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Biochimica et Biophysica Acta
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Variable fluorescence in green sulfur bacteria

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Received 16 August 2006; received in revised form 1 November 2006; accepted 7 November 2006
Available online 17 November 2006

Abstract

Green sulfur bacteria possess a complex photosynthetic machinery. The dominant light harvesting systems are chlorosomes, which consist of bacteriochlorophyll *c*, *d* or *e* oligomers with small amounts of protein. The chlorosomes are energetically coupled to the membrane-embedded iron sulfur-type reaction center via a bacteriochlorophyll *a*-containing baseplate protein and the Fenna–Matthews–Olson (FMO) antenna protein. The fluorescence yield and spectral properties of these photosynthetic complexes were investigated in intact cells of several species of green sulfur bacteria under physiological, anaerobic conditions. Surprisingly, green sulfur bacteria show a complex modulation of fluorescence yield upon illumination that is very similar to that observed in oxygenic phototrophs. Within a few seconds of illumination, the fluorescence reaches a maximum, which decreases within a minute of illumination to a lower steady state. Fluorescence spectroscopy reveals that the fluorescence yield during both processes is primarily modulated on the FMO-protein level, while the emission from chlorosomes remains mostly unchanged. The two most likely candidates that modulate bacteriochlorophyll fluorescence are (1) direct excitation quenching at the FMO-protein level and (2) indirect modulation of FMO-protein fluorescence by the reduction state of electron carriers that are part of the reaction center.

Keywords: Green sulfur bacteria; Bacteriochlorophyll fluorescence; Chlorosome; FMO-protein

1. Introduction

Green sulfur bacteria contain a unique photosynthetic machinery that permits them to thrive in an oxygen-free, sulfur-rich and often light-limited environment. The main light harvesting system of green sulfur bacteria, the chlorosomes, consist of bacteriochlorophyll (BChl) *c*, *d* or *e* oligomers. In contrast to the light harvesting systems employed in most other photosynthetic bacteria, the BChls in the chlorosomes are not ligated to proteins. The chlorosome is energetically coupled to the reaction center (RC) via the baseplate protein and the Fenna–Olson–Matthews (FMO) protein, both of which contain BChl *a*. Excitation migrates down an energy gradient from the BChl oligomers, baseplate protein, FMO-protein, to the RC by Förster-type energy transfer (see Table 1 for excitation and fluorescence maxima) [1,2]. It is widely accepted that the RC of the green sulfur bacteria is an iron–sulfur (FeS)-type RC in which iron–sulfur clusters serve as the electron acceptors [3,4].

Green sulfur bacteria are obligate anaerobes in which the photosynthetic apparatus is optimized for anaerobic and low-light conditions. These living conditions might explain that in the presence of oxygen, strong quenching of excitation reflected by a decrease in fluorescence is observed [5]. Fluorescence quenching that is dependent on the presence of oxygen or other oxidants and associated changes in redox potential has been observed in whole cells and was also characterized in isolated chlorosomes and isolated FMO-proteins [6–11].

Fluorescence emitted by the chlorosome is decreased in aerobic conditions [6,12]. A model for this BChl fluorescence quenching has been developed that is based on the excitation quenching ability of oxidized quinones. Oxidized quinones are known to be effective quenchers of excitation energy [13]. Two chemically distinct quinones are present in green sulfur bacteria.

Abbreviations: RC, Reaction center; Q-type RC, RC in which quinones are the final electron acceptors; FeS-type RC, RC in which an iron sulfur cluster is the final electron acceptor; Q_A, First quinone electron acceptor in Q-type RC; P840, Reaction of green sulfur bacteria; FMO-protein, Fenna–Olson–Matthews protein; BChl, Bacteriochlorophyll

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doi:10.1016/j.bbabio.2006.11.011

Table 1
Absorption and fluorescence maxima of photosynthetic components in green sulfur bacteria

Species	Complex	Absorption (nm)	Fluorescence (nm)
Cb. tepidum	BChl *c* oligomers	745–755	775
	FMO-protein	810	820
Cb. vibrioforme	BChl *c* and/or *d* oligomers	715–745 [28]	770
	FMO-protein	810	816
Pd. phaeum	BChl *e* oligomers	710–725 [28]	733
	FMO-protein	805	811
green sulfur bacteria	Baseplate	~795	~810
green sulfur bacteria	RC	~840	~855

Gloe et al. [28].

