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ARTICLE

Fluorescence induction reveals organization of antenna and reaction center in photosynthetic bacteria

László Gerencsér, Péter Maróti*

Department of Biophysics, University of Szeged, Szeged, Hungary

ABSTRACT The photochemical phase of the bacteriochlorophyll fluorescence induction generated by rectangular shape of laser diode illumination was measured in different organization levels (whole cell, chromatophore and isolated reaction center protein) of carotenoidless mutant of purple photosynthetic bacterium *Rhodobacter sphaeroides* R-26.1. While the antenna containing species showed large and positive variable fluorescence (F_v) relative to the constant (initial) fluorescence (F_0) (F_v/F_0 ~ 4.5 in whole cell), the isolated RC had smaller and negative change (F_v/F_0 ~ -0.6). The variable fluorescence of the cells increased steadily in the function of the age of the cultivated bacterium: F_v/F_0 ~ 2 for young cells and F_v/F_0 ~ 4.5 for old cells while the rise time of the fluorescence induction remained constant (~ 2 ms). In chromatophore, 7 times higher rate was measured than in isolated reaction center under identical experimental conditions. The results obtained under different conditions are interpreted by an extended version of the Lavergne-Trissl model where the simultaneously measured fluorescence inductions from the antenna and the RC can be separately expressed.
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KEY WORDS

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Photosynthetic organisms convert the light energy of the sun to other forms of free energy manifested mainly by chemical energy of useful chemical products. The overall yield of the conversion is low. Based on the amount of carbon fixed by a field of corn during a typical growing season, only about 1 - 2% of the solar energy falling on the field is recovered as new photosynthetic products. The photosynthetic efficiency of uncultivated plant is even less, about 0.2%. In sugar cane, which is one of the most efficient plants, about 8% of the light absorbed by the plant is preserved as chemical energy. In contrast to the relatively low efficiency of the overall photosynthesis, the yield of the initial photophysical processes (capturing the light by antenna, funneling to the reaction center (RC) protein and primary charge separation) is high: for example, close to 100% of the excited (bacterio)chlorophyll dimer of the RC (P*) makes charge separation (Wraight and Clayton 1974). The efficient photophysics is due mainly to the optimal organization of the antenna system and the RC.

The photosynthetic apparatus of the bacterium *Rhodobacter (Rba.) sphaeroides* (wild type) consists of three membrane-bound pigment-protein complexes: light-harvesting complexes I (LH I or B875) and II (LH II or B800-850), and the RC, along with the associated components required for subsequent electron transport and energy transduction (Fig. 1). The pigment-protein complexes B875 and B800-850 harvest solar energy and funnel it to the RC. Approximately 14 B875 complexes surround and contact the RC whereas the B800-850 complexes are positioned around the B875-RC complex (Cogdell et al. 2003; Roszak et al. 2003). The B800-850 complex has a minimal unit consisting of three BChl molecules and two carotenoids noncovalently bound to two low-molecular-weight hydrophobic apoproteins labeled as α and β (Zuber and Brunisholz 1993). Two of the BChls are bound in close proximity to each other in the protein and are responsible for the absorption at 850 nm, while the remaining monomeric BChl is responsible for the absorption at 800 nm.

The LHII antenna complex (B850 complex) of the carotenoidless mutant of *Rba. sphaeroides* (R-26.1) is similar in polypeptide composition to the wild-type B800-850 complex, but lacks both carotenoids and the 800 nm absorbing bacteriochlorophyll. The optical absorption spectra of cellular suspension show the typical broad and intense absorption band at ~865 nm due to the overlapping of both LHI and LHII bacteriochlorophyll molecules (B875 and B850, respectively). At 800 nm, a weak absorption peak can be detected due to the RC bacteriochlorophyll monomers.

