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Phycoerythrin 566 – a fluorescence study

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The cryptomonad biliprotein, phycoerythrin 566 from *Cryptomonas ovata*, has been studied by a variety of spectroscopic technics, including absorption, fluorescence, fluorescence polarization, and ultrafast fluorescence kinetics. The data were analyzed to obtain information about the transfer of excitons among the various chromophores (bilins) on the isolated protein. Ultrafast fluorescence studies were performed using a variety of interference filters to monitor emission after excitation at 532 nm. The fluorescence kinetics was found to be dependent on the emission wavelength being observed. The kinetic fits of the data suggest that 532-nm light excited two types of chromophores and that one type transferred energy to the other in about 30 ± 3 ps. This conclusion was based on the fitting of the fluorescence to two kinetic equations in which the rate of energy transfer out of one chromophore was equal to the rate of energy transfer into another chromophore. Emission from the protein occurred with a 1000-ps radiative lifetime. The fluorescence polarization spectrum of phycoerythrin 566 was consistent with several energy-transfer events between various energetically different chromophores. The various steady-state fluorescence results suggested that one type of chromophore, or a group of chromophores in equilibrium, was the final emitter. After absorption of photons, excitons were transferred with great efficiency (97%) between various spectroscopically distinct chromophores and were eventually emitted by the lowest-energy chromophores. These results were for a dimeric ($\alpha_2\beta_2$) protein at pH 6.0; at lower pH or lower protein concentration, the protein dissociated.

Introduction

Biliproteins are photosynthetic light-harvesting pigments found in blue-green algae, red algae and the cryptomonads. Phycoerythrin 566 is a biliprotein found in certain cryptomonads (*Cryptophycea*). Once a photon is absorbed by a photosynthetic pigment its energy must be transferred as an exciton, perhaps over a considerable distance, until it reaches one of the two photosynthetic reaction centers. Excitation on the tetrapyrrole chromophores (bilins) of a biliprotein must first migrate among the multiple chromophores of a discrete protein aggregate before being passed on to the next pigment in the chain. When isolated and purified, cryptomonad biliproteins are generally found to be dimeric oligomers of two subunits ($\alpha_2\beta_2$). Both of the subunits have one or more bilins (for a review of biliproteins, see Ref. 1).

The current objective is to study the exciton migration process on purified phycoerythrin 566 using various types of fluorescence. The isolated biliproteins emit some photons in high yield that in the intact photosynthetic system would be transferred to the next pigment in the chain. In addition, there is very-low-yield fluorescence from a bilin whose primary function is to transfer excitation to another bilin on the same aggregate. These low yield events can be detected by ultrafast time-resolved fluorescence if the techniques are sufficiently sensitive. This is the first time the kinetics of exciton migration is reported for any cryptomonad phycoerythrin. Steady-state fluorescence (excitation) polarization is also very useful in the study of isolated biliproteins because excitation-energy-transfer events are observable, since the polarization values depend on the angles between transferring dipoles.

Experimental

Cultures of *Cryptomonas ovata* UTEX 358, were obtained from the culture collection at the University of Texas at Austin. Cells of *C. ovata* were grown, harvested and stored frozen. Freezing and thawing lysed the cells and the water-soluble phycoerythrin 566 was released into the solvent, a pH 6.0, 0.1 ionic strength, sodium

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phosphate buffer. Following the previous protocol [2], the protein was then purified by a combination of ammonium sulfate precipitation, and gel-filtration chromatography on Ultrogel AcA54 (LKB Instruments, Rockville, MD) and Sepharose 6B (Pharmacia, Piscataway, NJ). Purified protein was dialyzed against distilled water and was lyophilized. It was then stored at about 4°C.

