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PHYCOERYTHROCYANINS FROM *Westiellopsis prolifica* AND *Nostoc rivulare*: CHARACTERIZATION OF THE PHYCOVIOLOBILIN CHROMOPHORE IN BOTH STATES*

P. S. MARUTHI SAI[1], S. SIEBZEHNRÜBL[2], S. MAHAJAN[1] and H. SCHEER[2]†
[1]School of Life Sciences, Jawaharlal Nehru University, New Delhi 110067, India and [2]Botanisches Institut der Universität, Menzinger Str. 67, 8000 München 19, Fed. Rep. Germany

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Abstract—Phycoerythrocyanin or fractions enriched in it have been isolated from the filamentous cyanobacteria, *Westiellopsis prolifica* ARM 365 and *Nostoc rivulare* ARM 212. Both show the photoreversible photochromism (difference maxima at 503 and 570 nm) characteristic of this pigment, which is related to the phycoviolobilin chromophore on the α-subunit. Native phycoerythrocyanin and its β-subunit show little if any reversible photochemistry in the 600–620 nm region, where the phycocyanobilin chromophores absorb maximally. Instead the phycocyanobilin chromophores are bleached irreversibly. At the same time, the data show that reversible photochemistry is a useful analytical tool to detect phycoerythrocyanin in cyanobacterial extracts. Fluorescence measurements indicate that: (i) the 510 nm absorbing isomer of the violobilin chromophore has only little fluorescence; and (ii) the energy transfer from the violobilin chromophores to the cyanin chromophores is efficient only in the 570 nm form.

INTRODUCTION

Cyanobacteria are the oxygen evolving photosynthetic prokaryotes possessing brilliantly coloured phycobiliproteins involved in light harvesting and excitation energy transfer to the reaction centres (Scheer, 1986). Phycoerythrocyanin (PEC)‡, one such biliprotein, also shows photoreversible photochemistry reminiscent of the plant photoreceptor, phytochrome, but with an orange/green rather than red/far-red action spectrum (Björn, 1979; Kufer and Björn, 1989; Siebzehnrübl *et al.*, 1989). It has been found in several filamentous cyanobacteria (Bryant *et al.*, 1976; MacColl and Guard-Friar, 1987; Bryant, 1982). It is structurally related to C-phycocyanin (C-PC) (Zuber, 1986; Dürring *et al.*, 1990), but the α-subunit carries an unusual phycoviolobilin§ instead of the phycocyanobilin chromophores which are present on the β-subunit only. Phycoerythrocyanin is already photoreactive in its native state (Kufer and Björn, 1989). Björn (1979) first suggested a relationship between phycochrome *b*, so far identified only in PEC containing blue-green algae, and the α-subunit of PEC. Siebzehnrübl *et al.* (1989) discussed in detail the photochemistry of PEC from *Mastigocladus* (*M.*) *laminosus* in various aggregation states.

In continuing a systematic study on the photoreactivity of PEC, we have now further characterized PEC from two other species, *e.g.* the filamentous and heterocystous cyanobacteria *Westiellopsis prolifica* ARM 365 and *Nostoc rivulare* ARM 212. Here we report the behaviour of PEC observed during photochemical changes in various states ranging from PEC in intact phycobilisomes to the isolated native forms. Further, we also present the fluorescence properties of the phycoviolobilin chromophore on the α-subunit of PEC in its two forms.

