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Demonstration of phycobilisome mobility by the time- and space-correlated fluorescence imaging of a cyanobacterial cell

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Abstract

The cell-wide mobility of PBSs was confirmed by synchronously monitoring the fluorescence recovery after photobleaching (FRAP) and the fluorescence loss in photobleaching (FLIP). On the other hand, a fluorescence recovery was still observed even if PBSs were immobile (PBSs fixed on the membranes by betaine and isolated PBSs fixed on the agar plate) or PBS mobility was unobservable (cell wholly bleached). Furthermore, it was proved that some artificial factors were involved not only in FRAP but also in FLIP, including renaturation of the reversibly denatured proteins, laser scanning-induced fluorescence loss and photo-damage to the cell. With consideration of the fast renaturation component in fluorescence recovery, the diffusion coefficient was estimated to be tenfold smaller than that without the component. Moreover, it was observed that the fluorescence intensity on the bleached area was always lower than that on the non-bleached area, even after 20 min, while it should be equal if PBSs were mobile freely. Based on the increasing proportion of the PBSs anti-washed to Triton X-100 (1%) with prolonged laser irradiation to the cells locked in light state 1 by PBQ, it was concluded that some PBSs became immobile due to photo-linking to PSII.

Keywords: Mobility; Phycobilisome; FRAP; FLIP; Photosystem I; Photosystem II

1. Introduction

To keep the excitation energy distribution between the photosystem I (PSI) and the photosystem II (PSII) in balance is one of the most important factors for photosynthetic efficiency of green plants and cyanobacteria, while how to regulate the energy distribution is related to the light state transition mechanism. In green plants, the major light-harvesting complex (LHCII) in the membrane can shuttle between PSI and PSII to balance the distribution of the excitation energy [1,2]. Similarly, it is believed that phycobilisome (PBS), the light-harvesting complex attached to the stromal surface of the thylakoid membrane in cyanobacteria, could transfer the excitation energy to PSI and PSII alternatively [3–5], although other possibilities have been proposed [6–9]. Recently, PBS mobility was proved to be a prerequisite for light state transition [10,11], while it had never been experimentally observed until 1997 when the fluorescence recovery of PBSs on the photobleached area of a cyanobacterial cell was observed by the use of a technique called FRAP (fluorescence recovery after photobleaching), from which the time-related shallowing and widening of the bleaching profile, ascribed to PBS mobility, was clearly observed [12]. However, later, the fluorescence recovery was suspected of some artificial results caused by the high-energy laser [13]. In fact, the phycobiliproteins in PBSs, also known as a kind of photosensitizers, could photosensitively produce highly reactive oxygen species or free radicals [14–16], which in turn may induce successive photochemical processes.

Abbreviations: PBS, phycobilisome; PSI, photosystem I; PSII, photosystem II; LHCII, light-harvesting complex II; FRAP, fluorescence recovery after photobleaching; FLIP, fluorescence loss in photobleaching; PBQ, phenyl-1, 4-benzoquinone

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Therefore, some complexities may be involved in FRAP. Alternatively, the fluorescence loss in photobleaching (FLIP) could be used to monitor the synchronous fluorescence loss on the non-bleached region [17], which would certainly demonstrate PBS mobility even if FRAP itself might be suspected of any artifact. In this work, by jointly using FRAP and FLIP, a cell-wide mobility of PBSs was definitely demonstrated in a cyanobacterium *Thermosynechococcus elongates* cell (about 1.5 μm across and 8 μm long). It was also found that some artificial factors induced by the full-power laser were involved in both FRAP and FLIP, i.e., the renaturation of the reversibly-denatured proteins contributing to the fluorescence recovery and the laser-scanning to the fluorescence loss. Furthermore, under prolonged irradiation to the cells by the full-power laser, photo-crosslink of PBSs, most likely to PSII, was observed.

2. Materials and methods

2.1. Cell culture and sample preparation

Cells of the thermophilic cyanobacterium *Thermosynechococcus elongatus* were grown in liquid culture in BG-11 medium and bubbled with 5% CO_2 in air [18]. Cells were grown at 45 °C under illumination with white fluorescence lamp at an intensity of 50 μmol m^{-2} s^{-1}. The cells harvested at the middle-logarithmic phase of growth were used for the experiments. Cells were pre-illuminated with blue light (to state 1) or orange light (to state 2) and then glycine betaine was added to a final concentration of 0.5 M (to lock the cells in the pre-induced states). For observation on the laser confocal fluorescence microscopy, the cells were immobilized onto 1.5% (w/w) agar plates containing growth medium or 0.5 M betaine buffer (for the betaine-treated cells) on a slide covered with a glass slip and located under objective lens. For measurements of the diffusion coefficients at different temperatures, the samples were placed on a temperature-controlled holder connected with circulated water bath to keep the samples at 25 °C and 45 °C respectively.

