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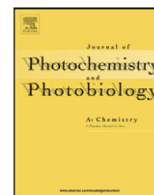

# Spectroscopic parameters of phycoerythrobilin and phycourobilin on phycoerythrin from *Gracilaria chilensis*

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ABSTRACT

The high efficiency of energy transfer by phycobiliproteins in phycobilisomes is an interesting and exciting biological phenomenon. The phycobilisome of *Gracilaria chilensis* contains three phycobiliproteins: phycoerythrin, phycocyanin and allophycocyanin. These proteins have their absorption and emission spectra overlapped allowing a non-radiative, direct and efficient transfer of the excitation energy among them, which is channelled along an energy gradient from the rods to the core and finally transferred to chlorophyll *a*. Phycobiliproteins are highly fluorescent proteins due to the presence of covalently bound phycobilins which provides them their functional properties. This work reports the experimental determination of the spectroscopic parameters for the chromophores present in R-phycoerythrin of *G. chilensis:* lifetime, quantum yield, and extinction coefficient of phycourobilin and phycoerythrobilin, using chromophorylated tryptic fragments containing one or two chromophores. These fragments were identified by mass spectrometry and characterized spectroscopically. These measurements provide the individual spectroscopic parameters of the three chromophores (two phycoerythrobilins and one phycourobilin) in the $\beta$ subunit and the two chromophores (phycoerythrobilins) bound to the $\alpha$ subunit considering the protein environment.

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## 1. Introduction

Phycobilisomes (PBS) are highly efficient accessory light harvesting protein complexes, responsible for the conduction of energy towards the photosynthetic reaction centers. They are exclusive for red algae and cyanobacteria [1]. This biological system has been studied with the purpose to understand the molecular basis for the high efficiency of the energy transfer through the protein complex [2]. The main components of phycobilisomes are the phycobiliproteins (PBPs), i.e. chromophorylated proteins through the covalent binding of lineal tetrapyrrols to specific cystein groups in the sequences [1]. All phycobiliproteins are formed by a basic $\alpha\beta$ heterodimer, that has been shown to oligomerize to $(\alpha\beta)_3$ trimers (allophycocyanin) or $(\alpha\beta)_6$ hexamers (phycocyanin and phycoerythrin) acquiring ring structures as it is shown in Fig. 1 for phycoerythrin. *Gracilaria chilensis* phycobilisomes contain allophycocyanin (APC, $\lambda^{A}_{max}$ 651 nm) in the core of the complex, from where radiate rods, formed by phycocyanin (PC, $\lambda^{A}_{max}$ 621 nm), closer to the core and phycoerythrin (PE, $\lambda^{A}_{max}$ 565 nm) at the far end of the rods [3,4], with their absorption and emission spectra overlapped, allowing a non-radiative, direct and efficient transfer of the excitation energy among them, which is channelled along an energy gradient from the rods to the core and finally transferred to chlorophyll a [5]. PBSs also contain linker proteins that stabilize the complex and contributes to the energy transfer [6]. Our approach is to dissect the process, calculating the constants for the resonance energy transfer ($K_{DA}$) for each pair of chromophores present in phycobiliproteins [7]. This calculation has been performed previously using the Förster approach for Phycocyanin from *G. chilensis* [8,9], however to analyze the function of one antenna, this study has to be performed also for the other component of the rod in *G. chilensis* PBS, Phycoerythrin for whose

*Abbreviations:* PBS, phycobilisome; PE, phycoerythrin; PC, phycocyanin; APC, allophycocyanin; FRET, Fluorescence Energy Transfer; $\lambda^{A}_{max}$, maximum absorption wavelength; $\varepsilon$, extinction coefficient; $\lambda^{E}_{max}$, maximum fluorescence emission wavelength; $\phi$, fluorescence quantum yield; $\tau$, fluorescence lifetime; $\lambda^{exc}$, excitation wavelength.