MATERIALS AND METHODS

Nostoc rivulare ARM 212 and *Westiellopsis prolifica* ARM 365 were obtained as agar slants from the National Facility for Blue-Green Algae, Indian Agricultural Research Institute, New Delhi, India. They were grown in 5–10 L batch cultures at 28°C under irradiation with fluorescent lamps (≈ 3000 lx) in BG-11 medium (Stanier *et al.*, 1971). After 10–12 days, the cells were harvested by vacuum filtration and washed thoroughly in potassium phosphate buffer (1 M, pH 7.0). Phycobilisomes (PBSomes) were isolated according to Gantt *et al.* (1979), or by the following modification of the procedure of Menon *et al.* (1988) which yields intact PBSomes from these species without mechanical breakage. Washed cells (1–1.2 g wet weight/10 mL) were suspended in potassium phosphate buffer (1 *M*, pH 7.0) containing PMSF (1 m*M*) and homogenized with a Potter homogenizer. Neat Triton-X 100 was added to 2%, or alternatively CTAB to 0.01%. The cell suspensions were vigorously stirred on a magnetic stirrer for about 4 h at room temperature, and then centrifuged (35 000 ***g***, at 20°C). The supernatants were carefully removed with Pasteur pipettes, and dialysed overnight at room temperature against the 1 *M* buffer. The preparations were centrifuged again (35 000 ***g***, at 20°C) to remove scattering particles, and checked for intactness of PBSomes by fluorescence spectroscopy. All the remaining

*Dedicated to Professor W. Wehrmeyer on the occasion of his 60th birthday.
†To whom correspondence should be addressed.
‡*Abbreviations*: APC, allophycocyanin; PBSomes, phycobilisomes; PC, phycocyanin; PEC, phycoerythrocyanin; PMT, photomultiplier tube.
§This chromophore nomenclature follows the one used for other biliprotein chromophores. In the literature, the PEC chromophore on the α-subunit has also been termed as phycobiliviolin, PXB (Bishop *et al.*, 1987).

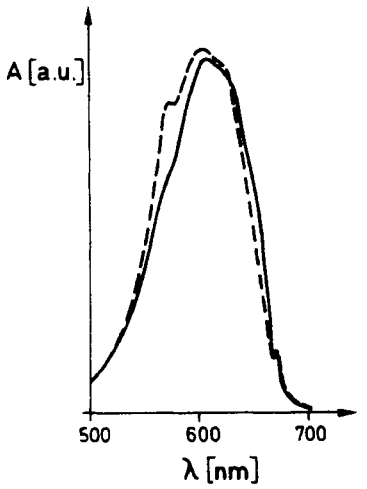

Figure 1. Absorption spectra of intact PBSomes in potassium phosphate buffer (1 *M*, pH 7.0) of *W. prolifica* (---) and *N. rivulare* (——).

steps were performed according to Gantt *et al.* (1979).

For obtaining PEC, the intact PBSomes were dissociated by dialysing overnight against potassium phosphate buffer (5 m*M*, pH 7.0) at 4°C. The dissociated PBSomes were applied onto DEAE cellulose (columns DE 52, Whatman) and constituent biliproteins were eluted with a step gradient (10–200 m*M* potassium phosphate buffer, pH 7.0). Alternatively, dissociated PBSomes were subjected to sucrose density ultracentrifugation (7–17% linear gradient, 48 h, 4°C, 238 000 *g*) and were separated into different fractions of biliproteins. All fractions indicating the presence of PEC (absorption band at ≈ 575 nm) were analysed for light induced absorbance changes (see below) and by SDS–PAGE (Laemmli, 1970) and pooled accordingly. Monomers of PEC were obtained by addition of KSCN to 1 *M* (MacColl *et al.*, 1981) to the PEC trimers.

Absorption spectra were obtained at room temperature unless specified otherwise. Steady state absorption spectra were recorded on either Shimadzu UV 260, Beckman DU-70, Hewlett–Packard 8451A diode array, or Shimadzu UV 3000 spectrophotometers fitted with thermostats. Absorption difference spectra were recorded on a Sigma-ZWS 11 dual wavelength spectrophotometer in split-beam mode with the cuvettes thermostatted at 15°C. This spectrophotometer, with the PMT mounted immediately behind the cuvette, was also used to measure the absorption of scattering samples. The steady state fluorescence excitation and emission spectra were recorded on Shimadzu RF 540, Perkin–Elmer MPF4 or Spex Fluorolog 221 spectrofluorometers in 90° geometry. Maximum absorption was $< 0.1\ cm^{-1}$ (square cells 1 × 1 cm). All spectra are corrected for instrument response.