Chlorobiumquinone, with a midpoint potential of +35 mV (pH 7) *in vitro* [14], is mainly found in the chlorosomes, while menaquinone, which is found within the cytoplasmic membrane [15], possesses a redox potential *in vitro* of −70 mV (pH 7). When chlorosome quinones were extracted, the redox-induced modulation of fluorescence intensity was only threefold, compared with up to 40-fold modulation in the presence of quinones [15]. This result also demonstrates that oxygen itself is probably not the quencher but appears to act via the oxidation of quinones. Several redox-active proteins have been identified in the chlorosome envelope [16] and might mediate the redox-modulation of a quencher, which likely is chlorobiumquinone [9,10]. The midpoint potential for fluorescence quenching in isolated chlorosomes has been reported to be between −100 mV in *Cb. vibrioforme* [9] and −140 mV in *Cb. tepidum* [7,17].

In addition to chlorosomes, isolated FMO proteins also exhibit a redox-dependent quenching behavior. Fowler and coworker [18] reported that complexes consisting of a RC and FMO-protein complexes of *Chlorobium* species show changes in FMO-fluorescence yield when these complexes were chemically reduced or oxidized. This initial observation was further explored [7,8,11], while the molecular origin of this redox effect on the FMO protein is not yet clear.

Changes in fluorescence behavior were observed in whole cells as well as isolated photosynthetic complexes. Oxygen was effective in decreasing the fluorescence yield and reductants (usually sodium dithionite) were added to achieve reduction and anaerobiosis and a fluorescence increase of the investigated system. Whether the fluorescence quenching on the chlorosome or FMO-protein level has a physiological relevance has not been clearly demonstrated. The quenching might protect the photosynthetic machinery, especially the FeS-type RCs, from the formation of oxygen radicals when cells are exposed to oxygen, [7,10,19,20]. However, before the aforementioned biochemical studies, modulation of fluorescence yield in green sulfur bacteria has been reported by Clayton [21] and Hoffman and Metzner [22] in a more phenomenological framework. The reported modulation was not dependent on the addition of oxygen or strong reductants, but occurred in physiological conditions with light being the only external modulating factor. However, this phenomenon has not been given any attention in the intervening years.

The interpretation of chlorophyll fluorescence has proven to be an extremely powerful tool to investigate photosynthesis in oxygenic phototrophs [23–25]. Oxygenic phototrophs contain RCs in which a quinone (Q_A) serves as a final electron acceptor (Q-type RCs) in addition to FeS-type RCs. In these organisms changes in fluorescence yield depend on the oxidation state of Q_A, which is redox-coupled to a membrane-bound pool of quinones. FeS-type RCs do not show variable fluorescence [23,26].

How is fluorescence modulated in green sulfur bacteria? In this report we provide a framework to answer this questions by investigating light-induced kinetics and spectral properties of fluorescence emission, under anaerobic conditions, in three green sulfur bacteria: *Chlorobium tepidum* (*Cb. tepidum*), *Chlorobium vibrioforme* (*Cb. vibrioforme*) and *Pelodictyon phaeum* (*Pd. phaeum*).

2. Materials and methods

2.1. Sample growth

Cb. tepidum was grown under a tungsten incandescent light at 42 °C in medium described by Wahlund and coworkers [27]. *Cb. tepidum* was grown under fluorescent light at 25 °C in the same medium as *Cb. vibrioforme* (NCBI 8327). *Pd. phaeum* was grown under fluorescent light at 25 °C using the same medium supplemented with 40 g NaCl per liter. Cells were harvested in late exponential or early stationary growth phase. A healthy looking leaf of *Ficus retusa* was picked from a tree outside the laboratory and dark-adapted for ~20 min before light exposure.

2.2. Steady state fluorometry

Steady state fluorescence spectra were recorded on a fluorometer (Photon Technology International, Birmingham, NJ, USA) equipped with an avalanche photodiode detector (Advanced Photonics Inc., Camarillo, CA). The detector was protected from stray light by a 440 nm interference filter in the excitation beam path and a 620-nm-long-pass filter in the detector beam path.

