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Fluorescence polarization studies on four biliproteins and a bilin model for phycoerythrin 545

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Abstract

Fluorescence (excitation) polarization spectroscopy in the wavelength region of the bilin chromophores was applied to phycoerythrocyanin (CV-phycocyanin), phycocyanins 645 and 612, and phycoerythrin 545. The cryptomonad biliproteins – phycoerythrin 545 and phycocyanins 612 and 645 – were studied as both protein dimers having an $\alpha_2\beta_2$ polypeptide structure and as $\alpha\beta$ monomers. The cyanobacterial phycoerythrocyanin (CV-phycocyanin) was a trimeric oligomer. The changes in polarization across the spectrum were attributed to transfers of energy between bilins. Cryptomonad biliproteins are isolated as dimers. The similarities between their steady-state fluorescence polarization spectra and those of the corresponding monomers suggested that the monomers' conformations were analogous to the dimers. This supports the use of monomers in the study of dimer bilin organization. The unusual polarization spectrum of phycoerythrin 545 was explained using a model for the topography of its bilins. Obtaining the emission spectra of phycoerythrin 545 at several temperatures and a deconvolution of the dimer circular dichroism spectrum also successfully tested the bilin model. Circular dichroism spectroscopy was used to determine which polarization changes are formed by Förster resonance energy transfers and which may be produced by internal conversions between high- and low-energy states of pairs of exciton-coupled bilins. Attempts were made to assign energy transfer events to the corresponding changes in fluorescence polarization for each of the four biliproteins.

Keywords: Cryptomonad biliprotein; Energy migration in biliprotein; Exciton coupling; Model for bilin topography

1. Introduction

Biliproteins are light-harvesting pigments that absorb electromagnetic radiation in regions of low chlorophyll absorption. The absorbed energy migrates from bilin to bilin and from high to low energy through the biliprotein. The bilins are segregated into different energies by virtue of various factors, including the chemical structures of the bilins and various bilin interactions. The properties of the biliproteins have been reviewed [1–6].

Fluorescence (excitation) polarization spectroscopy was the first method systematically used to examine energy migration in various biliproteins [7,8]. Biliproteins in different aggregation states have been studied by fluorescence polarization [8–12]. Polarizations significantly lower than +0.36–0.40 result from energy transfer, as rotational effects are eliminated [13].

Fluorescence polarization will be extended to a cyanobacterial biliprotein phycoerythrocyanin, which is isolated as an $\alpha_3\beta_3$ trimer. Each monomer of a

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trimer has three bilins: one phycoviolobilin and two phycocyanobilins. A renaming of this protein to CV-phycocyanin has been suggested [6]. Studies are reported on three cryptomonad biliproteins in both the dimer and monomer oligomeric states. A cryptomonad monomer ($\alpha\beta$) has four bilins. These spectra will be analyzed against proposed models for bilin organization. Cryptomonad protein monomers have been used extensively in attempts to understand the bilin topography of cryptomonad biliprotein dimers. These studies will test the value of this protocol. Circular dichroism (CD) spectra have been employed to better understand the nature of the changes in fluorescence polarization. The sum of these studies should result in proposals for a correspondence of an energy transfer event to a polarization change.

2. Materials and methods

Phycoerythrocyanin (CV-phycocyanin) was purified from laboratory harvests of *Anabaena variabilis* as described previously [14]. Phycoerythrin 545 and phycocyanins 612 and 645 were purified from laboratory harvests of *Rhodomonas lens*, *Hemiselmis verescens* and *Chroomonas* sp., respectively, by methods described previously [15].

Fluorescence polarization measurements were carried out on either a Perkin-Elmer MPF44A, or a Perkin-Elmer LS50B fluorescence spectrophotometer. Emission and excitation slits were generally set at 10 nm.

Trimers of phycoerthrocyanin and dimers of phycoerythrin 545 and polycocyanins 612 and 645 were in pH 6.0, 0.1 ionic strength, sodium phosphate buffer. Monomers of phycoerythrin 545 and phycocyanins 612 and 645 were prepared in 0.10 M sodium acetate at pH 4.5, 4.3 and 4.0, respectively. Gel filtration column chromatography was used to establish conditions for homogeneous monomers.

Fluorescence polarization (p) was calculated as described previously [16,17]. The usual protein concentration was 0.015 g/l, but concentrations of 0.010 and 0.005 g/l were occasionally employed. Samples were diluted to these concentrations immediately prior to the measurement.

Absorption spectra were obtained with a Beckman DU640 spectrophotometer, and CD spectra was taken on a JASCO 720 spectropolarimeter. CD spectra were obtained using a 5-mm light path. Protein concentrations for these experiments were typically 0.15 g/l.

The CD spectrum of phycoerythrin 545 dimer was deconvoluted by the PeakFit software (Jandel Scientific). Four band types were tried: Gaussian, Lorentzian, Voight, and Pearson VII. The program employs a Levenberg-Marquardt interactive fitting. The best r^2 value was selected as the best fit. The spectrum was in cm^{-1} for the fitting.