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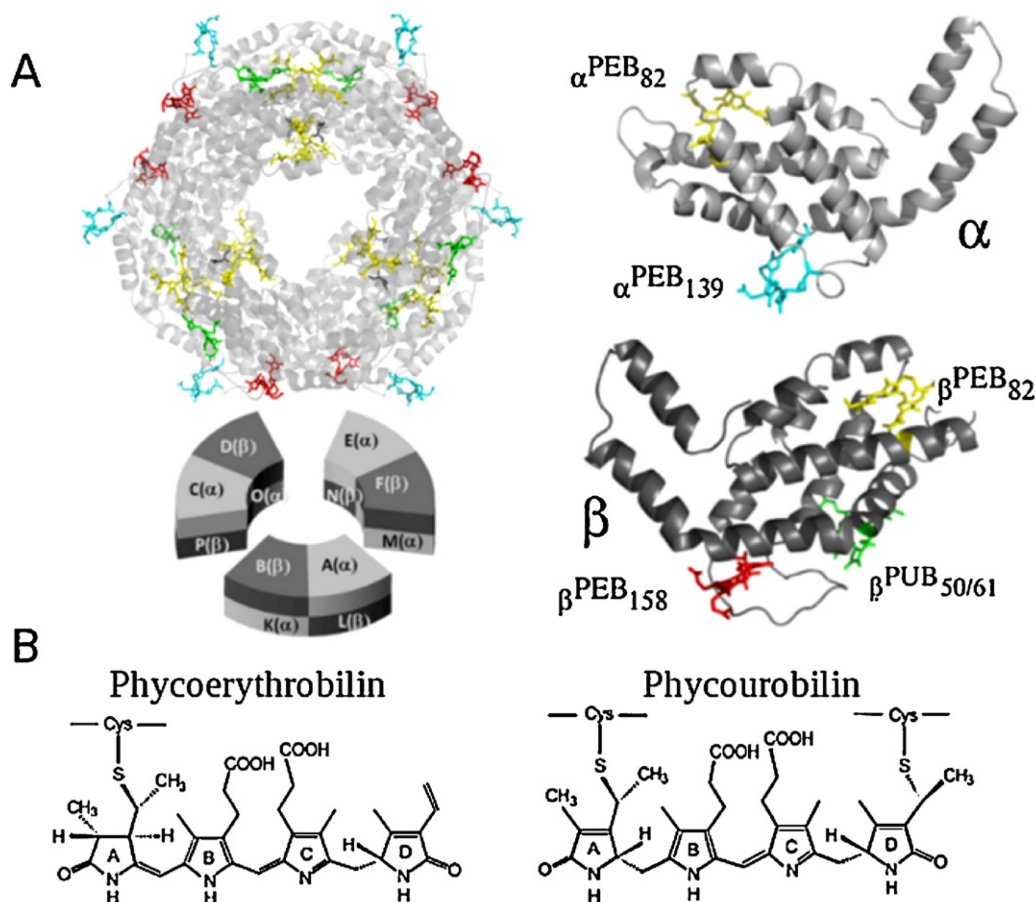



**Fig. 1.** (A) On the left panel, ribbon representation of the hexameric R-phycoerythrin (PDB code: 1eyx) [10], and on the right panel, $\alpha$ and $\beta$ subunits ribbon representation. The chromophores are presented as sticks. (B) Chemical structure of phycoerythrobilin (PEB) and phycourobilin (PUB).

chromophores, phycoerythrobilin and phycourobilin, the required spectroscopic parameter values were not yet available in the literature.

R-phycoerythrin is an oligomeric protein formed by $(\alpha\beta)_6$ hexamer organized in an annular architecture [10] as it is shown in Fig. 1A. This organization allows them to pile up to form the rods. Each $\alpha$ subunit contains 2 phycoerythrobilin (PEB) chromophores bound to C82 and C139 ($\alpha_{82}^{PEB}$, $\alpha_{139}^{PEB}$) by a thioester linkage. Each $\beta$ subunit contains two phycoerythrobilin bound to C82 and C158 ($\beta_{82}^{PEB}$, $\beta_{158}^{PEB}$) and one phycourobilin (PUB) bound to C50-61 ($\beta_{50/61}^{PUB}$).