For steady-state experiments, cells were diluted in a Coy Laboratory Products anaerobic chamber into fresh medium to obtain a red BChl peak absorption of ~0.1 in a 1 cm × 1 cm cuvette. The sample was sealed in the anaerobic chamber and remained sealed during the experiment. The excitation light (centered at 440 nm) intensity was modified by changing the excitation light slit width of the fluorometer.

For experiments using a flow-through cuvette, the cells were diluted to a red Bchl peak absorption of ~0.4 with fresh medium into a 1L Erlenmeyer flask that was equipped with an outlet pipe at the bottom. This outlet was attached to a rubber tube, which was closed using a metal clamp, and the Erlenmeyer flask top was sealed with a rubber stopper before removing the flask from the anaerobic chamber. The bottom outlet tube was attached to a flow-through cuvette through a hole in the center of the fluorometer by oxygen-impermeant black rubber tubing. A peristaltic pump was attached to the outlet of a 0.3 cm × 1 cm flow-through cuvette via tubing. The pump was used to exchange light-exposed samples with fresh dark-adapted sample from the Erlenmeyer reservoir. The entire setup was covered in black cloth to eliminate stray light. Excitation of 440 nm with an intensity of 150 μmol m^{-2} s^{-1} photons was applied by manually opening a shutter to the sample to induce changes in fluorescence.

2.3. Variable fluorometry

Variable fluorescence was determined using a 101 PAM fluorometer (Walz, Effeltrich, Germany) with custom built hardware and software. The standard PAM emitter-detector unit was used, which features a 650 nm

excitation and a fluorescence detection using a 700-nm long pass filter. A halogen light source with adjustable light intensity was used for actinic illumination. The relays that triggered the opening of the shutter that protected the sample from the actinic light had opening and closing times of 1–2 ms. Halogen light pulses were triggered by a Uniblitz shutter (Vincent and Associates, Rochester, NY) with opening and closing times of 2–3 ms. Upon onset of illumination the measuring light frequency was increased automatically from 1.6 kHz to 100 kHz. This increase in light intensity was insignificant compared to the actinic light. Shutter control and data acquisition were integrated into a custom-built control system using the software environment Labview (National Instruments, Austin, TX), under a Windows 98 (Microsoft, Redmond, WA) operating system. Samples were diluted in anaerobic conditions to obtain a BChl red peak absorption of ~0.15. Small, round flat-bottom vials (1.2 cm inner diameter) were filled with 1 mL of diluted green sulfur bacteria resulting in a fill level of about 1 cm. The flat bottom vials were sealed in the anaerobic chamber with fitting screw tops. The oxygen-free samples were placed onto the upright end of the fiber optic arm of the PAM fluorometer.

3. Results

The absorption spectra of green sulfur bacteria are dominated by the BChl oligomers contained in chlorosomes (Fig. 1). These oligomers are composed of BChl *c* in *Cb. tepidum*, BChl *c* and little or no BChl *d* in *Cb vibrioforme* (NCBI 8327) and BChl *e* in *P. phaeum*. Dependent upon whether BChl *c*, *d* or *e* make up the BChl oligomers, different absorption and fluorescence maxima are present (Table 1). The fluorescence spectra of anaerobic green sulfur bacteria also reveal another component of the photosynthetic apparatus, the FMO-protein. Because FMO-proteins from all species of green sulfur bacteria contain BChl *a,* the fluorescence spectrum of the FMO-protein is not as variable as the fluorescence spectrum of the BChl *c, d, e* oligomers found in different species. Compared to the chlorosomes and the FMO-proteins, the RCs possess a relatively small absorption and low fluorescence yield.

Green sulfur bacteria possess a variable fluorescence that can be modulated by exposure to light. A basal level of fluorescence is observed under weak measuring light and a variable fluorescence yield is found when the sample is illuminated. The fluorescence yield decreases to the basal fluorescence level within seconds after the actinic illumination is stopped. Upon illumination, all green sulfur bacteria show a complex modulation of BChl fluorescence (Fig. 2). Within a few seconds of illumination a high fluorescence level is reached. The amplitude of the initial peak increases with the actinic light intensity, but saturates at higher light intensities. The high initial fluorescence level decreases within 100 s to a stationary fluorescence yield. The decrease does not follow simple kinetics but is modulated and exhibits secondary peaks or transitional plateaus. The shape of the induction is influenced by many factors, including pre-illumination and culture conditions (data not shown). The overall modulation of fluorescence bears similarities to the modulation of fluorescence of a plant leaf (Fig. 3; for other examples, see reference [25]).