The photochemistry was tested by difference spectroscopy. After a saturating pre-irradiation (10 min, 500 nm light), the samples were irradiated for 8 min with 600 nm light. Reversibility was checked by post-irradiation for 10 min with 500 nm light. Quantitative data are given as $\Delta\Delta A$ values (%). Details of the assay are given by Siebzehnrübl *et al.* (1989).

RESULTS

Whole cells of both species of cyanobacteria show prominent absorptions around 675 nm (chlorophyll *a*) and 620 nm (PC) (not shown). A distinct shoulder around 580 nm in *W. prolifica* indicates the presence of PEC. In *N. rivulare*, the 620 absorption is broadened, but shows no distinct shoulder, which agrees with the low level of PEC (see below).

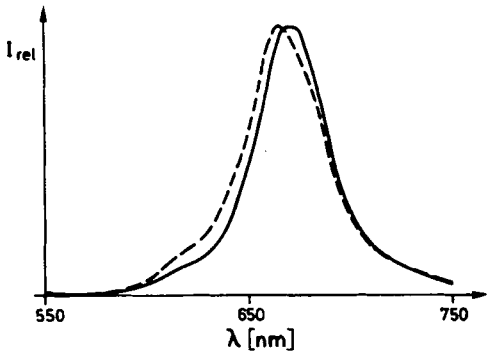

Figure 2. Fluorescence emission spectra of intact PBSomes from *W. prolifica* (---) and *N. rivulare* (——). Excitation at 540 nm, slit widths 5 nm, normalized to the same maximum emission.

The absorption spectrum of intact PBSomes from *W. prolifica* (Fig. 1) shows a maximum around 608 nm with a prominent red shoulder, in addition to the one at the short-wavelength side. The spectrum of the *N. rivulare* PBSomes is similar, but here the 575 nm shoulder is barely visible (Fig. 1). The fluorescence emission spectra of intact PBSomes (Fig. 2) were almost identical from both species with emission around 670–680 nm. This indicates that the PBSomes are energetically well-coupled (Gantt *et al.*, 1979). The intact PBSomes of *W. prolifica* were dissociated overnight and the extent of dissociation was checked by fluorescence emission spectroscopy (Fig. 3). Emissions in dissociated PBSomes at 580 and 645 nm, when excited at 545 nm, reveal the detachment of PEC, PC and APC with a concomitant loss of energy transfer from PEC to PC to APC. Note that the PEC fluorescence maximum is close to the wavelength of the absorption maximum of PEC in intact PBSomes.

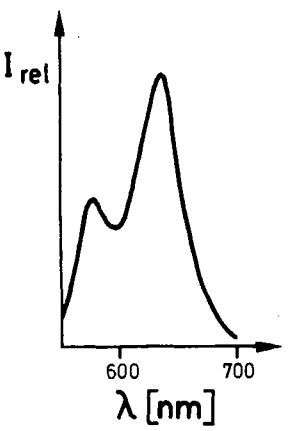

Figure 3. Fluorescence emission spectrum of dissociated PBSomes from *W. prolifica* in potassium phosphate buffer (5 m*M*, pH 7.0). Excitation wavelength 540 nm.

Table 1. Reversible photochemistry of PEC from *W. prolifica* and *N. rivulare* in different states. Irradiation protocol as in Figs. 6 and 7. $\Delta\Delta A$ denotes the ratio of the amplitude of the s-shaped difference band [see Fig. 6(a)] and the 575 nm absorption after saturating pre-irradiation with green light