Fig. 1B shows the chemical structure of these chromophores. It has been reported that the extended conformation that these chromophores show while attached to the protein changes to a closed conformation in solution [11], and that there is an associated change of their spectroscopic properties. Then, the environment of the protein is crucial to maintain the geometry of the chromophores and the spectroscopic properties of the PBPs, in such way that the determination of the photophysic parameters should involve the protein environment. In order to determine the fluorescence quantum yield ($\Phi$), fluorescence lifetime ($\tau$) and extinction coefficient ($\varepsilon$) of PEB and PUB in the extended conformation, chromophorylated tryptic fragments were used for the spectroscopic studies, thus preserving the protein environment as natural as possible. In this report we present a spectroscopic characterization of the PE hexamer, the $\alpha$ and $\beta$ subunits and tryptic chromopeptides, that were used to obtain the spectroscopic parameters for PUB and PEB that will be used afterwards, to study the energy transfer process in this system.

## 2. Materials and methods

### 2.1. Purification of $\alpha$ and $\beta$ subunits of R-phycoerythrin

*R*-phycoerythrin(R-PE) from *G. chilensis* was purified as described previously [12], and the separation of subunits and tryptic proteolysis was performed as reported in the literature [13] with some modifications as follows: 100 μl of the purified phycoerythrin (1 mg/ml) ($A_{566}/A_{280}$ = 5.32) were used to purify $\alpha$ and $\beta$ subunits by Reverse phase high-performance liquid chromatography using a Merck-Hitachi chromatographer coupled to a $C_{18}$ Lichrocart 250-4 column; the elution was performed with a gradient of solution A: 0.1% TFA in water and solution B: 0.1% TFA in acetonitrile at a flux of 0.8 ml/min. The details for the gradient used are shown in Fig. S1. The elution was followed at 280 nm using an UV–Vis detector.

### 2.2. Tryptic digestion of R-phycoerythrin

Chromophorylated fragments of phycoerythrin were obtained using a mild proteolysis with trypsin. 1 μl of 1 M HCl and 2 μl of trypsin (1 mg/ml) (Sigma–Aldrich, St. Louis, MO) were added to 100 μl R-PE (1 mg/ml, in 10 mM pH 7 phosphate buffer), and $NH_4HCO_3$ to a final concentration of 1 mg/ml. After 2 h at 37 °C, the solution was injected in the chromatographic system described above for the separation of the tryptic fragments. The details for the gradient used are shown in Fig. S1A. The chromophorylated fragments were studied by mass spectrometry at the University of Edinburgh. The identification of the peptides masses obtained

**Table 1**
Spectroscopic parameters of R-phycoerythrin, subunits and chromopeptides. $\lambda^{A}_{max}$ is the wavelength of maximum absorption, $\varepsilon$ is the extinction coefficient; $\lambda^{E}_{max}$ is the wavelength of maximum fluorescence emission; $\phi$ is the fluorescence quantum yield and $\tau$ is the fluorescence lifetime. In parentheses is given the wavelength of the reported value.

| Chromoproteins | $\lambda^{A}_{max}$ | $\varepsilon$ ($M^{-1}$ $cm^{-1}$) | $\lambda^{E}_{max}$ | $\phi$ | $\tau$ (ns) |
|---|---|---|---|---|---|
| R-phycoerythrin | 497, 538, 566 | $1.81 \times 10^6$ (566) | 575 | 0.78 | 2.75 |
| Subunit $\alpha$ | 554 | $1.36 \times 10^5$ (554) | 575 | 0.42 | 2.38, 3.92 |
| Subunit $\beta$ | 494, 560 | $1.05 \times 10^5$ (494) | 575 | 0.48 | 4.17 |
| Subunit $\gamma$ | 495, 555 | – | 529 | – | – |
| Chromopeptide A | 530 | $1.08 \times 10^5$ | 577 | 0.28 | 2.02 |
| Chromopeptide B | 560 | $9.7 \times 10^4$ | 595 | 0.25 | 2.12 |
| Chromopeptide C | 495, 530 | $1.83 \times 10^5$ | 529 | 0.20 | 3.78 |
| Fluorescein | 495 | | | 0.95 | 4.1 |

