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A picosecond time-resolved fluorescence study on the biliprotein, phycocyanin 645

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Phycocyanin 645, a photosynthetic chromoprotein isolated from a cryptomonad, has been studied by picosecond time-resolved fluorescence by comparing the results of excitation at three wavelengths, 565, 585 and 615 nm. These excitation wavelengths were selected to correspond as closely as possible using our instrumentation to the maxima of three of the four bands found by deconvolution of the absorption spectrum. These three deconvolution bands – 558, 585 and 624 nm – corresponded to the absorption maxima of chromophores that transfer their excitation energies at very high efficiencies to the lowest-energy chromophore. The fourth, lowest-energy, deconvolution band was at 650 nm, and this chromophore provided the main fluorescence emission of the protein. The decay kinetics for 565 and 615 nm excitation were at 8.9 and 14.7 ps, respectively, but excitation at 585 nm gave rise to a faster component at about 2 ps. Careful statistical analysis was performed to verify that the decays obtained by the various excitations were significantly different. These results were discussed in terms of a model in which the 585 nm and 650 nm bands were strongly coupled and shared delocalization energy. The remaining two bands – 558 and 624 nm – probably transferred excitons to the 650 nm band by very weak dipole coupling. If we assume the delocalization hypothesis to be valid, the 2 ps decay would be a measure of the internal conversion between the high- and low-energy exciton states of the delocalized pair.

Introduction

Biliproteins are light-harvesting and excitation-energy-transfer pigments active in photosynthesis in cyanobacteria, red algae and cryptomonads. These proteins harvest solar energy in spectral regions of low chlorophyll *a* absorbance and transfer the excitons at very high efficiency initially to the Photosystem II pigments in the thylakoid membrane. Cyanobacteria and red algae may have three spectrally distinct types of biliprotein in an individual organism – phycocyanin, phycoerythrin (or phycoerythrocyanin) and allophycocyanin – but a cryptomonad apparently may have only phycocyanin or phycoerythrin in any organism. The individual biliproteins are usually isolated as oligomeric proteins, each having several chromophores. The chromophores (bilins) are linear tetrapyrroles, which are covalently attached to cysteine residues of the apoprotein. There are two major bilins, phycocyanobilin and phycoerythrobilin, and at least three minor bilins. Minor bilins only occur together with one of the major bilins. Reviews of the biliprotein literature are available [1–7].

The transfer of excitons on a biliprotein occurs very rapidly and can be measured using appropriate instrumentation. Holzwarth [8] has reviewed the applications of picosecond time-resolved measurements to the study of biliproteins and other photosynthetic materials. In general, fluorescence and absorption kinetics measurements show that, after exciting the higher-energy bilins, excitons reach the lowest-energy bilins in the range of tens of picoseconds. The lowest-energy or fluorescing chromophores, which would transfer energy to the next component in the intact photosynthetic system, display decay times of 1 to 2 ns for isolated biliproteins.

The mechanism by which excitons are transferred between bilins is usually assigned to very weak coupling

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of dipoles [9]. Although this is probably appropriate in most cases, more recent evidence from a variety of steady-state spectroscopic approaches (CD, absorption, fluorescence and fluorescence polarization) suggested that some bilin pairs have energy delocalized (strong coupling) between them. Very weak and strong coupling represent extremes in the continuum of interaction strengths, and they are sufficient, at present, to describe the data on the biliproteins. Allophycocyanin and phycocyanins 645 and 612 have been the principal objects of these studies and all show behaviors suggesting possible delocalization [10–15]. Arguments opposing the existence of delocalization in the case of allophycocyanin have also been forwarded [16,17]. Furthermore, some bilins in C-phycocyanin have also been discussed as possible candidates for energy transfer by means other than very weak dipole coupling on the basis of analysis of X-ray crystallographic data [18,19].