Phycobilisomes were isolated from the cells as reported previously [19] with some modifications. The cells (1 g) suspended in 10 ml 0.75 M K-phosphate (pH 7.0) buffer were ultrasonically broken in ice bath. Triton X-100 was immediately added to the broken cells to a final concentration of 2% (w/w), and the mixture was incubated for 20 min with stirring at room temperature. Large fragments and cell debris were removed by centrifugation at 25,000×*g* for 30 min. Medium supernatant samples were collected by centrifugation twice for 30 min at 50,000×*g*. PBSs were concentrated by further centrifugation at 250,000×*g* for 2 h. Isolated PBSs were immobilized as described above for observation on the laser confocal fluorescence microscopy at room temperature (25 °C).

2.2. FRAP-FLIP Measurements

Olympus FV500 laser scanning confocal fluorescence microscope with a 60× oil-immersion objective lens was employed with a He–Ne red laser (633 nm, 10 mW) for photobleaching PBSs selectively. The laser light was passed through a 100-μm pinhole and focused onto the sample with a 60× oil-immersion objective lens. The vertical (*Z*) resolution was 0.27 μm and the resolution in the *x*–*y* plane was 0.20 μm. Fluorescence emission from the sample was separated from the excitation light (633 nm) by a 660 nm long-pass filter, passing through a 100-μm pinhole and detected by a cooled photomultiplier. The fluorescence image processing was implemented in MATLAB (MathWorks, Inc.). For synchronous measurements of FRAP and FLIP, a 1-μm-wide region at one end of cell was photobleached across the long axis of the cell and through the entire depth with 100% power laser for 12.5 s, and then the whole cell was imaged with 1% power laser, with that before photobleaching as a control. The images were recorded at 3-s intervals in the first 300 s and at 30-s intervals after then. The photobleaching at one end of the cell instead of that in the middle was for better observation of FRAP and FLIP.

2.3. FRAP measurements for determination of diffusion coefficient

For determination of diffusion coefficients, a 1-μm-wide strip in the middle of the cell was selectively bleached with 100% power laser for 1.2 s. Then, the fluorescence images were recorded at 3-s intervals with 1% power laser until 300 s, with the image recorded before photobleaching as a control. The width of the strip was verified by optical parameters. A cell was wholly photobleached by 100% power laser across the long axis of the cell line by line repeated 5, 15, 30 or 50 times to reach a 50%, 70%, 85% or 95% bleach depth respectively. Recovery of fluorescence was monitored by scanning the cell with 1% power laser at 3-s intervals until the fluorescence intensity reached a plateau.

2.4. Calculation of the diffusion coefficient

The lateral diffusion coefficient of PBSs was estimated by analysis of the recovery profile. The fluorescence recovery of the photobleached strip was fitted by Eq. (1), an empirical formula agrees within 5% with the solution of the diffusion equation in one dimension [20], while Eq. (2) contains both of the diffusion term and an exponential one.

$$I_{(t)} = I_{\text{final}}\left(1 - w^2(w^2 + 4\pi Dt)^{-1}\right)^{1/2} \quad (1)$$

$$I_{(t)} = I_r(1 - e^{-t/\tau}) + (I_{\text{final}} - I_r)\left(1 - w^2(w^2 + 4\pi Dt)^{-1}\right)^{1/2} \quad (2)$$

here, $I_{(t)}$ is the intensity as time *t*, with the time zero defined as the midpoint of the photobleaching; I_r and $(I_{\text{final}} - I_r)$ are the amplitudes of the two terms respectively with I_{final} defined as the maximal intensity reached after recovery; τ is the lifetime of the exponential term; *w* is the strip width and *D* is effective diffusion coefficient. For determination of the diffusion coefficients, "non-linear curving fitting" program in Origin software was used to fit the fluorescence intensities data to Eqs. (1) or (2).