**Table 2**
Overlap integrals ($I \times 10^{l8}$ $cm^4$) for the chromophores in R-PE [17].

| | Donor | | |
|---|---|---|---|
| Acceptor | PUB | $PEB_{530}$ | $PEB_{560}$ |
| PUB | 0.388 | 0.056 | 0.005 |
| $PEB_{530}$ | 6.080 | 1.962 | 2.568 |
| $PEB_{560}$ | 2.015 | 3.822 | 2.395 |

PUB: $\beta^{PUB}_{50/61}$. $PEB_{530}$: $\alpha^{PEB}_{82}$ and $\beta^{PEB}_{82}$. $PEB_{560}$: $\alpha^{PEB}_{139}$ and $\beta^{PEB}_{158}$.

from MALDI-TOF was performed using the MASCOT server (Matrix Science, London, UK). Phycoerythrin, $\alpha$ and $\beta$ subunits and the chromophorylated fragments were studied by absorption and emission spectroscopy.

### 2.3. Spectroscopy

The absorption spectra were recorded in 100 mM pH 7.0 phosphate buffer with JASCO V-650 spectrometer (Tokyo, Japan) with a Peltier system to regulate temperature using 10 mm cuvettes at 20 °C. Steady state and time resolved fluorescence measurements were performed on a K2 multifrequency phase shift and modulation spectrofluorometer (ISS Inc., Champaign, IL, USA) and software from ISS was used for data collection and analysis. For fluorescence emission spectra, the exciting light was a 300 W Xenon lamp (ILC Inc.). The values of quantum yield were obtained as described on [14]. Fluorescein in 0.1 M NaOH was used as standard solution ($\lambda^{exc}$ 496 nm, $\Phi$ = 0.95) [15].

A Laser Led (ISS, Champaign, IL) centered at 470 nm was used for excitation for the lifetime measurements. The emission was collected through a Schott OG520 long-pass filter (Schott Glass Technology, INC, Duryes, Pensylvania). The exciting light was polarized parallel to the vertical axis and the emission was viewed through a Glan–Thompson polarizer oriented at 54.7°. The phase and modulation data were analyzed either by assuming a sum of discrete exponential or continuous distribution models which assumed Lorentzian or Gaussian distributions. Fluorescein was used as standard with $\tau$ = 4.0 ns [16]. Sample temperature was controlled by an external bath circulator (Cole-Parmer, Chicago, IL, USA) and monitored before and after each measurement using an

