Chromophore Topography and Exciton Splitting in Phycocyanin 645

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ABSTRACT: The biliprotein phycocyanin 645 has been purified from a photosynthetic cryptomonad *Chroomonas* species. It is composed of two copies each of two polypeptides (α and β); each α polypeptide has one chromophore, and each β polypeptide has three. There are one cryptoviolin and two phycocyanobilins on each β polypeptide and one 697-nm bilin on each α polypeptide for a total of eight chromophores on the protein. Circular dichroism (CD) spectroscopy has been used to investigate the arrangement of these chromophores. Comparisons among the intact protein ($\alpha_2\beta_2$) and various urea-treated products have yielded a tentative model for chromophore topography. The six chromophores on the two β polypeptides are segregated into three pairs. The chromophores of each pair are close enough to experience electronic interactions. One pair, consisting of the two cryptoviolins, produces exciton splitting on the blue edge of the visible CD spectrum, and the two pairs of phycocyanobilins cause exciton splitting on the red edge of this spectrum. Deconvolution shows that the CD spectrum of each pair has a positive and a negative band, which are nearly conservative as expected for exciton coupling. The two chromophores on the α polypeptides are more isolated. The pairing of cryptoviolin chromophores occurs across two β polypeptides, but the more likely position of each of the two pairs of phycocyanobilins is probably not across a β–β interface but within a single β polypeptide. The exciton splitting events both increase the range of visible light absorption for the protein and establish the routes of exciton migration through the protein.

Photosynthesis is accomplished through the efficient operation of a large and complex array of molecules. The harvesting of light energy and the subsequent transfer of that energy to the photosynthetic reaction centers consume a large fraction of the total number of molecules in the photosynthetic matrix. The transfer of excitons is very rapid and efficient, and many chromophores can be involved in the process of moving each exciton relatively long distances from the site of absorption to the reaction center where the process of energy transduction is initiated. To facilitate the process of understanding how the arrangement of chromophores establishes the orderly function of this aspect of photosynthesis, we have selected a single and relatively small unit of photosystem II to examine in detail.

The system chosen is the biliprotein phycocyanin 645. This protein is purified in a form having the polypeptide structure $\alpha_2\beta_2$, and there are two or more different α polypeptides. Each α polypeptide has a single chromophore—called the 697-nm bilin—and each β polypeptide has one cryptoviolin and two phycocyanobilins. The properties of biliproteins have been reviewed (Gantt, 1979; Zuber, 1987; Scheer, 1981; Holzwarth, 1991; MacColl & Guard-Friar, 1987).

The visible absorption spectrum of this protein is fairly complex, but the visible circular dichroism (CD) spectrum is even more interesting, having a mixture of overlapping negative and positive bands. CD offers a unique approach to the study of chromophore topography since two or more chromophores that approach each other closely enough can interact to split their absorption bands into high- and low-energy forms. These split bands will be expressed in CD as negative and positive bands having equal intensities. Pairs of negative/positive CD bands may be caused by these coupled pairs or may simply result from the overlap of two independent bands that are coincidentally positive and negative. In order to decipher the visible CD of phycocyanin 645, we have studied the native spectrum by deconvolution methods and then examined the changes in the spectrum as the protein was partially or fully dissociated. Gel-filtration column chromatography methods were used to follow the dissociation process. These studies have resulted in the construction of a model for the topography of the eight chromophores and an appreciation of how exciton splitting may contribute to the function of the protein.

EXPERIMENTAL PROCEDURES

Phycocyanin 645 was purified from *Chroomonas* species by a combination of ammonium sulfate precipitation and gel-filtration column chromatography (MacColl & Guard-Friar, 1983). Purified protein was dialyzed into distilled water, lyophilized, and stored in a freezer. All experiments, except those with the separated α and β polypeptides, were performed in pH 6.0, 0.1 ionic strength, sodium phosphate buffer. Phycoerythrins 566 and 545 and phycocyanin 612 were isolated from *Cryptomonas ovata*, *Rhodomonas lens*, and *Hemiselmis virescens*, respectively, and purified as described above.