Fluorescence measurements were performed on a model MPF44A fluorescence spectrophotomer (Perkin-Elmer, Norwalk, CT). The excitation spectrum was corrected using a rhodamine B quantum counter. The fluorescence (excitation) polarization spectrum was calculated by $p = (I_{vv} - GI_{vh})/(I_{vv} + GI_{vh})$, where $G = I_{hv}/I_{hh}$; I is the intensity of the fluorescence excitation; and vh is excitation light polarized in the vertical direction and emission monitored in the horizontal (vv, hv, hh are defined anologously). The maximum absorbance of the solutions used for fluorescence measurements was 0.10 absorbance in a 1-cm light path, since, at this absorption, negligible reabsorption of emission occurs. Absorption measurements were performed on a model 320 spectrophotometer (Perkin-Elmer, Norwalk, CT). All experiments utilized pH 6.0 buffer unless otherwise noted. A few fluorescence experiments were carried out in pH 3.9, 0.1 ionic strength, sodium acetate buffer. All spectroscopic measurements were carried out at room temperature.

Ultrafast fluorescence measurements were performed as previously described [3,4]. Solutions of phycoerythrin 566 were irradiated with single 30-ps pulses at 532 nm by a active-passive mode-locked neodymium-yttrium-aluminum-garnet (Nd^{3+}YAG) laser. Fluorescence was detected with a low-jitter streak camera. The distribution of the kinetics parameters as a function of wavelength was determined by placing various interference filters in the collection optics. The interference filters (Ditric Optics, Inc.) had 10-nm bandwidths.

Sedimentation velocity experiments were performed on a model E analytical ultracentrifuge using absorp-