2.5. Photo-crosslink of PBSs

Cells suspended in growth medium in the presence of PBQ (150 μM) were exposed to intense laser (635 nm, 100 mW) for 10, 30, 60, 90 and 120 min. The PBS-thylakoid membrane complexes were isolated from unexposed and exposed cells and washed by Triton X-100 according to the previously reported method [11]. Absorption spectra were recorded on a UV-1601 ultra-vis spectrophotometer (Hitachi, Japan) before and after the laser irradiation.

3. Results and discussion

3.1. Cell-wide mobility of PBSs

To observe FRAP and FLIP at the same time, the cell-wide fluorescence images were recorded from the time immediately after photobleaching to about 20 min. Fig. 1 shows the

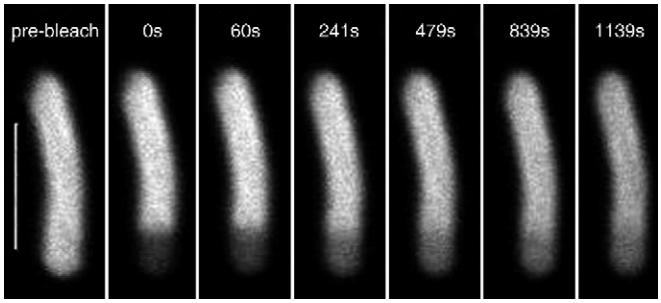

Fig. 1. The fluorescence images for a cell of thermophilic cyanobacterium *Thermosynechococcus elongatus* at selected times before and immediately after photobleaching of PBSs on one end of the cell. Scale bar: 5 μm.

fluorescence images of a cell at a series of selected time with that before bleaching as a control, from which the fluorescence intensity as a function of time and distance to the photobleached area was derived and shown in Fig. 2. Figs. 1 and 2 demonstrate a synchronous fluorescence loss on the non-bleached area with the fluorescence recovery on the bleached area, suggesting a cell-wide mobility of phycobilisomes.

Based on the fluorescence images of the cell, the plots of the fluorescence intensities on the cell to the time and to the distance along the long axis of the cell are shown in Fig. 3A and B respectively. Fig. 3 demonstrates that the fluorescence intensity on the bleached area increases mainly before 300 s but becomes nearly invariable after then, while the fluorescence intensity on the non-bleached area decreases continuously on the whole time scale. Apparently, the fluorescence loss after 300 s could not be ascribed to PBS mobility, implying more complexities involved in FRAP and FLIP.

Fig. 4A selectively shows those in Fig. 3B before 300 s, from which the total fluorescence yield of the cell was derived, as shown in Fig. 4B. The invariable fluorescence yield before 300 s suggests that the fluorescence recovery on the bleached area is just enough to compensate the fluorescence loss on the non-bleached area, which could be simply explained as PBS mobility but may also be a coincidence, as proved below.

3.2. Artificial contributions involved in the fluorescence recovery and loss

As mentioned above, the fluorescence loss on the non-bleached area from 300 s to 1200 s should not be ascribed to PBS mobility for the fluorescence intensity on the bleached area kept invariable at the period of time, but might be related to the repeated scanning with 1% power laser during the measurement. To clarify that, plots of cell-wide averaged fluorescence intensity for a cell without pre-photobleaching to the times of scanning and to the time are shown in Fig. 5A and B respectively. It was confirmed that the laser scanning-induced fluorescence loss did occur and was linearly proportional to the

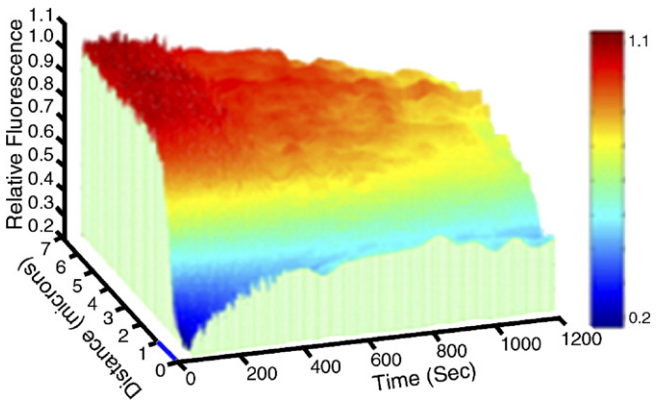

Fig. 2. The phycobilisome fluorescence intensities for a cell of thermophilic cyanobacterium *Thermosynechococcus elongatus* versus time and distance from the bleached region after selective photobleaching the PBSs on one end of the cell. Blue bar: bleached region. Color bar: relative fluorescence intensity. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