This work employs picosecond fluorescence measurements to examine exciton migration in phycocyanin 645 – isolated from the cryptomonad *Chroomonas* sp. – which we propose to occur by a combination of both very weak and strong dipole coupling. Phycocyanin 645 has an $\alpha_2\beta_2$ polypeptide structure, and the α subunits are heterogeneous in amino-acid composition. Each α subunit has one cryptobilin 697 (formerly called the 697 nm bilin) bilin and each β subunit has two phycocyanobilins and one cryptoviolin (or phycoviolobilin) bilins [20,21]. Phycocyanin 645 was selected because analysis of the steady-state spectroscopic data has enabled us to establish a well-defined model for exciton flow among its bilins [12]. The absorption spectrum can be deconvoluted into four bands; this result together with the protein structure suggests that the protein is divided into two identical halves [12]. The highest-energy bilin is probably the cryptoviolin. CD studies [10] have indicated that exciton delocalization may be occurring between pairs of bilins and, on the basis of these data, we have assigned the coupled pair to the 585 and 650 nm bands in our deconvolution. This assignment further suggests that the remaining two bands at 558 and 624 nm would transfer energy to this delocalized pair by very weak dipole coupling. Of course, there is undoubtedly some spectral overlap at all wavelengths among these absorption bands, but the deconvolution clearly suggests that an enrichment of the desired band can be obtained using excitation of 565, 585 and 615 nm. In the present report, we attempt to understand better the routes of exciton migration in this protein by selectively exciting the three higher-energy bands near their respective maxima and analyzing the picosecond time-resolved fluorescence. If we assume that our exciton migration hypothesis is valid, this strategy gives us a unique opportunity to observe both exciton transfer between pairs of chromophores by very weak dipole coupling and internal conversion between high- and low-energy states of a delocalized pair of chromophores on the same protein molecule.

Experimental

Chroomonas sp. was grown, harvested, and stored frozen. Harvests were lyzed into pH 6.0, 0.1 ionic strength, sodium phosphate buffer by two cycles of freezing and thawing. The phycocyanin 645 was purified by methods used previously [20]. Briefly, precipitation with 80% saturated ammonium sulfate and chromatography on Sepharose 6B and Ultrogel AcA54 were employed. The purified proteins was dialyzed into distilled water and lyophilized. All experiments were performed using the pH 6.0 buffer unless another buffer was indicated.

Time-resolved fluorescence measurements, with picosecond resolution, were performed using the frequency-domain method [22] using an instrument which operates from 4 to 6000 MHz [23]. The excitation source was a Rhodamine 6G dye laser, cavity dumped at 3.76 MHz. The pulse width of the dye laser was 5 ps, and the number of photons in the pulse was 10^8. The area of the beam was 1 mm^2. The dye laser was pumped with a mode-locked argon ion laser (Coherent, Innova 15). The emitted signals were observed with a microchannel photomultiplier, and the cross-correlation detection was performed outside the photomultiplier tube. The frequency-domain intensity data were fit to the time-resolved expression:

$$I(t) = \sum_i \alpha_i \, e^{-t/\tau_i} \tag{1}$$

where α_i are the pre-exponential factors; τ_i, the decay times; $\Sigma\alpha_i = 1.0$. The parameters were recovered by nonlinear least squares using the theory and software described elsewhere [24,25]. This instrumentation and data analysis has been shown to be useful down to 2 ps from the study of standard methods and materials [22,23,26,27]. Excitation was at 565, 585 and 615 nm on samples having absorbances, in a 1 cm light path, between 0.10 and 0.16. A cutoff filter at 640 nm was used for the emission. With these optical conditions, no background signal was observed.

The data were also fit to distributions of decay times [28]. In this case the time-resolved decays were described by:

$$I(t) = \int_{\tau=0}^{\infty} \sum_i g_i \alpha_i^0(\tau) \, e^{-t/\tau_i} \, d\tau \tag{2}$$

where τ_i are the decay times associated with each normalized distribution ($\alpha_i^0(\tau)$) and g_i the amplitude

of the ith component. The normalized distribution functions are assumed to be Lorentzian in shape,

$$\alpha_i^0(\tau) = \frac{1}{\pi} \frac{\Gamma_i/2}{(\tau - \bar{\tau}_i)^2 + (\Gamma_i/2)^2} \quad (3)$$

Where $\bar{\tau}_i$ and Γ_i are the central values and half-widths (fhw – full width at half-maximum intensity), respectively.

Irrespective of the assumed form of the intensity decay (Eqns. 1 or 2), the parameters describing the decay are obtained by minimizing the goodness-of-fit indicator, χ_R^2,

$$\chi_R^2 = \frac{1}{\nu}\sum_{\omega} \frac{\phi_\omega - \phi_\omega c^2}{\delta\phi} + \frac{1}{\nu}\sum_{\omega} \frac{m_\omega - m_\omega c^2}{\delta m} \quad (4)$$

where ω is the modulation frequency, $\delta\phi = 0.200$ and $\delta m = 0.005$ are the experimental uncertainties of phase shift ϕ and modulation m, ν is the number of degrees of freedom, and c indicates a calculated value.

