# Fluorescence Studies on R-Phycoerythrin and C-Phycoerythrin

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Biliproteins are photosynthetic light-harvesting proteins, which transfer excitons with high efficiencies over relatively long distances until they arrive at a photosynthetic reaction center. Purified R-phycoerythrin (isolated from a red alga) and C-phycoerythrin (isolated from a cyanobacterium), each of which contains several chromophores, were studied by a combination of fluorescence emission, fluorescence excitation polarization, and absorption methods. The polarization spectra of both these biliproteins showed that there was a minimum of two spectrally distinct sensitizing chromophores, which, after absorbing photons, transfer excitons to the lowest-energy (fluorescing) chromophores. Some of these spectroscopic data were used to deconvolute the absorption spectra into the spectra of the two sensitizing and one fluorescing chromophores. It was shown that the higher-energy sensitizing chromophore could readily transfer its excitation energy to the fluorescing chromophore using the lower-energy sensitizing chromophore as an intermediary. However, there was sufficient spectral overlap between the higher-energy sensitizing chromophore and the fluorescing chromophore so that direct transfer between them could not be ruled out.

**KEY WORDS:** Biliproteins; exciton migration in photosynthesis; phycoerythrins; fluorescence polarization.

## INTRODUCTION

The initial event in photosynthesis is the absorption of a photon by a pigment, which is one of a large complex array of pigments. The next step is the migration of this exciton through the array toward one of the two photosynthetic reaction centers. Different organisms have varying combinations of these pigments. In all cases, it appears that the migration process occurs with a very high efficiency. The chromophores, which are involved in light harvesting and exciton migration, usually are segregated into various protein complexes. Exciton migration may then be considered in terms of transfer between proteins or transfer between chromophores contained in a particular protein. In this study, particular proteins, biliproteins, are isolated and purified from their organisms and studied by various spectroscopic methods.

Biliproteins are pigments found in cyanobacteria, red algae, and cryptomonads. In the red algae and cyanobacteria, these proteins are arranged in discrete organelles, phycobilisomes, which are located on the outer surface of the thylakoid membranes. The phycobilisomes are highly structured, usually consisting of an allophycocyanin core and six rods, which contain phycocyanin and phycoerythrin. The phycoerythrins possess the highest-energy chromophores—phycoerythrobilins and, sometimes, phycourobilins—and are situated furthest from the thylakoid membrane. Photons captured by the highest-energy chromophores on the phycoerythrins migrate to the lowest-energy chromophores on the same

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protein, then consecutively to other phycoerythrins, phycocyanins, allophycocyanins, chlorophyll *a*–protein complexes in the thylakoid membrane, and finally, a reaction center. In the reaction centers, the excitons are converted to chemical energy. The properties of biliproteins and phycobilisomes have been reviewed [1–7].

Two biliproteins, R-phycoerythrin and C-phycoerythrin, have been purified for this study from a red alga and a cyanobacterium (blue-green alga), respectively. C-Phycoerythrin is usually isolated as a hexameric $(\alpha_6\beta_6)$ protein aggregate having phycoerythrobilin as its only chromophore, while there are five phycoerythrobilins on each monomer $(\alpha\beta)$ of an aggregate. The phycoerythrobilins can interact with the apoprotein and perhaps with each other to form different energy levels. R-Phycoerythrin has both phycoerythrobilins and phycourobilins. This biliprotein is usually isolated having an $\alpha_6\beta_6\gamma$ protein subunit structure. The chromophores are linear tetrapyrroles, containing no metal ions. The chromophores are covalently linked to the apoprotein by one or two thioether bonds.

Teale and Dale [8] and Dale and Teale [9] produced the first systematic fluorescence study of biliproteins. Recently, picosecond time-resolved laser fluorescence and absorption studies have saliently extended the knowledge obtained on the exciton migration processes of these pigments [reviews in Refs. 10 and 11]. In this study we use fluorescence emission, fluorescence polarization, and absorption techniques to add to the understanding of exciton migration for these two purified phycoerythrins.