3. Results and discussion

3.1. Fluorescence polarization

The fluorescence polarization spectrum of phycoerythrocyanin (CV-phycocyanin) trimers showed two changes in polarization in the spectral regions where the absorption spectrum showed the overlap of bands (Fig. 1). Emission was monitored at 640 nm for these measurements. The change in polarization

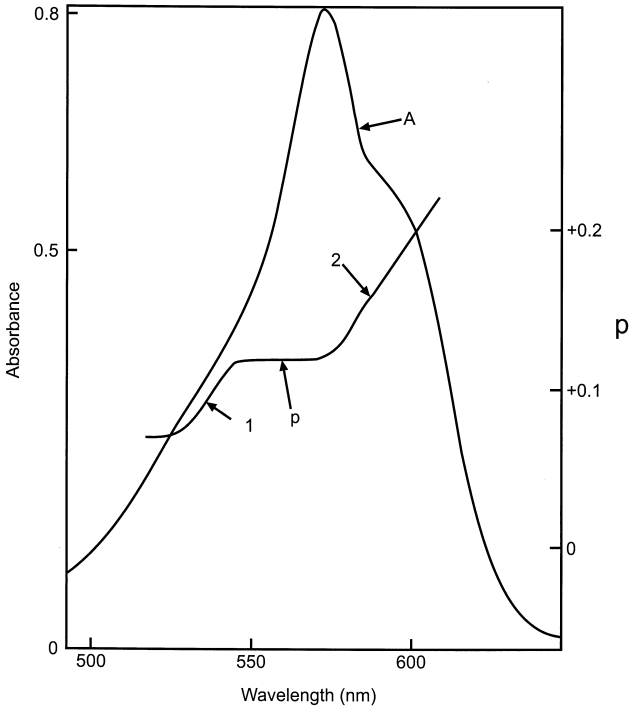

Fig. 1. Fluorescence polarization (p) and absorption (A) spectra of trimers of phycoerythrocyanin (CV-phycocyanin). Protein was in pH 6.0 buffer.

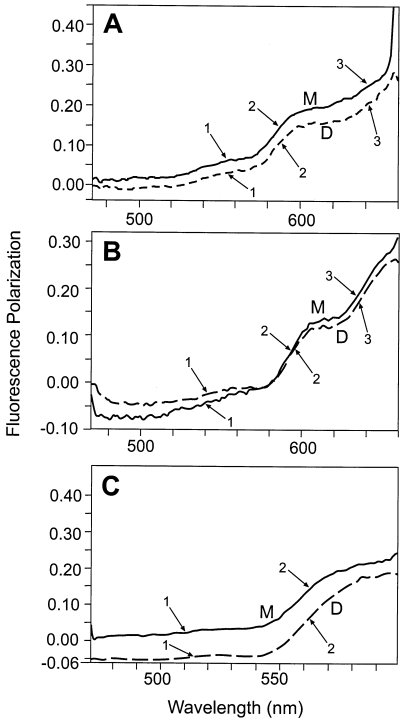

Fig. 2. Fluorescence polarization spectra of monomers (M) and dimers (D) of (A) phycocyanin 612, (B) phycocyanin 645, and (C) phycoerythrin 545. Arrows point to polarization changes.

indicates energy transfer between energetically distinct bilins.

For phycocyanin 612, dimers and monomers were studied (Fig. 2A). The fluorescence polarization spectra of monomers and dimers each show three changes in polarization in the identical wavelength regions. Monomers of phycocyanin 612 have been used to analyze the bilins' organization [17]. The conformation of monomers should resemble that of dimers for this analysis to be valid. If monomers were more like disordered protein, their utilization in the study of bilin organization in dimers would be invalid. The fluorescence polarization results suggest that the four bilins of the monomer are arranged very similarly to the bilins in the monomeric units of the dimers. This conclusion is valid because energy transfer between chromophores is extremely sensitive to the orientation and distance between bilins [18].

The fluorescence polarizations of phycocyanin 612 monomers and dimers were obtained at a series of protein concentrations (Fig. 3). All three concentrations showed very analogous transitions, and this result eliminated the possibility of any artefact occurring from using a protein concentration that was too high. In addition, emission was monitored at either 670 nm or 690 nm and both emissions yielded identical polarization spectra (data not shown). The polarization spectrum of phycocyanin 612 dimers (Figs. 2A and 3) was qualitatively different from the spectrum obtained previously [19] for dimers. This spectrum (Fig. 2A, arrow 3) clearly shows a polarization change near the red edge of the spectrum that was not observed earlier.

Using phycocyanin 645, the fluorescence polarization spectra of dimers and monomers showed three changes in the same wavelength regions (Fig. 2B). Again, the excellent correspondence of monomer and dimer polarization spectra suggests that using monomers to derive the bilin topography in dimers [17] is an acceptable procedure. Fluorescence polarization of dimers of phycocyanin 645 (Fig. 2B) agreed with the previous results [20,21]. The monomer spectrum has not been reported previously.