Sample	Buffer*	Difference etrema (nm)		$\Delta\Delta A$† (%)	Reversibility
		Orange	Green		
W. prolifica					
Intact PBSomes	1 *M*	—	—	—	—
Dissociated PBSomes	5 m*M*	567	503	6.1	
PEC(X)	50 m*M*	570	508	84.8	Total
PEC	100 m*M*	570	508	17	Total
PEC	100 m*M*/KSCN 1 *M*	567	505	41	Total
N. rivulare					
Intact PBSomes	1 *M*	—	—	—	—
Dissociated PBSomes	5 m*M*	569	505	3.3	Total
PEC‡	1 *M*	571	506	2.5	—
PEC‡	100 m*M*	569	506	23.8	Total

*Potassium phosphate buffer, pH 7.
†Absorption difference amplitude, nomalized to A_{max} of sample, see Siebzehnrübl *et al.* (1989).
‡Fraction enriched in PEC, see text and Figs. 4 and 6(c).

During the isolation of PEC from *N. rivulare* PBSomes, a separate band containing PEC was observed in the region of 0.5–1.0 *M* on the sucrose density step gradients apart from the main PBSome band in the region of 2.0 *M*. The PEC containing band, when checked for the photochemistry indicated ≈ 2.5% photochemical signal ($\Delta\Delta A$, Table 1), but we could observe a photochemistry similar to that of PEC monomers when KSCN was added to 1 *M*. We thus report the presence of PEC from *N. rivulare*.

The dissociated PBSomes were subjected to chromatography on DEAE–cellulose ion exchanger for separation of component phycobiliproteins. The absorption spectra of various fractions of PEC are shown in Fig. 4. The fractions of PEC from *W. prolifica* eluting at 50 m*M* phosphate buffer, resemble spectroscopically that of an α-subunit of PEC after 600 nm irradiation (Kufer and Björn, 1989) (Fig. 4). It lacks the characteristic absorption of the two cyanobilin-chromophores on the β-subunit. The nature of this fraction, which contains additional polypeptides, is presently under investigation. The subsequent fractions eluting at 100 m*M* phosphate have an absorption like that of PEC trimers from *Mastigocladus laminosus* (Siebzehnrübl *et al.*, 1989). The absorption spectrum of the *N. rivulare* pigment fraction eluting first from DEAE–cellulose is shown in Fig. 4. It is dominated by PC (λ_{max} = 620 nm), but now shows a prominent shoulder at 575 nm indicative of PEC.

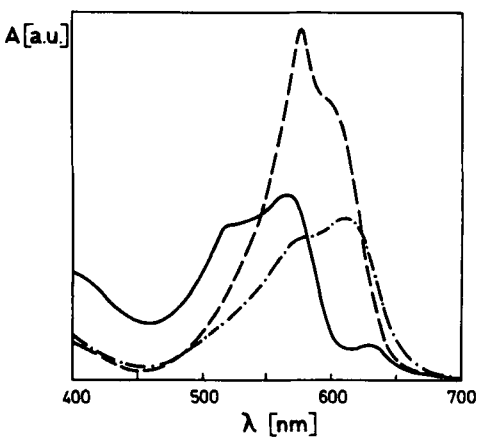

Figure 4. Absorption spectra of PEC fractions obtained on DEAE–cellulose columns. PEC(X) in potassium phosphate buffer (50 m*M*, pH 7.0) from *W. prolifica* (——). PEC from *W. prolifica* in potassium phosphate buffer (100 m*M*, pH 7.0) (---). PEC containing fraction from *N. rivulare* in potassium phosphate buffer (100 m*M*, pH 7.0) (·-·-·).

The enrichment of PEC was also followed by its photoreversible photochromic response to orange (600 nm)* and green light (500 nm). Photochemical changes in dissociated PBSomes and all the fractions of PEC from *W. prolifica* and *N. rivulare* in which in the presence of PEC was indicated by absorption are summarized in Table 1. The most intense absorption change was observed with PEC(X) (Fig. 5) from *W. prolifica*. The amplitude of the difference signals [Fig. 6(a)] is 5 times that of the trimeric PEC from the same organism. However, the shape, exact positions of the maxima, and relative areas of the positive and negative difference bands are somewhat different in the main PEC fraction from

*The photochemical response of PEC is consistently higher upon irradiation at 600 than at 580 nm, although the absorption is much higher at 580 nm. It is possible that this is caused by the presence of intermediates in the photoprocess which are in photochemical equilibrium with the 570 nm form of PEC, or by an "uphill" energy transfer from the lower lying phycocyanobilin chromophores.