CD measurements were done on a JASCO J-720 spectropolarimeter. Most experiments in the visible range were done at a 0.10 mg/mL protein concentration in a 5-mm light path, and at 23.0 °C. Multiple scans were averaged to improve the signal-to-noise ratio. Far-UV measurements to obtain spectra for the estimation of secondary structure were done with a 0.5-mm light path. Calculations of protein secondary structure were done using software provided by JASCO (Yang *et al.*, 1986). Spectra were analyzed down to 190 nm. All spectra shown here were treated by subtracting the solvent spectra (base line) obtained under identical conditions to the protein spectra. The buffer used was pH 6.0, 0.1 ionic strength,

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sodium phosphate buffer. This buffer has low absorbance in the far-UV. The spectrum was then noise-reduced using JASCO software that filters out high-frequency components. The instrument was calibrated with 0.06% (w/v) ammonium camphorsulfonate.

UV/VIS absorption studies were done either using a Beckman DU640 at 23.0 °C or a Perkin-Elmer Model 320 spectrophotometer at ambient temperature.

Spectroscopic deconvolutions were done using the PeakFit software from Jandel Scientific. The approach used was nonlinear and iterative. The program will not be considered converged unless the sum of the squares for the fit is unchanged within eight significant figures for five iterations. The experimental curves were fitted with either Gaussian, Voight, Pearson VII, or Lorentzian equations and with mixtures of two of these equations.

The α and β polypeptides were separated by ion-exchange chromatography at pH 2.2 (Guard-Friar & MacColl, 1986). The α and β polypeptides were eluted from the resin with 5 and 10 M urea, respectively. Sodium dodecyl sulfate gel electrophoresis experiments showed that both polypeptides were entirely purified. Renaturation experiments consisted of diluting the polypeptide solutions with a 0.5% acetic acid solution, pH 1.9–2.2, to make them 1 M in urea. The 1 M urea solutions were then allowed to incubate in the cold for several days.

Samples in 2.5 m urea and in pH 6.0 buffer without urea were analyzed by high-performance liquid chromatography performed on a Waters 625 LC system equipped with a Waters 996 photodiode array detector. Samples were injected manually using a Rheodyne injector onto a Waters Protein Pak 200SW column suited for separation of biopolymers in the 500–60 000 molecular weight range. The column (8.0 mm × 300 mm) was equilibrated in pH 6.0 buffer and run under isocratic conditions at a flow rate of 0.8 mL/min with an operating back-pressure of 350 psi. All injections were 50 μL at a concentration of 0.1 g/L. Chromatograms were recorded by monitoring wavelengths between 200 and 700 nm and analyzed using Waters Millennium software. The photodiode array detection was used to measure the absorption spectra at specific retention times during the elution. In particular, spectra at the maximum absorbances and at the points most likely to be a different species from that at the maximum were analyzed.

RESULTS AND DISCUSSION

Visible CD of Phycocyanin 645. The visible CD spectrum of phycocyanin 645 (Figure 1) shows an intense negative band to the red at 643 nm (15 542 cm^{-1}), an indication of a seemingly very weak negative band in the blue side of the spectrum, and in between a group of positive bands. Jung *et al.* (1980) have suggested that the negative band at 643 nm and a positive band at 584 nm (17 118 cm^{-1}) may be formed by exciton splitting. Their publication presented the first experimental evidence for exciton splitting in any biliprotein, and its appearance was followed by arguments and evidence for and against this phenomenon in this and other biliproteins (Malak & MacColl, 1991; Csatorday *et al.*, 1984, 1988; Xie *et al.*, 1992; Sharkov *et al.*, 1992; Beck & Sauer, 1992; Gillbro *et al.*, 1992; Holzwartz *et al.*, 1990; Sauer & Scheer, 1988; Huang *et al.*, 1987; MacColl *et al.*, 1980, 1981).

Deconvolution of this CD spectrum (Figure 1) was accomplished by a trial and error process in which four types of bands (Gaussian, Lorentzian, Voight, and Pearson) were tested either alone or in binary combinations. The best fit