Although the components of the light harvesting system are tightly packed and energetically well coupled, small portion of the transferred energy is escaped in form of (bacterio)chlorophyll fluorescence. If the photosynthetic organism is exposed to sudden change from dark to light of constant intensity, complex kinetics of the (bacterio)chlorophyll

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*Corresponding author. E-mail: pmaroti@sol.cc.u-szeged.hu

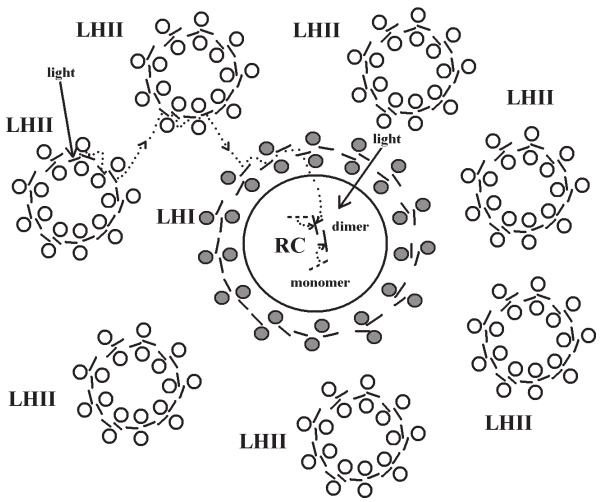

Figure 1. Schematic view of the structural arrangement of the bacterial photosynthetic light gathering complex in *Rba. sphaeroides* cells. The reaction center protein (RC) is surrounded by the core (LHI) and peripheral (LHII) light harvesting complexes. The porphyrine rings of the BChls are represented by short lines in RC (dimer, P and monomer (dashed line)), in LHI (B875) and in LHII (B800-850). The antenna BChls are sandwiched between the inner and outer arrays of the transmembrane α-helices (filled and open circles in LHI and LHII, respectively, end-on view). The complexes are shown in plane of membrane. Both the antenna and the RC pigments can be excited by light. The way of migration of the exciton from the place of absorption (antenna pigment and/or monomer in RC) to the dimer of the RC is indicated by dotted line. (Constructed after Koblizek et al. (2005).)

fluorescence (fluorescence induction) can be observed. The phenomenon was discovered by Müller (1874) and quantitatively studied for the first time by Kautsky and Hirsch (1931) in oxygenic phototrophs and by Vredenberg and Duysens (1963) in anoxygenic phototropic purple bacteria. It has become one of the most frequently used tools in photosynthesis research of green plants to study the organization of the light harvesting system and the molecular mechanisms of energy capture and conversion to chemical energy (Dau 1994; Govindjee 2004; Cser and Vass 2007). In comparison with chlorophyll fluorescence, the use of fluorescence induction of bacteriochlorophyll is much more limited, probably due to the widespread use of kinetic absorption spectrometers to monitor the redox reactions in photosynthetic bacteria (Osváth et al. 1996; Trissl 1996, 1999; Koblizek et al. 2005).

In this work, we measured kinetic traces of fluorescence induction of the carotenoidless mutant of *Rba. sphaeroides* (R-26.1) in different levels of organization (whole cells, chromatophores and isolated RCs) in the (sub)millisecond time range under rectangular shape of intense illumination. The variable fluorescence of the bacterium showed remarkable variation during cultivation. The simplest possible explanation includes simultaneous excitation and detection of fluorescence from both RC and antenna and variable energetic coupling between the components of the light gathering system. This view offers qualitative reasons for the observed widespread changes of the kinetics of bacteriochlorophyll fluorescence induction of the photosynthetic bacteria. By comparison of the rise times of the fluorescence induction in chromatophores and in isolated RC under otherwise identical conditions, the effect of antenna size and the absorption cross section parameters can be directly analyzed in the light harvesting process.

Materials and Methods

Whole cells

The cells of the carotenoidless mutant (R-26.1) of the photosynthetic bacterium *Rba. sphaeroides* were anaerobically cultivated in the light of four tungsten lamps (40 W) under 1.3 mW/cm^2 light intensity measured by Spectra Physics 404 power meter that had flat spectral response between 450 nm and 900 nm. The bacteria were harvested in different phases of the growth. The concentration of the cells (N) was determined either by counting the number of cells under microscope or by measuring the optical density (OD_{obs}) in a 1 cm cuvette at 535 nm (where the cell has minor absorption, therefore the scattering dominates). The following polynomial approach of calibration (Italiano 2007) was used:

$$OD_{obs}(535\ nm) = 0.0119 + 1.61 \cdot N - 0.548 \cdot N^2 + 0.0817 \cdot N^3$$

where N is normalized to 10^9 active cells/ml (that corresponds to $OD_{obs} = 1.16$).

