Photophysical properties of *Prochlorococcus marinus* SS120 divinyl chlorophylls and phycoerythrin in vitro and in vivo

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Abstract ***Prochlorococcus marinus*** **SS120 is an ecologically important and biochemically intriguing marine cyanobacterium. In addition to divinyl chlorophylls (DV-Chls)** ***a*** **and** ***b*** **it possesses a particular form of phycoerythrin (PE), but no other phycobilins and therefore no complete phycobilisomes. Here, a spectroscopic characterisation of these DV-Chls and PE is provided. Comparison of fluorescence quantum yields, excited state lifetimes and absorption characteristics indicate similar light-harvesting properties of the DV-Chls as their monovinyl counterparts. PE, which is present only in tiny amounts, was purified and considerably enriched. A phycourobilin to phycoerythrobilin ratio of 3:1 chromophores per (αβ) PE monomer is suggested. The in vitro fluorescence lifetime of PE is 1.74 ns. In vivo time-resolved fluorescence measurements with synchrotron radiation were used to investigate the possible role of PE in light-harvesting. The fluorescence decay time for PE is about 550 ps, indicating an unusually slow excitation energy transfer. The decay time slowed to 1 ns after addition of glycerol to cell cultures. The contribution of PE to total light-harvesting capacity was estimated to be about one (αβ) PE monomer per 330 DV-Chl** ***b*** **molecules. Thus, the capacity of PE to function primarily as a photosynthetic light-harvesting pigment in** ***P. marinus*** **SS120 is low.**

Key words: Excitation energy transfer; Fluorescence lifetime and quantum yield; Phycoerythrin; Spectrofluorimetry; Synchrotron radiation; *Prochlorococcus*

1. Introduction

Prochlorococcus is an abundant marine oxyphotobacterium. Compared to closely related cyanobacteria, it has an unusual pigment composition [1–3]. Instead of (monovinyl) chlorophyll (Chl) *a* and phycobilisomes *Prochlorococcus* accumulates divinyl-Chls (DV-Chls) *a* and *b*. DV-Chls are usually biosynthetic precursors of Chls and apart from *Prochlorococcus* they are found in significant amounts only in a Chl biosynthesis mutant of maize [4,5]. In addition, *Prochlorococcus marinus* SS120 and several other strains contain phycoerythrin (PE) but lack the phycobiliproteins phycocyanin and allophycocyanin [6–8].

Some other oxyphotobacteria contain Chls *a* and *b* (*Prochloron didemni* and *Prochlorothrix hollandica*) or Chls *a* and *d* (*Acaryochloris marina*) as major light-harvesting pigments (see Partensky and Garczarek for a review [9]). In all these organisms the accessory pigments are bound to special Chl-binding proteins (prochlorophyte chlorophyll *b*-binding proteins, PCBs) [10]. Bibby et al. [11] have recently shown that the PCBs of *Prochlorococcus* form a concentric 18-meric ring around trimeric photosystem I. Other cyanobacteria form a similar structure around photosystem I only in response to iron stress [12,13]. This structure consists of the protein IsiA (CP43′), which is phylogenetically related to PCBs [14]. In *P. marinus* SS120 PCBs are constitutively present. Thus, the DV-Chl *a*/*b*–PCB light-harvesting protein complex of these oxyphotobacteria and of other cyanobacteria under iron stress is structurally and evolutionarily very different from the well-characterised phycobilisomes of cyanobacteria or membrane-intrinsic light-harvesting complexes of chlorophyta and algae.

Association of *Prochlorococcus* PE with thylakoid membranes has been shown [7] and evidence for excitation energy transfer (EET) has been presented [15]. However, as shown by immunogold labelling studies [7] only tiny amounts of PE are present in cells, which argues against a significant role of PE as light-harvesting pigment in *Prochlorococcus*. Regulatory effects on phycobiliprotein contents exerted by external stimuli such as light quantity and nitrogen deprivation, which are well known from phycobilisome-bearing cyanobacteria, have not been observed in *Prochlorococcus* [16,17].