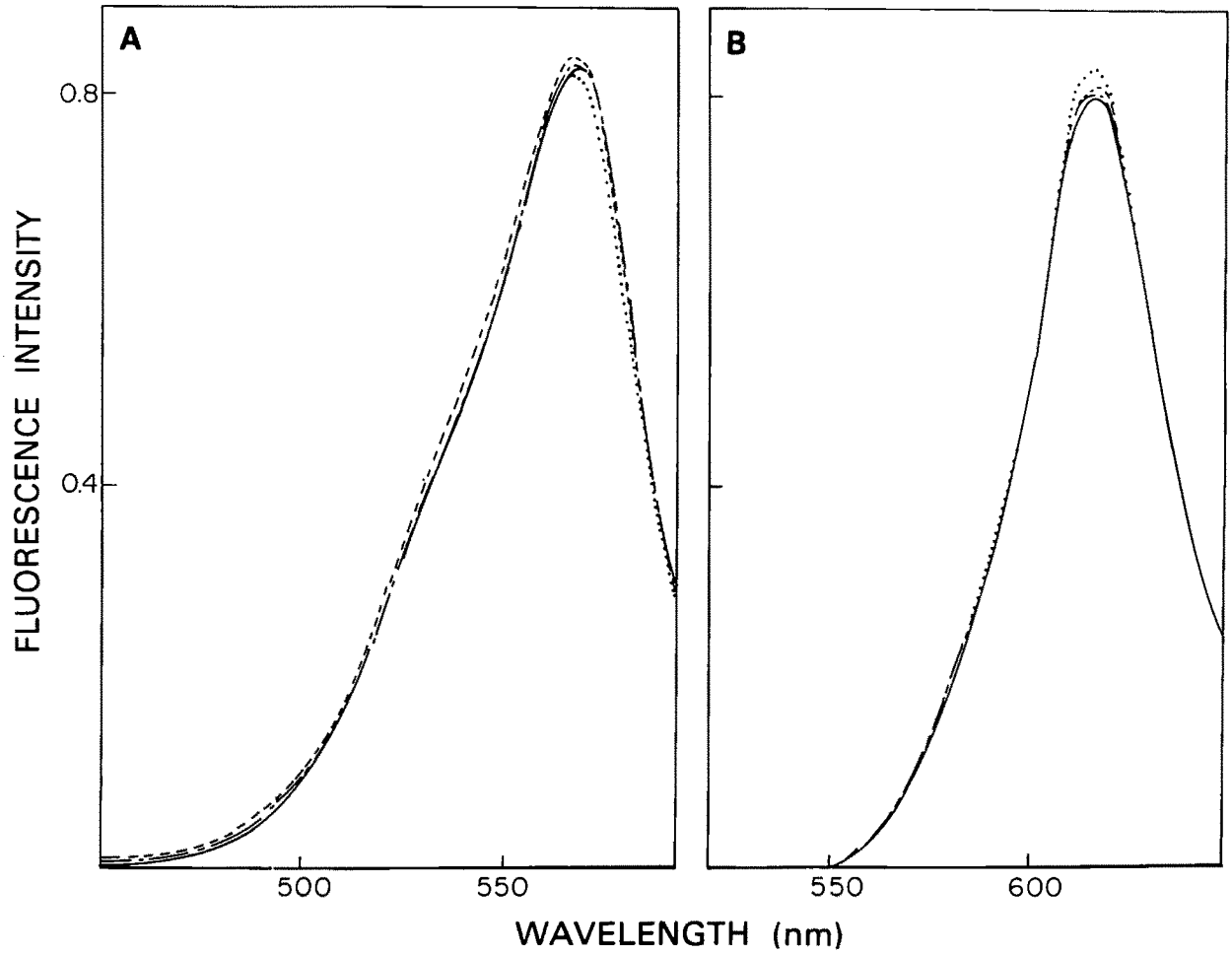

Fig. 1. Fluorescence spectra (uncorrected) of phycoerythrin 566. Spectra were taken in pH 6.0, sodium phosphate, buffer, and at ambient temperature. For the emission (B) spectra, the excitation wavelengths were 480 (— - —), 520 (· · · · · ·), 560 (— — —) and 600 (———) nm, and for the excitation (A) spectra emission wavelengths of 600 (- - - - - -), 620 (· · · · · ·), 650 (— - —), and 680 (———) nm were used.

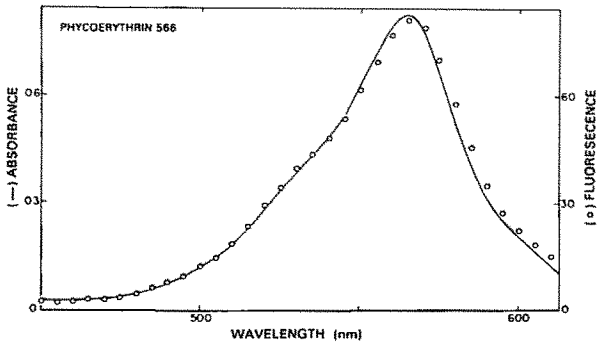

Fig. 2. Corrected fluorescence excitation spectrum and visible absorption spectrum of phycoerythrin 566. Spectra were taken on a solution in pH 6.0, sodium phosphate, buffer at ambient temperatures. Emission was set at 640 nm.

tion optics and the photoelectric scanner. Sedimentation was carried out at 52000 r.p.m. for phycoerythrin 566 (0.1 mg/ml) at pH 3.9 and 6.0.

Results

Phycoerythrin 566 was observed to have an absorption maximum at a longer wavelength than the other cryptomonad phycoerythrins (545 and 555 nm). The fluorescence emission spectra of phycoerythrin 566 in pH 6.0 buffer were determined as a function of excitation wavelength between 480 and 600 nm. In addition, the emission spectra were compared using 525 and 350 nm excitations. In every case, when the emission maxima were set to an equal intensity, the emission spectra were virtually identical. Likewise, the excitation spectra as a function of emission wavelength from 600 to 680 were identical (Fig. 1). The excitation spectrum of phycoerythrin 566 was corrected by use of a rhodamine