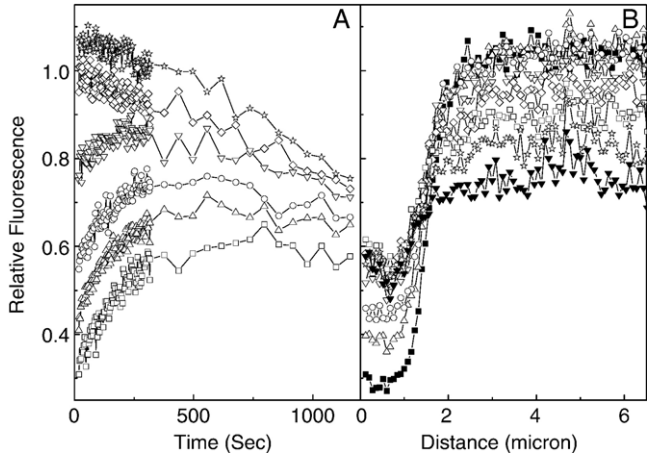

Fig. 3. (A) The plots of the fluorescence intensities versus time for those regions with the distance of 0.6 μm (△), 0.7 μm (○), 1.0 μm (▽), 1.5 μm (◇) and 6.0 μm (☆) to the bleached region (□). (B) Plots of the fluorescence intensities versus the distances along the long axis of cell at $t=0$ (■), $t=60$ s (△), $t=120$ s (○), $t=241$ s (▽), $t=479$ s (◇), $t=720$ s (□), $t=960$ s (☆), $t=1139$ s (▼) after photobleaching of PBSs on one end of the cell.

scanning times. Furthermore, the fluorescence loss caused by the laser scanning mainly occurred in the first 300 s (Fig. 5B) for the scanning interval was only 3 s which was used to obtain a precise estimation of the diffusion coefficient [21,22]. However, compared the two curves in Fig. 5, it can be seen that the laser scanning-induced fluorescence loss is still not enough to count for that in a pre-photobleached cell. Furthermore, the fluorescence loss for the latter is linearly proportional to the time (Fig. 5B) but not to the scanning times (Fig. 5A), suggesting some intrinsic processes in the photo-bleached cell. It should be noticed that the only difference between the latter and the former is with or without pre-photobleaching, therefore, one reasonable deduction is that the additional fluorescence loss for the latter might be ascribed to photo-damage of the cell or some reactive photochemical products diffused into the non-bleached area to induce some successive reactions [23].

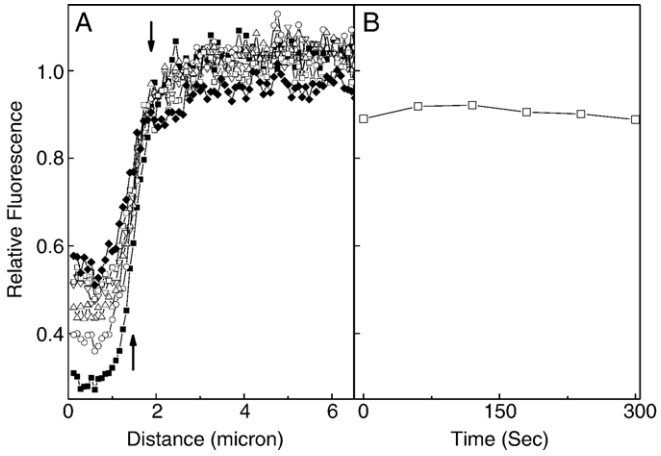

Fig. 4. (A) The profiles of fluorescence intensity for a cell of thermophilic cyanobacterium *Thermosynechococcus elongates* at various time after photobleaching at one end of the cell. $t=0$ (■), $t=60$ s (○), $t=120$ s (△), $t=180$ s (▽), $t=240$ s (□), $t=300$ s (♦). (B) The average fluorescence intensities over all the pixels of the cell to the time above were plotted.