Phycoerythrin 545 has four chromophores on each monomer and eight in a dimer as do phycocyanins 612 and 645. However, the fluorescence polarization spectra of dimers and monomers exhibited only two polarization transitions (Fig. 2C) as compared to the polarization spectra of the other cryptomonad bili-

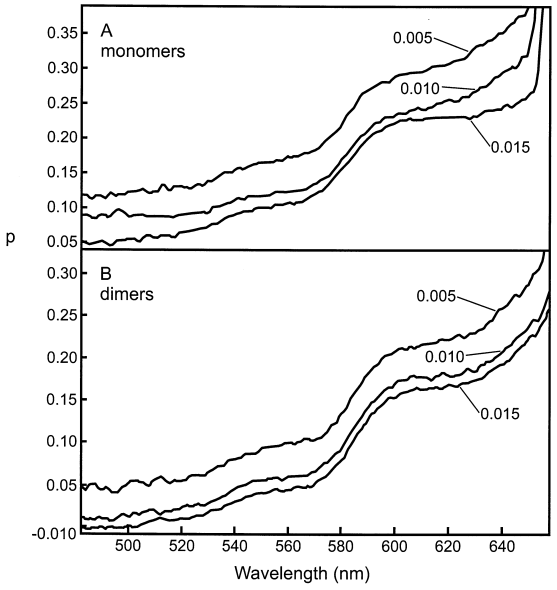

Fig. 3. Fluorescence polarization of phycocyanin 612 dimers and monomers as a function of protein concentrations. Concentrations in g/l are indicated on each spectrum.

proteins in which each had three polarization changes. Nonetheless, as found for phycocyanins 612 and 645, the monomer and dimer polarization spectra of phycoerythrin 545 were similar (Fig. 2C). Further, more detailed comparisons between the fluorescence polarization spectra of these monomers and dimers will follow, and they will continue to support the conformational similarity of monomers and dimers of cryptomonad biliproteins.

3.2. Bilin model for phycoerythrin 545

A study of the CD spectra of phycoerythrin 545 [17,22] allowed the suggestion of a model for the bilin topography of dimers. The eight bilins are arranged with two identical exciton-coupled bilin pairs within each monomeric unit, an exciton-coupled pair of bilins across the monomer-monomer interface, and a more isolated bilin on each monomeric unit (Fig. 4).

When a chromophore transfers energy to another chromophore by the radiationless Förster resonance process, the donor and acceptor chromophores tend to retain their isolated spectra. However, when a chromophore pair engages in exciton coupling, the spectra of the chromophores are split into high- and low-energy bands. The excitation energy is delocalized in the pair. This model [17,22] nicely explains the fluorescence polarization spectra of phycoerythrin 545 dimers and monomers. The highest-energy bilin, which is more isolated, transfers energy by Förster resonance as observed in the high-energy part of the polarization spectra (Fig. 2C, arrow 1). The intramonomer- and intermonomer-coupled pairs of bilins have split spectra in the same wavelength region, and saliently overlap each other (Fig. 4). For the fluorescence polarization spectra, the lower-energy transition is the result of more than one energy transfer (Fig. 2C, arrow 2). The polarization spectrum of phycoerythrin 545 dimers agreed with a spectrum taken previously [23] and the monomer spectrum was obtained for the first time.

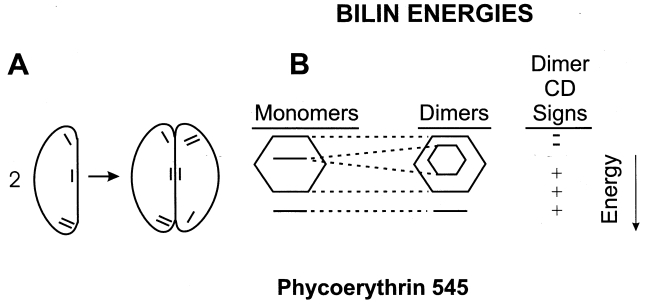

Fig. 4. A possible model for the bilin topography of monomers and dimers of phycoerythrin 545. The lines on the models (A) represent bilins. The energy levels (B) joined by brackets are those which are suggested to be caused by an exciton-coupled pair of bilins. The two proposed types of exciton-coupled pairs of bilins for dimers, intermonomer and intramonomer, are observed to extensively overlap each other. Energy transfer between an isolated bilin and another bilin uses the Förster resonance method. For the exciton-coupled pairs of bilins, excitation energy is delocalized between the high- and low-energy bands of the split spectra and the delocalized energy moves to the low-energy band by internal conversion.