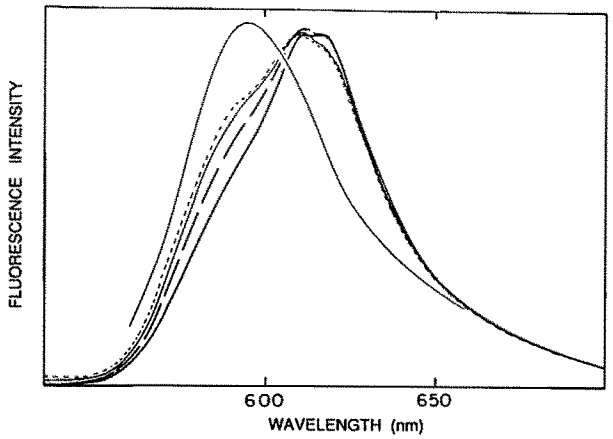

Fig. 3. Fluorescence emission spectrum (uncorrected) of phycoerythrin 566 at pH 3.9. ($A_{566} = 0.10$) and at pH 6.0 as a function of protein concentration. The various spectra are set to equal intensity at their emission maxima. The concentrations at pH 6.0 were as follows: heavy line, $A_{566} = 0.10$, about 0.01 mg/ml; long dashes, $A_{566} = 0.05$, 0.005 mg/ml; thin line, $A_{566} = 0.025$, 0.0025 mg/ml; short dashes, $A_{566} = 0.0125$, 0.00125 mg/ml. Excitation was performed at 525 nm.

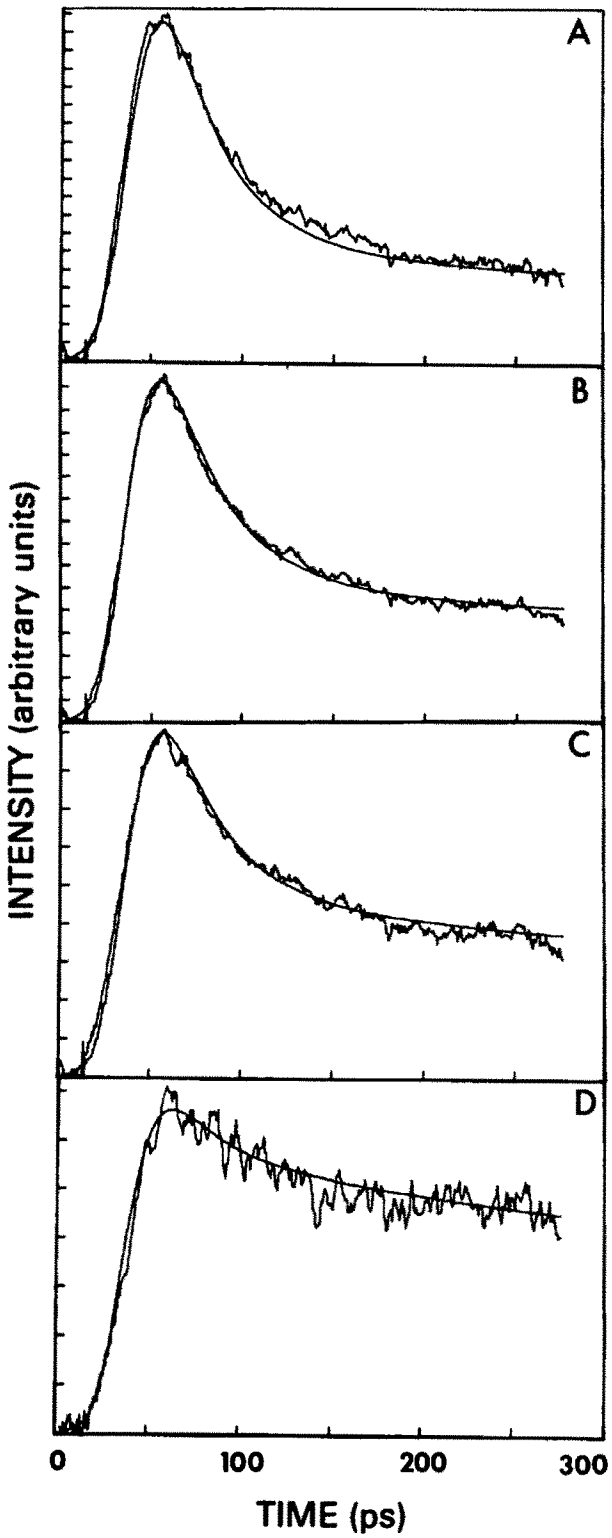

Fig. 4. Picosecond fluorescence of phycoerythrin 566. The following interference filters were used: (A) 570 nm; (B) 580 nm; (C) 590 nm; (D) 700 nm. Other experiments were also performed using interference filters of 600 and 650 nm as well as those using no interference filter (data not shown). The best fit by our protocol is indicated by a solid line. The values for N_A and N_D used in these particular cases are: (A) 0.10, 0.90; (B) 0.13, 0.87; (C) 0.17, 0.83; (D) 0.33, 0.67.

