Bilin Organization in Cryptomonad Biliproteins

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ABSTRACT: The bilin organization of three cryptomonad biliproteins (phycocyanins 612 and 645 and phycoerythrin 545) was examined in detail. Two others (phycocyanin 630 and phycoerythrin 566) were studied less extensively. Phycocyanin 645 and phycoerythrin 545 were suggested to have one bilin in each monomeric ($\alpha\beta$) unit of the dimer ($\alpha_2\beta_2$) isolated from the others, and the remaining six bilins may be in pairs. One pair was found across the monomer−monomer interface of the protein dimer, and two identical pairs were proposed to be within the monomer protein units. For phycocyanin 612, a major surprise was that a pair of bilins was apparently not found across the monomer−monomer interface, but the remaining bilins were distributed as in the other two cryptomonad proteins. The effect of temperature on the CD spectra of phycocyainin 612 demonstrated that two of the bands (one positive and one negative) behaved identically, which is required if they are coupled. The two lowest-energy CD bands of phycocyanin 612 originated from paired bilins, and the two higher-energy bands were from more isolated bilins. The paired bilins within the protein monomers contained the lowest-energy transition for these biliproteins. Using the bilins as naturally occurring reporter groups, phycocyanin 612 was shown to undergo a reversible change in tertiary structure at 40 °C. Protein monomers were shown to be functioning biliproteins. A hypothesis is that the coupled pair of bilins within the monomeric units offers important advantages for efficient energy migration, and other bilins transfer energy to this pair, extending the wavelength range or efficiency of light absorption.

The energy of photosynthesis is the product of the activities of light-harvesting pigments. These chromoproteins absorb light, and the energy migrates with great efficiency to a reaction center of photosystem I or II. In the reaction centers, a series of electron transfers cause the energy from absorbed photons to be converted to chemical energy. The efficiency of the migration of energy from the distal sites of absorption to these reaction centers is produced by networks of chromophores that allow very fast transfers of energy from one chromophore to another. Energy will travel from an area of high energy to an area of low energy through these assemblies.

Cryptomonad biliproteins are light-harvesting pigments that are purified with eight chromophores covalently attached to a dimeric protein ($\alpha_2\beta_2$). The chromophores are open-chain tetrapyrroles, called bilins. There are seven different cryptomonad biliproteins (*1*−*4*). One biliprotein is found in each cryptomonad. Five of these spectroscopic types were examined. Three biliproteins (phycocyanins 645 and 612 and phycoerythrin 545) were examined in more detail, and a limited study was carried out with phycoerythrin 566 and phycocyanin 630. Efforts were made to test and extend previous studies with phycocyanin 645 and to develop models for the organization of the bilins for phycocyanin 612 (*5*−*7*) and phycoerythrin 545. Key data for phycocyanin 630 and phycoerythrin 566 allowed suggestions of certain possible bilin models. The properties of biliproteins have been reviewed (*8*−*16*).

The protocols employed include various types of optical spectroscopy and the study of the dissociation of protein dimers to monomers ($\alpha\beta$). A paramount issue is whether certain bilins associate as pairs, or whether bilins are more isolated entities. The presence of bilin pairs within protein monomers, or across the monomer−monomer interface, is a salient distinction. The properties of protein monomers were considered.

EXPERIMENTAL PROCEDURES

The sources of phycocyanin 645, phycocyanin 612, phycoerythrin 545, and phycoerythrin 566 were *Chroomonas* sp., *Hemiselmis virescens*, *Rhodomonas lens*, and *Cryptomonas ovata*, respectively. The protozoa were grown in Albany and their biliproteins extracted and purified (*5*−*7*). Phycocyanin 630 was obtained from an axenic culture of *Chroomonas* HP9001. The organism was isolated from the Choptank River, a tributary of the Chesapeake Bay (*17*). This cryptomonad was grown in Georgetown, SC, and its bili-

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protein extracted and treated as described previously (*18*). Biliproteins were either stored under 80% saturated ammonium sulfate or lyophilized after extensive dialysis into water.

C-Phycocyanin was obtained from harvests of the cyanobacterium *Phormidium luridum*. The protein was purified by ammonium sulfate fractionation and hydroxylapatite column chromatography. The protein was considered purified when the A_{620}/A_{280} was ≥4.0. R-Phycoerythrin was isolated from the red alga *Porphyra tenera* and was received purified (Pierce).