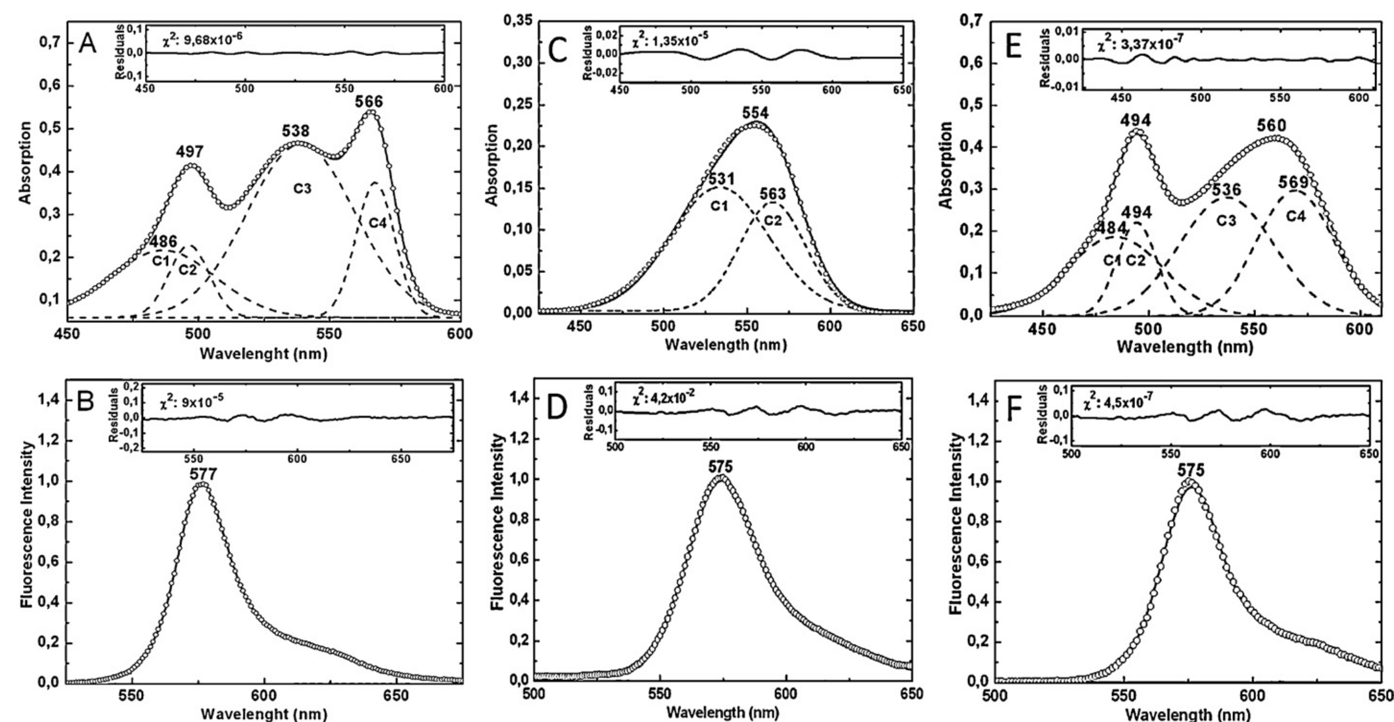
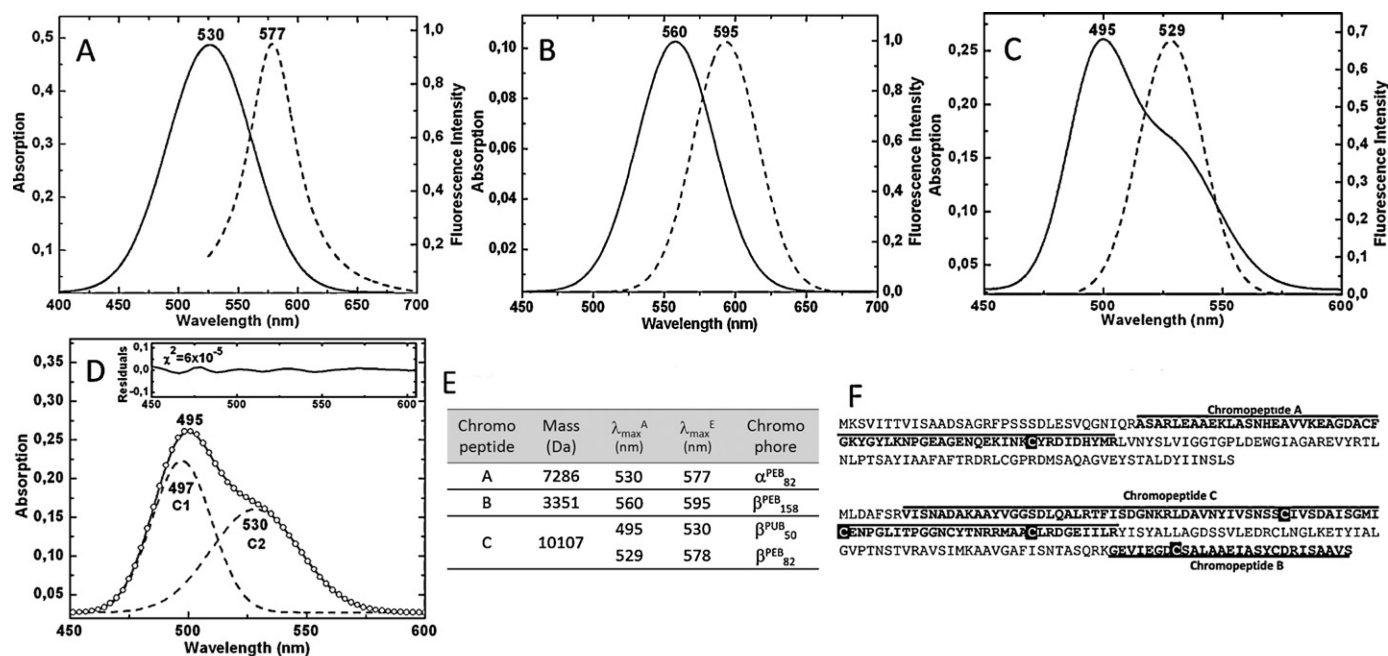