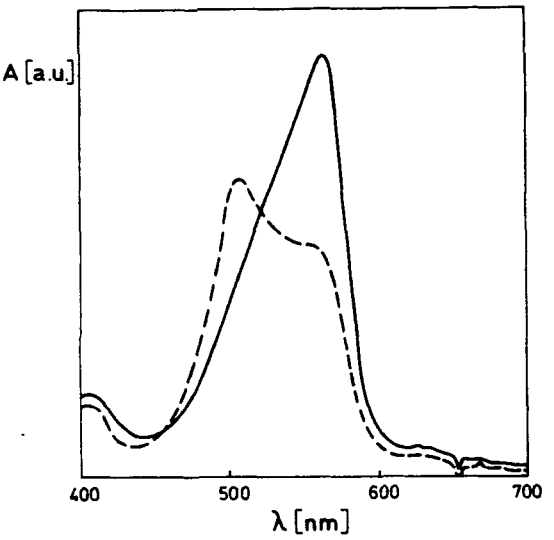

Figure 5. Absorption spectra of native PEC(X) from *W. prolifica* after saturating pre-irradiation with 500 nm (———) and saturating irradiation with 600 nm light (----) (8 min each).

this organism [Fig. 6(b)]. Apart from these variations, the difference spectra are similar to those observed in PEC from *M. laminosus* (Siebzehnrübl *et al.*, 1989; Kufer and Björn, 1989). The difference signal of the enriched fraction from *N. rivulare* clearly shows that this organism contains PEC too, albeit in much smaller amounts [Fig. 6(c)].

The intense absorption changes observed in PEC(X) from *W. prolifica* prompted us to study the contribution of the α-subunit of PEC in its two forms to the fluorescence. For this, the 510 nm absorbing form was first enriched by orange pre-irradiation. Fluorescence emission spectra were then recorded for excitation at 540 and 480 nm. The band shape and position of the emission is the same when this sample is excited at 480 and 540 nm (Fig. 7). However, although the absorption at 480 nm is as intense as that at 540 nm (see Fig. 5), the emission is greatly diminished upon 480 nm excitation. These fluorescence yields parallel the absorption of the 565 nm absorbing form of the phycoviolobilin chromophore, which is much larger at 540 nm than at 480 nm. By contrast, the 510 nm absorbing form of the chromophore has much higher absorption at 480 nm than at 540 nm (Scheer, unpublished). This indicates that the 565 nm absorbing form of phycoviolobilin chromophore contributes predominantly to fluorescence, whereas the 510 nm form is "silent".