Measurements of optical spectra were usually carried out at 0.15 or 2.5 g/L protein, except for those for fluorescence. Dimers were in pH 6.0, 0.1 ionic strength, sodium phosphate buffer. Monomers were obtained at lower pH values in 0.10 M sodium acetate (*6*, *7*).

Visible CD spectra were measured with a 0.5 cm light path, and CD spectra in the ultraviolet (UV) from 180 to 260 nm were recorded using a 0.02 cm light path for 0.15 g/L solutions. Protein secondary structure was obtained from CD spectra (*19*). For temperature experiments, samples were equilibrated at each temperature for 10 min before spectra were taken.

Fluorescence spectra were usually obtained using a protein concentration of 0.015 g/L. In addition, lower protein concentrations of 0.010 and 0.005 g/L were occasionally used. The fluorescence (excitation) polarization (*p*) spectra were calculated from (eq 1)

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

where I is the fluorescence intensity, G is an instrument correction factor calculated as I_{HV}/I_{HH}, and VV and VH were vertical excitation and vertical emission polarizers and vertical excitation and horizontal emission polarizers, respectively, and HH and HV were defined similarly. The solutions were prepared immediately before the measurement to avoid possible denaturation at these low protein concentrations.

Dynamic light scattering measurements were performed using a DP-801 instrument (Protein Solutions). The samples were illuminated at 780 nm using a solid-state laser. The experiment was used to obtain the diffusion coefficient (D) of the protein. The hydrodynamic radius (R_H) can be calculated from the Stokes−Einstein equation (eq 2):

$$R_H = \frac{6\pi\eta D}{kT} \quad (2)$$

where η, k, and T are the viscosity of the solvent, the Boltzmann constant, and the absolute temperature, respectively. The frictional coefficient (f) was obtained with the Einstein−Sutherland equation (eq 3):

$$D = \frac{kT}{f} \quad (3)$$

The diffusion coefficient was corrected to the value at 20 °C with water (eq 4):

$$D_{20,w} = D\frac{293}{T}(\eta_T/\eta_{20})(\eta_B/\eta_w) \quad (4)$$

where η_T is the viscosity of water at T, η_{20} is the viscosity of water at 20 °C, η_B is the viscosity of buffer at T, and η_w is the viscosity of water at the same temperature as the buffer. The frictional coefficient of a sphere having the same volume as the unhydrated molecule, f_o, was calculated from

$$f_o = 6\pi\eta\left(\frac{3m\upsilon}{4\pi}\right)^{1/3} \quad (5)$$

where m is molecular mass/Avogadro's number and υ is the partial specific volume of the protein. A ratio, f/f_o, can then be calculated as a measure of molecular shape, although the water of hydration and surface roughness may also influence the value.

Time-resolved fluorescence was performed at the Center for Fluorescence Studies in Baltimore by two-photon methods described previously (*20*−*25*). A mode-locked Ti:sapphire laser was used for the two-photon experiments. The laser provided 80 fs pulses (measured by an autocorrelator) with a 10 nm spectral distribution at 80 MHZ pulse repetition. Excitation was at 800 nm for phycocyanin 612. The high peak power of the laser output was able to produce a two-photon excitation, where a bilin absorbed two photons simultaneously. The solutions had an absorption of 0.2 at the maxima and were in pH 6.0 buffer. The filters used were 630 nm interference and 720 nm short-wavelength glass. The choice of 800 nm for the excitation wavelength avoided unwanted one-photon excitation. The phase angle and modulation of the emission were related to the intensity decay parameters and modulation frequency by equations described previously (*24*). The resolution for these experiments was about 1 ps.

The α- and α′-polypeptides were separated from the β-polypeptide and from each other using ion exchange chromatography and a urea gradient at pH 2.2. Solutions of 2.0, 3.0, 4.0, 5.0, 6.0, and 10 M urea were prepared at pH 2.2 in 0.4% acetic acid. Bio-Rex 70 cation exchange resin was prepared in 0.4% (volume/volume) acetic acid and adjusted to pH 2.2 with hydrochloric acid. The resin was packed into a column 4 cm high and 1.5 cm in diameter. A 5 mg portion of phycocyanin 612 was dissolved at pH 2.2 and applied to the column.

Gel filtration column chromatography was performed as described previously (*7*).