**Fig. 2.** Absorption and fluorescence emission spectra of R-phycoerythrin and their subunits including their decomposition in Gaussian sub-bands. (A) Absorption spectrum of R-PE. (B) Fluorescence emission spectrum of R-PE ($\lambda^{exc}$ 566 nm). (C) Absorption spectrum of $\alpha$ subunit. (D) Fluorescence emission spectrum of $\alpha$ subunit ($\lambda^{exc}$ 554 nm). (E) Absorption spectrum of $\beta$ subunit. (F) Fluorescence emission spectrum of $\beta$ subunit ($\lambda^{exc}$ 494 nm). The numbers above the curves represent the wavelengths of maximum absorption. The open circles represent the experimental spectrum, and the solid line gives the best fit obtained using different components (C1–C4) with Gaussian distributions. The contributions from the individual peaks are given by the broken lines. The differences between the sum of the components and the experimental curve are shown as residuals in the figure inserts.




| Chromo peptide | Mass (Da) | $\lambda_{max}^{A}$ (nm) | $\lambda_{max}^{E}$ (nm) | Chromo phore |
|---|---|---|---|---|
| A | 7286 | 530 | 577 | $\alpha^{PEB}_{82}$ |
| B | 3351 | 560 | 595 | $\beta^{PEB}_{158}$ |
| C | 10107 | 495 | 530 | $\beta^{PUB}_{50}$ |
| | | 529 | 578 | $\beta^{PEB}_{82}$ |

**Fig. 3.** (A)–(C) The absorption (full line) and fluorescence emission (broken line) spectra of isolated chromopeptide A, B, and C, respectively (for fluorescence emission, $\lambda^{exc} = \lambda^{A}_{max}$. (D) Corresponds to the deconvolution of the absorption spectrum of fragment C. The numbers above the curves represent the wavelengths of maximum absorption. The open circles represent the experimental spectrum, and the solid line gives the best fit obtained using different components (C1–C2) with Gaussian distributions. The contributions from the individual peaks are given by the broken lines. The differences between the sum of the components and the experimental curve are shown as residuals in the figure inserts. (E) and (F) The mass, $\lambda^{A}_{max}$, $\lambda^{E}_{max}$, the chromophore composition and the sequences of the MALDI-TOF identified proteolytic fragments.

Omega digital thermometer (Omega Engineering Inc., Stanford, CT, USA).

The deconvolution of all the spectra was performed using the QTI plot software in Linux environment, using a Gaussian model analysis of the components. Residuals and $\chi^2$ were used for the evaluation of the data. The values for the spectroscopic parameters obtained here are shown in Table 1. The overlaps integrals between donor emission spectra and acceptor absorption spectra were calculated as [17] and are shown in Table 2.