To date, there is only scarce information on photophysical properties of DV-Chls and *Prochlorococcus* PE. The extinction coefficients for DV-Chls *a* and *b* were determined [5], whereas values for excited state lifetimes and fluorescence quantum yields of DV-Chls *a* and *b* remained unknown. Here, these values are reported together with a characterisation of *Prochlorococcus* PE following ca. 1000-fold enrichment. Furthermore, time-resolved fluorescence measurements with intact cells using synchrotron radiation were performed

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Abbreviations: DV-Chl, divinyl chlorophyll; EET, excitation energy transfer; PCB, prochlorophyte chlorophyll *b*-binding protein; PE, phycoerythrin

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to determine fluorescence decay times and to estimate the contribution of PE to the total light-harvesting capacity of this organism.

2. Materials and methods

2.1. Cultures and growth conditions

Cultures of *P. marinus* SS120 (=CCMP 1375) (courtesy of Prof. S.W. Chisholm and Dr L.R. Moore) were grown at 20°C in PCR-S11 medium (Partensky et al. [3]) under 8 μmol quanta m^{-2} s^{-1} continuous blue light.

2.2. Purification of pigments

Cells were harvested by centrifugation at 10 000 rpm for 10 min using a Sorvall GSA rotor. DV-Chls *a* and *b* were extracted with 100% acetone from cell pellets. The pigments were purified by HPLC on a Waters Spherisorb 5 μm ODS2 4.6×250 mm analytical column using a 33 min linear gradient between 0% and 66.7% solvent B (ethyl acetate) in solvent A (acetonitrile/water/triethyl amine 900:100:1).

For PE purification cell pellets from a 20 l culture were resuspended in an adequate volume of extraction buffer (20 mM HEPES pH 8.0, 10 mM NaCl). Insoluble proteins and cell debris were pelleted by centrifugation at 10 000×*g* for 10 min at 4°C. The supernatant was once more centrifuged in an L8-70M Beckman ultracentrifuge at 50 000 rpm for 30 min at 4°C. Soluble proteins were concentrated using Centricon YM-10 concentrators (Amersham) or in a SpeedVac. PE was further purified by non-denaturing IEF adapted from the procedure of Jackowski and Przymusinski [18] with the following modifications: gels were polymerised with APS and TEMED instead of riboflavin, 2% ampholines (Amersham-Pharmacia; pH 4.0–6.5 instead of pH 3.5–5.0) were used. This procedure resulted in an about 1000-fold enrichment of PE. All spectroscopic measurements with PE were done in 10 mM Tricine buffer (pH 7.8).

2.3. Steady-state fluorescence measurements

Room temperature fluorescence emission spectra of all pigments were recorded using a Fluorolog FL-112 spectrofluorimeter with a 1680 emission double monochromator (Jobin-Yvon, Longjumeau, France). Fluorescence of DV-Chls was excited at three different wavelengths in the Q_y region (585, 595 and 615 nm). PE was excited at 495 nm. Absorption spectra were measured using a Lambda 900 spectrophotometer (Perkin-Elmer). Fluorescence quantum yields (Φ_F) were calculated according to Eq. 1 from the corrected fluorescence spectra as integrated on a wavenumber scale taking reabsorption and the absorbance at the respective excitation wavelength into account:

$$\Phi_u = (A_{st}/A_u)(F_u/F_{st})(n_u^2/n_{st}^2)\Phi_{st} \quad (1)$$

where Φ, A, F, n are the fluorescence quantum yields, absorption at the excitation wavelength, integrated fluorescence intensity (on a wavenumber scale) and refractive index of the medium of the unknown pigment (u) and the standard (st), respectively. Chl *a* in diethyl ether ($\Phi_F = 0.32$ [19]) and rhodamine 6G in ethanol ($\Phi_F = 0.95$ [20]) were used as standard.

2.4. Time-resolved fluorescence decay measurements

Fluorescence lifetimes (τ_F) of isolated pigments were measured at the respective fluorescence emission maxima with the same solutions as above using a multidecay spectrometer operating in boxcar mode. Excitation was performed either by a nitrogen laser MSG 800 (LTB Berlin; λ_{exc} 337 nm, pulse width 400 ps) or by a dye laser UDL 200 (LTB Berlin; tuned to the respective pigment's absorption maximum, pulse width <400 ps). The spectrometer is described in detail elsewhere [21]. Data were numerically analysed by deconvolution with a sum of exponentials as model function.