Results and Discussion

The visible absorption spectrum of phycocyanin 645 (pH 6.0, 0.1 I, sodium phosphate buffer) has maxima at 645 and 583 nm and a shoulder at 625 nm, while the fluorescence emission spectrum is much less complex with a maximum at 661 nm (Fig. 1). The emission spectrum (not corrected) shown was obtained by exciting the sample of phycocyanin at 565 nm, and the emission spectra are identical for all other excitation wavelengths. The picosecond fluorescence data are shown as a plot of phase angle and modulation versus frequency for excitation of phycocyanin 645 at 585 nm (Fig. 2). Three components are found to give the best decay-time fit for the time-resolved fluorescence data of phycocyanin 645 after excitation at 565, 585 or 615 nm (Table I). The response to the changes in excitation wavelength is plotted as alpha versus tau (Fig. 3). We also analyzed the data using a model in which the intensity decay is fit to Lorentzian distribution of decay times [28,29]. The results of the distribution analysis is shown in Fig. 3. The data could be fit with bimodal distribution, resulting in values of χ_R^2 (legend in Fig. 3) which are essentially equivalent to those found with the three decay time model (Table I). This means that the experimental data cannot be used to select either model as correct: both models are consistent with data.

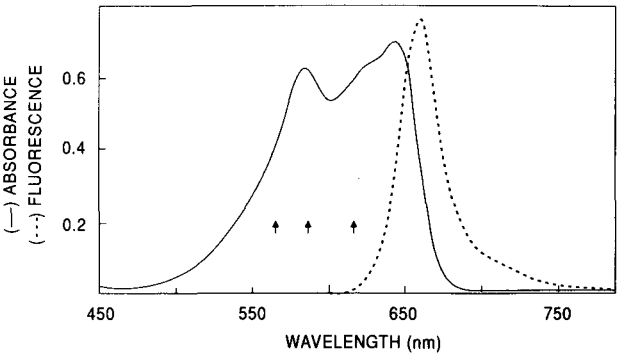

Fig. 1. Absorption (–) and fluorescence (— — —) spectra of phycocyanin 645. Spectra were taken at room temperature, and the protein was dissolved in pH 6.0, 0.1 I, sodium phosphate buffer. The fluorescence emission spectrum was obtained by exciting the sample at 565 nm. Arrows show wavelengths of laser excitation for kinetics experiments.

TABLE I

Decay times of the fluorescence emission from phycocyanin 645

Excitation wavelength (nm)	τ_i (ns)	−C.I. [a]	+C.I.	α [b]	Int.$_i$ [c]	χ_R^2
565	0.0089	0.0073	0.0110	0.859	0.04	3.0/12.1 [d]
	1.0044	0.9521	1.0669	0.090	0.44	
	2.0778	1.9809	2.1934	0.051	0.52	
585	0.0020	0.0018	0.0022	0.962	0.04	1.4/20.6
	0.6324	0.5629	0.7115	0.014	0.17	
	1.6417	1.6039	1.6847	0.024	0.79	
615	0.0147	0.0115	0.0179	0.758	0.03	2.8/18.2
	1.0797	1.0508	1.1107	0.164	0.50	
	2.0598	2.0089	2.1072	0.078	0.46	

[a] C.I. are confidence intervals, 67%. When the data for 585 nm excitation are cross-fit, χ_R^2 become 18.9 and 77.1 using data from excitations of 565 and 615 nm, respectively, for three-decay-time fits. The data were further analyzed fixing the fit at 1 ps, and χ_R^2 was then calculated to be 15.1, 10.6 and 1.4 for excitation at 615, 565 and 585 nm, respectively. When this procedure was employed fixing the fit at 10 ps, χ_R^2 was then found to be 5.0, 3.0 and 4.7, respectively.

[b] α is a pre-exponential factor from the time-resolved expression $I(t) = \Sigma_i \alpha_i \, e^{-t/\tau_i}$

[c] Int. is the steady-state intensity: $\text{Int.}_i = \alpha_i\tau_i / \Sigma_i \alpha_i \tau_i$

[d] The second value is χ_R^2 from a two-decay-time fit. Values of $\delta\phi = 0.2^0$ and $\delta_m = 0.005$ were used in all pertinent analyses in this paper. A four-decay-time fit failed to improve χ_R^2 over a three-decay-time fit.