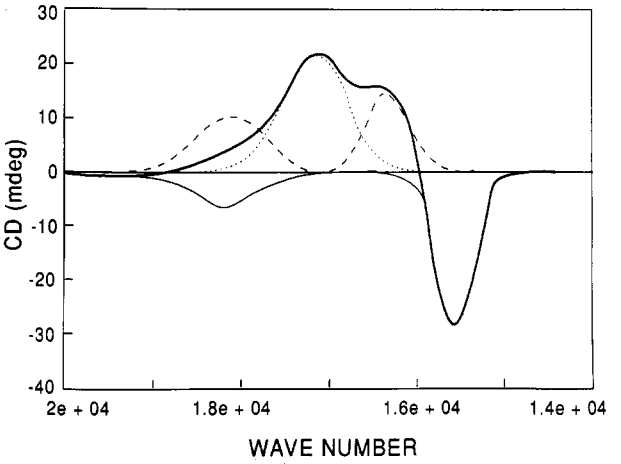

FIGURE 1: Deconvolution of CD of phycocyanin 645. The fit was made with four Gaussian bands and one Voight band (the blue-edge negative). The Voight band could be replaced by a Lorentzian band with only little change in the fit. The r^2 for this fit was 0.99975. From high to low energy, the maxima for each band were 18 169.8, 18 079.3, 17 118, 16 327.9, and 15 542.4 cm^{-1}. The heavy solid line is the experimental data.

Table 1: Deconvolution of the CD Spectrum of Phycocyanin 645

band type	max wavenumber (cm^{-1})	max wavelength (nm)	area (mdeg·cm^{-1})	sign
Gauss	15542.4	643	16000	−
Gauss	16327.9	612	8863	+
Gauss	17118.0	584	19945	+
Gauss	18079.3	553	9646	+
Voight	18169.8	550	8562	−

judged by deviation of the sum of the components to the actual spectrum was found to be four Gaussians and one Voight (or Lorentzian) for the blue-edge component. The most intriguing aspect of this fit was that the blue-edge negative band could be coupled to a positive band of similar rotational strength found at slightly lower energy by the deconvolution protocols; a combination of a negative and a positive CD band is required if exciton splitting occurs. The deconvolution showed that the areas of the two bands at 584 and 643 nm already thought to be coupled were also similar in rotational strength (Table 1), as expected for a pair of CD bands formed from exciton splitting. A caveat is that complex spectra usually can be fitted in more than one way. We look for solutions that correspond to the number of chromophores or bands expected with various models for the protein. For the positive band at 553 nm, in particular, additional analysis or experimental study is important.

Urea Treatment of Phycocyanin 645. To probe the factors producing this CD spectrum, the protein was treated with varying concentrations of the protein denaturant urea (Figure 2). At relatively high urea concentrations (5 M), the protein should be completely dissociated and the polypeptides denatured from their normal conformation. The visible CD spectrum is, in general, greatly reduced in intensity under these conditions. At 1 M urea, there is no noticeable change, but at intermediate urea concentrations significant changes in the CD spectrum occur, and it may be expected that there is only partial dissociation and unfolding of the protein at these concentrations. At concentrations of urea near 2.5 M, the CD spectrum has a most interesting appearance (Figure 3) in that the weak negative band at 525 nm is apparently gone. The rest of the spectrum is lower in intensity. The absorption spectra at 0 and 2.5 M urea did not show similar unique features (Figure 4). The deconvolution of the CD

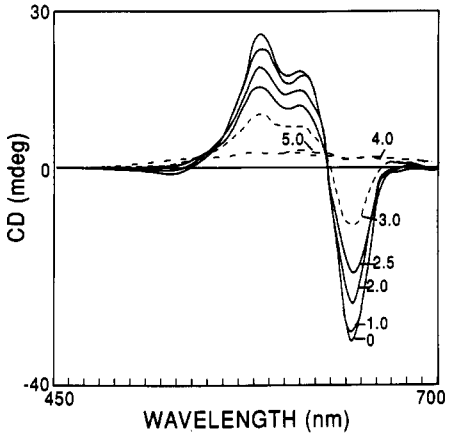

FIGURE 2: Effect of urea concentration on the visible CD of phycocyanin 645. Solutions were 0.10 mg/mL protein at pH 6.0 and 23.0 °C. The molar concentration of urea is shown on each spectrum.