During illumination the variable fluorescence does not recover to the dark adapted high fluorescence yield during short (2 s) dark periods. Maximum fluorescence yield was restored after several minutes of dark adaptation (Fig. 3). The time required to complete the recovery is similar to the time required for recovery of fluorescence in higher plants.

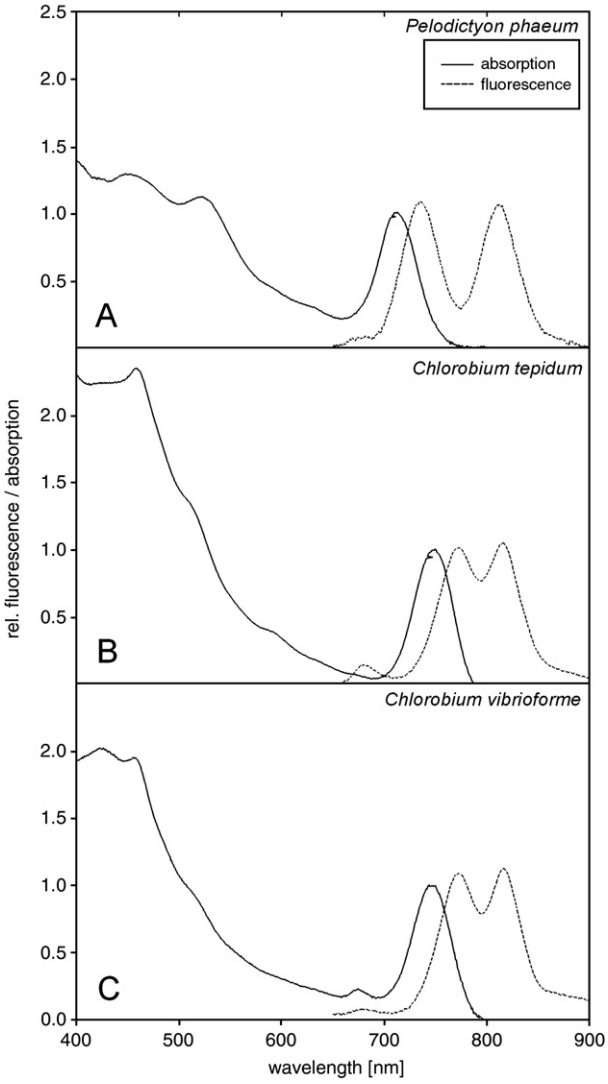

Fig. 1. Absorption (solid lines) and fluorescence (dashed lines) spectra of green sulfur bacteria. (A) *Pd. phaeum* (B) *Cb. tepidum*, (C) *Cb. vibrioforme*. Corrected fluorescence spectra were recorded in anaerobic conditions using an excitation wavelength of 440 nm at room temperature.

The spectral changes during the first seconds of illumination were investigated by following the wavelength characteristics of BChl oligomers and FMO-protein in different green sulfur bacteria species (Fig. 4). Because the samples need to be replaced after each experiment with fresh sample for each acquired wavelength, the experiment was repeated three times for each wavelength to demonstrate reproducibility. In all three organisms the fluorescence at BChl oligomers and FMO-protein is modulated. However, the modulation at the wavelength that corresponds to the FMO-protein of respective species is the main modulator of fluorescence yield. The modulation observed at wavelengths specific for the BChl oligomers may not reflect variation in