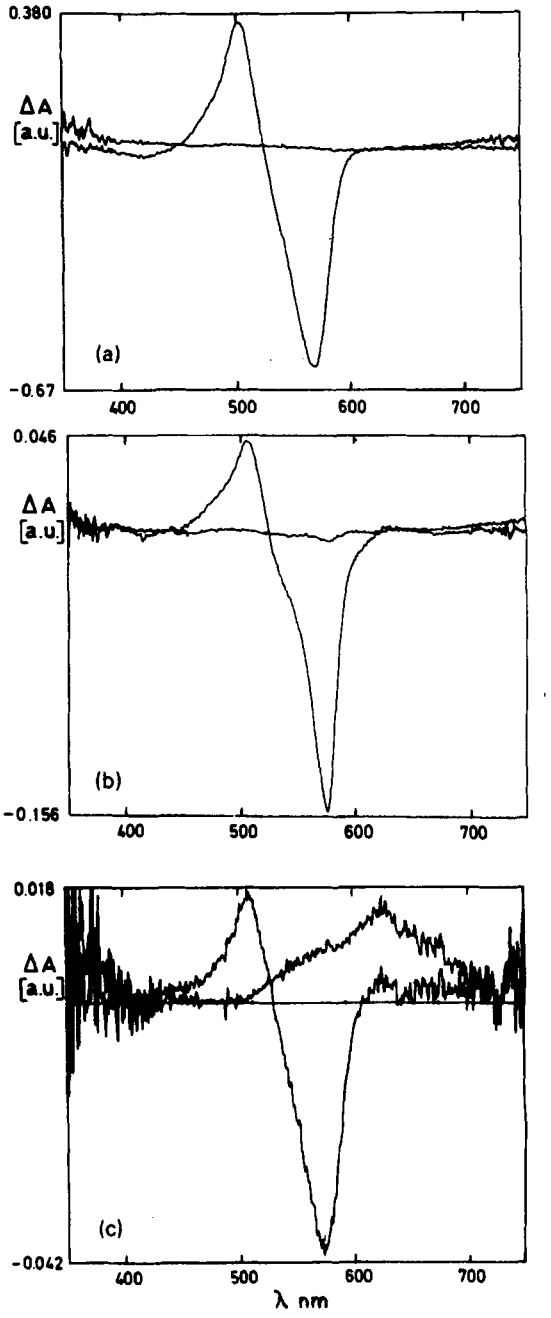

Figure 6. Absorption difference spectra (orange-irradiated *minus* green-irradiated samples) of PEC(X) (sample shown in Fig. 5) (a) and of PEC containing fractions from *W. prolifica* (absorption spectra shown in Fig. 4) (b) and *N. rivulare* (c). The "baselines" represent the difference spectra of the ones obtained after a green–orange irradiation sequence, *minus* that of the original.

DISCUSSION

Action spectra have been obtained for a variety of light-controlled responses including chromatic adaptation and photomorphogenesis, and the putative photoreceptors have accordingly been called adaptochromes and phycomorphochromes, respectively (Bogarad, 1975). The search for photoreversibly photochromic pigments yielded at least four different phycochromes, phycochrome *a*, *b*, *c* and *d*, analogous to the higher plant photoreceptor phytochrome. Among these, phytochrome *b* gives the largest signals which have ever been detected in

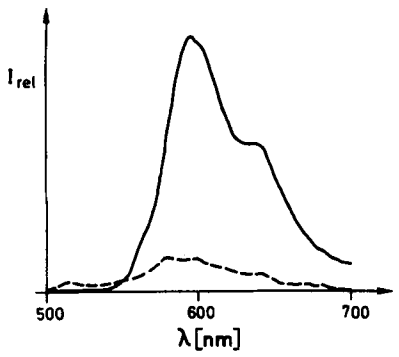

Figure 7. Fluorescence emission spectra of PEC(X), pre-irradiated at 600 nm, excited at 480 nm (---) and 540 nm (——). Slitwidths were maintained at 5 nm for both excitation and emission.

whole cells. This pigment has so far only been found in the PEC containing cyanobacteria, and a relationship or even identity between the PEC α-subunit and this phytochrome *b* has already been suggested (Björn, 1979; Kufer and Björn, 1989; Siebzehnrübl *et al.*, 1989). The α-subunit of PEC contains a rare bilin chromophore, phycoviolobilin, which contains a conjugation system intermediate in length between phycocyanobilin and phycoerythrobilin (Bishop *et al.*, 1987). Phycoerythrocyanin is known to dissociate more rapidly than the other cyanobacterial biliproteins (Bryant, 1982) and disaggregation has been shown to be closely related to the amount of reversible photochemistry in PEC from *M. laminosus* (Siebzehnrübl *et al.*, 1989).