RESULTS AND DISCUSSION

Absorption and CD Spectra. It has been proposed that pairs of bilins produce exciton splitting (*26*) and delocalization of energy for phycocyanins 645 and 612 and phycoerythrin 545 (*5*−*7*, *27*). The CD spectra of these three cryptomorad biliproteins have both positive and negative bands (Figure 1). This type of CD suggested the possibility of exciton splitting between bilins. Certain noncryptomonad biliproteins, e.g., C-phycocyanin, do not exhibit exciton splitting for their bilins as demonstrated by their CD spectra. The CD of C-phycocyanin has only positive bands (Figure 1). The CD and absorption spectra of the bilins of five biliproteins were obtained (Figure 1). The main bilins in phycocyanins 612 and 645 are phycocyanobilins, and their properties will be compared with those of C-phycocyanin, which contains phycocyanobilins. The aggregation state of this preparation of C-phycocyanin is mostly trimers ($\alpha_3\beta_3$). Phycoerythrin

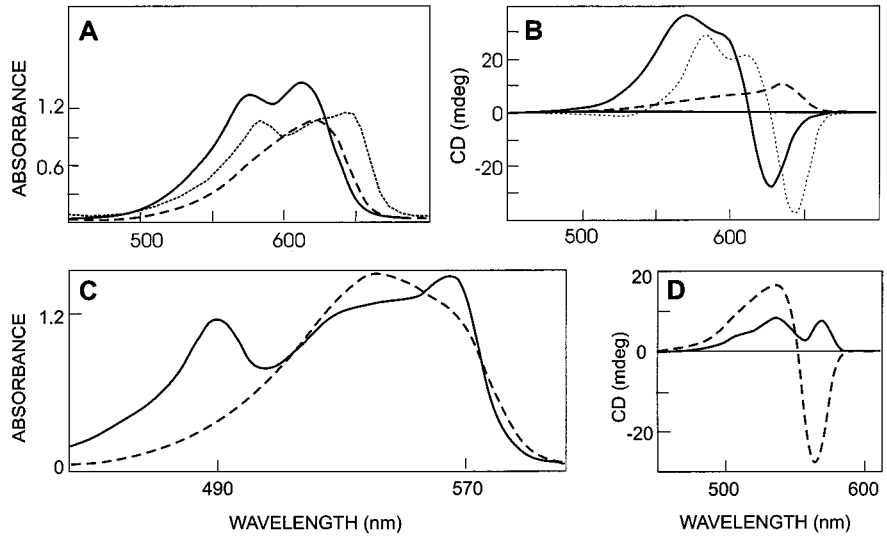

FIGURE 1: Absorption (A) and CD (B) spectra in the visible range for phycocyanin 612 (—), phycocyanin 645 (···), and C-phycocyanin (– – –) and the absorption (C) and CD (D) spectra for phycoerythrin 545 (– – –) and R-phycoerythrin (—). All solutions were at pH 6.0 with 0.15 g/L proteins at 20 °C. A 5 mm light path was employed for CD. Note that the CD spectrum of phycocyanin 645 has two negative bands, one on the red edge and another less intense band on the blue edge.

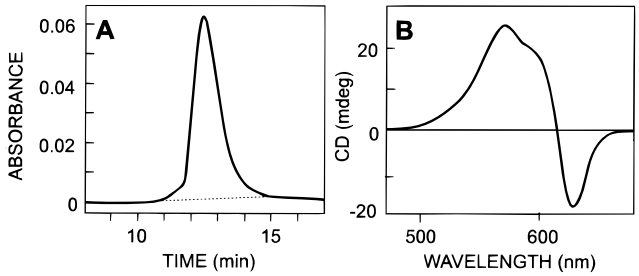

FIGURE 2: Monomers ($\alpha\beta$) of phycocyanin 612. Solutions were at pH 4.3 with 0.15 g/L protein. Temperatures were 20 °C for CD and ambient temperature for gel filtration: (A) gel filtration column chromatography and (B) CD in a 5 mm light path.

545 contains mainly phycoerythrobilins. Its properties will be compared with those of R-phycoerythrin, which contains a major quantity of this same bilin. This R-phycoerythrin has a CD spectra, which did not indicate exciton splitting (Figure 1). The R-phycoerythrin has an $\alpha_6\beta_6\gamma$ oligomeric structure.

Location of the Bilin Pair. For phycocyanin 612, it was of interest to determine if the reputed bilin pair existed within protein monomers, or across the monomer−monomer interface of the protein. For certain noncryptomonad biliproteins, X-ray crystallography results showed that the closest distance between two bilins in protein trimers was across a monomer−monomer interface (*28*, *29*).