In vivo time-resolved fluorescence decay measurements were carried out using the synchrotron at CLRC Daresbury Laboratory, Warrington, UK. Incubation of *Prochlorococcus* cells in 50% glycerol/growth medium was done to dissociate PE from the thylakoid membranes as described previously [15]. The synchrotron was operated in single-bunch mode and measurements were performed on Station 13.1. Narrow-bandwidth excitation was defined by a double monochromator. Emission wavelengths were defined with 10 nm bandwidth interference filters. Fluorescence decays were recorded using a single-photon timing system with 1024 channels (11.8 ps per channel). A prompt–response signal (FWHM about 600 ps) was recorded using a scattering sample. Decays were analysed using HETANL software (Center for Fluorescence Spectroscopy, Baltimore School of Medicine at the University of Maryland).

2.5. Relative absorption cross-section and concentration of PE

The steady-state fluorescence (F) of a pigment is the product of the fluorescence amplitude (I_F) and the fluorescence lifetime (τ_F) [22]:

$$F = I_F \tau_F \quad (2)$$

I_F is proportional to the absorption cross-section (α) and inversely proportional to the natural lifetime (τ_0) of the respective pigment [22]:

$$I_F = C\alpha/\tau_0 \quad (3)$$

where C is a constant of proportionality.

The natural lifetime (τ_0) was calculated as:

$$\tau_0 = \tau_F/\Phi_F \quad (3a)$$

from fluorescence lifetimes (τ_F) and the fluorescence quantum yields (Φ_F) of each pigment in solution.

The absorption cross-section of PE relative to DV-Chl *b* in the cells can therefore be estimated:

$$\alpha_{PE}/\alpha_{DV-Chl} = (I_{F\,PE}\tau_{0\,PE})/(I_{F\,DV-Chl}\tau_{0\,DV-Chl}) =$$

$$F_{PE}\,(\tau_0/\tau_{a\,PE})/F_{DV-Chl}\,(\tau_0/\tau_{a\,DV-Chl}) \quad (4)$$

Data for I_F and average fluorescence lifetimes (τ_a, calculated from the resolved lifetime components weighted according to their amplitudes), of DV-Chls and PE were obtained from cells in the presence of glycerol, with excitation at 495 nm. F_{PE}/F_{DV-Chl} was estimated from a steady-state fluorescence emission spectrum, using the fluorescence peaks at 575 and 680 nm. $\tau_{a\,PE}/\tau_{a\,DV-Chl}$ was estimated using the average fluorescence lifetimes at 575 and 680 nm from fluorescence decays with excitation at 495 nm. The relative concentration of PE and DV-Chl *b* ([PE]/[DV-Chl]) was estimated from $\alpha_{PE}/\alpha_{DV-Chl}$ by dividing by the ratio of extinction coefficients ($\varepsilon_{R-PE}/\varepsilon_{DV-Chl}$) at 495 nm. Since the extinction coefficient for *Prochlorococcus* PE has not been determined yet, a typical value for R-PE recalculated for a monomeric (αβ) subunit was used, see also Table 1 [23].

Table 1
Spectroscopic properties of *Prochlorococcus* pigments and data used to estimate the contribution of PE to the light-harvesting capacity of the cells

	DV-Chl *a*	DV-Chl *b*	PE
Fluorescence quantum yield (Φ_F)	0.36 ± 0.04	0.10 ± 0.01	0.1 ± 0.02
Fluorescence lifetime (τ_F) (ns)	6.0 ± 0.2	2.9 ± 0.2	1.74 ± 0.08
Natural lifetime (τ_0) (ns)	17 ± 2	29 ± 2	19 ± 2
Millimolar extinction coefficient (ε) (mM^{-1} cm^{-1}) at 495 nm[a]	–	27.75	237
Relative steady-state fluorescence (I_F) with 495 nm excitation	1.00	n.d.	0.26
Average fluorescence lifetime (τ_a) (ns) in *Prochlorococcus* cells	0.20	n.d.	1.30

n.d., not determined.
[a]Calculated from data in Fig. 1 using the maximum extinction coefficient from ref. [5] for DV-Chl *b* and from ref. [23] recalculated for monomeric (αβ) R-PE.