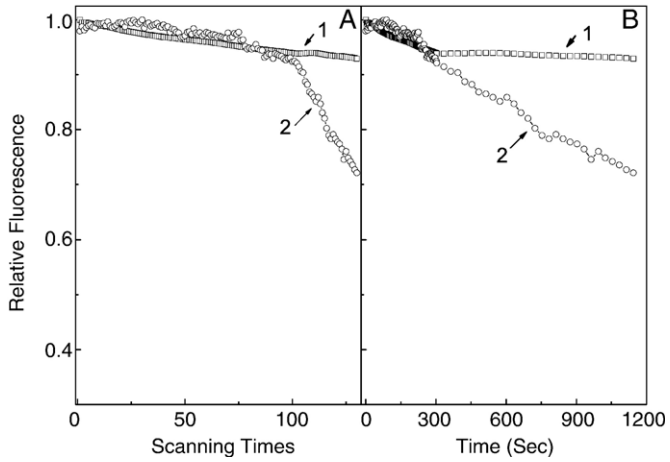

Fig. 5. The fluorescence intensities for the cells of thermophilic cyanobacterium *Thermosynechococcus elongates* plotted to the scanning times (A) and time (B). The cells were scanned at 3-s intervals in the first 300 s and 30-s intervals till 1200 s by 1% power laser. The fluorescence intensities were averaged over all the pixels of the cells without pre-photobleaching (□), and over the non-bleached region of the cell with pre-photobleaching on the opposite distal region of 1 μm (○). The curve 1 (open circle) was normalized to the curve 2 (open square).

The nearly invariable fluorescence yield of the cell on the first 300 s (Fig. 4B) is obviously contradictious to the laser scanning-induced bleaching (Fig. 5), implying that some other processes may occur synchronously to compensate the scanning-induced fluorescence loss. In other words, the fluorescence recovery on the bleached area may not completely from PBS diffusion. To search for whether the fluorescence recovery on the photobleached area involves any artificial factor, the fluorescence recovery was measured for a locally photobleached cell pretreated with betaine and the isolated PBSs fixed firmly onto agar plate, as shown in Fig. 6A, and also the cell wholly photobleached to various bleach depths, as shown in Fig. 6B from which a plot of the recovery percentage to bleach depth was shown in Fig. 6C.

It was observed previously that betaine could fix PBSs firmly on the thylakoid membrane in the cyanobacterium *Spirulina platensis* [10,24], so did in the thermophilic cyanobacterium *Thermosynechococcus elongates* (data not shown). Apparently, the PBS mobility should not be observable for the wholly photobleached cells, neither for the isolated PBSs fixed on agar nor for the cells pretreated with betaine, however, a fast fluorescence recovery could be observed for all of the cases. It was known that excess light energy could cause denaturation of proteins [25], while the denaturation extent, roughly denatured permanently and temporarily or reversibly, must depend on the illumination dosage (or illumination time). In this case, the reversibly denatured proteins may undergo automatic renaturation. The fast recovery component possesses an invariable time constant but variable amplitude in inverse proportion to the illumination time, as shown in Fig. 6B and C, suggesting that prolonged illumination would make more proteins denatured permanently. It was reported that the renaturation process could be fitted by single exponential [26]. The recovery in Fig. 6A and B was fitted with a time constant of about 5±1 s.

It was reported that phycobiliproteins in PBSs could photosensitively produce free radicals or reactive oxygen species under illumination [15,16]. In principle, the photobleaching of PBSs is due to a configuration transformation of the tetrapyrrole chromophores from the open-chain one into the cyclohelical one [27], which is originated from the conformational changes of the apoproteins. Some of the conformational changes may be temporary and reversible [25,28,29], which would recover via the renaturation of the proteins. On the other hand, any cleavage of the covalent bonds in the pigments (tetrapyrroles) or the proteins should be irreversible but might recover via biological repairing [30]. In fact, the laser dose-dependent fluorescence recovery in Fig. 6B well suggests that both of the reversible and irreversible denaturation was involved in the photobleaching.

Based on the considerations above, the fluorescence recovery (F_R) on the bleached area should include three parts of contributions, i.e., PBS mobility (F_M), renaturation (F_A) and scanning-induced bleaching—the minus term (F_S). Quantitatively, it can be described as $F_R = F_M + F_A - F_S$. The fluorescence recovery on the bleached area at the 300 s was 22.7% estimated from Fig. 4A while that owing to the protein renaturation under the same bleach depth was 11% from Fig. 6C, therefore 11.7% of the fluorescence recovery could be ascribed to PBS diffusion for the scanning-induced bleaching terms canceled out by the subtraction. Similarly, the averaged fluorescence loss on the non-bleached area at the 300 s was 9% estimated from Fig. 4A, contributed by both PBS diffusion and laser scanning. For the fluorescence loss was nearly the same (5%) at the first 300 s no matter under a case with or without pre-photobleaching (Fig. 5), the fluorescence loss owing to diffusion was about 4%. Further, the non-bleached area was estimated as large as 3 times of the bleached one from Fig. 4A, therefore, the total fluorescence loss on the non-bleached area was about 12%, nearly equal to the PBS-diffusion-dependent fluorescence recovery on the bleached area. Therefore, no