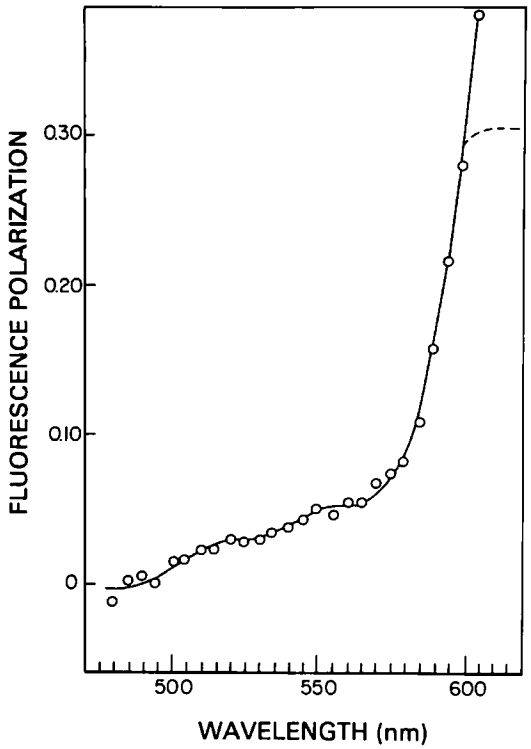

Fig. 5. Fluorescence (excitation) polarization spectrum of phycoerythrin 566. Sample was dissolved in pH 6.0, sodium phosphate, buffer. Emission was set at 610 nm.

B quantum counter (Fig. 2). It was shown to be very similar to the absorption spectrum of the biliprotein.

At pH 3.9, the fluorescence emission spectrum was shifted to lower wavelengths (Fig. 3). The sedimentation coefficient of the protein at pH 3.9 was 3.6 S compared to a value of 5.1 S at pH 6.0. At pH 6.0, the fluorescence emission spectrum was examined as a function of protein concentration (Fig. 3).

The ultrafast fluorescence studies of phycoerythrin 566 in pH 6.0 buffer were performed by exciting the protein at 532 nm at a fluence of $3 \cdot 10^{13}$ photons/cm^2. A series of interference filters at 560, 570, 580, 590, 600, 650 and 700 nm was used to study the emission. The longer-wavelength filters showed a progressively increasing contribution of much longer lifetime (Fig. 4). Solutions having an absorption of 0.10–01.5 in a 1.0 or 0.5 mm light path at 532 nm were used in these studies.

The fluorescence (excitation) polarization spectrum of phycoerythrin 566 was likewise obtained in pH 6.0 buffer using steady-state technics. The polarization was very complex (Fig. 5). Solutions having an absorption of 0.10 at 566 nm in a 1-cm light path were used in these studies.

Discussion

The ultrafast kinetics experiments showed that excitons are transferred in 30 ps between certain bilins of phycoerythrin 566. These bilins are spectroscopically different. The fluorescence emission spectrum of phycoerythrin 566 was found to be independent of excitation wavelength (Fig. 1). Likewise, the excitation spectrum was unchanged when obtained at various emission wavelengths (Fig. 1). In addition, the absorption and corrected fluorescence excitation spectra (Fig. 2) were essentially identical. Excitation energy transfer among the various bilins, therefore, must take place very efficiently until the exciton arrives at the final emitter. The final emitter is perhaps a single chromophore or a group of chromophores that were in equilibrium in terms of their excitation, and it may not be a group of independent and nonidentical chromophores.