3. Results and discussion

3.1. Steady-state spectroscopic data

Elucidating the photophysical properties of DV-Chls *a* and *b* as well as of PE is of interest for understanding EET in the photosynthetic apparatus of *Prochlorococcus*. Absorption and fluorescence emission spectra of highly purified DV-Chls *a* and *b* (in diethyl ether) as well as of PE are shown in Fig. 1A–C. Both, DV-Chl *a* and DV-Chl *b* exhibit two prominent absorption maxima at 436 and 660 nm as well as at 463 and 645 nm, respectively, in agreement with previous reports [2,5]. Emission maxima (665 and 650.5 nm for DV-Chl *a* and DV-Chl *b*, respectively) display a small Stokes shift relative to the absorption maxima. The emission maximum of PE is at 569 nm with a shoulder at about 620 nm. The absorption spectrum of PE shows two maxima at 495 nm (corresponding to the chromophore phycourobilin) and at 549 nm (phycoerythrobilin). Using the extinction coefficients for phycoerythrobilin (53.7 mM^{-1} cm^{-1} at 550 nm) and phycourobilin (94 mM^{-1} cm^{-1} at 495 nm) as determined in [24] a deconvolution of the absorption spectrum would result in a phycourobilin to phycoerythrobilin ratio of 2.9. However, in contrast to the non-denaturing conditions used here, extinction coefficients for phycourobilin and phycoerythrobilin were previously determined in 8 M urea at pH 3 [24]. Hence, the obtained value can only serve as a rough approximation and a phycourobilin to phycoerythrobilin ratio of 3 appears to be likely in agreement with the capability to bind four chromophores per (αβ) PE monomer in *P. marinus* SS120 [6].

Fluorescence quantum yields of DV-Chls and PE as calculated according to Eq. 1 are given in Table 1. The same procedure was executed with Chl *b* yielding a Φ_F equal to previously published data (not shown, cf. also [25]). Noteworthy, the obtained Φ_F values for DV-Chls are almost identical to their Chl counterparts (0.36 and 0.32 for DV-Chl *a* and Chl *a* [26] as well as 0.10 and 0.12 for DV-Chl *b* and Chl *b* [27], respectively). A Φ_F of 0.1 for *Prochlorococcus* PE is unusually low as compared to other PEs previously determined for *Nostoc* sp., *Porphyridium cruentum*, *Gastroclonium coulteri* and *Anabaena variabilis* [23,28].

Fluorescence lifetimes were measured to further characterise the light-harvesting properties of DV-Chls and PE (see Fig. 2 and Table 1). Analysis of the fluorescence traces was based on monoexponential decays (upon either, nitrogen or dye laser excitation). Inclusion of further kinetic components did not significantly improve the fits. Again, striking similarities between Chls (6.0 ns for Chl *a* and 3.0 ns for Chl *b*) and DV-Chls (6.0 ns for DV-Chl *a* and 2.9 ns for DV-Chl *b*) were observed. Isolated PE is characterised by a fluorescence lifetime of 1.74 ns which is slightly shorter as observed for PEs of *Nostoc* sp. and *P. cruentum* [28].

3.2. Fluorescence decay kinetics of PE in P. marinus SS120

Figs. 3 and 4 show fluorescence decays for whole cells recorded at 570 nm (corresponding to the PE emission maximum with the excitation wavelength set to 495 nm, the absorption maximum of phycourobilin [29]). Fig. 3 displays the decay in the absence of glycerol. The decay can be deconvoluted into two components with $\tau_1 = 550$ ps and $\tau_2 = 3.06$ ns with fractional amplitudes of 0.88 and 0.12, respectively. The predominant decay time of 550 ps is slower than that generally observed for energetically coupled phycobilins with 70–90 ps [30,31]. However, it is faster than that for the isolated PE (Table 1) and for isolated phycobilins in general [32]. This confirms that EET from PE to an acceptor occurs as was suggested previously [15], though the slow lifetime indicates