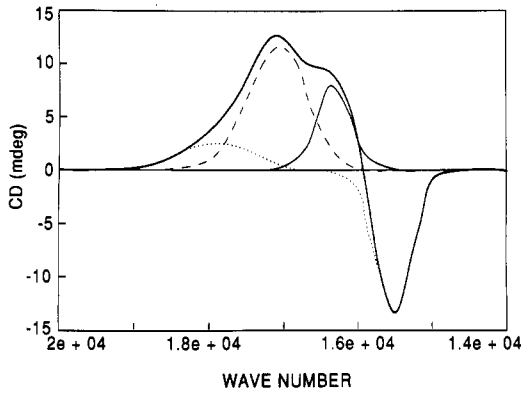

FIGURE 3: Deconvolution of the CD spectrum of phycocyanin 645 in 2.5 M urea. Solution was 0.10 mg/mL protein at pH 6.0 and 23.0 °C. All component bands were Gaussian. The r^2 for this fit was 0.99992. The heavy solid line is the experimental data.

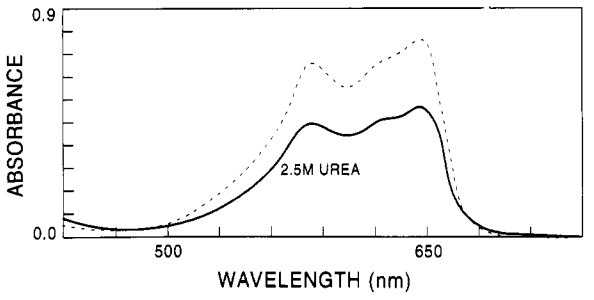

FIGURE 4: Visible absorption spectra of phycocyanin 645. Spectra were taken in a 1-cm light path at 23 °C. Samples were in pH 6.0 buffer or pH 6.0 buffer plus 2.5 M urea for 24 h.

spectrum of phycocyanin 645 in 2.5 M urea showed that the best fit consisted of four Gaussian bands (Figure 3). The disappearance of this negative CD band under these fairly mild denaturation conditions suggests that the band is strongly dependent on the native protein structure.

Gel-filtration chromatography showed elution in 2.5 M urea to be very similar to the single band observed in the absence of urea (Figure 5). The center of the band in 2.5 M urea eluted at the same molecular weight as native protein, and its visible absorption spectrum was observed by the photodiode array detector of the chromatographic system to be that of undissociated protein. The elution band in 2.5 M urea was broader than the band with no urea, and the slower edge of this band had an altered visible absorption spectrum. It can be concluded that the protein in 2.5 M urea has its conformation changed and has been dissociated to a small

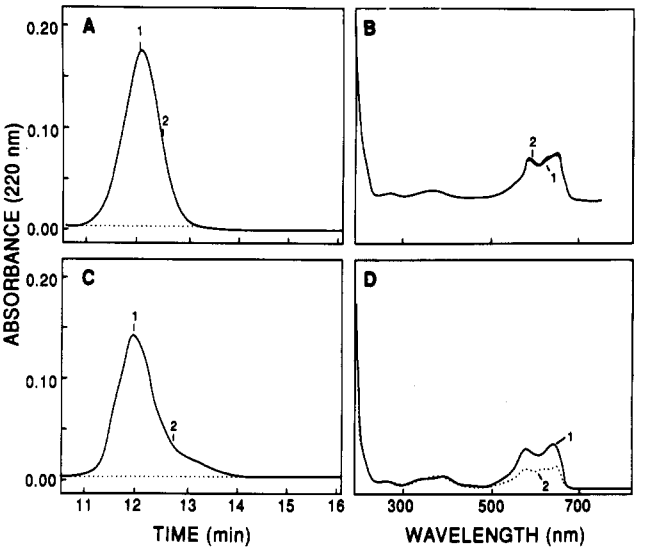

FIGURE 5: Gel-filtration chromatography of phycocyanin 645. (A) Elution of protein, no urea, pH 6.0; (B) spectra of protein with no urea at two elution times; (C) elution of protein, 2.5 M urea, pH 6.0; (D) spectra of protein, 2.5 M urea at two elution times. In both examples, the elution times were picked to exhibit spectra at the maximum absorbance (1) of the elution and at the time that gives the greatest chance to be different (2) from the maximum.