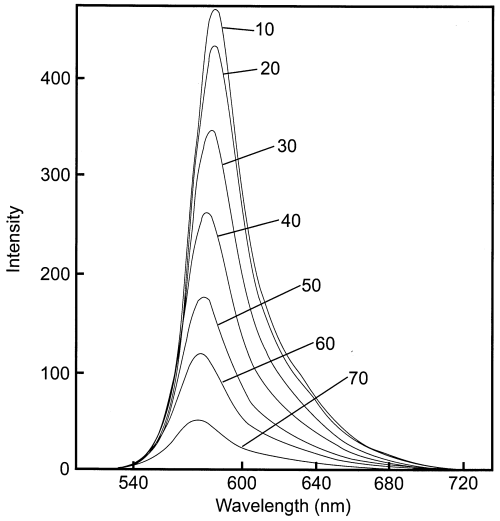

Fig. 5. Fluorescence emission spectra of phycoerythrin 545 dimers. The number on each spectrum is the temperature, °C. Protein concentration was 0.015 g/l in pH 6.0 buffer. After each temperature was reached, the emission spectrum was obtained after a 10-min wait. Excitation wavelength was 500 nm. Slits were both at 6 nm.

All biliproteins have an emission spectrum resulting from radiation from the lowest-energy bilins. For phycoerythrin 545, the emission maximum of dimers was found at 585 nm (Fig. 5). For the polarization measurements of phycoerythrin 545, the emission was monitored at 620 nm or 640 nm, with identical results at either wavelength (data not shown).

Monomers of phycoerythrin 545 [17] are proposed to lose the intermonomer pair of coupled bilins that are reputed to exist for dimers (Fig. 4). How will this affect the fluorescence polarization? The polarization

spectrum of monomers still should probably resemble that for dimers because the more isolated bilins, which are produced when monomers form, transfer energy to the intramonomer-coupled pairs in approximately the same wavelength region as did the proposed intermonomer-coupled pair of bilins.

At a series of temperatures, the emission spectra of phycoerythrin 545 dimers had the emission maximum shifted gradually from 585 nm at 10–20°C to 576 nm at 70°C (Fig. 5). If the proposed model (Fig. 4) is accurate, the emission at 10–20°C comes from the lower-energy band produced by a pair or pairs of exciton-coupled bilins. These pairs should become uncoupled as the higher temperatures begin to disorder the protein, and the result of the loss of exciton splitting would produce a blue shift in the emission maxima (Fig. 4), which now would arise from isolated bilins. Since this is the observed result (Fig. 5), this is a modest confirmation of the proposed model (Fig. 4). A more complete study of the effects of temperature on the optical spectra of phycoerythrin 545 is available [22]. These studies [22] showed that the protein was largely disordered at 60°C.

Spectral deconvolution can also be used to test the proposed bilin model. Five component bands were found to be an excellent fit of the CD spectrum of phycoerythrin 545 dimers (Fig. 6). Three of the component bands are positive and two are negative. CD spectra are particularly useful in the study of interacting chromophores. When two chromophores undergo strong interaction their spectra may undergo exciton splitting. The CD spectrum of such a chromophore pair is distinctive, having a positive and a negative band. These bands may be conservative with the positive and negative bands having equal intensities. For phycoerythrin 545 dimers, the bands, labeled by 2s, correspond to the expected spectrum of the intermonomer exciton-coupled pair of bilins. The bands labeled by 1s correspond to the expected [22] CD spectrum for the intramonomer pair of bilins as predicted from the model and illustrated by the fluorescence polarization spectrum (Fig. 2C). These two different pairs of bilins have spectra that extensively overlap each other (Fig. 4). The band labeled by 3 is not coupled and, as predicted by the model (Fig. 4), is the highest-energy bilin. The bilin model, if accurate, would generate a CD spectrum fit by five components – two negatives and three positives – as observed in this deconvolution (Fig. 6). The deconvolution for the CD of monomers of phycoerythrin 545 was best fitted by four components (data not shown). The loss of a band is speculated to be produced by separation of the pair of bilins that were coupled between monomers. Phycocyanin 645 monomers and dimers illustrate the same deconvolution characteristics (Fig. 7).

The difference CD spectrum for phycoerythrin 545 of dimers minus monomers gave a positive CD ex-

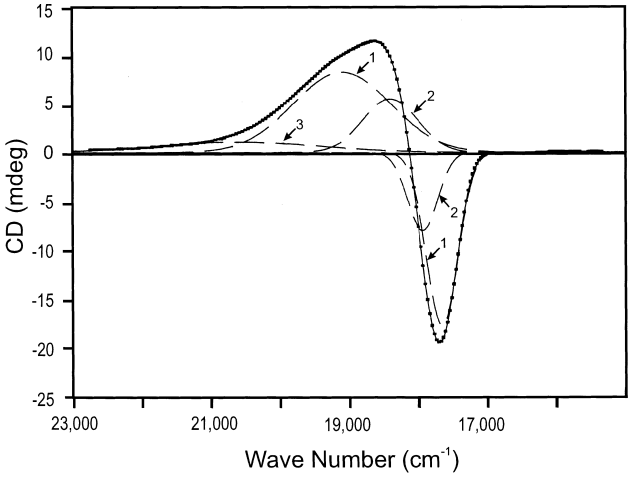

Fig. 6. CD spectrum of phycoerythrin 545 dimers and its deconvolution. The highest-energy component is a Voight and the rest are Gaussians. The component bands are at 566, 577, 543, 523 and 486 nm. The r^2 for this fit is 0.99984. The experimental curve (solid line) and the sum of the components (dots) are observed to be in excellent agreement.