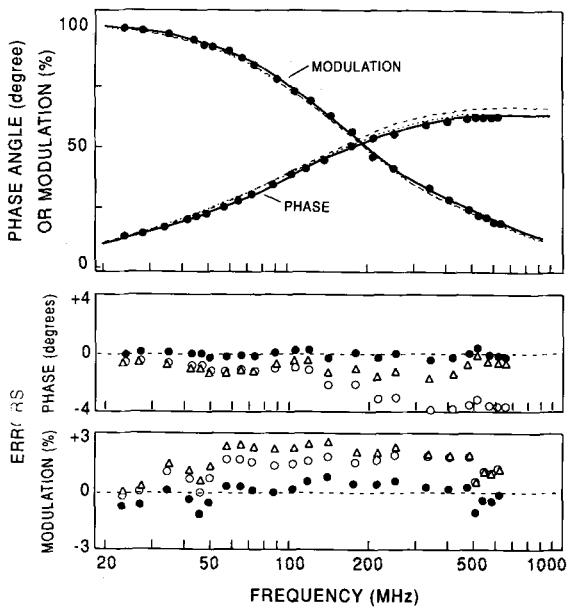

Fig. 2. Plot of phase angle and modulation versus frequency for excitation of phycocyanin 645 at 585 nm. The dotted and dashed curves represent best fits for excitation wavelengths 565 and 615 nm, respectively. The bottom panels show cross-fit data at 565 nm (○) and 615 nm (△) excitations to 585 nm (●) excitation (see Table I).

We believe the bimodal distribution model, and in particular the lifetime distribution plots, provides useful visualization of the complex intensity decay. In previous picosecond work with various biliproteins, it is usually maintained that the fastest decay represents the transfer of excitons from sensitizing to either fluorescing, or to other lower-energy sensitizing chromophores [30–37]. It is important in this study to be able to estimate the uncertainty of the decay times at each excitation wavelength. Because of the complexity of this measurement the approach is to examine the

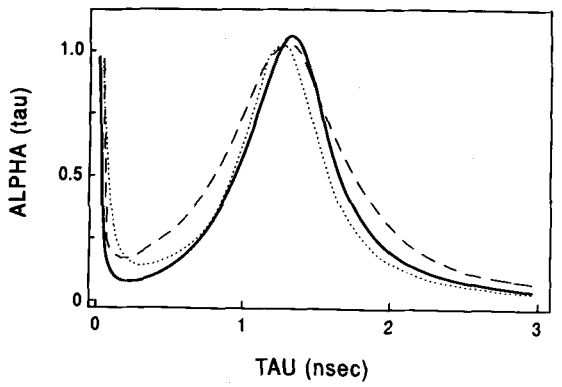

Fig. 3. Bimodal distribution of decay times for phycocyanin 645 upon excitation at 565, 585 and 615 nm. The following conditions were used: for 565 nm excitation, $\bar{\tau}_1 = 0.01$, $\bar{\tau}_2 = 1.278$, $\text{fhw}_1 = 0.001$, $\text{fhw}_2 = 0.936$; for 585 nm excitation, $\bar{\tau}_1 = 0.002$, $\bar{\tau}_2 = 1.256$, $\text{fhw}_1 = 0.001$, $\text{fhw}_2 = 0.642$; for 615 nm excitation, $\bar{\tau}_1 = 0.015$, $\bar{\tau}_2 = 1.320$, $\text{fhw}_1 = 0.001$, $\text{fhw}_2 = 0.660$. For excitation at 565, 585 and 615 nm, the χ^2_{RN} were 3.1, 2.6 and 3.1, respectively. The curves are 585 nm (·····), 565 nm (— — —), and 615-nm (———) data.

χ^2_{RN} surface of each decay time (Fig. 4). These plots are made by fixing the decay times at the values on the X axis and adjusting the other parameters to minimize $\chi^2_{R_N}$. A very conservative estimate of the uncertainties (67% confidence interval) is shown by the horizontal line (Fig. 4). Excitation at 565, 585 and 615 nm of phycocyanin 645 yield fast components of 9, 2 and 15 ps, respectively. The uncertainty in the 2 ps value is less than 2 ps, and this value appears likely to be faster than the other two picosecond values since the uncertainty in the 9 ps result is about 4 and that of the 15 ps value is about 6.