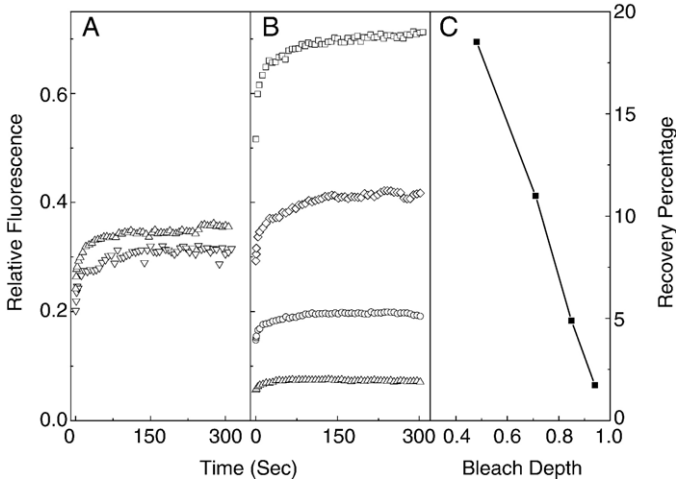

Fig. 6. (A) The fluorescence recovery on the photobleached area of a cell treated with betaine (△), and the isolated PBSs fixed on the agar (▽). (B) The fluorescence recovery of the cell wholly photobleached with deferent bleach depth: 50% (□), 70% (◇), 85% (○), and 95% (△). (C) The recovery percentage versus the bleach depth derived from graph B.

matter the complexities were involved in the fluorescence recovery, the PBS mobility was confirmed. On the other hand, the fluorescence recovery on the bleached area should contain the contributions not only by PBS mobility but also by renaturation of the reversibly denatured proteins.

3.3. Determination of the diffusion coefficient

To estimate the diffusion coefficient, a strip of 1-μm wide was selectively photobleached at the middle of a cell to a bleach depth of about 70%, and then the fluorescence recovery was monitored from the time zero (the time immediately after 1.2 s photobleaching) to 300 s, as shown in Fig. 7. Fitting the fluorescence recovery data to Eq. (1), a diffusion coefficient of $(1.7\pm0.4)\times10^{-10}$ cm^2 s^{-1} was derived, while, fitting the data to Eq. (2), a diffusion coefficient of $(2.5\pm1.2)\times10^{-11}$ cm^2 s^{-1} was derived. It can be seen that the difference of the diffusion coefficients is as large as nearly tenfold due to, with or without, consideration of the fast recovery component. Besides, it can also be seen that the two components are necessary for a good fitting of the data.

Because thermophilic cyanobacterium *Thermosynechococcus elongatus* was cultured at 45 °C, it may be argued that the diffusion coefficient determined at room temperature (25 °C) may not reflect the PBS mobility at the culture temperature. In fact, it was reported previously that the PBS mobility was temperature-independent from 10 °C to 40 °C for another cyanobacterium [21]. Anyway, the diffusion coefficients of PBSs in *Thermosynechococcus elongates* cells were determined at both 25 °C and 45 °C based on the averaged fluorescence recovery on a strip of 0.2-μm width in the middle of the cell after 1.2-s photobleaching (to a bleach depth of 50%). Fitting the two groups of data in Fig. 8 to Eq. (2) separately, the diffusion coefficients of $(1.4\pm0.6)\times10^{-11}$ cm^2 s^{-1} at 25 °C and $(1.8\pm1)\times10^{-11}$ cm^2 s^{-1} at 45 °C were derived, confirming that PBS mobility was temperature-independent. It should be