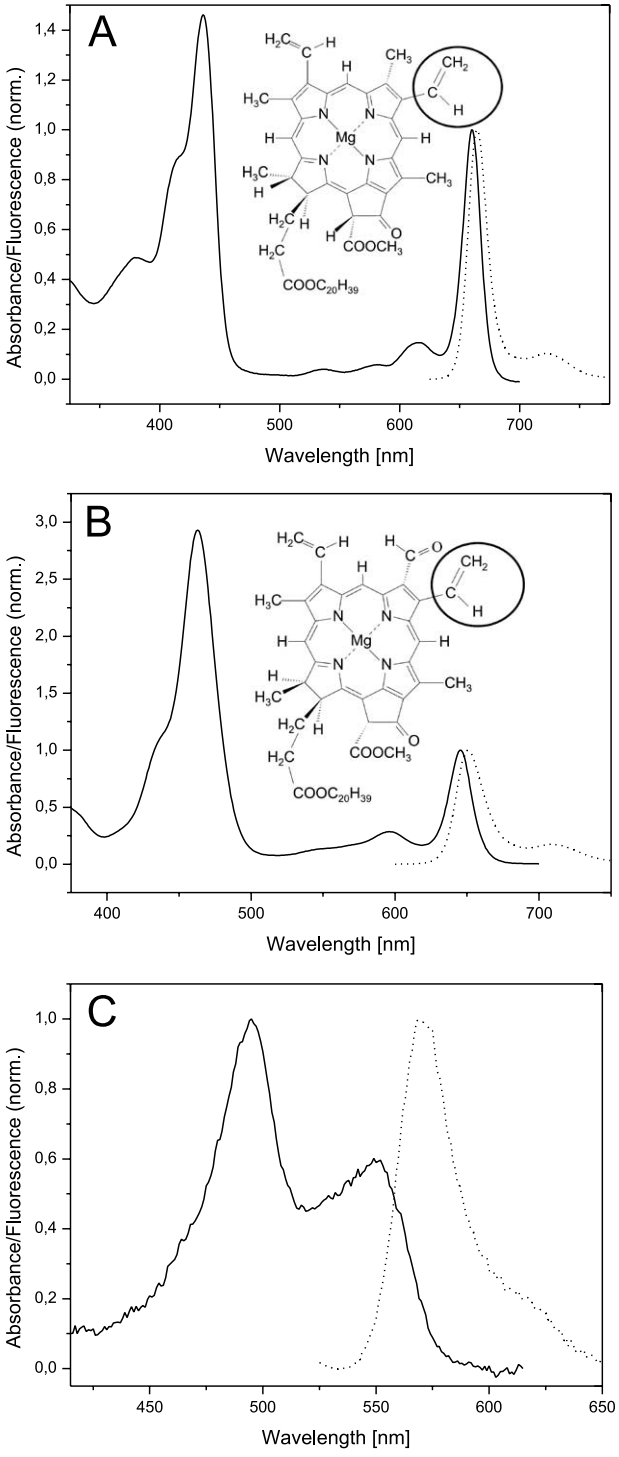

Fig. 1. Absorption (solid line) and fluorescence emission spectra (dotted line) of DV-Chls *a* and *b* (A,B) in diethyl ether and of PE in 10 mM Tricine buffer, pH 7.8 (C). Fluorescence was excited at 440 nm (DV-Chls) or at 495 nm (PE). Spectra were normalised to the Q_y absorption (DV-Chls) or the phycourobilin maximum (PE) as well as their emission maxima. Inserts in A and B show the chemical structures of the respective DV-Chls. Differences with monovinyl counterparts are indicated.