The ultrafast kinetics were analyzed by the following equations:

$$\frac{dN_D}{dt} = N_{oD}\sigma_{oD}I(t) - N_D(k_{DA} + 1/\tau_{Do})$$

$$\frac{dN_A}{dt} = N_{oA}\sigma_{oA}I(t) + N_D k_{DA} - N_A/\tau_{Ao}$$

where N_D and N_A (number/cm^3) were the local densities of singlet excitations of the donor and acceptor species, respectively, within the protein, and the other parameters are defined below. These equations were solved numerically. The excitation function $I(t)$ was essentially a 532-nm Gaussian pulse (in photons/cm^2 per s) but the experimentally measured value was used directly in integrating these equations. The absorption cross sections, $\sigma_{oA} = \sigma_{oD} = 6.4 \cdot 10^{-17}$ cm^2, were taken to be that of the two chromophores at 532 nm. N_{oD} and N_{oA} were the local ground-state donor concentrations, approx. $3.0 \cdot 10^{19}$ cm^{-3} (a parameter not crucial to the linear kinetics). Finally, k_{AD}, τ_{Do} and τ_{Ao} were fitting parameters corresponding to, respectively, a pairwise rate of transfer (in s^{-1}) and donor and acceptor radiative decay times (in s) in absence of transfer.

The data fits were done at first by simply choosing parameters and selecting solutions that appeared best by eye. The coefficients of N_D in the two equations were independently identical to within experimental error, with the value $(30 \pm 3$ ps$)^{-1}$, indicating a rate of transfer $k_{DA} = (3.0\text{–}3.7) \cdot 10^{10}$ s^{-1} and a donor lifetime of at least 0.1 ns. The acceptor lifetime was found to be $\tau_{Ao} = 1.0$ ns $\pm$ 0.1 ns. In all the fits, the use of a theoretical Gaussian fit to the actual experimental pulse did not change the fitted parameters to within the experimental uncertainty. The total fluorescence in this range may be satisfactorily fitted to the sum of various fractions of the donor and acceptor emissions. The best fit of the data demonstrated that there were two chromophores excited, and that $\sigma_{oD} = \sigma_{oA}$. One chromophore transferred excitation to the second with $k_{DA} = (30 \pm 3$ ps$)^{-1}$ or $k_{DA} = (3.0\text{–}3.7) \cdot 10^{10}$ s^{-1} for both equations as given above. The rate of transfer out of the donor was equal to the rate of transfer into the acceptor chromophore.

The fitting protocol indicated that very similar results were obtained by both the 700-nm interference filter and from analysis of the total fluorescence emission. This finding is caused by the very low quantum yields of the higher-energy chromophores. The total emission is, therefore, dominated by the high fluorescence emission yield of the final emitters.

The picosecond time-resolved methods were able to measure the very small amounts of emission coming from nearly non-fluorescing chromophores. Analysis of this fluorescence demonstrated that 532-nm light was exciting at least two types of chromophores. These results indicated that one of these chromophores – the higher energy one – transferred excitation energy to the other in 30 ± 3ps. Support for this assignment came from the finding that the 30 ps^{-1} transfer rate out of the donor matched the 30 ps^{-1} transfer rate into acceptor bilin. These measurements further showed that emission from the final accepting chromophore decayed with about a 1 000 ps lifetime. The salient reduction of the 1 000-ps lifetime to 30 ps was another demonstration of the high efficiency of the energy-transfer process. Using this value together with the 1 000-ps emission lifetime, we calculated a 97% efficiency of energy transfer from the donor to acceptor chromophore by $\tau_{D,A}/\tau_D = 1 - E$, where $\tau_{D,A}$ was the lifetime of donor in presence of acceptor, τ_D was the lifetime of donor in the absence of acceptor and E was the transfer efficiency.

Hanzlik et al. [3] had shown previously in picosecond studies on phycocyanin 612 that 532-nm light excited essentially a single chromophore. It was postulated that this one chromophore transferred excitation to the second chromophore in much less than 7 ps. The data clearly showed that this second chromophore was de-excited by transferring its energy to other chromophores in 7–10 ps. The absorption band of phycoerythrin 566 is blue-shifted relative to phycocyanin 612, and the 532-nm laser light will excite chromophores having energies nearer the middle of the absorption envelope. The phycoerythrin 566 (Fig. 4) results were consistent with the phycocyanin 612 findings in that both support a complex energy-transfer situation in which certain chromophores probably function as both acceptors and donors, but instead of only a single chromophore being initially excited there was a dual excitation by the laser. Another cryptomonad biliprotein, phycocyanin 645, has been studied by picosecond fluorescence and absorption methods [5,6]. As in the cases of phycoerythrin 566 and phycocyanin 612, these studies found a fast decay (8 or 15 ps) that was consistent with energy transfer between chromophores.