Chromatophores

Chromatophores were obtained by mechanical rapture (sonication) of the cells harvested in the stationary phase of cell growth as previously described (Maróti and Wraight 1988). By this procedure, most of the water-soluble c type cytochromes were lost. The chromatophores were used immediately or kept at -14°C in a glycerol-containing (60:40 (v/v)) buffer (50 mM sodium glycyl-glycine (Fluka)) at pH 7.5. The absorption at 860 nm is the measure of the bacteriochlorophyll content in the chromatophore that can be determined spectroscopically following extraction in polar solvents (acetone/methanol, 7:2 v/v) and using an extinction coefficient of 75 $M^{-1}cm^{-1}$ at 775 nm.

Reaction center protein

The reaction center protein was yielded from chromatophore by standard procedure reported earlier (Maróti and Wraight 1988).

Fluorescence measurements

For recording the kinetics of the fluorescence upon rectan-

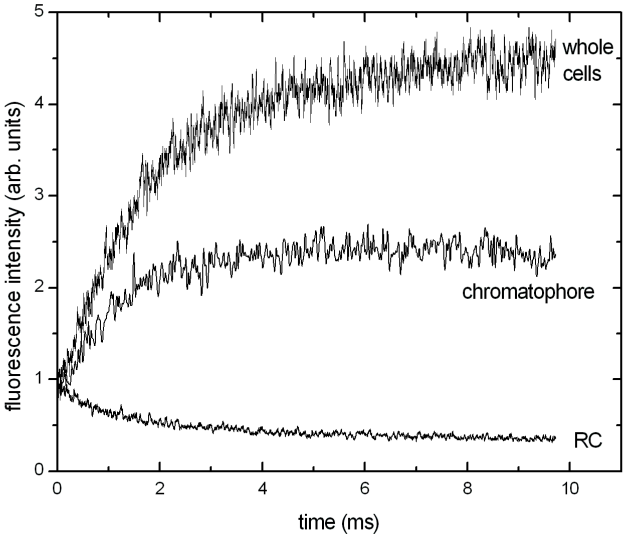

Figure 2. Typical fluorescence induction kinetics in whole cells, chromatophore and isolated RC. The fluorescence levels were normalized to the same initial values (F_0) and were excited by different light intensities to bring the kinetics to the same time scale.

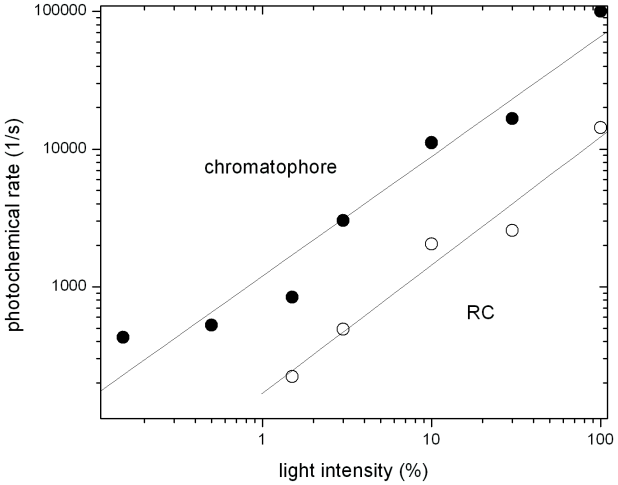

Figure 3. Comparison of the rates of the photochemical phase of the fluorescence induction excited by the same light intensities in chromatophores (●) and isolated RCs (○). The light intensities were attenuated by calibrated neutral density filters. In double logarithm representation, the slopes are 0.87 (chromatophore) and 0.93 (RC) and the rate is 7 times higher in chromatophore than in isolated RC under identical light intensity.

gular shape of illumination, we used a home-built spectrofluorometer with laser diode excitation (808 nm, maximum power 1 W) described previously (Osváth et al. 1996). The duration and intensity of the laser pulse could be adjusted arbitrarily. Fluorescence ($\lambda > 850$ nm) was detected at 90° with respect to the excitation light. The optical densities of the samples in the 1x1 cm cuvette were kept low (OD (865 nm) ~ 0.4) to minimize the secondary effects (scattering, re-absorption of BChl fluorescence, secondary fluorescence, etc.). Addition of 120 μM terbutryne (Chem Service) to RC or chromatophores blocked the electron transfer between the quinones and assured the transfer of only one electron to the acceptor quinone complex. The fluorescence kinetic traces were recorded after single illumination (duration 1-10 ms) at room temperature.