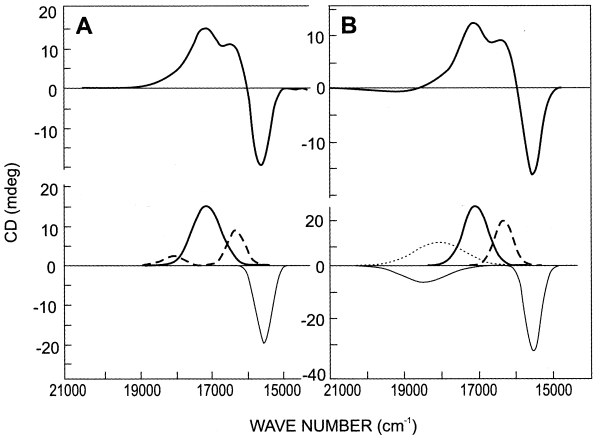

Fig. 7. CD spectra of dimers (B) and monomers (A) of phycocyanin 645 and their deconvolutions. The tops of A and B are the CD spectra. The bottoms are the deconvoluted bands.

Table 1
Bilins of phycocyanins 645 and 612 and phycoerythrin 545 [5,26]

Protein	Polypeptide	Bilin[a]	N-terminal position
Phycoerythrin 545	α	DBV (7)	19
	β	PEB (6)	50, 61[b]
		PEB (6)	82
		PEB (6)	158
Phycocyanin 645	α	MBV (9)	19
	β	DBV (7)	50, 61
		PCB (8)	82
		PCB (8)	158
Phycocyanin 612	α	PCB (8)	19
	β	DBV (7)	50, 61
		PCB (8)	82
		PCB (8)	158

[a]MBV, mesobiliverdin; DBV, 15,16-dihydrobiliverdin; PCB, phycocyanobilin; PEB, phycoerythrobilin. In parentheses is the number of conjugated double bonds in each attached bilin. The greater the number of conjugated double bonds the lower the energy of the spectrum. The α polypeptides for all three biliproteins have the lowest-energy, or one of the lowest-energy, bilins. [b]The doubly bonded bilin at positions β50, 61 for all of the three biliproteins is the least conjugated, or one of the least conjugated, bilins. In the proposed models [17,22,24] for phycoerythrin 545 and phycocyanin 645, the bilins at β50, 61 are coupled across the monomer-monomer interface. For all three biliproteins, the α19 bilins are paired with one of the other β bilins within a monomer. For phycoerythrin 545, phycocyanin 612 and phycocyanin 645, the β bilin coupled to the bilin at α19 would be a PEB, a PCB, and a PCB, respectively. The choice of the α19 bilins to be involved in the reputed intramonomer pair is speculative. The proposal is based on the agreement between the amino acid sequences of the β polypeptides of cryptomonad biliproteins and red algal phycoerythrins [26]. Since the β polypeptides of red algal phycoerythrins do not appear to have paired bilins [27–29], it is tentatively assumed that the β polypeptides of cryptomonad biliproteins, likewise, will lack bilin pairing within that polypeptide, and the involvement of the α19 bilin is implicated.

tremum at 538 nm and a negative at 564 nm [22]. The deconvolution shows the intramonomer split bands for dimers having a negative CD extremum at 557 nm and a positive extremum at 543 nm (Fig. 6). There is a reasonably close agreement between the spectra measured from difference CD and predicted from the deconvolution from the split bands, since both methods have errors associated with them. The difference spectrum also contains a contribution for the bilins uncoupled when dimers convert to monomers, and the deconvolution used band shapes that undoubtedly differ from actual CD band shapes.

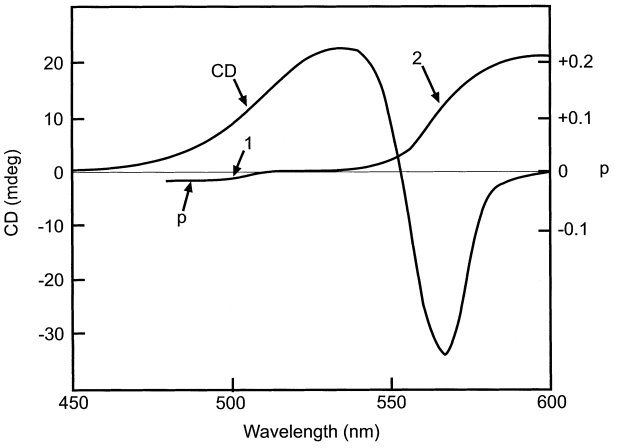

Fig. 8. CD and fluorescence polarization spectra of phycoerythrin 545 dimers. The numbers indicate changes in polarization.