Dale and Teale [7,8] applied fluorescence polarization spectroscopy to the study of biliproteins. They suggested that changes in the polarization were due to excitation-energy-transfer-events between two spectrally distinct types of chromophore. The fluorescence polarization spectrum of phycoerythrin 566 (Fig. 5) was in basic agreement with this concept, but in addition to the large polarization change occurring between 570 and 600 nm, smaller polarization changes between 490 to 520 nm and 530 to 550 nm were now considered important and relate to a series of exciton transfer events between several spectrally distinct chromophores within the isolated protein. The complexity of the fluorescence polarization spectrum supported the ps fluorescence kinetics results of two spectrally distinct types of chromophores absorbing at 532 nm. These changes in polarization occur in wavelength regions where shoulders are observed in the visible absorption spectrum (Fig. 2).

All the experiments discussed above were for phycoerythrin 566 in pH 6.0 buffer. The emission spectrum shifted about 10 nm to the blue of the pH 6.0 band and was hypochromic at pH 3.9 (Fig. 3). With some other biliproteins, it had been reported that these slightly acidic solvents produced partial dissociation of the oligomeric protein [1]. Likewise, as the protein concentration was lowered at pH 6.0, a new band was observed at shorter wavelengths (Fig. 3). Sedimentation velocity experiments showed the sedimentation coefficient of phycoerythrin 566 dropped from 5.1 to 3.6 S at pH values 6.0 and 3.9, respectively. The pH 6.0 sedimentation value is characteristic of a dimeric subunit structure [1], and the protein partially dissociated at pH 3.9 or lower protein concentrations. The various steady-state fluorescence measurements were, therefore, performed at the highest protein concentration allowable, which avoids the possibility of reabsorption of emitted light, so that a solution of dimers was present. Much higher protein concentrations were used in the ps experiments ensuring that dimers were present.

This is the first report of the rate of exciton migration within a phycoerythrin. Several ultrafast kinetics measurements on phycocyanins have found rates similar to the 30 ps found for phycoerythrin 566 [1,9–12]. For cryptomonad phycocyanins, reports of 7–15 ps have appeared [3,5,6]. For phycocyanins 612 and 645, models for the exciton migration throughout the entire isolated protein have been proposed [13,14]. These models suggested that a series of transfer events occurred before the energy was finally released as fluorescence. The mechanism for energy transfer for these phycocyanins included a combination of very weak dipole coupling and delocalization of excitation between certain chromophore pairs. The fluorescence polarization spectrum indicated that phycoerythrin 566 was also complex in its behavior. The coincidence of the corrected fluorescence excitation and the absorption spectra, the independence of the excitation and emission spectra to the wavelengths used to monitor and excite, respectively, and the 30-ps rate of transfer suggested that all the chromophores contributed to the final emission and that cer-

tain high-energy chromophores contributed by very efficiently transferring excitation energy to other chromophores. The high-energy chromophores emitted extremely low yields of fluorescence. These emissions from the high-energy chromophores were undetected in our steady-state measurements but were, of course, observed in our time-resolved experiments. The chromophores that did emit fluorescence in high yields included the lowest-energy chromophores of the protein. These different chromophores were either chemically distinct, or the same chromophore in a different environment [1]. In order to maximize spectral overlap and allow most efficient energy transfer, we assume that the transfer events indicated in the fluorescence polarization spectrum occur between the pairs of chromophores that are energetically closest to one another. The fluorescence polarization spectrum (Fig. 5) was consistent with absorption maxima of the chromophores, which were involved in the initial exchange of an exciton after 532-nm excitation, being very roughly at 530 and 565 nm.

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