Results

After switching on the exciting light of constant intensity, the fluorescence of BChl *a in vitro* (in solution) appeared immediately and its intensity did not changed during the excitation. On the contrary, BChl *a*-containing photosynthetic organisms show complex time-courses of the fluorescence. The fastest and the more obvious phase of the fluorescence induction is the photochemical phase whose rise time is controlled by the exciting light intensity. We will concentrate here on this phase only. As BChl *a* pigments are major constituents of the bacterial RC and the light harvesting (antenna) system, one can expect different fluorescence induction kinetics in different organization levels of the bacterium. Indeed, we observed significantly different types of curves in isolated RC, membrane fragments (chromatophores) and in whole cells (Fig. 2).

One of the major differences is the direction of change of the fluorescence in whole cells (or chromatophore) and in isolated RC. While the fluorescence increases in the antenna-containing species, it decreases if the antenna is removed. There is a small spectral shift in fluorescence: the peaks of the fluorescence are at 890 nm and 920 nm for the antenna and the RC, respectively (De Klerk et al. 1969; Osváth et al. 1996). Significant difference is observed in the levels of variable fluorescence (F_v) relative to that of initial (constant) fluorescence (F_0): the ratio F_v/F_0 is high for whole cells (> 4) and much less (≈ 2.5) in chromatophore. As the kinetic traces were detected after single illumination, the signal-to-noise ratio is not high enough to make clear-cut distinction between the exponential and sigmoidal shape of the fluorescence induction in whole cells or chromatophores (Trissl 1996, 1999). The decay of fluorescence yield in isolated RC follows the exponential kinetics of charge separation (Osváth et al. 1996).

The photochemical nature of the fast phase of the fluorescence induction is demonstrated by determination of the rate of rise (chromatophore) or decay (RC) at different light intensities (Fig. 3). Attenuation of the exciting light intensity was achieved by calibrated neutral density filters. In double logarithm representation, straight lines with slopes close to 1.0 were obtained for both chromatophores and isolated RC indicating that only photochemical reactions determine the rise (chromatophore) and decay (RC) of the fluorescence in

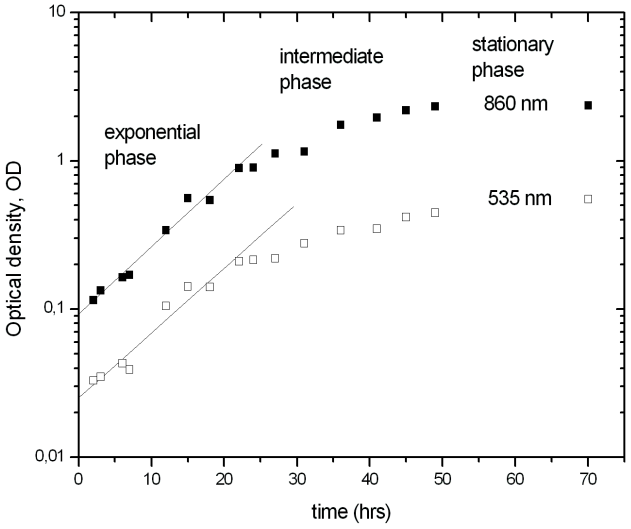

Figure 4. Growth curves of the culture. After inoculation of the cells, the culture is set to light. The optical densities measured in 1 cm cuvette at wavelengths of 860 nm and 535 nm are characteristics of the BChl content and cell population of the culture, respectively. The duplication time of culture in the exponential phase is 6 hrs. OD(535 nm) = 0.1 corresponds to $5.5 \cdot 10^7$ active cells/ml (see M&M).

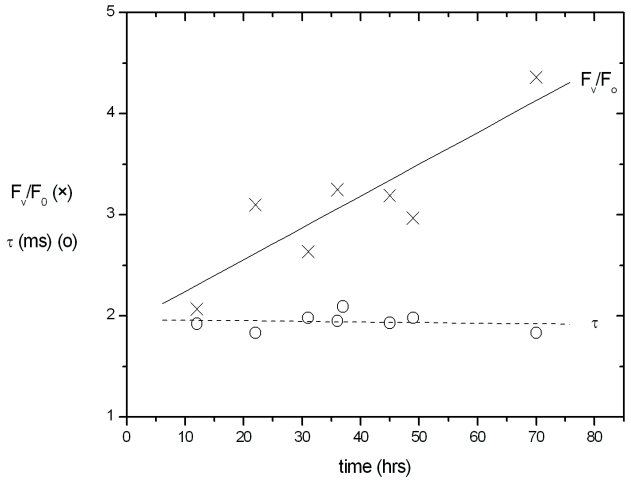

Figure 5. Ratio of the variable to initial fluorescence (F_v/F_0) and rise time (τ) of the fluorescence induction during growth of the culture.