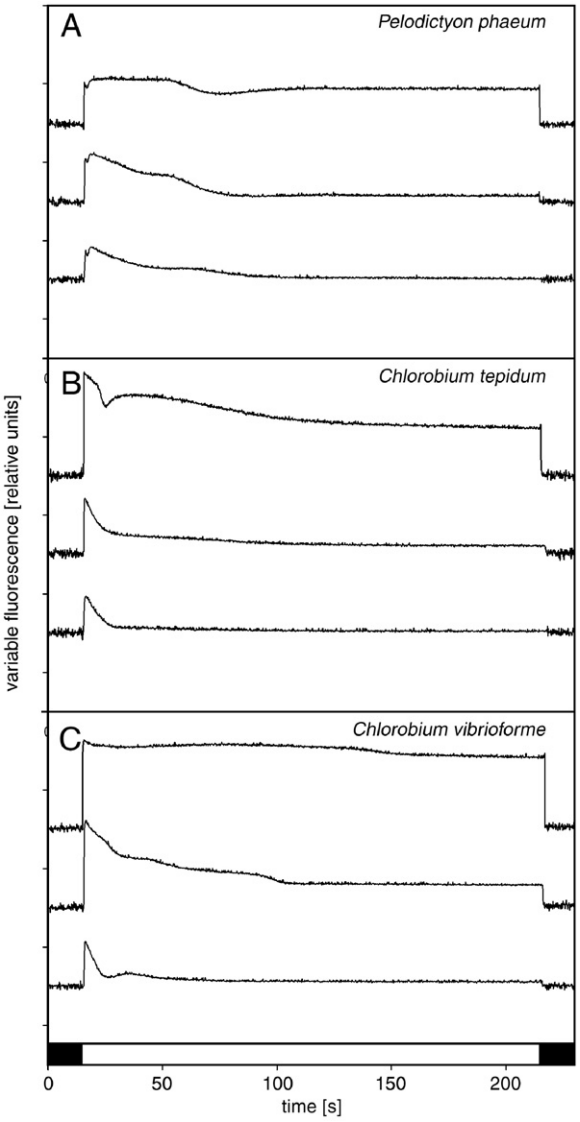

Fig. 2. BChl induction kinetics. Induction kinetics of (A) *Pd. phaeum,* (B) *Cb vibrioforme* and *Cb tepidum* upon illumination. Cells were dark-adapted for at least 30 min. Data is normalized to the dark fluorescence level (=1). Induction kinetics at different light intensities are shifted by one unit. Illumination started at 15 s and lasted for 200 s. Light intensities from top to bottom in each panel are 1350, 33 and 7 μmol m^{-2} s^{-1} photons.

the BChl fluorescence at all, but rather might also reflect a modulation of the FMO-protein fluorescence, because the width of the excitation and emission light modulating slits were increased to allow time-sensitive detection of the kinetics.

To investigate the spectral components of the fluorescence modulation after several minutes of illumination (Fig. 2), the fluorescence spectra of samples in steady-state under different light intensities (2.4 to 150 μmol photons/m^2 * s of blue light) were recorded (Fig. 5). In the green sulfur bacteria species investigated, the fluorescence yield of the BChl oligomers does not change under different light intensities, while the fluorescence of the FMO-proteins is modulated. At high light intensities the FMO-protein fluorescence is higher than at low light intensities.

4. Discussion

The overall modulation of BChl fluorescence in green sulfur bacteria shows a striking similarity to Chl *a* fluorescence modulation of oxygenic phototrophs [25]. The complex Chl *a* fluorescence of oxygenic phototrophs is modulated primarily by the redox state of the first quinone electron acceptor in Q-type RC [9], but also by the generation of additional protective quenchers [29–31] and redistribution of light harvesting systems [32,33]. Are there indications for several different fluorescence modulating mechanisms in green sulfur bacteria?

The sigmoidal shape of the fast fluorescence transient after illumination might be indicative of a cooperative process. The shape of the fluorescence induction is influenced by pre-illumination, background illumination, temperature and culture conditions (data not shown). The decrease in fluorescence that occurs within a few minutes after illumination might be caused by the same mechanism that modulates the fast increase in fluorescence yield. In this case, fluorescence quenchers depleted during the first seconds of illumination may recover during further illumination. The other possibility is that one or several additional quenching mechanisms may be induced. The timescale of the fluorescence decrease (few minutes) are typical of a physiological response like state transitions [32,33] or activation of the carbon fixation cycle observed in oxygenic phototrophs [34]. The modulation of steady state fluorescence at the FMO-protein level indicates that in these experiments we are probing only a single modulation mechanism, as chlorosome fluorescence is not changed, and that this mechanism is very likely in equilibrium with some aspect of cellular physiology.

By contrast differences in time scale between the light-induced fast fluorescence increase and light-sustained slow decrease in fluorescence yield might indicate that at least two different mechanisms participate in fluorescence modulation.