To accomplish this goal, monomers of phycocyanin 612 were needed. Monomers of various biliproteins have been obtained at slightly acidic pH values (*16*), but monomers had not been produced for phycocyanin 612. The dimers (molecular mass of 58 000 Da) are known to be stable at pH 6.0, and lower pH values were applied to phycocyanin 612. Gel filtration column chromatography showed some monomers dissociated to individual α- and β-polypeptides at pH 4.0. At pH 4. 3, stable and homogeneous monomers at 0.15 g/L were obtained (Figure 2) as demonstrated by their elution time compared with those of proteins with known molecular masses. Gel filtration column chromatography of phycoerythrin 545 at pH 4.5, a pH where monomers had previously been produced (*7*), showed the same elution time that phycocyanin 612 exhibited at pH 4.3. Monomers (molecular mass of 29 000 Da) of phycocyanin 612 have an $\alpha\beta$ polypeptide structure.

Table 1: Secondary Structure Estimates from CD for Phycocyanin 612

	% composition			
protein[a]	α-helix	β-sheet	β-turn	other
pH 6.0, 20 °C, dimers	48.0	12.2	21.7	18.6
pH 6.0, 40 °C	48.7	11.9	21.8	17.1
pH 4.3, 20 °C, monomers	53.0	11.1	20.2	15.9

[a] The protein concentration was 0.15 g/L for all samples.

The CD spectrum of monomers of phycocyanin 612 (Figure 2) still showed the characteristic appearance of exciton splitting since the spectrum had positive and negative bands. The proposed coupled pair of bilins was, therefore, within the monomeric protein units. This same result was obtained for phycoerythrin 545 (*7*) and phycocyanin 645 (*6*).

For several other cryptomonad bilproteins, the α-polypeptide has been shown to be composed of two or more types, having the same bilin (*30*−*32*). Using an ion exchange column and a urea gradient, phycocyanin 612 was also shown to have a least two α-polypeptides (data not shown). The monomers are a mixture of $\alpha\beta$ and $\alpha'\beta$.

To fully accept these results, the protein monomers of phycocyanin 612 must be shown to resemble the protein dimers and not be disordered protein. Two approaches to this problem were taken. The secondary protein structures were obtained by CD in the ultraviolet, and the fluorescence polarization spectra of the bilins were obtained. Monomers and dimers of phycocyanin 612 were shown to be similar in secondary structure (Table 1). Fluorescence polarization spectra show the transfers of energy between bilins. These spectra show monomers and dimers to be very similar (data not shown). They both have energy transfer transitions in exactly the same wavelength regions. This similarity is very important because a function of the protein is energy transfer, and these spectra show protein dimers and monomers perform this function in an analogous manner.

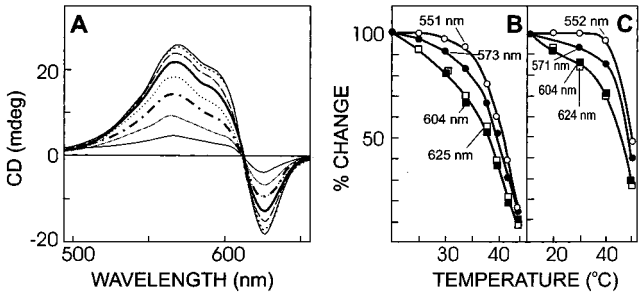

FIGURE 3: CD of phycocyanin 612 as a function of temperature: (A) spectra of monomers, (B) band decay of monomers, and (C) band decay for dimers. In panel A, the highest positive extreme and lowest negative extreme were at 20 °C. The remaining bands in order were at 25, 30, 36, 38, 40, 42, and 44 °C.

Phycocyanin 612 has four bilins in a protein monomer, and three changes in polarization are possible as energy is transferred from bilin to bilin through the protein. Three transitions were observed for both monomers and dimers.

