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FLUORESCENCE AND CIRCULAR DICHROISM STUDIES ON THE PHYCOERYTHROCYANINS FROM THE CYANOBACTERIUM *Westiellopsis prolifica*

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Abstract—Two phycoerythrocyanin (PEC) fractions have been obtained from the phycobilisomes of the cyanobacterium *Westiellopsis prolifica* ARM 365. They have been characterized by absorption, fluorescence and circular dichroism spectroscopy. One of them is spectroscopically similar to a PEC trimer known from other organisms. Whereas efficient energy transfer from its violin (α-84) to the cyanin (β-84, 155) chromophores is efficient in the trimer $(\alpha\beta)_3$, it is impeded after dissociation to the monomer ($\alpha\beta$). A second fraction of PEC which we earlier termed PEC(X) (Maruthi Sai *et al., Photochem. Photobiol.* **55**, 119–124, 1992), exhibited the spectral properties similar to that of the α-subunit of PEC from *Mastigocladus laminosus*. With this highly photoactive fraction, the circular dichroism spectra of the violobilin chromophore in both photoreversible states were obtained.

INTRODUCTION

Cyanobacteria are oxygen-evolving photosynthetic prokaryotes possessing brilliantly colored phycobiliproteins.[1–3] In association with so-called linker polypeptides[4] they form special antenna complexes, the phycobilisomes (PBSomes),[5]‡ which are involved in the light harvesting and excitation energy transfer to the reaction centers.[6,7] Two of the biliproteins, *viz.* phycocyanin (PC) and allophycocyanin (APC) are common to PBSomes from all species. Most of the cyanobacteria contain either phycoerythrin (PE) or phycoerythrocyanin (PEC) as a third biliprotein.[1,8,9] The latter has been suggested as a taxonomic marker for a group of filamentous nitrogen-fixing species.[10]

Phycoerythrocyanin is special among the biliproteins because, apart from being an antenna pigment, it shows a reversible photochemistry reminiscent of that of the photoreceptor, phytochrome, of higher plants. It is structurally related to PC.[3,11] One distinct difference is the substitution of the α-84 cyanobilin by a violobilin chromophore, whereas the two cyanobilin chromophores of the β-subunit are still present.[3,12] The photochemistry of this pigment has been related to the α-subunit carrying the phycoviolobilin (PVB) chromophore.[13–17] It can also serve as an analytical tool to detect low levels of PEC.[17]

Many cyanobacteria show photomorphogenetic responses.[18] The receptors have been suggested to be biliproteins. Attempts to isolate them from the large background of antenna pigments have yielded several pigments termed phycochromes.[2,19] Phycochrome *b* is present only in PEC-containing species and probably is identical to the α-subunit of this pigment.[13,14,16] There is presently no good evidence, however, identifying PEC and in particular its α-subunit as a photomorphogenetic receptor. The variations in its properties, from a photochemically inactive antenna component to a pigment with photochemical activities comparable to those of phytochrome, make it nonetheless an intriguing evolutionary model in which the boundary conditions relevant to these properties can be studied.

Westiellopsis prolifica ARM 365 is a branched filamentous, heterocystous nitrogen-fixing cyanobacterium isolated from Indian soils. It is moderately halotolerant and halophilic (up to 400 m*M* of NaCl). In a screening of several species of cyanobacteria it was found to contain rather large amounts of PEC. In continuation of our earlier reported findings from this organism,[17] we have now characterized two PEC fractions through fluorescence and circular dichroism (CD) spectroscopy. One of them, PEC(X), allowed us for the first time to obtain good CD data on the violobilin chromophore in both states.

MATERIALS AND METHODS

Westiellopsis prolifica ARM 365 was obtained on agar slants from the National Facility for Blue-Green Algae, Indian Agricultural Research Institute, New Delhi, India. The organism was grown in BG-11 medium[20] as described previously.[17] PBSomes were isolated from fresh cells of this organism either according to Gantt *et al.*[21] or Maruthi Sai *et al.*[17] The integrity and the purity of the PBSome preparations were checked by fluorescence emission spectroscopy[5] and sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE),[22] respectively.