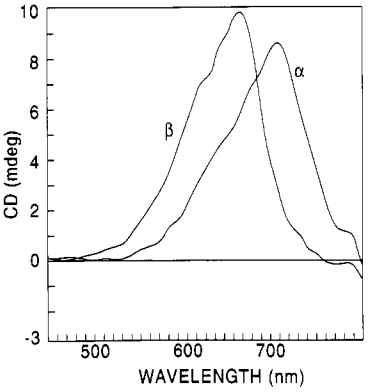

FIGURE 6: CD spectra of the separated α and β polypeptides of phycocyanin 645. The α polypeptide was in 5 M urea at pH 2.2 and 23.0 °C, and the β polypeptide was in 10 M urea at pH 2.2 and 23.0 °C. A 2-mm light path was used for both samples.

extent. These two changes are required to account for both the elution band becoming heterogeneous and the changes in the CD spectrum (Figures 3 and 5).

CD of the Phycocyanin 645 Polypeptides. The α and β polypeptides of phycocyanin 645 were separated by ion-exchange chromatography at pH 2.2 (Guard-Friar & MacColl, 1986). The α polypeptide is obtained in 5 M urea and the β in 10 M urea. At acidic conditions, these chromophores will be fully protonated and their visible absorption bands will be shifted to lower energies. The CD spectra of these polypeptides showed only positive bands, and neither negative CD band from the $\alpha_2\beta_2$ spectrum was observed (Figure 6). The disappearance of the two negative bands (Figures 1 and 6) under conditions of conformational change may indicate that the existence of both these bands depends on the positions of the chromophores in the native protein.

In order to further observe the factors necessary for the formation of the CD spectrum of phycocyanin 645, dilute acetic acid was added to the separated and denatured α and β polypeptides until they were each in about 1 M urea. At 1 M urea, the structure of phycocyanin 645 is stable (Figure 2), and these conditions should promote partial renaturation.

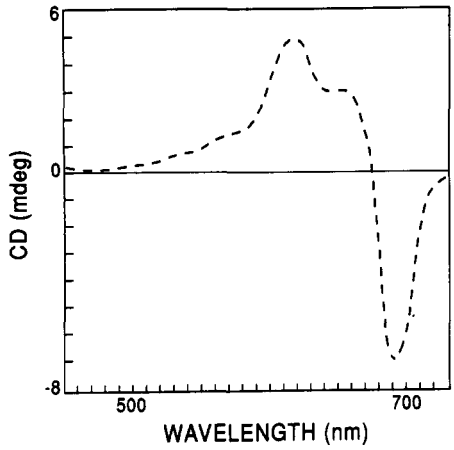

FIGURE 7: CD spectrum of the β polypeptide of phycocyanin in 1 M urea at pH 1.9 and 23 °C. A 5-mm light path was used.

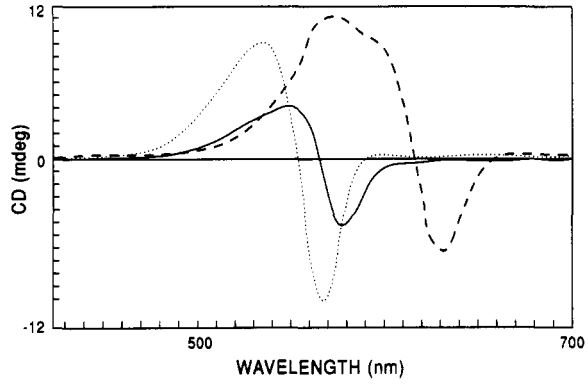

FIGURE 8: CD spectra of phycocyanin 612 and phycoerythrins 545 and 566. All proteins were 0.10 mg/mL at pH 6.0 and 23.0 °C. A 5-mm light path was used. The identification of each curve is as follows: dotted, phycoerythrin 545; dashed, phycocyanin 612; and solid, phycoerythrin 566.

The solutions were allowed to remain in 1 M urea, pH 1.9, for several days in the cold, and the CD spectrum was observed. Gradually, the β polypeptide assumed a conformation in which a salient negative CD band was observed at the red edge of the spectrum (Figure 7) along with the appearance of a higher-energy positive band. The concurrent appearance of both a negative and a positive CD band is cogent evidence for exciton splitting. The CD spectrum of the α polypeptide was relatively unchanged by the 5 M to 1 M urea shift (data not shown). The small CD negative on the blue side of the native spectrum was not formed under those conditions.

Comparison with Other Biliproteins. The very weak negative band on the blue side of the CD spectrum of phycocyanin 645 may provide important clues to the organization of the protein. This may be a rare or even unique opportunity because three other cryptomonad biliproteins were examined and showed no blue-edge negative CD bands (Figure 8).