3.3. CD and fluorescence polarization

To further understand the fluorescence polarization spectra of cryptomonad biliproteins, the CD and fluorescence polarization spectra of dimers of phycoerythrin 545 (Fig. 8), phycocyanin 612 (Fig. 9), and phycocyanin 645 (Fig. 10) were compared.

The fluorescence polarization of phycoerythrin 545 dimers showed a major change in polarization in the wavelength region between positive and negative CD bands (Fig. 8, arrow 2). This correspondence supports the proposal that this change in polarization many result from internal conversion from the high- to the low-energy bands of coupled bilin pairs. The much smaller polarization change (Fig. 8, arrow 1) at higher energies appears to be the transfer of energy from the non-coupled bilin.

Considering phycocyanin 645, the highest-energy polarization change (Fig. 10, arrow 1) occurred between about 520 nm and 550 nm. There is a negative CD band in the wavelength region, but the corresponding positive band is presumed to be hidden by the stronger positive CD band at 584 nm. This hidden positive band was revealed by deconvolution methods (Fig. 7). This negative/positive pair of CD bands is viewed as perhaps being formed by interaction of a closely spaced pair of bilins across the monomer-monomer interface of the dimers. The neg-

Table 2
Changes in fluorescence polarization for four biliproteins

Biliprotein	Oligomer	Fluorescence polarization	Energy transfer[a]
Phycoerythrocyanin (CV-phycocyanin)	$\alpha_3\beta_3$	Fig. 1, arrow 1	PVB[b] to PCB
		Fig. 1, arrow 2	PCB to PCB
Phycoerythrin 545	$\alpha_2\beta_2$	Fig. 8, arrow 1	PEB to coupled pair
		Fig. 8, arrow 2	1. intramonomer-coupled pair
			2. intermonomer-coupled pair[c]
			3. Förster resonance between two coupled pairs
	$\alpha\beta$	Fig. 2C, arrow 1	PEB to coupled pair
		Fig. 2C, arrow 2	1. intramonomer-coupled pair[c]
			2. PEB to coupled pair
Phycocyanin 612	$\alpha_2\beta_2$	Fig. 9, arrow 1	DBV to another DBV or to PCB, or both
		Fig. 9, arrow 2	1. PCB to coupled pair
			2. perhaps DBV to coupled pair
		Fig. 9, arrow 3	intramonomer-coupled pair
	$\alpha\beta$	Fig. 2A, arrow 1	DBV to PCB
		Fig. 2A, arrow 2	PCB to coupled pair
		Fig. 2A, arrow 3	intramonomer-coupled pair[c]
Phycocyanin 645	$\alpha_2\beta_2$	Fig. 10, arrow 1	intermonomer-coupled pair[c]
		Fig. 10, arrow 2	1. Förster resonance transfer between coupled pairs[c]
			2. intramonomer-coupled pair
		Fig. 10, arrow 3	PCB to coupled pair
	$\alpha\beta$	Fig. 2B, arrow 1	DBV to coupled pair
		Fig. 2B, arrow 2	intramonomer-coupled pair[c]
		Fig. 2B, arrow 3	PCB to coupled pair

[a]Förster resonance transfers are assumed if energy transfer is not within a coupled pair, as for Fig. 1, arrows 1 and 2, Fig. 8, arrow 1, Fig. 9, arrows 1 and 2, Fig. 10, arrow 3. The term coupled pair indicates a pair of bilins that are close enough and oriented so that their spectra is split into high- and low-energy bands. For a coupled pair, internal conversion allows radiationless energy flow from the high- to low-energy band. For cases where Förster resonance between two difference coupled pairs is suggested, for one coupled pair excitation energy is viewed as moving to the low-energy band and from there energy is transferred by Förster resonance to the other coupled pair.

[b]PVB, phycoviolobilin; PCB, phycocyanobilin; PEB, phycoerythrobilin; DBV, 15,16-dihydrobiliverdin.

[c]The intermonomer-coupled pairs of bilins will be the highest-energy bilins of phycocyanin 645, which are the DBV, and for phycoerythrin 545 they are PEB. For all three cryptomonad biliproteins, the intramonomer-coupled pairs will consist of the bilin on the α polypeptide coupled to a bilin on the β polypeptide. The coupled β bilin will be PEB, PCB, and PCB for phycoerythrin 545, phycocyanin 612, and phycocyanin 645, respectively.

ative band of this pair disappears when monomers are formed [24]. The other proposed coupled bilin pair located within the protein monomers gives positive/negative CD bands at lower energies (Fig. 10, arrow 2).

For phycocyanin 612, the lowest-energy polarization change (Fig. 9, arrow 3) occurred in the wavelength region between a negative and a positive CD band. CD studies have suggested that these two bands are formed by an intramonomer exciton-coupled pair of bilins [17,25]. This polarization change is, therefore, associated with energy processed within the split bands. The other two polarization changes may represent energy transfers by Förster resonance from more isolated bilins, directly or indirectly, to the coupled pair of bilins.