The component phycobiliproteins were obtained from intact PBSomes. The latter were dialyzed overnight at 4°C against 5 m*M* potassium phosphate buffer, pH 7.0. The extent of dissociation was checked by fluorescence emission spectroscopy (shift of emission maximum from ≈675 to ≈645 nm). The dissociated PBSomes were applied to DEAE cellulose columns (Whatman, DE 52), and the biliprotein fractions were eluted by increasing concentrations of the buffer. The individual fractions thus obtained were analyzed spectroscopically and through SDS-PAGE[22] and were pooled accordingly.

Steady-state absorption spectra were recorded on either a Shi-

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‡*Abbreviations:* APC, allophycocyanin; CD, circular dichroism; PBSome, phycobilisome; PC, phycocyanin; PCB, phycocyanobilin; PE, phycoerythrin; PEC, phycoerythrocyanin; P_{fr}, phytochrome in its far-red form; P_r, phytochrome in its red form; PVB, phycoviolobilin; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis.

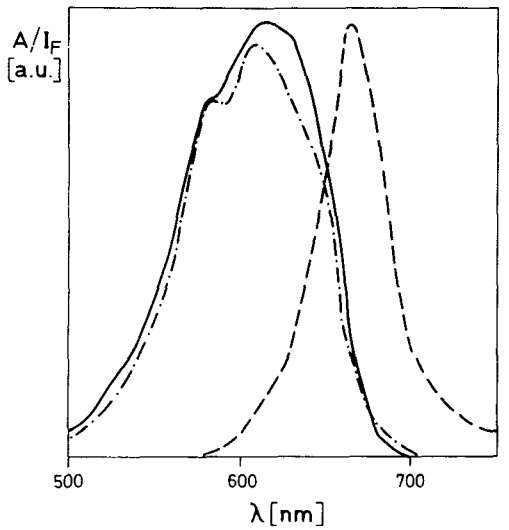

Figure 1. Absorption spectrum of intact phycobilisomes in potassium phosphate buffer (1 *M*, pH 7.0) (——). Fluorescence excitation spectrum, λ_{em} = 710 nm (–·–·–). Emission spectrum, λ_{exc} = 540 nm (– – –). All slit widths 5 nm.

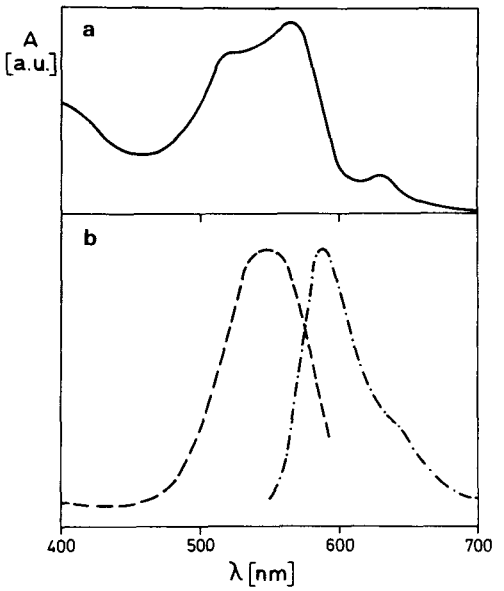

Figure 2. (a) Absorption spectrum of PEC(X) in potassium phosphate buffer (50 m*M*, pH 7.0). (b) Fluorescence excitation spectrum of PEC(X) in the same buffer, λ_{em} = 590 nm (– – –). Emission spectrum of PEC(X), λ_{exc} = 540 nm (–·–·–). All slit widths 5 nm.

madzu UV 260 scanning spectrophotometer or a Hewlett Packard 8451 diode array spectrophotometer. Fluorescence excitation and emission spectra were recorded on a Spex Fluorolog 221 spectrofluorometer in 90° geometry (1 × 1 cm cells, bandwidths 5 nm, maximum absorption. C measurements were performed on a Jobin-Yvon dichrograph V spectropolarimeter. All spectroscopic measurements were performed at room temperature. The photochemistry of PEC(X) was induced by irradiation at 500 or 600 nm as described earlier.[15,17]

RESULTS

The absorption spectrum (Fig. 1) of the isolated PBSomes from *W. prolifica* has a pronounced shoulder at 580 nm, which relates to the presence of PEC, apart from the common pigments PC (600–630 nm) and APC (650 nm). The second derivative yields the absorption maxima of the underlying chromophores at 580, 610, 630 and 650 nm (not shown). The fluorescence excitation spectrum of the PBSomes resembles that of the absorption spectrum, thus showing that the PBSome preparations are energetically well coupled. This is further corroborated by the fluorescence emission spectrum, which is qualitatively independent of excitation wavelength. In particular, excitation at 540 nm yields an emission around 675 nm, confirming the efficient energy transfer from PEC to APC in this system (Fig. 1).