## EXPERIMENTAL

C-Phycoerythrin was isolated and purified from the cyanobacterium, *Phormidium persicinum*. The alga was grown in our laboratory, harvested, and stored frozen. The biliprotein was extracted with pH 6.0, 0.1 I, sodium phosphate buffer from the lysed cells. Treatment with this buffer dissociated the phycobilisomes into their component biliproteins. The purification of C-phycoerythrin employed precipitation with ammonium sulfate followed by gel filtration using a column of Sepharose 6B (Pharmacia) and Ultrogel AcA45 (LKB Instruments). Samples having the best ratios of 560-nm absorbances to both 620 and 280 nm were selected. Each procedure was repeated at least a second time. All purification steps employed the pH 6.0 buffer, and this buffer was supplemented with 0.01% sodium azide in the column chromatography protocols. Protein was precipitated with 50% saturated ammonium sulfate and stored in a refrigerator

between steps of the purification. Proteins having the highest $A_{560}/A_{280}$ ratios and correct visible absorption spectra were considered purified. Purified protein was run on 12% acrylamide gels after treatment with detergent, stained by Coomassie blue, and shown to be free of contamination. Finally, the purified protein was exhaustively dialyzed into double-distilled water and lyophilized. The lyophilized protein was stored in a freezer until needed. Just prior to a measurement, the protein was dissolved in pH 6.0 buffer and centrifuged at low speed to remove insoluble material.

The R-phycoerythrin was a commercial product isolated and purified from the red alga, *Gastroclonium coulteri* (Chemical Dynamics Corp., South Plainfield, New Jersey). It was obtained as precipitate under ammonium sulfate and stored in a refrigerator until needed. The precipitated protein was centrifuged and the ammonium sulfate solution discarded. The protein was then dissolved in pH 6.0 buffer and dialyzed exhaustively into pH 6.0 buffer to remove residual ammonium sulfate. After completion of dialysis, the solution was centrifuged at low speed to remove insoluble material. It was then used immediately. Its visible absorption spectrum and the ratio of its visible maximum to the absorbance at 280 nm showed the protein to be highly purified. After a day of experiments, the protein was precipitated with 50% saturated ammonium sulfate and stored in a refrigerator.

Absorption spectra were obtained on a Model 320 spectrophotometer (Perkin–Elmer). The fluorescence spectra were obtained on a Model MPF 44a spectrofluorimeter (Perkin–Elmer). All measurements were carried out at ambient temperatures. Fluorescence measurements were made on samples having absorbances of 0.15 in a 1-cm light path at their visible absorption maxima. This absorbance was in the range where no reabsorption of emission was detectable. Lower absorbances were avoided because of the tendency of biliproteins to dissociate into smaller aggregates at low protein concentrations [12]. Additionally, to minimize the dissociation possibility, the protein was prepared at a much higher concentration. Then just prior to fluorescence measurement, the solution was quantitatively diluted with pH 6.0 buffer to produce the 0.15 absorbance. A fresh dilution was carried out for each measurement.

## RESULTS AND DISCUSSION

### C-Phycoerythrin

Pioneering fluorescence studies on biliproteins demonstrated that there were two functional types of

chromophores present in phycocyanins and phycoery-thrins isolated from cyanobacteria and red algae [8,9]. The sensitizing (s) chromophores transferred excitons with a high efficiency to the fluorescing (f) chromophores, which fluoresced with fairly high yields. In the intact system, the f chromophores would transfer excitons to the next pigment in the migration chain. Since the s chromophores did not transfer excitons with 100% ef-ficiency, some very small portion of their excitation was also released as fluorescence.

The very small amounts of fluorescence emitted by the high-energy s chromophores were sufficient in quan-tity to be detected by some picosecond laser systems. The picosecond studies of this emission and absorbance changes have confirmed the s-to-f chromophore model and allowed quantitation of the transfer events [13–21]. For example, a particular aggregate of B-phycoerythrin, isolated from a red alga, exhibited three lifetimes after excitation at 540 nm of 15, 620, and 2710 ps [21]. The 15-ps lifetime was assigned to exciton transfer from s to f chromophores. The 2710-ps lifetime was the decay by purely radiative events from the f chromophores. No systematic picosecond study of either R-phycoerythrin or C-phycoerythrin has appeared.

Another germane picosecond fluorescence result was obtained using phycocyanin 612 isolated from a cryp-tomonad [14]. Here, the fluorescence emission spectra were recorded at various times on the picosecond scale after excitation of the highest-energy chromophores. The results produced the first experimental evidence that ex-citons were transferred between two energetically dis-tinct s chromophores prior to migration to the f chromophores.