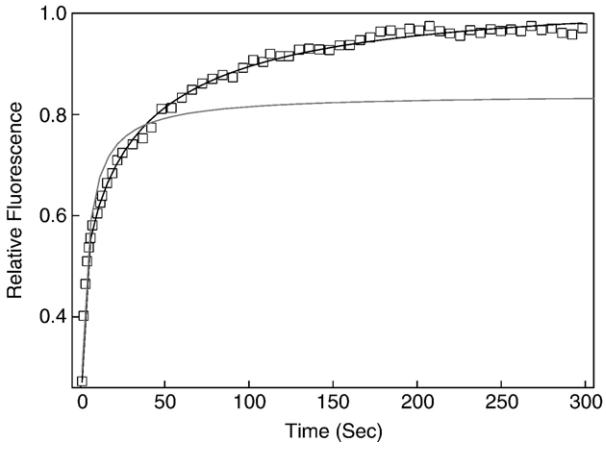

Fig. 7. Curve fitting to the fluorescence recovery on the 1-μm-wide strip in the middle of a cell after photobleaching. Fluorescence recovery data were marked by open squares; curve fitted of Eq. (1) (gray line) and Eq. (2) (black line) to the experimental data displayed kinetics allowing the determination of a single diffusion coefficient for PBSs. The standard deviations are given from a mean of $n=6$ independent determinations of different cells with 70–80% bleach depth.

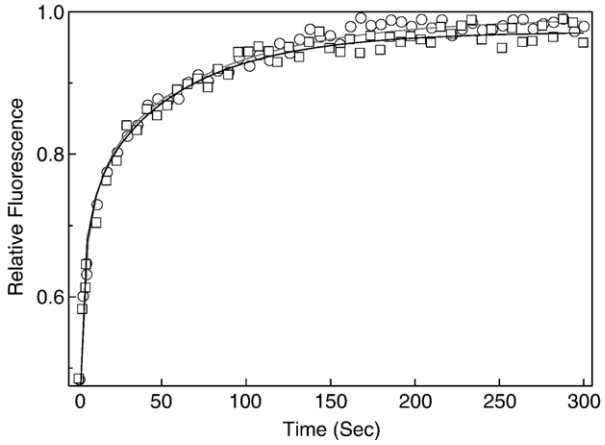

Fig. 8. Curve fitting to the fluorescence recoveries on the 0.2-μm-wide strip in the middle of cell after photobleaching. Fluorescence recovery data were monitored at 25 °C(□) and at 45 °C(○); Curve fitted of Eq. (2) to the experimental data monitored at 25 °C (black line) and to that at 45 °C (gray line) displayed kinetics allowing the determination of the single diffusion coefficients for PBSs. The standard deviations are given from a mean of $n=6$ independent determinations of different cells with 50% bleach depth.

indicated that estimation of the diffusion coefficients involves some approximation and the laser-scanning-induced fluorescence loss may introduce additional error (5%), however, the comparable diffusion coefficients suggest that the approximations are acceptable.

3.4. The "permanent bleaching trance"

From Fig. 3B and Fig. 4A, it can be seen that the fluorescence intensity on the photobleached area is always lower than that on the non-bleached area, i.e., a "permanent bleaching trace" caused by the full-power laser. Estimated by the diffusion coefficient, the distribution of PBSs, also the fluorescence intensity should have been cell-wide equal at 300 s if PBS diffusion were completely free. The "permanent bleaching trace" may lead to deduction that some photobleached residues of PBSs became immobile due to photocrosslink to the proteins in the membrane. In fact, intermolecular crosslink is a common photochemical reaction for proteins [31,32]. As an alternative, it may be argued that some phycobilisomes are intrinsically immobile. Anyway, in that case, the "permanent bleaching trance" should have been independent of bleaching dosage. However, the 12.5 s photobleaching (Figs. 3B and 4A) produced a more significant immobile fraction than the 1.2 s one (Fig. 7), suggesting that PBSs are not intrinsically immobile. It is well known that PBSs could be washed off from the thylakoid membrane by detergents [10,19] while not if they are photo-linked to some membrane proteins. For FRAP measurement, only one cell is the photobleaching focused to, anyway, one cell is not enough for any biochemistry analysis. As an alternative, 10 ml suspension of the cells in culture medium under stirring was irradiated by a laser of 635 nm (100 mW) for minutes to hours, unfortunately, all of the PBSs was washed off even prolonging the irradiation to 2 h. It is known that 635 nm light excites mainly PBSs so