The intact PBSomes were dissociated into the component biliproteins by dialysis against phosphate buffer of low ionic strength. The dissociated PBSomes were then separated on DEAE cellulose into five fractions: PEC(X), PEC, APCI, PC and APCII, in order of increasing ionic strength. For the present work only the two PEC-containing fractions were further characterized. The fast-moving fraction, which is generally not adsorbed to the column, resembles that of the α-subunit of PEC and has previously been called PEC(X).[17] Although the biochemical nature of PEC(X) is still not clear at present, it is obtained reproducibly. The absorption, fluorescence excitation and fluorescence emission spectra are shown in Fig. 2. The latter ($\lambda_{max} \approx 590$ nm) is independent of excitation wavelength. The absorption and the excitation spectra show no coincidence: In particular the absorption shoulder ($\lambda_{max} \approx 510$ nm) is missing in the excitation spectrum, indicating that the underlying short-wavelength form of the violobilin chromophore does not fluoresce efficiently. The spectra of PEC(X) show consistently a small absorption band around 630 nm. This is most likely due to a degraded (oxidized) chromophore, because (1) the absorption increases irreversibly upon prolonged irradiation and (2) it fluoresces poorly, as do chromophores uncoupled from specific protein interaction.[23]

The intense photochemistry of PEC(X) prompted us to investigate the CD properties of it in both photoreversible states. The CD spectra of the PEC(X) sample, preirradiated to saturation with 500 and 600 nm light, are both dominated by an intense positive CD signal with a maximum at 565 nm, corresponding to the absorption maximum of the 570 nm form (Fig. 3). However, the amplitude of the signal is decreased by approximately 35% after 600 nm preirradiation. There is, in particular, a steep decline at the short-wavelength side of this band, indicating a negative band in the 500 nm region. This is more clearly seen in the difference spectrum, which shows a poorly resolved structured band with components at ≈515 and ≈565 nm. Because both are of the same sign and similar amplitude in the difference spectrum, they must be of opposite signs and similar amplitudes in the CD spectra. Because the sign of the 570 nm form is positive, that of the 500 nm form is negative.

The absorption, fluorescence excitation and emission spectrum of the second, slower-moving PEC fraction eluting at 100 m*M* buffer (Fig. 4, solid lines) resembles spectroscopically that of the trimeric PEC reported from other species.[9,15,24] The fluorescence emission is qualitatively independent of the excitation wavelength, and there is reasonable agreement between the excitation and absorption spectra,

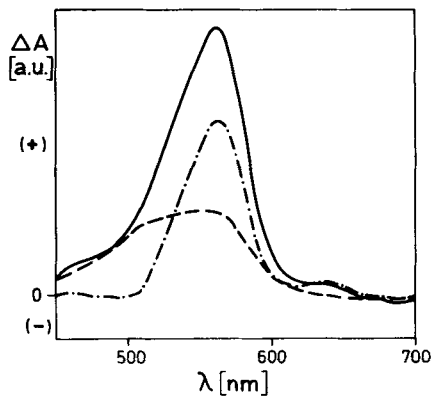

Figure 3. Circular dichroism spectrum ($\Delta A = \epsilon_L - \epsilon_R$) of PEC(X) after saturating green (600 nm) (——) and orange (500 nm) preirradiation (–·–·–). Difference spectrum, green minus orange preirradiation (– – –).

indicating that the constituent subunits are well coupled and exhibit efficient energy transfer.

Addition of chaotropic agents like KSCN (1 *M*) to the second fraction, which is known in other species to dissociate trimeric biliproteins to monomers,[1] leads to distinct spectral changes in the second fraction. The absorption of the PCB chromophores is shifted from 598 nm to 615 nm (Fig. 4a). There is also an enhanced absorption in the near-UV spectral region, which is indicative of a conformational change of the chromophore(s) to a more cyclic geometry.[23] The emission spectrum shows two distinct maxima upon excitation at 540 nm, indicating at least partial uncoupling and independent emission of the violobilin (590 nm) and the cyanobilin (640 nm) chromophores (Fig. 4b). The excitation spectra differ accordingly with the emission wavelength (Fig. 4c).