Otherwise, these cryptomonad biliproteins are highly similar. They all usually have the $\alpha_2\beta_2$ polypeptide structure and the eight chromophores. Perhaps, their conformations are distinct. Using CD in the far-UV, the secondary structures of three cryptomonad biliproteins were estimated (Table 2). The method of Yang *et al.* (1986) was used for the estimation. Again, the structures of three spectroscopically distinct cryptomonad biliproteins are shown to be quite similar. The weak negative band on the blue edge of the CD spectrum of phycocyanin 645 provides the opportunity to use spectroscopy to make inquiries about chromophore topography. The structural agreement among phycocyanin 645 and other

Table 2: Comparison of Estimated Secondary Structures of Phycoerythrins 566 and 545 and Phycocyanin 645

	% estimated from CD			
protein	helix	β-sheet	β-turn	random
phycoerythrin 566	42.8	33.7	0.0	23.5
phycoerythrin 545	43.1	32.7	1.3	22.9
phycocyanin 645	40.0	31.9	4.2	24.0

cryptomonad biliproteins suggests that the chromophore arrangements among these several proteins may be very similar as well.

A Model. The appearance of two negative bands in the CD spectrum of phycocyanin 645 provides a basis to investigate the interactions of chromophores and thereby obtain a construct of chromophore topography. Both negative bands are totally absent in the spectra of the separated and denatured α and β polypeptides. The red-edge negative band seems more robust than the one on the blue edge since it survives the 2.5 M urea treatment and is apparently re-formed when the 10 M urea is diluted to 1 M for the β polypeptide. The reappearance of the red-edge negative band in a solution containing only the β polypeptide demonstrates that the chromophore on the α polypeptide plays no part in the events producing this band. The region of the spectrum where the blue-edge negative band is located is where cryptoviolin chromophores—which have higher-energy transitions compared with phycocyanobilins—are expected, and they also are located on the β polypeptide.

The red-edge negative and the positive band at 584 nm have previously (June *et al.*, 1980) been shown to be candidates for a pair of split bands from interacting chromophores. The deconvolution of the CD spectrum shows them to be most similar in intensities of any other pair of bands. The deconvolution draws attention to the fact that the blue-edge negative at 550 nm and the possible positive band at 553 nm are very similar in rotational strengths. These four bands may then be formed by pairs of closely-spaced chromophores interacting to produce exciton splitting. For the red-edge negative band and its corresponding positive band, the chromophores will be the phycocyanobilins on the β polypeptides. The remaining component band at 612 nm in the native spectrum would, by default, be assigned to the chromophore on the α polypeptide. The lack of any remaining component band available for assigning to isolated phycocyanobilins suggests that there are two pairs of these red-edge-interacting chromophores (Table 3). The higher intensities of these bands compared with the other component bands also support this idea, but do not prove it.

Since there is one cryptoviolin on the β polypeptide, the interaction causing the blue-edge negative band at 550 nm and the positive band at 553 nm will be for the two chromophores on adjacent β polypeptides. The possibility that there are two cryptoviolins on identical subunits—one having a positive and the other a negative CD spectrum—is not credible. The red-edge negative and its corresponding positive band at 584 nm can be formed in two ways: either both phycocyanobilins on the same β polypeptide interact, or the phycocyanobilins interact across the boundary of the two β polypeptides. There is circumstantial evidence to support the former concept. This pair of CD bands survives the partial dissociation treatment that completely abolishes the blue-edge negative band. Second, a red-edge negative band is found under conditions where only the β polypeptide is present, and it would at least be easier to visualize chromophores on the same subunit approaching normal conditions after denatur-

Table 3: Assignments of Chromophores to the $\alpha_2\beta_2$ Polypeptide Structure of Phycocyanin 645

chromophore	polypeptide	no. of chromophores per polypeptide	component CD max (nm)	assignment
phycocyanobilin (PCB)	β	2	643, 584	pair of PCB's produces red-edge exciton splitting; each of these pairs is on the same β polypeptide; each β polypeptide has one of these pairs
cryptoviolin (CV)	β	1	550, 553	pair of CV's causes blue-edge exciton splitting; one member of pair is on each β polypeptide
697-nm bilin (697B)	α	1	612	more isolated chromophores; transfer excitons to the β polypeptide by very weak dipole–dipole coupling