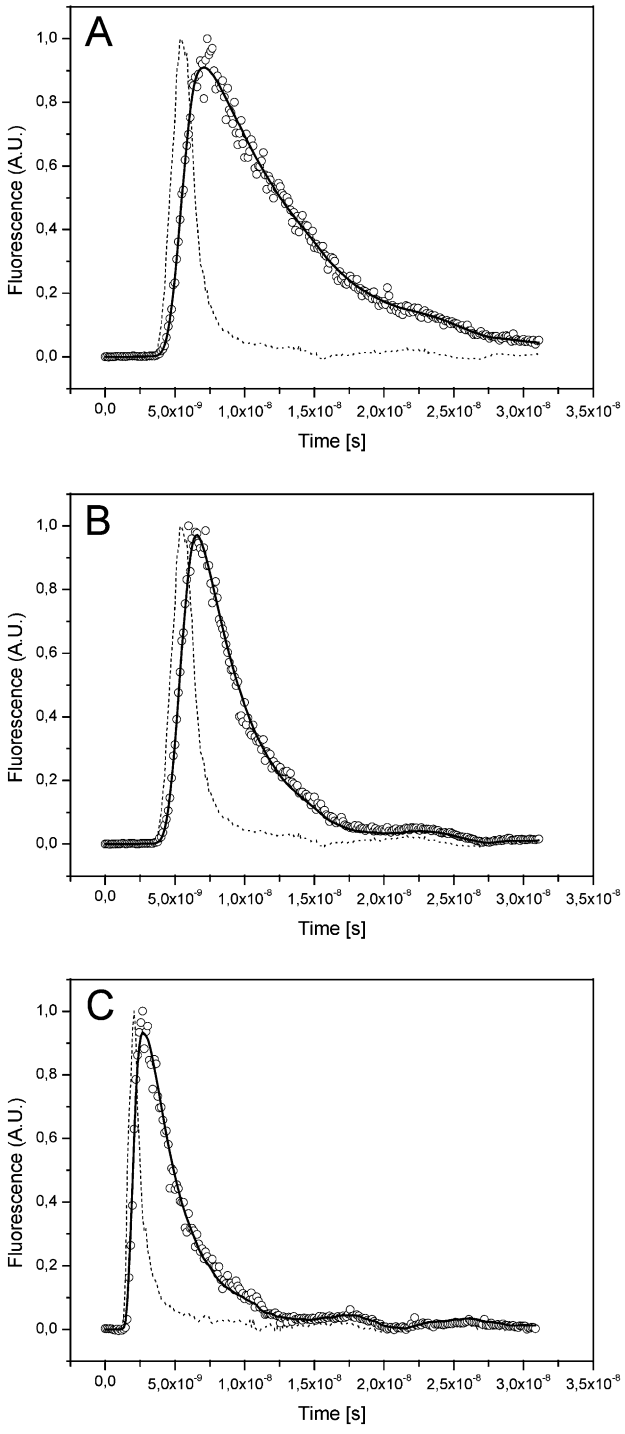

Fig. 2. Fluorescence decay curves of DV-Chls *a* and *b* in diethyl ether (A,B) and of PE in 10 mM Tricin buffer, pH 7.8 (C). Fluorescence was excited at 337 nm (A,B) or at 500 nm (C). Fluorescence decays were measured in the emission maxima at 665 and 650.5 nm for DV-Chl *a* and DV-Chl *b*, respectively, or at 569 nm for PE. Open circles: fluorescence decay, solid lines: fit, dotted lines: system prompt–response.

that this EET is not very efficient. Upon the addition of glycerol, the decay can also be fitted with two components, with $\tau_1 = 1.0$ ns and $\tau_2 = 2.4$ ns (Fig. 4). Fractional amplitudes were quite similar to those found without glycerol treatment whereas lifetimes of the major component show an obvious slow-

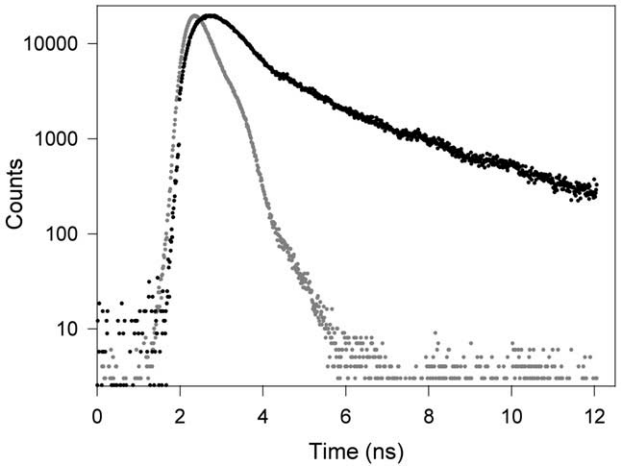

Fig. 3. Fluorescence decay of PE in *Prochlorococcus* cells. Excitation 495 nm, emission 575 nm. Black points: fluorescence decay, grey points: system prompt–response.

down from 550 ps to about 1 ns. This lifetime is closer to those measured for decoupled phycobilins [32].