Yet another minor type of photochemistry related to a reversible line broadening will be reported in a forthcoming publication.

DISCUSSION

One aim of the present investigations was to verify the energy transfer of the PEC from *W. prolifica* in association with other biliproteins within the PBSomes and in its isolated native form. Photochromic chromophores pose problems in energy transfer, because two forms are always present, and photochemistry competes with energy transfer. The reasonable matching of the fluorescence excitation and absorption spectra of the intact PBSomes and the fluorescence emission maximum around 675 nm suggest that our PBSome preparations are energetically well coupled. In particular, PEC is coupled to APC, *e.g.*, PEC is a true antenna pigment. To ensure that this pool was investigated and not a potential pool of free PEC, all further fractionations started from PBSomes.

The first fraction is PEC(X). One advantage of PEC(X) is that due to its intense photochemistry it allows us to characterize the properties of the α-84 chromophore in more detail. Previously, this has been difficult because with one exception,[25] even the monomers of PEC showed only moderate enrichments of either form after saturating irradiation with 500 or 600 nm light. With the repeated isolation of

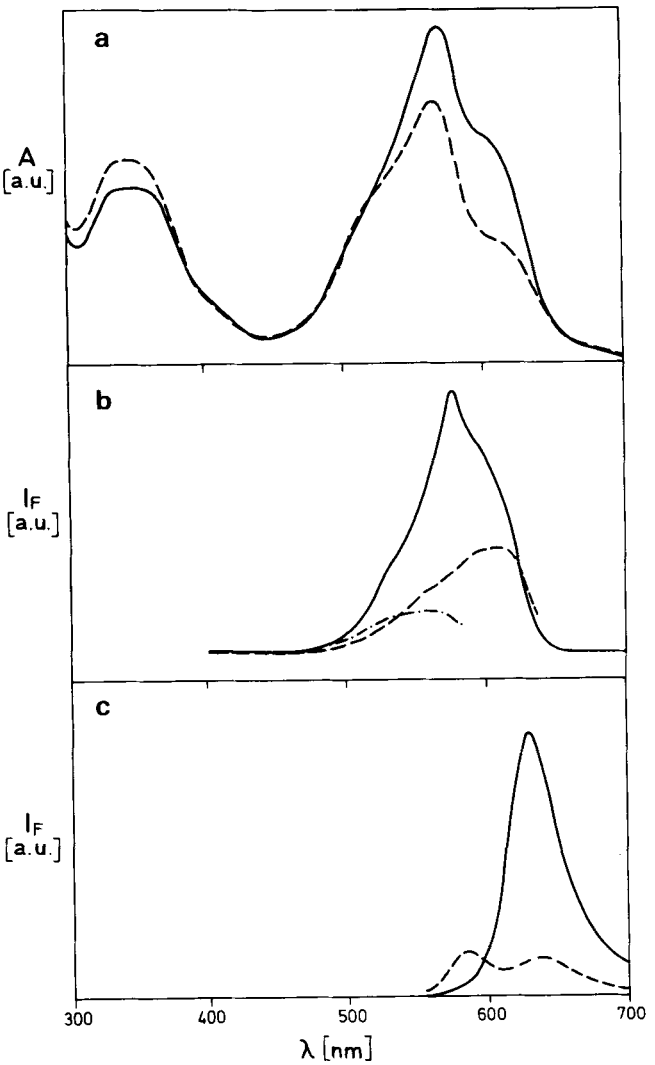

Figure 4. (a) Absorption spectrum of PEC in potassium phosphate buffer (100 m*M*, pH 7.0) (——) and in the same buffer containing additionally KSCN (1 *M*) (– – –). The spectrum in 1 *M* KSCN has been corrected for dilution. (b) Fluorescence excitation spectrum of PEC in potassium phosphate buffer (100 m*M*, pH 7.0, λ_{em} = 630 nm) (——). Excitation spectrum after addition of KSCN (1 *M*), λ_{em} = 590 (– – –) and 630 nm (–·–·–). The spectrum in 1 *M* KSCN has been corrected for dilution. (c) Fluorescence emission spectrum of PEC in potassium phosphate buffer (100 m*M*, pH 7.0) (——) and of the same solution after addition of KSCN (1 *M*) (– – –). Spectra are normalized to the same absorption at the excitation wavelength of 540 nm. All slit widths 5 nm.