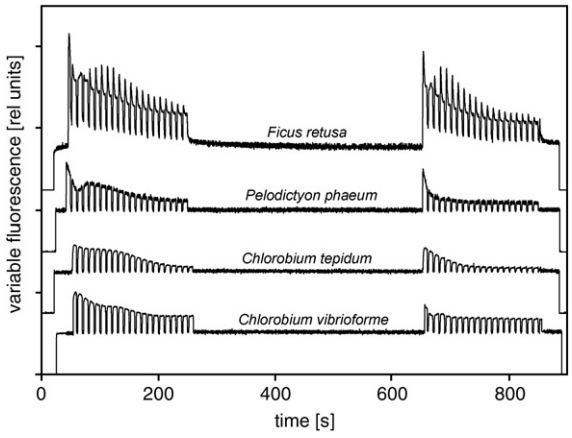

Fig. 3. Modulation of Chl and BChl fluorescence yield upon illumination. Fluorescence induction kinetics of the plant *Ficus retusa* (aerobic) and green sulfur bacteria *Pd. phaeum*, *Cb. tepidum* and *Cb. vibrioforme* (anaerobic). Samples were dark-adapted for at least 30 min. Data were normalized to the dark fluorescence level (=1). The illumination was interrupted every 8 s for a 2 s dark period. After a 6 min dark period, the sample was illuminated a second time. Light intensity: 150 μmol m^{-2} s^{-1} photons.

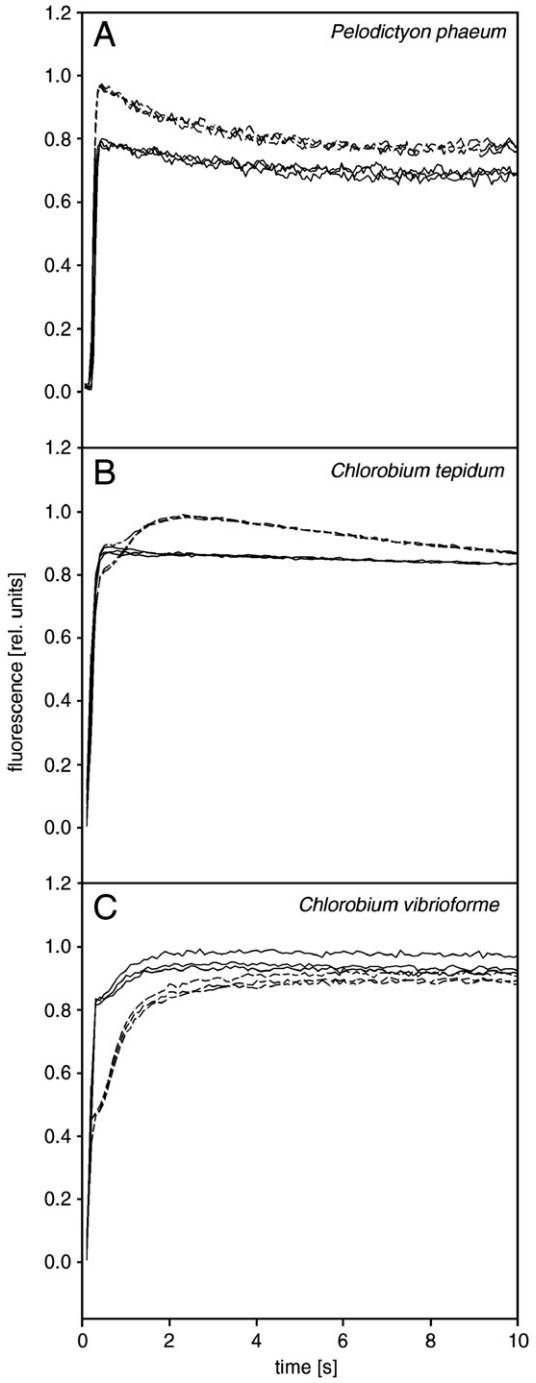

Fig. 4. Spectral characteristics of induction kinetics. Fluorescence emission specific for BChl oligomers and FMO-protein of *Pd. phaeum*, *Cb. tepidum* and *Cb. vibrioforme* in anaerobic conditions. Fluorescence was monitored after opening a shutter that protected the sample from 150 μmol m^{-2} s^{-1} photons light (centered at 440 nm). Fluorescence emission typical of FMO-protein is shown in as dashed lines. Fluorescence emission typical for the BChl oligomers is shown as solid lines.

The observed modulation reported here have all been acquired at low redox potential where the chlorosome is fully active and is different from previously reported fluorescence quenching at the chlorosome level [6,10,12].