The comparisons between CD and fluorescence polarization allow the polarization changes to be better understood. The polarization changes are suggested to arise either from transitions between the high- and low-energy bands of exciton split bands (e.g., arrow 3, Fig. 9), or by Förster resonance energy transfer between more isolated bilins (e.g., arrow 2, Fig. 9) for various biliproteins.

A caveat is that CD is the only available evidence for exciton coupling in cryptomonad biliproteins.

The more rigorous proof for exciton coupling could come from the analysis of the crystal structure by X-ray diffraction followed by theoretical evaluations using the distances and orientation of the bilins, or by various methods in ultrafast time-resolved fluorescence or absorption. None of this information is currently available for any cryptomonad biliprotein.

3.4. Assignment of polarization changes

A general goal with the fluorescence polarization spectra of these biliproteins is to consider each possible energy transfer event and to understand the polarization change that reflects the specific energy transfer. There are three bilins for each monomer in phycoerythrocyanin (CV-phycocyanin). It would be expected that two changes from energy transfers between spectrally distinct bilins would be possible across its fluorescence polarization spectrum. Two transitions were observed (Fig. 1), and they are assigned to transfers from phycoviolobilin to phycocyanin (Fig. 1, arrow 1) and from high-energy phycocyanobilin to low-energy phycocyanobilin (Fig. 1, arrow 2).

The selection of which bilin is higher in energy begins with their structures. The key in determining energy is the number of conjugated double bonds. Phycoviolobilin was assigned to a higher-energy transition than phycocyanobilin because phycoviolobilin has fewer conjugated double bands.

The structures of cryptomonad bilins have been reviewed [5]. For phycocyanin 645, there are three bilins, mesobiliverdin, phycocyanobilin, and 15,16-dihydrobiliverdin, having nine, eight, and seven conjugated double bands, respectively. The mesobiliverdin and one of the two phycocyanobilins are viewed as being coupled within the monomer (Tables 1 and 2). Two 15,16-dihydrobiliverdins are proposed to be paired in protein dimers across the monomer-monomer interface. For phycocyanin 612 there are two bilins, phycocyanobilin and 15,16-dihydrobiliverdin. There are three phycocyanobilins per monomer. For phycoerythrin 545, there are two bilins, phycoerythrobilin and 15,16-dihydrobiliverdin. There are three phycoerythrobilins per monomer, each having six conjugated double bands.

For phycocyanin 612 dimers, there are four bilins on a monomeric unit. Three polarization changes across its spectrum were observed (Fig. 9). The highest-energy polarization transition (Fig. 9, arrow 1) is proposed to be by Förster resonance form 15,16-dihydrobiliverdin either to the other 15,16-dihydrobiliverdin or to the isolated phycocyanobilin (Table 1). The next polarization change (Fig. 9, arrow 2) is Förster resonance transfer from an isolated phycocyanobilin, and perhaps also from 15,16-dihydrobiliverdin, to a coupled pair of phycocyanobilins, and the lowest-energy polarization change (Fig. 9, arrow 3) is suggested to be produced by internal conversion between the high- and low-energy bands of the proposed intramonomer-coupled pair of bilins. The 15,16-dihydrobiliverdin is the bilin that has been sug-

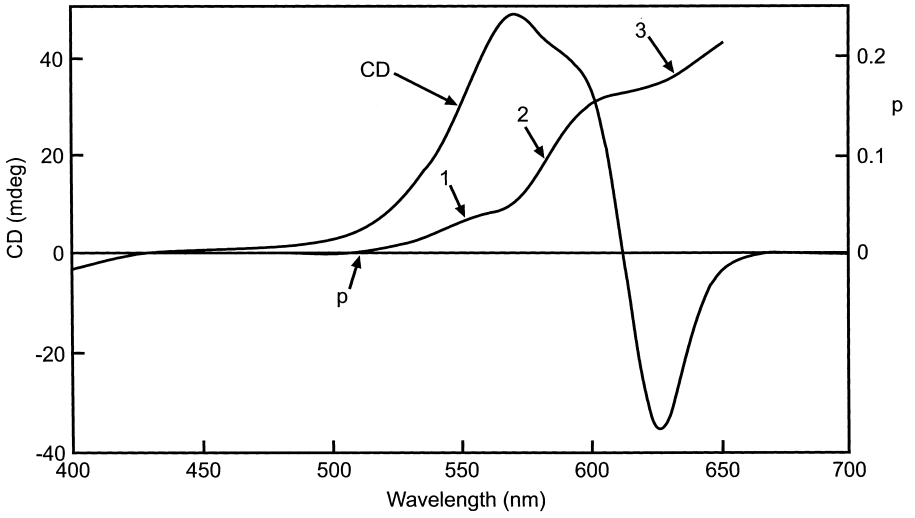

Fig. 9. CD and fluorescence polarization spectra of phycocyanin 612 dimers. The numbers indicate changes in polarization.