PEC(X) from *W. prolifica*, there is now a reliable source for a highly photoactive PEC preparation. Spectroscopically, PEC(X) resembles the α-subunit of PEC.[16,26] It lacks the characteristic absorption of the two cyanobilin chromophores on the β-subunit at 598 nm. Judging from its absorption it seems to contain two fractions with the violobilin chromophores in either state, *i.e.*, one absorbing at $\approx$510 nm and one at $\approx$565 nm.[17] In the previous study there were already indications that only one of the two chromophores is significantly fluorescent, *i.e.*, the 570 nm absorbing one. This has been substantiated in this study. In no case was it possible to observe an emission in the 520–540 nm range, where the 500 nm form of the chromophore is expected to fluoresce.

The high enrichment of the two forms by suitable preirradiations made it also possible to obtain information about their CD properties. The 570 nm form has a positive CD signal, roughly similar in magnitude and identical in sign to the other phycobiliprotein chromophores in their native environments. By contrast, the CD of the 500 nm form is negative and somewhat decreased in amplitude.

Both the fluorescence and CD changes among the forms can be compared to those of the plant photomorphogenetic receptor, phytochrome, which is also a biliprotein. It absorbs at longer wavelengths than PEC due to its more extended conjugated double-bond system but otherwise shows remarkable similarities. Phytochrome in its red form (P_r), which contains a 15Z chromophore, has a positive long-wavelength signal and is fluorescent; phytochrome in its far-red form (P_{fr}), which contains a 15E chromophore, has a negative CD signal and is not fluorescent.[27] These features are like the ones reported here for the 570 and 500 nm forms of the PEC(X), respectively. There remains one major spectroscopic difference. In phytochrome, the 15E isomeric form P_{fr} is red-shifted as compared to the 15Z isomeric form P_r, whereas the corresponding relative spectral positions are inverted in the PEC forms. Here, the nonfluorescent 500 nm form (probably 15E) is blue-shifted as compared to the 570 nm form (15Z, corresponding to P_r). In geometric isomers of bile pigments in solution, the 15E isomer is generally blue-shifted.[28] The blue shift of the 500 nm PEC(X) form is then "conservative," whereas the red shift of the P_{fr} is "unusual." Its molecular origin, which is important for the color perception of plants, is still unknown. Apart from this special phytochrome feature, the similarities are remarkable.

The second PEC fraction is the integral PEC resembling that of the trimeric PEC from other species.[8,24] The similarities in the absorption and the excitation spectra of PEC in potassium phosphate buffer (100 m*M*, pH 7.0) indicate that the PVB and PCB chromophores are energetically well coupled. The emission of PEC monomers from *W. prolifica* is double peaked (λ_{max} is $\approx$590 and $\approx$634 nm) and hence indicates a loss of energy transfer from the PVB chromophore on the α-subunit to the chromophores on the β-subunit. In trimeric PEC the emission comes from the β-subunit only. One major difference between the monomers of PEC and PC is that the most distant chromophores (α-84 and β-84) have rather different spectral properties. In PC, their absorptions and emissions are each only 6 nm apart.[29] Steady-state emission spectroscopy then can hardly distinguish among the emissions of the two chromophores. By contrast, the emissions of the α-84 (=violobilin) and β-84§) (=cyanobilin) chromophores of PEC are approximately 40 nm apart, which is sufficient to clearly distinguish their emissions in PEC monomers. Hence, any leakage fluorescence is expected to show up in steady-state emission spectra. The amount of fluorescence from α-84 is nonetheless surprisingly high, in view of an $\approx$100 ps transfer time to β-84 estimated from the X-ray structure[11] and a total lifetime of the ensemble fluorescence of about 1.5 ns. This indicates a more extensive uncoupling of the chromophores than in PC.

§In view of the similar structures of the two β-subunit chromophores in PC and PEC,[11] β-84 has been tacitly assumed the lowest energy chromophore.

In conclusion, the data show that PEC(X) from *W. prolifica* is a reliable source for a highly photoactive PEC preparation, which allows the investigation of the chromophore in both states. These two states show rather distinct similarities to the two states of phytochrome. They further show that energy transfer in PEC is more sensitive to environmental variations and is probably negligible for the 500 nm chromophore.

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