## 3. Results and discussion

### 3.1. Spectroscopic analysis

In order to obtain the spectroscopic parameters for the chromophores in R-phycoerythrin and to provide an interpretation to the results obtained, the spectra of purified R-PE and the purified subunits were recorded. Fig. 2A shows the absorption spectrum of R-PE, displaying three maxima at 497, 538 and 566 nm, typical for PEs that contains PUB ($\lambda^{A}_{max}$ 495) and PEB ($\lambda^{A}_{max}$ 530 and 560 nm) [18].

The deconvolution of the absorption spectrum of R-phycoerythrin showed the presence of 4 physicochemically different components: C1 and C2 were assigned to PUB, indicating that in R-PE, one of them corresponds to the PUB present in the $\beta$ subunit and the other could be assigned to the $\gamma$ subunit which copurified in small amount with phycoerythrin (Fig. 2A); C3 and C4 were assigned to PEB in slightly different conformation [18]. The emission spectrum showed a very intense emission at 577 nm with a wide shoulder at app. 600 nm due to vibronic transitions, described by MacColl et al. [19] and recently modeled by Novoderezhkin et al. [20]. No mirror image is observed between the absorption and emission spectra of R PE due to the fact that the absorption shows bands from the different pigments of the protein, and the fluorescence occurs only from the lower transition of the chromophore after efficient energy desactivation pathways [21].

The $\alpha$ subunit absorption spectrum (Fig. 2C) shows a lonely maximum an $\lambda^{A}_{max}$ 554 nm, but the deconvolution detected two maxima at $\lambda^{A}_{max}$ 531 and 563 nm. The $\alpha$ subunit emission spectrum shows also a maximum at $\lambda^{E}_{max}$ 575 and a shoulder at app. 593 nm (Fig. 2D), very similar to the R-PE fluorescence spectrum (Fig. 2B).

The $\beta$ subunit absorption spectrum shows two absorption maxima at $\lambda^{A}_{max}$ 494 and 560 nm. The spectrum deconvolution revealed 4 components, two of them associated to the PUB absorption (C1 and C2) and two to the PEB absorption (C3 and C4) with $\lambda^{A}_{max}$ 536 and 569 nm (Fig. 2E). The emission maxima ($\lambda^{E}_{max}$ 575 nm, Fig. 2F) is similar with the maximum obtained for phycoerythrin. The absorption spectra of the isolated subunits confirmed their chromophoric composition (Fig. 2); an absorption maximum at 494 nm in the $\beta$ subunit, not present in the $\alpha$ subunit was assigned to PUB. A lonely emission peak is observed at 575 nm for both subunits when excited at 530 nm, showing that only PEB would be responsible for the final emission of R-PE.

There are two possible approaches to measure the spectroscopic parameters of each equivalent chromophores in absence of energy transfer processes, decoupling the donor–acceptor pair or isolating the chromophores. In this case, a mild proteolysis and isolation of the chromophorylated peptides was performed (chromopeptides *A*, *B* and *C*, Fig. S1). The identification of the chromopeptides was made by mass spectrometry. The masses and sequences of the fragments isolated are shown in Fig. 3E and F. Every mass was tested with and without considering the masses of the bilins. The characterization and confirmation of the identity of the chromophores present in each fragment was performed by careful analysis of the absorption and emission spectra of each fragment (Fig. 3A–C). Any change in the conformation of the bilins would be detected by a change in the absorption or emission wavelength maxima [11]; this is important considering that the spectroscopic parameters

were obtained from chromophorylated peptides in which the environment could have been modified by the procedure, nevertheless no important change was detected. Fragment *A* showed one absorption maximum at 530 nm and was assigned to $\alpha_{82}^{PEB}$ [18]. Fragment *B* also shows one absorption maximum at 560 nm and one emission maximum at 595 nm. This chromopeptide was assigned, by its sequence, to $\beta_{158}^{PEB}$. Fragment *C*, nevertheless, showed two absorption maxima at 497 nm and a shoulder at 529 nm and one emission maximum at 530 nm upon excitation at 470 nm to avoid the transfer process. According to the mass spectrum, this fragment contains $\beta_{82}^{PEB}$ and $\beta_{50/61}^{PUB}$. The decoupled spectrum of $\beta_{50/61}^{PUB}$ (*C*1 in Fig. 3D) was used to determine the spectroscopic parameters of PUB.

