in a cell suspension

Quinone	BChl *c* fluorescence half-quenching concentration[a] (μM)	BChl *a* fluorescence half-quenching concentration[b] (μM)
Shikonin	0.6	15
5HNQ	0.9	100
AcMNQ	4	> 200
HEMNQ	9	500
2HNQ	200	> 2000

[a]Excitation at 720 nm and emission at 754 nm.
[b]Excitation at 800 nm and emission at 883 nm.

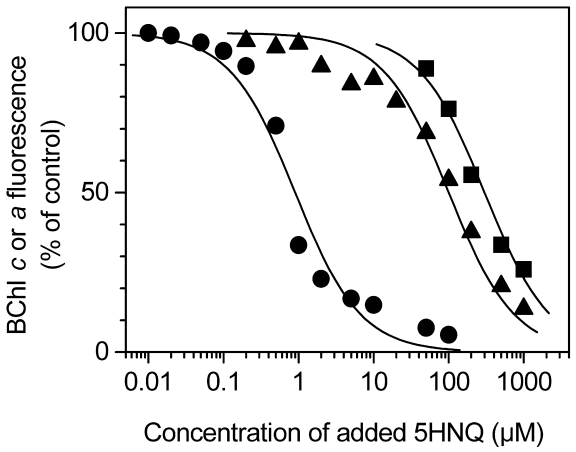

Fig. 2. Quenching of BChl *c* fluorescence (excitation at 720 nm and emission at 754 nm, circles) and BChl *a* fluorescence (excitation at 800 nm and emission at 883 nm, triangles; excitation at 815 nm and emission at 883 nm, squares) in washed and aerated cells by 5HNQ. The data are normalized to the fluorescence intensity when no quinone was added. The curves show Stern–Volmer fits.

quenching concentration was 300 μM for 5HNQ (Fig. 2, squares). This slightly higher value compared to that for 800-nm excitation may suggest that the different BChl *a* chromophores have slightly different susceptibility to 5HNQ, or that only one quenching process occurs with 815-nm excitation whereas two quenching processes occur in tandem with 800-nm excitation.

3.2. 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. Fig. 3 shows the extent of cytochrome *c* oxidation at different flash intensities when either BChl *c* or BChl *a* was specifically excited. (The filter used for BChl *a* excitation resulted in excitation of most of the B808–866 membrane complex and only some of the chlorosomal BChl *a*.) In control cells, half-saturation was obtained at a lower flash intensity when BChl *c* was excited (approximately 4%) than when BChl *a* was excited (approximately 30%), which is expected because the BChl *c* antenna is larger than the BChl *a* antennas. 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 but little affected when BChl *a* was excited (Fig. 3). This difference is consistent with the large difference in half-quenching concentration values stated in the previous section which predicts that the BChl *c* antenna is efficiently quenched with 50 μM 5HNQ whereas the BChl *a* antennas are not.

Fig. 4a 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.) Fig. 4b shows the fluorescence intensity of BChl *c* determined on the same samples used for Fig. 4a. The results show that the inhibition of BChl *c*-coupled cytochrome oxidation correlated with the