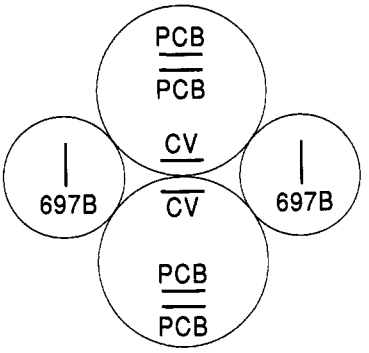

FIGURE 9: Model for the chromophore distribution in phycocyanin 645. The symbols used are the following: PCB, phycocyanobilin; CV, cryptoviolin; 697B, the 697-nm bilin. The 697-nm bilin is located on an α polypeptide, and the two phycocyanobilins and the one cryptoviolin are found on a β polypeptide.

ation as opposed to the chromophores on different polypeptides returning to very similar positions during β polypeptide aggregation to those found in native protein. We assign the phycocyanobilin–phycocyanobilin interaction to chromophores located on the same polypeptide but note that this assignment is not rigorously proven. A model follows from these experiments in which there are three pairs of chromophores and two more isolated chromophores (Figure 9).

Energy migration could proceed by very weak dipole–dipole coupling (Förster, 1948; Knox & Gülen, 1993) between the more isolated chromophores on the α polypeptide and the red-edge pairs and between the different pairs. Very weak coupling tends not to change the spectra of monomers. Between the high- and low-energy bands of a more strongly coupled pair, energy can be transferred by internal conversion.

The splitting of bands into high- and low-energy forms has important effects on the exciton migration scheme. For example, without the splitting of the spectra of phycocyanobilins, the lowest-energy chromophores might be ones on the α polypeptides. Now it can be proposed that the lowest-energy band, which results in emission from the isolated protein and at least one source of energy transfer from the protein in the intact system, is the lower-energy band resulting from band splitting of the pairs of phycocyanobilins. As suggested previously (Malak & MacColl, 1991), the 612-nm chromophore will transfer energy to the exciton split band at 643 nm. The blue-edge exciton bands will, also, transfer energy to the red-edge exciton bands. In addition, the absorption spectrum is broadened both from the splitting of phycocyanobilins to the red and from the splitting of cryptoviolins to the blue. This spectroscopic broadening increases the energy range of light harvesting available to the protein. The improved range for the absorption of solar energy may provide the organism with advantages in situations of varying types of available light.

CONCLUSION

For the biliprotein C-phycocyanin, the precise positions of its chromophores are known from X-ray crystallography (Schirmer *et al.*, 1986, 1987; Duerring *et al.*, 1991), and these results have led to seminal progress in understanding the function of this protein and the closely-related protein allophycocyanin (Sauer & Scheer, 1988; Beck & Sauer, 1992; Scharnagl & Schneider, 1989; Holzwarth *et al.*, 1987, 1990; Sharkov *et al.*, 1992; Gillbro *et al.*, 1992; Xie *et al.*, 1992; Debreczeny *et al.*, 1993). There are no corresponding crystallographic data available for any cryptomonad biliprotein. Femtosecond and picosecond time-resolved studies have shown that ultrafast transitions occur in C-phycocyanin and allophycocyanin after excitation (Xie *et al.*, 1992; Gillbro *et al.*, 1993; Holzwarth *et al.*, 1987, 1990; Sharkov *et al.*, 1992a,b; Beck & Sauer, 1992). These results have yielded a contested viewpoint concerning the mechanisms that produce these transitions. The transition may be a manifestation of energy transfer by very weak dipole–dipole coupling (Förster, 1948) between two chromophores—a mechanism that tends to retain the spectroscopic characteristics of the monomers, internal conversion between states produced by exciton splitting of the monomer spectrum, or other processes. CD spectroscopy, however, clearly shows the appearance of splitting in phycocyanin 645. Moreover, the CD results suggest that most of the chromophores of this protein may be involved in exciton splitting. The model consists of two different pairs of chromophores that may be engaged in exciton splitting. Two of the chromophores are in a more isolated position in the protein. More information is required before exciton splitting is totally established for this protein. Time-resolved experiments initiated by exciting the lowest-energy component at 550 nm would be interesting. Other avenues of possible progress on the study of chromophore topography for phycocyanin 645 would include a continuation of the approach used here of combining biochemical methods and spectroscopy.

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