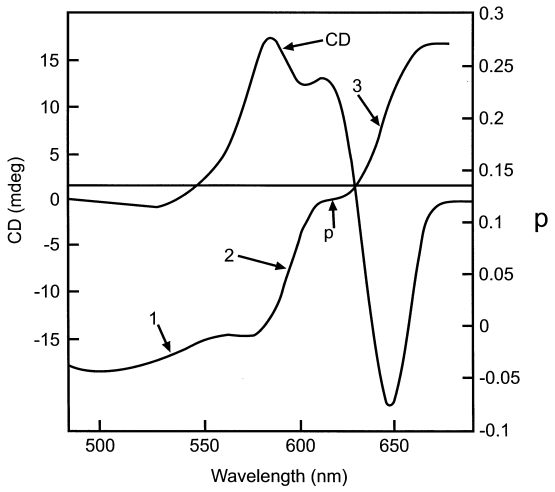

Fig. 10. CD and fluorescence polarization spectra of phycocyanin 645 dimers. The numbers indicate changes in polarization.

gested to be paired and exciton-coupled across the monomer-monomer interface in dimers of phycocyanin 645. It has been proposed that phycocyanin 612 dimers do not possess the CD characteristic of an intermonomer-coupled pair of bilins [17]. Nonetheless, even if these two bilins are not exciton-coupled, they could still transfer energy between each other by the Förster resonance mechanism. The absence in the CD spectrum of the characteristics of the intermonomer bilin pair could be caused by the increased distance between the bilins or by a change in orientation within the pair.

For phycocyanin 645 and phycoerythrin 545, the proposed bilin models [17,22,24] advanced speculation that a pair of identical bilins interact across the monomer-monomer interface of its protein dimer to become exciton-coupled. CD of monomers suggests another pair of bilins exists within monomers. Therefore, these proteins would possess two different coupled pairs of bilins and this intermonomer interaction would produce another change in the fluorescence polarization spectra. Therefore, the expected number of observed polarization changes is four rather than three.

The fluorescence polarization spectra of phycoerythrin 545 (Fig. 8) and phycocyanin 645 (Fig. 10) do not exhibit four obvious transitions. The assignment of energy transfer events will be, therefore, problematic. For phycoerythrin 545, only two polarization changes were observed. The higher-energy transition is assigned to Förster resonance transfer from a phycoerythrobilin to a coupled pair of bilins (Fig. 8, arrow 1). The lower-energy transition in polarization (Fig. 8, arrow 2) is reputed to consist of the overlaps of three-difference energy transfers [17]. They are speculated to be 15,16-dihydrobiliverdin coupled to a phycoerythrobilin within a protein monomer, two other phycoerythrobilins coupled across the monomer-monomer interface, and Förster resonance transfer between these two different coupled pairs (Fig. 4). For phycocyanin 645 dimers, instead of four polarization changes, three were observed (Fig. 10). A speculative explanation for the apparently missing polarization change is that the transfer out of the higher-energy coupled pair of bilins overlapped a nearby polarization change (Fig. 10, arrow 2, Tables 1 and 2).

How are the understandings of the relationship between fluorescence polarization and energy transfer affected by formation of protein monomers? For phycocyanin 612, no change is anticipated since the bilin arrangement in monomers is presumed to be nearly identical to that in the monomeric units of the dimer. This is in agreement with the observed results (Fig. 2A).

For phycocyanin 645 and phycoerythrin 545, monomers will lose the predicted exciton splitting across the monomer-monomer interfaces. This event will reduce the predicted number of fluorescence polarization changes from four to three. However, since neither of these proteins actually showed four obvious transitions in the dimers, a critical test of this prediction is not possible. For phycoerythrin 545, the bilins under consideration are under the envelope of other bilins and the conversion to isolated bilins in monomers would likewise be under the others (Fig. 4) and therefore, the fluorescence polarization spectra might not differ; this similarity was observed (Fig. 2C). Considering phycocyanin 645, the highest-energy fluorescence polarization change for dimers (Fig. 10, arrow 1) was presumed to be caused by exciton splitting between the two bilins interacting across the monomer-monomer interface (Fig. 7). For monomers, this interaction was totally terminated, but the fluorescence polarization spectrum still shows a transition in the same wavelength region (Fig. 2B, arrow 1). Although the intermonomer-coupled pair is a nonentity in monomers, the 15,16-dihydrobiliverdins will still be present on separated monomers

and will still transfer their excitation energy. Using Förster resonance transfer, these bilins will transfer energy, and this resulting transfer produces the change in polarization at this high energy (Fig. 2B, arrow 1). For phycocyanin 645 monomers, the four bilins should show three changes in polarization, and three changes were observed. Two of these polarization changes would be from Förster resonance transfer from the two more isolated bilins, and the remaining change would be attributed to processes between the high- and low-energy bands of the intramonomer-coupled pair of bilins.

A summary of these results (Table 2) gives the currently available estimates of the genesis of these various changes in fluorescence polarization for phycoerythrocyanin (CV-phycocyanin), phycoerythrin 545, and phycocyanins 612 and 645.

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