this time range. As the conditions for excitation (λ = 808 nm) and observation (λ > 850 nm) were the same (there is only slight difference in spectral properties of the fluorescence from the antenna and the RC), the ratio of the observed rates should be characteristics of the antenna function. Under these conditions, the presence of the antenna complex in chromatophore assured 7 times higher rate of the photochemistry than in isolated RC.

The photochemical phase of the fluorescence induction is the signature of organization of BChl pigments in the photosynthetic apparatus. One of the most demonstrative examples is the change of fluorescence characteristics during assembly (Koblizek et al. 2005) and age (de Klerk et al. 1969) of the photosynthetic unit. To show these variations of numbers, connectivity and distribution of the pigments during cultivation of R-26.1 strain of *Rba. sphaeroides,* we present typical growth curve measured after inoculation of the cell into culture medium in photochemostat (Fig. 4). The cell population is proportional to the turbidity of the sample measured by the optical density (OD) at 535 nm. At this wavelength, the absorption is negligible and the observed optical density is mainly due to light scattering. After proper calibration, the value of OD(535 nm) can be used to determine the number of cells (see M&M). Similarly, the optical density at the peak of the absorption spectrum (860 nm) is the measure of the BChl-a content of the cells, and its concentration can be determined by extraction (see M&M). Three distinct phases can be defined. In the exponential phase, the cells are dividing at maximum rate (the doubling time is 6 hrs), in the intermediate phase, only a portion of the cell population is growing at maximum rate and in the stationary phase, the net growth rate is zero. As the two curves of OD(860) and OD(535) run parallel during the growth phases, the ratio of the amount of BChl-a synthesized and the number of cells remains almost constant. Similarly, the rate of the photochemical rise of the variable fluorescence does not show major change during the growth (Fig. 5). In contrary, striking increase in the ratio of variable to constant fluorescence is observed. The older are the cells the greater is the ratio. While F_v/F_0 is only 2 in younger cells, it increases above 4.5 in older cells. However, care should be taken to assign the increase of F_v/F_0 solely to the age of the bacterial cells. Alternative effect of decreasing exciting light intensity may have its role. As the cells are growing under constant light conditions and the culture becomes more dense in the intermediate and stationary phases, the intensity of exciting light reaching a given cell decreases. It has been long known that decreased light intensity can increase the BChl-a synthesis and can modify the distribution of the pigments (Cohen-Bazire et al. 1956).

Discussion

Generally, the reason of the studies of the fluorescence induction is to translate the observed characteristics of the kinetics into physiological parameters. The theory of fluorescence induction of antenna containing species (mainly for PSII) has been elaborated in great details including two major transitions of the RC: photochemistry ($PQ \rightarrow P^+Q^-$), and radical-pair mechanism ($P^* \leftrightarrow P^+H^-$) and restricted transfer of excitation energy from closed RC to an open RC (Lavergne and Trissl 1995). Based on this sophisticated theory, the functional absorption cross-section and connectivity of the photosynthetic complexes (see the shape of the induction curve), the

quantum yield of primary charge separation (see F_v/F_0) and the efficiency of light harvesting by the photosynthetic units (see the rate of the rise) can be deduced. In the presence of antenna, its fluorescence will dominate the observed signal as the absorption cross section (size) of the antenna is much larger than that of the RC. In these cases, the open RC serves only as trap for the excitons in the antenna pigment bed and the fluorescence from RC is not detected.

Here, we try to extend the simplified version of the Lavergne-Trissl model by consideration of the contribution of the fluorescence originating from the RC. It can have not only theoretical but also practical significance in weakly coupled system of RC and antenna, where the two components of the observed fluorescence induction may be commeasurable. Based on this extended model, reasonable explanation can be given to fluorescence changes observed in our experiments. Only qualitative view will be given but it can be treated in quantitative terms, as well.