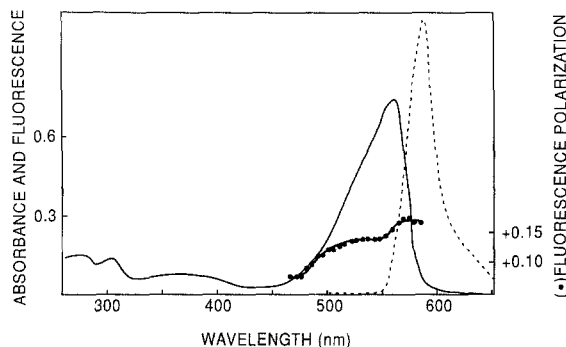Various spectroscopic properties of C-phycoery-thrin (Fig. 1) have been studied to determine whether the protein has more than one type of s chromophores and to find their properties. The fluorescence polariza-tion spectrum across the first excited state of the protein was calculated as follows:

$$P = \frac{I_{VV} - GI_{VH}}{I_{VV} + GI_{VH}} \quad (1)$$

where $I_{VV}$ is the intensity of fluorescence excitation mea-sured with both polarizers in the vertical position, $I_{VH}$ is the intensity of fluorescence excitation measured with the excitation polarizer vertical and the emission polar-izer horizontal, and $G$ is the instrument correction con-stant, which was calculated as $I_{HV}/I_{HH}$ for a particular emission wavelength. The emission monochromator was set at 620 nm and the excitation spectra recorded with the proper polarizer setting. The polarization spectrum for C-phycoerythrin in pH 6.0 buffer showed three po-larization regions (Fig. 1). The f chromophores were found starting at about 550 nm and longer, a lower-energy s chromophore between about 490 and 560 nm, and a high-energy s chromophore ranging between about 470 and 500 nm. The differences in polarization for these chromophores was produced by the angle between ab-sorption and emission oscillators.

## R-Phycoerythrin

The visible absorption spectrum of R-phycoerythrin was much more detailed than that of C-phycoerythrin (Fig. 2). Two maxima and one pronounced shoulder were evident at 494, 565, and 545 nm, respectively. The 494-nm band mainly was produced by phycourobilin and the remaining two bands were different energetic states of phycoerythrobilin. The two highest-energy bands were a clear demonstration that at least two sensitizing chrom-



Fig. 1. The absorption (——), fluorescence (- -), and fluorescence excitation polarization (●) spectra of C-phycoerythrin. All spectra were obtained in pH 6.0 buffer at room temperature.
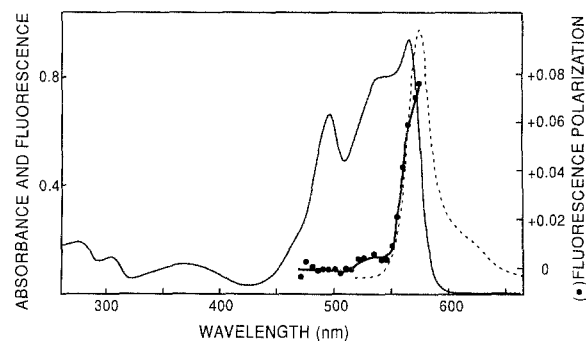


Fig. 2. The absorption (——), fluorescence (- -), and fluorescence excitation polarization (●) spectra of R-phycoerythrin. All spectra were obtained in pH 6.0 buffer at room temperature.

ophores were present. In addition, the polarization spectrum also showed three regions that corresponded precisely to those in the absorption spectrum (Fig. 2). The emission spectrum was not complex and resembled that found for C-phycoerythrin (Fig. 2), and this comparison among the spectra demonstrated that the emission resulted from only a portion of the chromophores on an isolated biliprotein. This sharp difference for R-phycoerythrin between the shape of the visible absorption spectrum and the fluorescence emission allowed early investigators first to propose that biliprotein excitons migrated to the lowest-energy chromophores prior to emission [22]. It is possible that a single chromophore might have produced more than one absorption band in the visible region of the spectrum. This concept was considered unlikely, because when each chromophore was isolated free of overlapping absorbances from other chromophores, they all exhibited single transitions in the visible wavelength region [3,4]. In the intact protein, it was likely that they would, also, show single absorption bands.