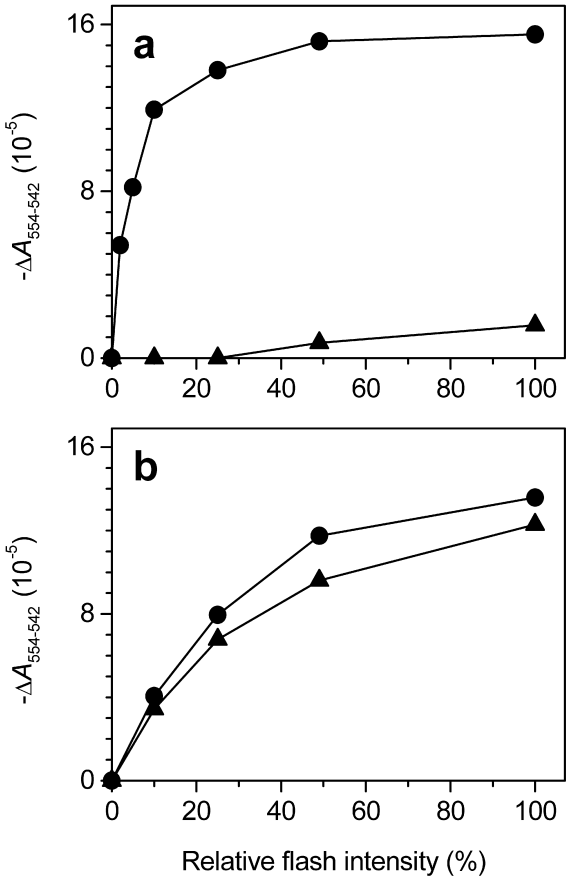

Fig. 3. Extent of flash-induced cytochrome *c* oxidation ($-\Delta A_{554-542}$) in diluted cell culture measured in the absence (circles) or presence (triangles) of 50 μM 5HNQ and when either (a) BChl *c* or (b) BChl *a* was specifically excited. Each data point represents an average of 40 flashes per wavelength recorded on a 10-ms time scale and separated by 20 s.

quenching of BChl *c* fluorescence and that both were almost eliminated at 5HNQ concentrations above 8 μM.

In the experiments for Fig. 5, 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. In contrast, the BChl *a*-coupled cytochrome oxidation in the experiments for both Fig. 4 and Fig. 5 was similar in controls and quinone-supplemented samples (data not shown).

3.3. Inhibition of cytochrome c re-reduction

After flash-induced cytochrome *c* oxidation in whole cells, the cytochrome was re-reduced by cellular reductants on a millisecond timescale. The rate of this re-reduction was inhibited by addition of quinone. The quinone concentrations that caused a 50% decrease of the initial rate of cytochrome *c* re-reduction in whole cells were determined as follows: 20 μM for 5HNQ and shikonin, 0.4 mM for AcMNQ, and >1 mM for HEMNQ and 2HNQ. This inhibition can probably be explained either by oxidation of the endogenous menaquinone pool or by inhibition of electron transfer in the cytochrome bc_1 complex by the added quinones.

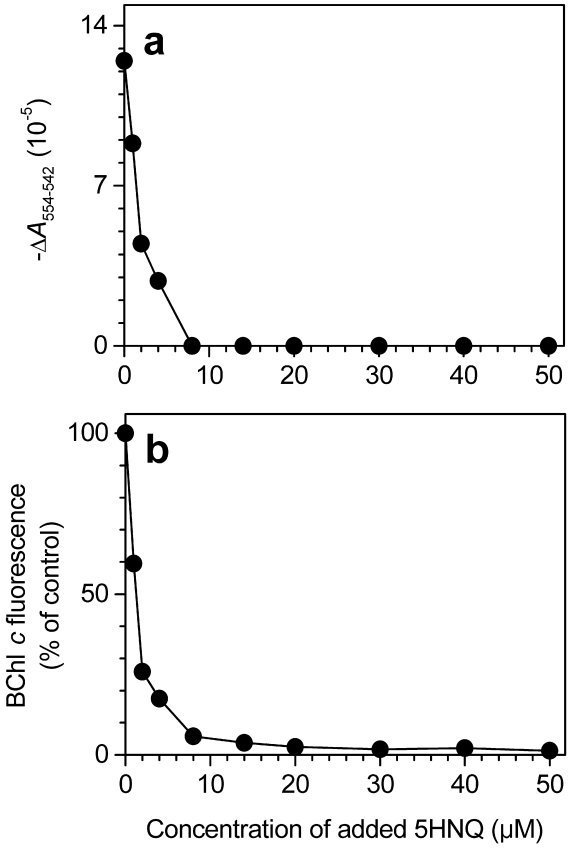

Fig. 4. Effect of various concentrations of 5HNQ on (a) the extent of flash-induced cytochrome *c* oxidation ($-\Delta A_{554-542}$) and (b) BChl *c* fluorescence in diluted cell culture. Cytochrome *c* oxidation was measured as described in Fig. 3, except that BChl *c* was excited with 10% flash intensity. Steady-state fluorescence intensities of BChl *c* were measured as in Fig. 2.