The present study was undertaken in order to test if this relationship also holds for PEC from other cyanobacteria. The maximum photochemical signal was found in PEC(X) fractions from *W. prolifica*, followed by (in this order) PEC monomers, trimers and dissociated PBSomes, and a vanishing signal in the intact PBSomes. These observations regarding the photochemistry of PEC in different states of aggregation from *W. prolifica* are in agreement with those reported for *M. laminosus* by Siebzehnrübl *et al.* (1989). However in the latter, a minor signal was found in isolated PBSomes, whereas we could not observe any photochemistry of PEC in intact PBSomes from *W. prolifica* and *N. rivulare*. This substantiates the suggestion that PEC photochemistry is very small or even absent in the PBSome environment. The decrease of photochemistry in higher aggregates has been attributed either to a decreased mobility of the phycoviolobilin chromophore which inhibits the rotation of ring D necessary for isomerization, or to a competition of energy transfer to lower energy phycocyanobilin chromophores with photochemistry (Siebzehnrübl *et al.*, 1989). The pronounced increase of the signal in PEC fractions from *N. rivulare*, when comparing the 1 and 0.1 *M* buffer solutions supports the former mechanism (Table 1). Furthermore, the studies on PBSomes and biliproteins from *N. rivulare* indicate the presence of PEC in this organism.

The amount of PEC is known to depend on growth conditions; it is, in particular, reduced in high light, but it is also dependent on species (Bryant, 1982). In view of the taxonomic problems in filamentous nitrogen fixing cyanobacteria, PEC has recently been suggested as a marker pigment (Kufer *et al.*, 1990). The results obtained here indicate that reversible photochemistry is a sensitive analytical tool to detect PEC in low amounts and it may thus be helpful in the assignment of species. To test this assay, we have investigated the dissociated PBSomes and isolated phycoerythrins from *Anabaena variabilis* ARM 310. In agreement with its assignment, no photosignal was observed (data not shown).

A question, which to our knowledge has not yet been addressed, concerns the photophysics of the two forms of the phycoviolobilin chromophore on the α-subunit, *viz.* its fluorescence properties and its involvement in energy transfer. The very pronounced enrichment of either one of them (depending on pre-irradiation) in PEC(X) from *W. prolifica*, prompted us to study the problem with this pigment. The fluorescence emission properties (Fig. 7) of PEC(X) fractions after 8 min of 600 nm illumination indicates that only one form, the long-wavelength absorbing one, is fluorescent. The PEC(X) when excited at 545 nm emits at 590 nm, characteristic of the native α-subunit of PEC. The orange pre-irradiated PEC(X) also emits at the same wavelength.

However, the intensity of the emission is reduced, and it is further reduced upon excitation at 480 nm. This indicates that the isomer of α-subunit absorbing at 510 nm acts only as an absorber, but has a fast radiationless deactivation channel similar to free bile pigments (Scheer, 1981; Braslavsky *et al.*, 1983). With a photoconversion yield of 4% (Siebzehnrübl, 1990), this channel dominates the excited state relaxation of chromophores in the 510 nm form. Accordingly, the involvement of this form in energy transfer in integral PEC or PBSomes may be reduced. It is presently not clear whether the PEC(X) fraction occurs as a free biliprotein in cyanobacteria or is an isolation artefact. Phycoerythrocyanin is known to dissociate more readily than other biliproteins, which could also explain the large phycochrome *b* related amplitudes found *in vivo* (Björn, 1979). It may then be that PEC(X) exits as a free biliprotein *in vivo*.

To summarize our findings, we conclude that in all species of cyanobacteria studied the photochemistry is dependent on the aggregation state of PEC. The negligible photochemical responses of PEC in intact PBSomes suggest that it (or at least the component possessing the long-wavelength absorbing α-chromophore) functions only in light harvesting and energy transfer. Our main obser-

vation, that the 510 nm absorbing isomer of PEC(X) does not fluoresce, questions its role in light harvesting for energy transfer purposes and strengthens the possible involvement in light perception when it exists as a free biliprotein.

Finally, it should be noted that the induction of photochemistry of PEC can be used as a sensitive assay for its presence in cyanobacteria, since it is the only biliprotein reported so far to exhibit photochemical responses in the native state.

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