The fluorescence emission spectra of R-phycoerythrin and C-phycoerythrin were investigated by exciting the proteins at various wavelengths. The emission spectra were identical in all cases for each protein. This result suggested that, unlike the s chromophores, the f chromophores may be a homogeneous collection of chromophores. For R-phycoerythrin, the emission spectra were obtained from excitations between 460 and 560 nm in 20-nm intervals, and for C-phycoerythrin in 20-nm intervals between 480 and 560 nm (data not shown).

## Deconvolutions

The possible routes of exciton migration through R-phycoerythrin and C-phycoerythrin were considered from a functional deconvolution of the absorption spectra using the fluorescence excitation polarization data. The method of energy transfer between pairs of chromophores in biliproteins was normally considered to be a very weak dipole–dipole coupling [23], although some discussion has been given to certain pairs of chromophores, in particular, biliproteins, being involved in strong or intermediate dipole–dipole coupling [11,24–29]. In the very weak coupling case, the efficiency of energy transfer was related to several factors including modified spectral overlap between the emission spectra of a donor and the absorption spectra of a receptor, the orientation of these dipoles, and the center-to-center distance between donor and acceptor chromophores. The contribution of spectral overlap to the energy migration pathways can be considered after the absorption spectra were deconvoluted

into the three components corresponding to two types of sensitizing and one type of fluorescing chromophores. The absorption spectrum (Fig. 1) of C-phycoerythrin was deconvoluted by modifying a method introduced for biliproteins assuming one type s and one type f chromophore [8,9]:

$$A_{LE} = \frac{A[3 - p_{LE}]\,[p - p_{HE}]}{[p_{LE} - p_{HE}]\,[3 - p]} \qquad (2)$$

$$A_{HE} = A - A_{LE} \qquad (3)$$

where $A_{LE}$ is the calculated absorbance of the lower-energy component in a pair of chromophores, $A$ is the experimental absorbance, $A_{HE}$ is the calculated absorbance of the higher-energy component, and $P_{LE}$, $P_{HE}$, and $p$ are the corresponding polarizations. These papers showed the derivation of the equations and the assumptions employed [8,9]. The calculations for the three components were done separately on the two pairs, and the calculated component spectra obtained (Fig. 3). The resulting component spectra were useful for our purposes but should not be considered rigorously correct. The shape of the emission spectrum of the intact protein was adopted as that of the s chromophore, and it was positioned so that a Stokes shift of 18 nm was present from the maximum of the absorption band of the higher-energy s chromophore to the emission maximum (Fig. 3). This Stokes shift was equal to the one found for the total protein. The assumption concerning the shape of the emission spectra of the s chromophores was based on the general consistency of the shapes of the emissions
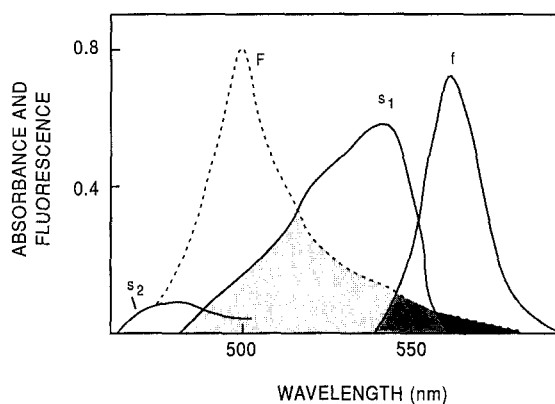


Fig. 3. The calculated absorption spectra of the three deconvolution components of C-phycoerythrin and the proposed fluorescence emission (····) from the highest-energy chromophore. The three shaded areas corresponded to regions of overlap of the emission with absorbances of (from left to right) the higher-energy s chromophore, both the high-energy s and the f chromophores, and the f chromophore.

from all biliproteins regardless of which chromophore was the emitter [8,9]. The three shaded regions corresponded to overlaps between the fluorescence of higher-energy s chromophore and the absorbance of the lower-energy s chromophore; the absorbance of both the lower-energy s chromophore and the f chromophore; and the absorbance of just the f chromophore.

The absorption spectrum of R-phycoerythrin (Fig. 2) was analyzed in the same manner, and the absorption spectra of the three components obtained (Fig. 4). The absorption maxima of the components and their characteristic fluorescence polarization values are found in Table I. The deconvolution for the two phycoerythrins demonstrated a salient property of the biliproteins. Even though C-phycoerythrins possessed phycoerythrobilins as their only chromophore and R-phycoerythrins had both phycoerythrobilin and phycourobilin, the two proteins had component absorption maxima at similar wavelengths (Table I). This ability of the apoprotein to modulate the characteristics of the tetrapyrroles is a key to the light-harvesting capabilities of the proteins. Another example of this ability was C-phycoerythrin and cryptomonad phycoerythrin 566 [30]. In this example, very similar native absorption spectra were achieved by very different chromophore contents.