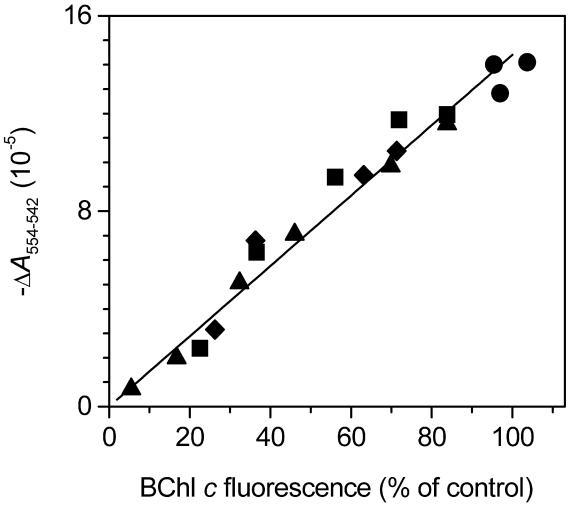

Fig. 5. Correlation between the extent of flash-induced cytochrome *c* oxidation upon excitation of BChl *c* ($-\Delta A_{554-542}$) and BChl *c* fluorescence in diluted cell culture. Cytochrome *c* oxidation was measured as in Fig. 4 and fluorescence as in Fig. 2. Different degrees of fluorescence quenching 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).

Because the initial half-time of re-reduction was approximately 80 ms in control cells and the intervals between flashes were 20 s, we concluded that the inhibition of re-reduction caused by the added quinones did not interfere with the measurements of the extent of cytochrome *c* oxidation in this study.

3.4. Flash-induced cytochrome c oxidation in isolated membranes

When BChl *c* was excited at 720 nm, the ratio of BChl *a* fluorescence at 803 nm to BChl *c* fluorescence

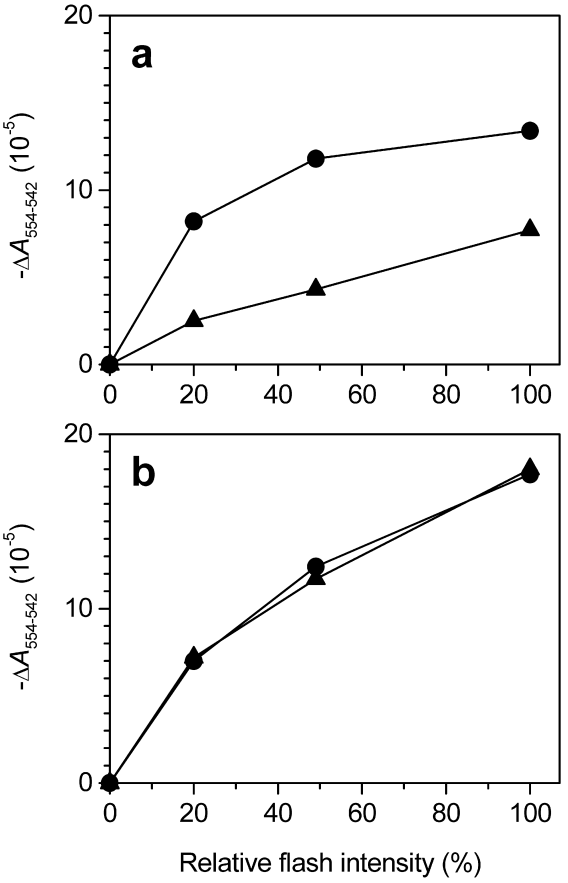

Fig. 6. Extent of flash-induced cytochrome *c* oxidation ($-\Delta A_{554-542}$) in a membrane suspension poised at a redox potential of +100 mV in the absence (circles) or presence (triangles) of 100 μM 5HNQ and when either (a) BChl *c* or (b) BChl *a* was specifically excited. Each data point represents an average of 10 flashes per wavelength recorded on a 256-ms time scale and separated by 30 s.

at 754 nm was higher in our membrane preparation than in whole cells (data not shown). This indicates that transfer of excitation energy from chlorosomal BChl *a* to membranous BChl *a* was impaired in the membrane preparation. In addition, when flash-induced cytochrome *c* oxidation obtained by BChl *c* excitation in the membrane preparation (Fig. 6a, circles) was compared to whole cells (Fig. 3a, 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 *Cfx. aurantiacus* [17] and *Cb. tepidum* [7]. Due to this uncoupling problem we have focused our experiments on whole cells.

Nevertheless, we observed a similar effect of added quinone in the membrane preparation to that in whole cells. 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, but not affected when BChl *a* was excited (Fig. 6).

4. Discussion

Natural and synthetic quinones are potential inhibitors of electron transfer in membrane complexes, such as the cytochrome bc_1 complex [18] and photosystem II-like reaction centers [19]. Quinones are also well-known quenchers of chlorophyll fluorescence in organic solution [20] and several authors have observed that exogenous quinones are capable of quenching the fluorescence from antenna chlorophyll in chloroplasts and algae [21–27]. 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 Q_B binding site by the added quinone.