The significant differences between the decay of 585 nm excitation and the other two decays are further demonstrated by cross-fitting the data (Table I). When these calculations are performed, very large increases in χ^2_R are noted. The three-decay time fits were tested by fixing the decays at 1 or 10 ps (Table I). The resulting changes in χ^2_R show that values of the decay times calculated at each excitation wavelength are very good. Clearly, the decay of the 585 nm excitation is very different compared with the other two decays. In addition, the excitation at 585 nm will not be absorbed solely by the one chromophore since the deconvolution shows some smaller absorbance by other bands at this wavelength. Therefore, the 2 ps result must be considered higher than the actual value for this transition. A 2 ps result could come from either internal conversion between the two states of a delocalized pair, or from exciton transfer by very weak coupling between two more isolated bilins. As already discussed, CD studies [10] support, but do not rigorously prove, the delocalization hypothesis. Holzwarth et al. [38] have discussed the possibilities of measuring internal conversion for another biliprotein, allophycocyanin. An alternate interpretation of these data might suggest that some of these absorption bands are second excited states of a

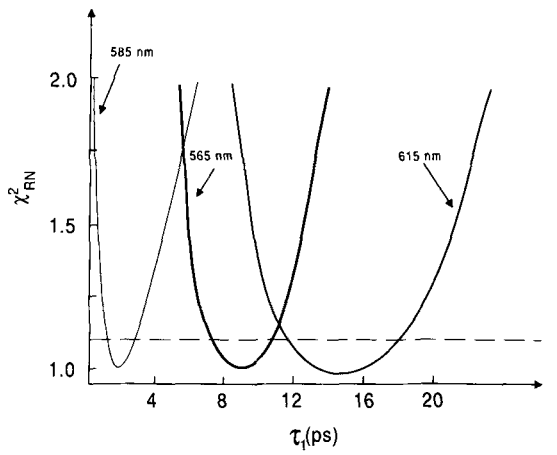

Fig. 4. Dependence of $\chi^2_{R_N}$ on the picosecond decay times of phycocyanin 645 upon excitation at wavelengths of 565, 585 and 615 nm. The horizontal lines give the range of values encompassed by the 67% confidence level of the minima of the curves.

single chromophore. This explanation is less likely because whenever isolated chromophores are studied these bands are not observed.

The slowest transition is measured to be between 1.6 and 2.1 ns. Extensive results from biliproteins – from both steady-state and picosecond methods – usually give values between 1 and 2 ns for the decay of the lowest-energy fluorescing chromophores. This 1–2 ns transition would be much shorter in the intact photosynthetic system, since the fluorescing chromophore would be linked to the next chromophore in the migration matrix. The middle decay time (Table I) has also been observed in other picosecond fluorescence studies on biliproteins [30,34–37].

These data can be discussed in terms of an hypothesis in which a flow of excitons occurs independently from the two sensitizing chromophores at 558 and 624 nm to the delocalized pair of bilins, which are the fluorescing chromophores (Fig. 5). The decay times are substantially different, 8.9 and 14.7 ps for the 558 and 624 nm bands, respectively. Clearly the excitons from the 558 nm band are not funneled through the 624 nm band to any major extent. Both halves of the protein would exhibit identical behavior. Are any other pairwise energy transfers possible? Although our data do not provide direct support, there are other transfers that probably occur and are allowed by sufficient spectral overlap between the fluorescence from the donor and the absorption of the acceptor. These transfers could be from the delocalized pair to the 624 nm chromophores on each half of the protein dimer, from bilins on one of the dimer halves to bilins on the other, and perhaps a small amount of transfer from the 558 to the 624 nm chromophores. If the distance and dipole orientations are favorable, these transfer would occur to some extent. These picosecond fluorescence results present evidence for the main migration pathways and for what would be the net result of the migration. Energy transfer models have recently been examined for the biliprotein allophycocyanin [38]. The findings for phycocyanin 645 differ saliently from those proposed for phycocyanin 612, where transfer from one sensitizing chromophore to another is possibly a main pathway of the exciton migration [39,40].

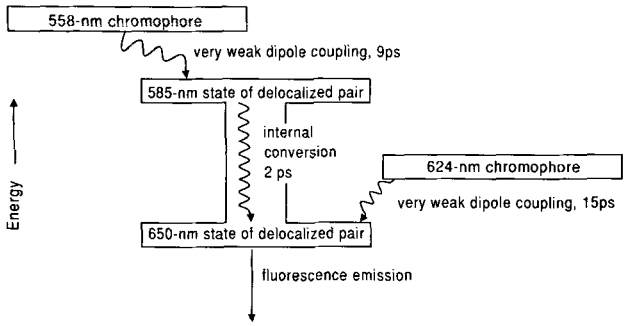

Fig. 5. Schematic model for the flow of excitons through phycocyanin 645. These four chromophores represent half of the total and the other half would exhibit identical behavior. Note that the occurrence of internal conversion has not been rigorously proven, and the 2 ps event could correspond to exciton transfer by very weak dipole coupling. CD studies [10] support the internal conversion hypothesis.

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