The assumed emission spectra from the higher-energy s chromophores of both biliproteins overlapped very strongly with the absorption spectra of the lower-energy s chromophores. The modified spectral overlap can be calculated:

$$J = \frac{\int F_D \ (\lambda) \ a_A \ (\lambda)\lambda^4 \ d\lambda}{\int F_D \ (\lambda) \ (\lambda) \ d\lambda} \tag{4}$$
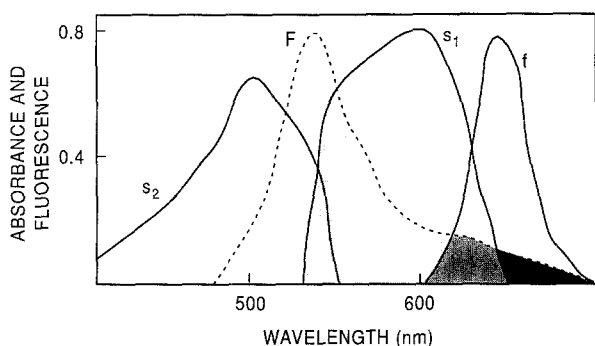


**Fig. 4.** The calculated absorption spectra of the three deconvolution components of R-phycoerythrin and the proposed fluorescence emission (····) from the highest-energy chromophore. The three shaded areas corresponded to regions of overlap of the emission with absorbances of (from left to right) the higher-energy s chromophore, both the high-energy s and the f chromophores, and the f chromophore.

**Table I.** Fluorescence Polarization and Deconvolution Data for R-Phycoerythrin and C-Phycoerythrin

| Proteins | Chromophores | $p^a$ | Absorption maximum |
|---|---|---|---|
| R-Phycoerythrin | s (high energy) | −0.0010 | 495 |
| | s (low energy) | +0.00200 | 541 |
| | f | +0.0700 | 566 |
| C-Phycoerythrin | s (high energy) | +0.0500 | 480 |
| | s (low energy) | +0.111 | 542 |
| | f | +0.141 | 561 |

[a]The fluorescence excitation polarization found for several biliproteins in cases where there was no energy transfer was always +0.4 [11], which corresponds to an angle of 23° between absorbing and emitting dipoles for a single chromophore. The equation giving this relationship was $p = \dfrac{(3 \cos^2\theta - 1)}{(\cos^2\theta + 3)}$, and it should be noted that this calculation assumed that the fluorescence occurred from chromophores that did not move during the lifetime of the excited state and that there was no energy transfer from these states. The former condition has been shown to hold for nondenatured biliproteins, and the latter condition was shown to be valid in selected cases where a limited number of chromophores occurred on a biliprotein [3].

where $J$ is the modified spectral overlap in $M^{-1}cm^3$, $F_D$ is the intensity of the emission of the donor normalized to unity, $a_A$ is the molar absorptivity of the acceptor in $M^{-1}cm^{-1}$, and $\lambda$ is the wavelength. Since the deconvolution was not rigorous quantitatively, the calculation of $J$ was not carried out. Qualitatively, the inspection of the overlaps (Figs. 3 and 4) clearly showed that excitons could be transferred from the high-energy to the low-energy s chromophores with a very high efficiency if the distance and dipole orientations were also favorable. The fairly small volumes of the proteins and the angles suggested by the polarization spectra (Figs. 1 and 2) guaranteed that these conditions were met. Transfer directly to the f chromophores was likewise allowed from the overlap perspective. The extent of these overlaps (Fig. 3 and 4) demonstrated that the approximations made during the analyses could not have seriously affected the conclusions. Sidler et al. [31] have entirely sequenced the amino acids of C-phycoerythrin. Their data determined the positions of all five tetrapyrroles on the chain of amino acids for the α and β subunits. They have speculated that the additional chromophores not found on C-phycocyanin were located on the periphery of the three dimensional structure of the protein and would be sensitizing chromophores. An X-ray diffraction study of crystals of this protein would yield more information on the routes of exciton migration.

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