3.3. EET from PE to DV-Chls

In order to investigate whether EET from PE to DV-Chls might occur, fluorescence decays were recorded with excitation at 495 nm (corresponding to the phycourobilin absorption maximum) and emission at 680 nm (corresponding to the DV-Chl *a* emission maximum). In principle, if energy is being transferred from PE to DV-Chls, the fluorescence decay kinetics of DV-Chl *a* should include a component corresponding to the 550 ps fluorescence lifetime of PE [33]. Fig. 5 shows the fluorescence decays observed at 680 nm in the presence and absence of glycerol. As expected for an intact photosynthetic system [32] the DV-Chl *a* fluorescence decay kinetics were extremely complex. It was not possible to resolve all the kinetic components present or to identify a 550 ps component corresponding to the PE fluorescence decay. Furthermore, there was no difference in the fluorescence decay kinetics when glycerol was added (Fig. 5). Since the addition

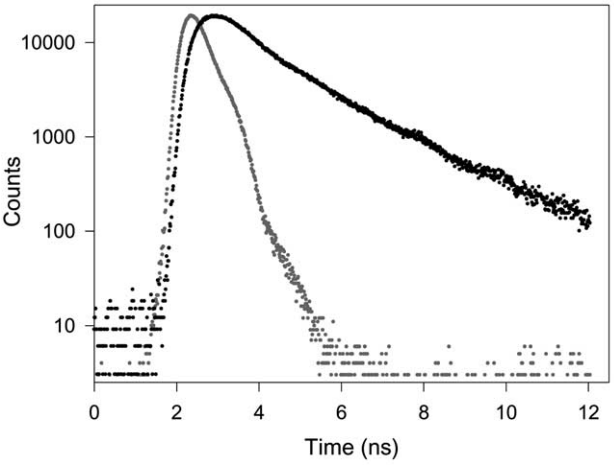

Fig. 4. Fluorescence decay of PE in *Prochlorococcus* cells in the presence of glycerol. Excitation 495 nm, emission 575 nm. Black points: fluorescence decay, grey points: system prompt–response.

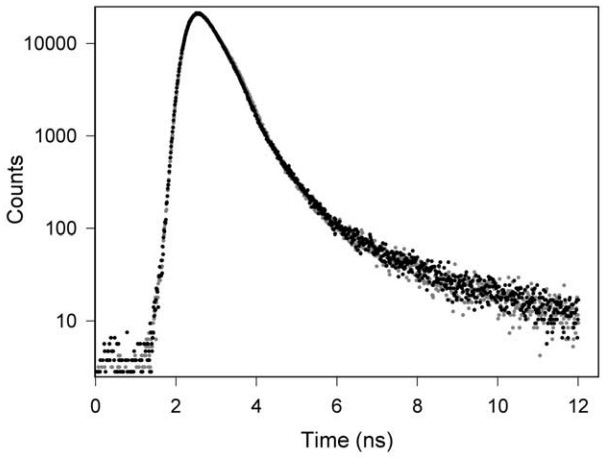

Fig. 5. Fluorescence decay of DV-Chl *a* in *Prochlorococcus* cells. Excitation 495 nm, emission 680 nm. Black points: cells in the absence of glycerol, grey points: cells in the presence of glycerol.

of glycerol caused a marked change in the PE fluorescence decay kinetics (Section 3.2), a corresponding change in the DV-Chl *a* fluorescence decay kinetics should be expected if PE contribution to DV-Chl *a* excitation were significant. Thus, we conclude that PE cannot serve as a major light-harvesting pigment in *Prochlorococcus*, even at its peak absorption wavelength (495 nm). When the cells are excited at 495 nm, the vast majority of the photons that are used for photosynthesis are absorbed by DV-Chl *b* and/or carotenoids.