In the present work, we have shown that when 5HNQ, AcMNQ, or shikonin was added to a suspension of whole cells of *Cfx. aurantiacus*, flash-induced cytochrome *c* oxidation was only inhibited when the BChl *c* antenna was excited and not when the BChl *a* antennas were excited (Figs. 3–6). Therefore, the inhibition was due to interactions between the added quinone and the BChl *c* antenna and not between the added quinone and components in the cytoplasmic membrane. We have also shown that the BChl *c* fluorescence was quenched at quinone concentrations much lower than needed to quench BChl *a* fluorescence (Fig. 2 and Table 1) and that the quenching of BChl *c* fluorescence correlated with the inhibition of flash-induced cytochrome *c* oxidation (Figs. 4 and 5). We therefore concluded

that the added quinones inhibited flash-induced cytochrome *c* oxidation in *Cfx. aurantiacus* by specifically quenching the excited BChl *c* antenna.

Approximately 25–100 times higher concentrations of added quinone were needed to quench BChl *a* fluorescence than to quench BChl *c* fluorescence (Fig. 2 and Table 1). The explanation for this difference probably involves the different organization of BChl *c* and BChl *a* in green bacteria. Assuming that the quenching mechanism involves close contact between a quinone molecule and an excited chlorophyll molecule, one possible explanation is that the BChl *a* molecules are less accessible than the BChl *c* molecules to the added quinone. Another possible explanation is based on that the BChl *c* molecules are organized in very large, strongly exciton-coupled aggregates which the BChl *a* molecules are not [1,3]. This difference may increase the probability of interaction between the quinone molecules and excited BChl *c* because the BChl *c* excitons are distributed over a much larger number of molecules than the BChl *a* excitons.

We used AcMNQ and HEMNQ in the present study because these quinones are soluble, low-molecular structural analogs of chlorobiumquinone and 1′-hydroxymenaquinone-7, respectively (Fig. 1). Both of these isoprenoid quinones are present in chlorosomes from *Cb. tepidum* [9] (assuming that the 'polar menaquinone' previously detected in green sulfur bacteria is identical to 1′-hydroxymenaquinone-7 [9,28]). Our observation that AcMNQ was an efficient quencher of the BChl *c* antenna in *Cfx. aurantiacus* thus supports the idea that chlorobiumquinone serves a quenching function in green sulfur bacteria under oxic conditions. Because HEMNQ also quenched the BChl *c* antenna in *Cfx. aurantiacus*, although less efficient than AcMNQ, 1′-hydroxymenaquinone-7 may also be of some importance as quencher in green sulfur bacteria.

In organic solution, the ability of quinones to quench chlorophyll fluorescence is determined mainly by the redox midpoint potential of the quinone/semiquinone couple with quinones of higher midpoint potential being better quenchers [20]. However, it is not clear what determines the ability of quinones to quench BChl fluorescence in chlorosomes. In previous studies with isolated chlorosomes from *Cfx. aurantiacus*, we attributed a high quenching ability of an added quinone to a combination of properties, including high midpoint potential, high hydrophobicity, and the nature of the substituents on the quinone ([11] and S. Tokita, N.-U. Frigaard, M. Hirota, K. Shimada, K. Matsuura, unpublished data). 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. The quinones listed in Table 1 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 (all quinones in Table 1 have octanol–water partition coefficients greater than 20 [29]). The observed abilities of the quinones in Table 1 to quench BChl *c* fluorescence are consistent with the above-mentioned criteria: shikonin and 5HNQ have the highest midpoint potentials and are very hydrophobic and these quinones are the best quenchers; 2HNQ has the lowest midpoint potential and is the least hydrophobic quinone and the worst quencher. The observations that chlorobiumquinone in green sulfur bacteria is located inside the chlorosomes, is highly hydrophobic, and has a significantly higher midpoint potential than menaquinone therefore support the idea that it functions as an indigenous quencher.

In conclusion, our results show that addition of low concentrations of certain quinones to *Cfx. aurantiacus* induced a quenching of the BChl *c* antenna under aerobic conditions without affecting the BChl *a* antennas 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. Our present work may also provide a useful model system for further studies of the redox effect in green sulfur bacteria.

Acknowledgements

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naphthoquinone. This work was supported by grants-in-aid from the Ministry of Education, Science and Culture, Japan (Decoding the Earth Evolution program and International Joint Research) and a special grant (1999) from Tokyo Metropolitan University. N.-U.F. was supported by a scholarship from the Carlsberg Foundation.

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