Because of the promptness of modulation, the light-induced fast modulation is likely to be caused by a mechanism involving electron carriers in the RC or electron carriers coupled to redox reactions of the RC. A correlation of fluorescence yield with bleaching of the RC (P840) and cytochrome oxidation state has been established [35–37]. Sybesma and Olson [35] showed that at high light intensities the fluorescence yield of FMO-protein is higher than at low intensities, while fluorescence yield emitted

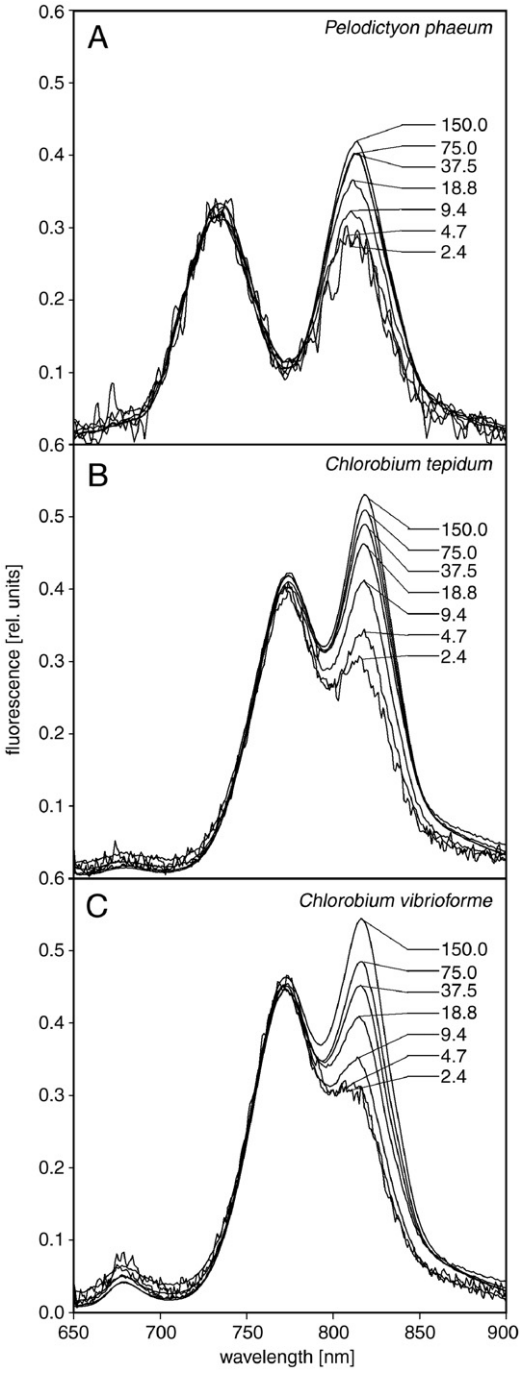

Fig. 5. Steady state fluorescence spectra. Steady state fluorescence spectra of anaerobic *Pd. phaeum*, *Cb. tepidum* and *Cb. vibrioforme* under different light intensities (2.4–150 μmol m^{-2} s^{-1} photons of blue light). The spectra were normalized by a factor that is proportional to the intensity of the excitation beam (excitation centered at 440 nm).

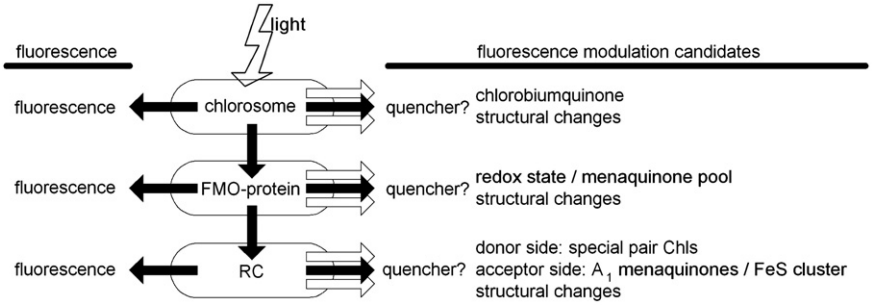

Fig. 6. Schematic representation of excitation transfer and potential excitation quenchers in green sulfur bacteria. Energy absorbed by the chlorosome reaches the RC via the FMO protein. Excitation transfer between these complexes and transfer to known and hypothetical quenchers are indicated by arrows.

from the chlorosomes remains constant under both conditions. Furthermore, the fluorescence yield increase at the FMO-protein level is correlated with an increase in P840 bleaching [36]. These results are in line with the interpretation that fluorescence yield of the FMO-protein is modulated by the redox-state of the RC special pair chlorophyll. If the RC is in the oxidized state (P840 bleached) it is a less effective quencher of FMO-protein excitation compared to a RC in the (re)reduced state.