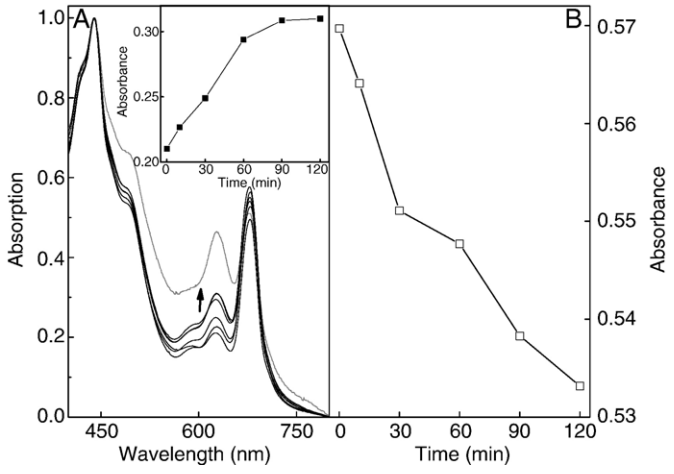

Fig. 9. (A) The absorption spectra of the PBS-thylakoid membrane complexes isolated from the cells irradiated by 635 nm laser for different time from 0 to 120 min and further washed by Triton X-100 (1%) (black lines), from which a plot of the absorbance to irradiation time was derived and shown in the inset. The gray line is for the control. (B) The absorbance of PBSs in cells versus the irradiation time.

leads to state 2 at which PBSs become detached from PSII but are not structurally connecting with PSI [10,24,33]. Therefore, the current result is a further confirmation for the previously proposed model [11]. Indeed, to the cells locked in the light sate 1 by PBQ [34,35], the anti-washed PBSs became more and more with prolonging the irradiation by a 635 nm laser (100 mW), as shown by the absorbance in Fig. 9A.

From Fig. 9A, it can be seen that without irradiation nearly all of the PBSs are washed off while the anti-washed PBSs are proportional to the irradiation time before 1 h but then reached "saturation" from 1 to 2 h. In fact, laser irradiation could cause two effects at the same time, i.e., the photo-linking (Fig. 9A) and the photobleaching (Fig. 9B). Consequently, it can be concluded that the photo-linking did occur during the prolonged photobleaching, and PBSs would be specifically photo-linked to PSII. As mentioned above, the photo-linking occurred under 12.5 s photobleaching to a certain cell but did not under 2-h irradiation to a large volume of cells even though the laser power for the latter was high (100 mW). For cyanobacterium, generally, orange light induces cells to light state 2 while blue light to state 1, however, it was also reported that state 1 could be achieved by prolonging exposure to high orange light [36], which is understandable for further orange-light illumination to a cell at the state 2 which may drive PBSs back to PSII (toward state 1). Besides, the time to achieve a light state would certainly depend on the illumination time and intensity. Therefore, during photobleaching, that the full-power laser was focused to a certain area of a cell might drive the cell into state 1 during the 12.5-s illumination, which in turn led to a photo-linking. On the other hand, during the irradiation to a large volume of cells under stirring, although the irradiation time to the cells was as long as 2 h, but to a certain cell it was not long enough to lead to state 1, that is why the photo-linking would not occur under the latter case. It should be indicated that the conclusion of PBSs photo-linking specifically to PSII is derived due to what occurred specially at the PBQ-fixed state 1, however, to prove it, further intensive researches are necessary to obtain the molecular details of the photo-linked products.

4. Conclusions

Firstly, a cell-wide mobility of PBSs was definitely demonstrated in cyanobacterial cells by synchronous monitoring FRAP and FLIP. Secondly, the fluorescence recovery of the photobleached area did involve an artificial factor induced by the full-power laser, which was ascribed to the renaturation of reversibly denatured proteins but not to the diffusion of PBSs. The diffusion coefficients estimated with or without consideration of the contribution by the renaturation are $(2.5\pm1.2)\times10^{-11}$ cm^2 s^{-1} and $(1.7\pm0.4)\times10^{-10}$ cm^2 s^{-1}, i.e., a nearly tenfold difference, suggesting the contribution to the fluorescence recovery is not negligible. Thirdly, besides PBS mobility, the fluorescence loss on the non-bleached area also involved artificial factors ascribed to the laser scanning and photo-damage to the cell. Fourthly, the "permanent bleaching trace" on the photobleached area after 20 min suggested that some photobleached residues of PBSs became immobile probably due to a photo-linkage to the proteins in the membrane, most likely PSII, which was further confirmed by the fact that more PBSs became anti-washed to detergent with prolonging the irradiation time.

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