### 3.2. Determination of the spectroscopic parameters of PEB and PUB

It has been reported that in the phycoerythrin multichromophoric system, it is possible to distinguish different chromophore populations based on their spectroscopic behavior. The first population is represented by PUB with absorption maxima close to 495 nm, the second population is represented by the PEBs with absorption maximum close to 530 nm ($PEB_{530}$) and last one represented by the PEBs with absorption maximum close to 560 nm ($PEB_{560}$). The differences observed could be assigned to a change in the chromophores stereochemistry, associated to the observed deviation in the planarity between rings A and B, which may produce a change in the conjugated system [18].

The data from chromopeptide *A* was used to determine the spectroscopic characteristics for $\alpha_{82}^{PEB}$ and $\beta_{82}^{PEB}$ chromophores, because as it was discussed above, their spectroscopic behavior is similar. For $\alpha_{139}^{PEB}$ and $\beta_{158}^{PEB}$ it has been also reported that they show the same spectroscopic characteristics, the spectrum of chromopeptide *B* was used for their parameters determination. The spectroscopic parameters of PUB were determined from the decoupled spectrum *C*1 of chromopeptide C (Fig. 3D).

The spectroscopic parameters $\varepsilon$, $\phi$ and $\tau$ determined for each chromophore are shown in Table 1. The values determined for R-PE and for the $\alpha$ and $\beta$ subunits are also shown. The values obtained are in the order of magnitude expected for these type chromophores [22–24]. In comparison with the information available for phycocyanobilin, $\phi$ and $\tau$ are higher in phycoerythrobilin; $\varepsilon$, nevertheless is very similar [23,25]. The quantum yield for R-PE from *G. chilensis*: $\phi = 0.78$ is similar to those reported for other species [26]. Less similar values have been reported using different experimental approaches [18,22,23]; experimental values of the spectroscopic parameters for phycocyanobilin have been obtained by replacing sequentially the cysteines in the $\beta$ subunit, producing subunits with one chromophore, in this way avoiding the energy transfer. This was possible for cyanobacteria, nevertheless that approach was not select for *G. chilensis* R-PE, because of the difficulty to express this eukaryotic protein in bacteria.

The values obtained here for the chromophorylated peptides, because of the protein environment, should represent closely the spectroscopic parameters of each chromophore in the complete protein. The fluorescence lifetime ($\tau$) for all the studies reported for PBPs in PBS of different species are in the range of 2–4 ns and are similar to those of PEB in this PE and in phytochromes [27,28].

The overlap integral of the absorption spectra of acceptors and emission of the donors were calculated from the spectra determined for the fragments identified A, B, and C and they are shown in Table 2. These values indicate that PUB has a low possibility of homo transfer to another PUB, but a strong chance to transfer to PEB ($\alpha_{82}^{PEB}$ or $\beta_{82}^{PEB}$, identified as $PEB_{530}$).

All these experimental results permitted to determine the constants, $K_{DA}$, between every pair of chromophores, considering the 24 PEB and 6 PUB chromophores in a molecule of R-phycoerythrin from *G. chilensis*.

In natural conditions, PE is located in the far end of the rods and it is the most chromophorylated protein. Every chromophore in the hexamer, should be receiving and transferring energy according to their spectroscopic properties. $\alpha_{139}^{PEB}$ and $\beta_{158}^{PEB}$ are the chromophores more exposed to solvent, more prone to dissipation of the energy. $\beta_{50/60}^{PUB}$ because of its spectroscopic properties should always be a donor in the energy transfer process towards $\beta_{82}^{PEB}$ or $\alpha_{82}^{PEB}$. The availability of the spectroscopic parameters for all the chromophores present in a PBS, including PEB and PUB reported in this article will allow a better understanding of the energy transfer process in PBS.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jphotochem.2011.02.012.

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