Is this correlation conclusive evidence that the redox state of the P840 special pair modulates FMO-protein fluorescence yield? Not necessarily—the number of RCs with an oxidized P840 might also increase in parallel to the RC electron acceptors becoming more reduced. In fact, in Q-type RCs of oxygenic photosynthesis, where an increase in fluorescence also correlates with P680 bleaching in many conditions, the modulator of fluorescence is the electron acceptor Q_A [38]. In green sulfur bacteria, fluorescence yield and P840 bleaching each show a different light saturation behavior [36], where P840 bleaching is saturated at 30% of maximal fluorescence yield. This differential saturation behavior might be indicative of a fluorescence modulation that is dependent on the redox state of the electron acceptor of P840. At present it is not clear whether a quinone or an FeS-cluster is accepting electrons from P840. While stable charge separation seems to occur in RCs that do not contain quinones [39,40], an electron paramagnetic resonance study showed the photoproduction of a quinone radical [41]. Interestingly, there are indications that the RC menaquinones of green sulfur bacteria appear only loosely bound to the RC and might exchange with the menaquinone pool [42].

Isolated FMO-protein shows a redox-dependent fluorescence modulation. Dithionite (−450 mV) is efficient in increasing FMO-protein fluorescence yield, whereas sulfide, ascorbate (58 mV), dithiothreitol (−330 mV) and sodium borohydride (−400 mV) only slightly affect FMO-protein fluorescence yield [8]. These authors note that the most purified FMO-proteins show the most modulation in fluorescence yield between oxidized and reduced conditions. Furthermore, anaerobic buffers with different redox-potentials were efficient in modulating fluorescence, demonstrating that fluorescence quenching occurs independently of molecular oxygen. The mechanism of FMO-fluorescence modulation remains unknown.

Independent of their possible role as fluorescence modulators in the RC, oxidized quinones are known to possess the capability to quench excitation from excited chlorophylls [43]. An excitation energy quenching mechanism that involves a transient charge separation has been proposed. In this mechanism excited chlorophyll donates an electron to a quinone and is subsequently re-reduced by the quinone [44]. Takamiya [45] reported an increase in photooxidation of menaquinone but not chlorobiumquinone under anaerobic conditions. Therefore, it appears possible that fluorescence yield is modulated by direct interaction of menaquinones with FMO-protein BChls and/or RC BChls. The physiological control of the redox state of the menaquinol pool is very complex. The redox state of the menaquinol pool in the dark is poised by oxidation and reduction of a number of enzyme complexes that interact with cellular metabolism. When light is applied this equilibrium is disturbed by RC-driven quinone oxidation (via cytochrome and the bc complex) and a new equilibrium is reached after a few minutes. The complex modulation of fluorescence yield induced by illumination might reflect aspects of the modulation of the quinone pool reduction state.

Light-induced fluorescence modulation in green sulfur bacteria exhibits similarities to that in oxygenic phototrophs. This is quite astonishing given the very different organization of the photosynthetic apparatus in both groups of organisms. A diagram that shows overall excitation transfer and possible quenchers modulating fluorescence yield in green sulfur bacteria is presented in Fig. 6. Although the mechanisms of fluorescence modulation in green sulfur bacteria are still unclear, this modulation appears to contain a richness of information comparable to the chlorophyll *a* fluorescence in oxygenic phototrophs. Further deciphering fluorescence modulation in green sulfur bacteria might provide a much needed tool to elucidate the still enigmatic photosynthetic apparatus and physiology of green sulfur bacteria.

Acknowledgements

We would like to thank Govindjee for help with identifying relevant literature and valuable discussion of the manuscript. This research was supported by US

Department of Energy grant #DE-FG-02-04ER15550. Martin Hohmann-Marriott acknowledges support through the Jerome Aronson Fellowship from Arizona State University.

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