The exciting photon can be absorbed both by one of the antenna BChl molecules and by the RC itself determined by their absorption cross sections (Fig. 1). The excited state (exciton) in the antenna is rapidly distributed in the pigment bed and finally finds the way to the open RC (redox pigment state is PQ_A) that becomes closed ($P^+Q_A^-$). The trap will be similarly closed if the photon is directly absorbed by the RC. These processes are accompanied by loss of the excited states by different other mechanisms including fluorescence. The observed variable fluorescence originates both from the RC and from the antenna and reflects the redox status of the RC.

In accordance with Figure 2, the antenna fluorescence is the inverse process of the trapping mechanism: initially when the photochemical trapping is most effective, the fluorescence is low (F_0). Later, when the trap disappears, the fluorescence is high (F_M). The rise time of the fluorescence increase depends on the effectiveness of the light harvesting and the extent of the rise (F_M/F_0) on the coupling of the light harvesting pigments to the RC. Both quantities could be modified in our experiments.

In intact cells, we measured larger variable fluorescence than in chromatophores (Fig. 2). During mechanical fracture of the cells and break of the natural membranes into fragments (chromatophore), the connection of the light harvesting system is impaired and the open RC became less effective trap for the excitons in the antenna. As the role of competitive processes (including also fluorescence) increased in comparison to photochemistry, the constant fluorescence (originating from detached pigments of the antenna) enhanced in expense of the variable fluorescence.

Similar changes were observed during aging of the cells. The BChl pigments might have severe re-distribution from young to old cells as the old cells showed significantly larger fluorescence induction (Fig. 5). In young cells, the pigments are arranged in loose structures that can be visualized by loosely attached and/or not properly well developed LHII units around the core complex (Koblizek et al. 2005). In old cells, the light harvesting complex becomes more compact assuring effective transfer of electronic excitation energy to the open RC. This process is accelerated not only by age of the cell but the decrease of the internal light intensity, as well.

Contrary to the relatively simple picture of the antenna fluorescence, the temporal change of the RC fluorescence is more difficult (Fig. 2) and less elaborated (Osváth et al. 1996). The observed fluorescence can arise from one BChl dimer (P), two BChl monomers and some residual (unknown) pigments left after protein purification. Initially, the RC is open and the dimer is ready to absorb photon and emit fluorescence. However, if the RC becomes closed ($P^+Q_A^-$), the dimer cannot be excited anymore and cannot emit fluorescence. The simple drop of the RC fluorescence during closure of the RC is modified by the BChl monomers in the RC. They contribute to the more efficient utilization of the photon (open RC) and to the residual RC fluorescence (closed RC). If they absorb the photon, they would transfer the electronic excitation energy to the closely placed dimer of the open RC. Thus, the monomers increase the absorption cross section of the RC. However, if the RC is closed, they emit the electronic excitation energy in form of fluorescence whose quantum yield depends on the redox states of the nearby cofactors (P, Q_A and Q_B, see Osváth et al. 1996). The fluorescence intensities from the dimer (P) and the BChl monomers in $P^+Q_A^-$ redox state of the RC are almost the same (Fig. 2).

The antenna fluorescence is usually much more intense than the RC fluorescence due mainly to two reasons: 1) the antenna has about 100 times more BChl pigments than the RC and 2) the quantum yield of the BChl fluorescence in the antenna (1-2%, van Grondelle and Duysens 1980, but $1 \cdot 10^{-3}$, Borisov and Godik 1972) is much larger than in the RC ($4 \cdot 10^{-4}$, Zankel et al. 1968). The difference is due to the effectiveness of the photochemical trap in the two cases. If the absorption cross section of the antenna is artificially decreased then commeasurable fluorescence can be observed from the antenna and the RC. In our experiments, the wavelength of the laser diode excitation was 808 nm that matched the BChl monomers of the RC but not the LHII of R-26.1 that lacked the B800 pigment. Under this condition, the photochemical rise of the fluorescence induction of the chromatophore was only 7 times faster than in isolated RC. The large number of pigments in the antenna was compensated by the favorable excitation of the RC. The positive outcome of this experiment encourages us to use this method to lower the fluorescence level of the antenna close to that of the RC and to detect the simultaneous fluorescence induction from the antenna and the RC. This will assure firm experimental background to test the quantitative consequences of the extended Lavergne-Trissl model.

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