3.4. Estimation of the PE/DV-Chl b ratio and the relative absorption cross-sections at 495 nm

Due to a strong absorption of DV-Chl *b* and carotenoids at 495 nm, absorption spectra of *Prochlorococcus* cells show no clearly resolved peak that can be attributed to PE [2]. Fluorescence emission from PE can be detected, but steady-state fluorescence emission spectra can only be used to estimate pigment content in conjunction with information on fluorescence decay kinetics, since an abundant pigment with a short fluorescence lifetime could have the same steady-state fluorescence yield as a scarce pigment with a long fluorescence lifetime. Therefore, it has not previously been possible to determine the PE content of *Prochlorococcus* cells. Fluorescence lifetime data (Section 3.2) as well as values for the extinction coefficients and natural lifetimes of PE and DV-Chls (Table 1) were used to calculate the PE contribution to the total light-harvesting capacity at 495 nm using Eqs. 2–4 (Section 2). From Eq. 4, the relative absorption cross-section of PE in the cells is estimated to be about 0.026, i.e. about 2.6% of the photons absorbed at 495 nm are absorbed by PE and the remainder by DV-Chls and carotenoids. The ratio of PE to DV-Chls in the cell can now be calculated using the extinction coefficients at 495 nm (Table 1), giving a PE/DV-Chl *b* ratio of about 1/330. The typical phycobilin/Chl (chromophore) ratio is about 1/2 for a cyanobacterium [34]. Consequently, PE is present only in tiny amounts as compared to other cyanobacteria.

3.5. Conclusions

Considering the spectral distribution of light (peaking at 495 nm) penetrating to depths at which the low light-adapted *Prochlorococcus* ecotype occurs [35], the advantage of retaining DV-Chls (biosynthetic precursors of Chls in plants and algae) but also PE as antenna pigments is immediately obvious from their absorption spectra (Fig. 1). In particular, DV-Chl *b* displays a strong absorption in this particular spectral region. However, whereas data presented here indicate similar light-harvesting capacities for DV-Chls and Chls, they cast doubt on a light-harvesting function of PE in *Prochlorococcus*.

We have confirmed previous observations that *Prochlorococcus* PE transfers energy to an acceptor, and that PE becomes energetically decoupled after addition of glycerol. However, our data indicate that this EET is very slow. No lifetime component at 680 nm that could be ascribed to EET from PE to DV-Chls was detectable. However, the amplitude of a lifetime component at 680 nm that would result from EET from PE to DV-Chls can be estimated using a simple kinetic scheme and the relative fluorescence amplitudes of PE and DV-Chls: it would be about 0.7% of the total. This is far too small to detect. Thus, it cannot be decided whether PE transfers energy to DV-Chls or to another acceptor such as a carotenoid. If PE does transfer energy to DV-Chls, it is present in such small amounts in *Prochlorococcus* that it cannot be a significant light-harvesting component. Even at the peak absorption wavelength of 495 nm, only about 2.6% of photons will be absorbed by PE. Furthermore, the EET is slow and therefore rather inefficient, so that the effective contribution of PE to total light-harvesting capacity at 495 nm is unlikely to be more than about 1.8%, taking the quantum yield for EET into account.

However, active genes encoding PE have been conserved in *Prochlorococcus* genotypes from such diverse geographical origins as the subtropical Pacific Ocean [8] or the Sargasso Sea [6,7] whereas genes encoding other phycobiliproteins have been wiped out during a short period of time [36] in which the genus *Prochlorococcus* may have evolved from closely related marine cyanobacteria such as *Synechococcus* [37,38].

A light-harvesting function for PE in *P. marinus* SS120 was proposed previously [15]. However, those measurements did not permit a quantitative evaluation of the overall PE contribution to light-harvesting. Data presented here indicate that the capacity of PE to function primarily as a photosynthetic light-harvesting pigment in *P. marinus* SS120 is low due to the slow EET, the tiny amounts present and the relatively poor Φ_F of PE. Thus, it is suggested that PE might have an additional role, possibly as some kind of receptor for example in a phytochrome-type signal transduction process.

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