Which Bands Are Coupled? The CD spectrum of phycocyanin 612 monomers showed a single negative band on the red edge and a number of higher-energy positive transitions. To ascertain which positive band, if any, may be coupled to the negative band, a temperature study of the CD spectrum of the protein monomer was undertaken. If two bands are part of an exciton pair, the excitation energy is delocalized between them and the two bands behave identically. If one band changes, then the other must change accordingly. As the temperature was increased, the CD spectra gradually changed (Figure 3A). When the changes in the CD maxima of the four bilin bands were individually plotted, the lowest-energy positive band decreased at exactly the same extent as the negative band, while the other two higher-energy positive bands declined differently (Figure 3B). The band maxima, which are plotted, were obtained from the four components suggested from deconvolution of the CD (*33*). This analysis did not take into account overlap between component bands (data not shown). The most important overlap is between the bands at 573 and 604 nm. At 604 nm, there is a 9% contribution from the band at 573 nm. Given the difference in decays between the two bands, this 9% overlap would not significantly affect the plots of the data. This result is the very best evidence for exciton splitting of one bilin pair, and clearly shows that the 604 and 625 nm CD bands are involved. The two other bilins at 551 and 573 nm are uncoupled in monomeric protein.

Monomers have a collection of interesting properties. They have very similar secondary structure compared to that of dimers (Table 1). Monomers have an altered bilin spectrum when compared to that of dimers (Figure 2), but the essential identity of the fluorescence polarization spectra of dimers and monomers shows that the bilins are maintained at identical relative distances and orientations with respect to each other. The tertiary structures of dimers and monomers differ, but not globally. The monomers are more easily denatured than dimers, however (Figure 3). The monomer−monomer interaction, or the better access of water to the surface of monomers, may provide the augmented dimer stabilization.

In previous studies using $KMnO_4$ with dimers of phycocyanin 645, it was found that two CD bands also behaved similarly (*34*). These two CD bands that may be produced by paired bilins were the lowest-energy negative band at 643 nm and the positive band at about 584 nm. Unlike the findings for phycocyanin 612 (Figure 3), the lowest-energy positive band at 612 nm was not coupled to the negative band.

Comparisons of CD. It is of interest to study the proteins under conditions where they are known to be homogeneous dimers (~58000 Da molecular mass). For phycoerythrin 545, this condition was found to be 2.5 g/L at pH 6.0 (*7*). For phycocyanin 645, a 0.4 mg/mL solution at pH 6.0 was also homogeneous dimer (*6*).

To verify a solution of homogeneous dimers for phycocyanin 612, a 2.5 g/L solution (pH 6.0) was subjected to experiments using dynamic light scattering. Dynamic light scattering has three criteria for homogeneity of a polymer solution: a small range of values for the hydrodynamic radius of the protein, a small standard error for the measurement, and an excellent fit of a standard curve to the data. All three of these criteria demonstrated that phycocyanin 612 was a homogeneous preparation (data not shown).

A diffusion coefficient was obtained for phycocyanin 612 from the dynamic light scattering measurements (Table 2). Previously, a sedimentation coefficient ($s^{o}_{0,w}$) of 4.1 S was obtained (*35*). The diffusion coefficient (D) was corrected to $D_{20,w}$, and the Svedberg equation (eq 6) was used to calculate the molecular mass (M):

$$M = \frac{sRT}{D(1 - \upsilon\rho)} \tag{6}$$

where R is the ideal gas constant, T is the absolute temperature, υ is the partial specific volume, and ρ is the density of the solution. The value for molecular mass agreed with the expected value for protein dimer (Table 2). The value of f/f_o measures the ratio of the frictional coefficient of the protein to the frictional coefficient of a sphere having the same volume as an unhydrated molecule. The large value of f/f_o suggests a nonspherical protein, but surface roughness and bound water can also affect the frictional coefficient.

The ratios of the area of the positive CD to the area of the negative CD were obtained for these three dimeric proteins (Table 3). For phycocyanin 645, the CD had two negative bands, one on the red edge and a very weak band on the blue edge. These negative bands were summed, as were the areas of all the positive bands. For the three biliprotein dimers, the positive CD bands were significantly greater than the negative bands.

The shape of the CD spectra for phycocyanin 630 and phycocyanin 566 also showed that some of the bilins could perhaps be coupled (Figure 4). It was not possible in this study to have these proteins at 2.5 g/L, but at pH 6.0, they should have been mostly protein dimers.

All five cryptomonad proteins showed the possible presence of bilin clusters, and there were apparently two classes of behavior. Phycocyanins 612 and 630 were in one class as they showed much higher ratios of positive to negative CD than the others (Table 3).

Is There a Second Coupled Pair of Bilins? In addition to the bilin pair suggested to be within the protein monomer units of the protein dimer, evidence has been presented for a second coupled pair of bilins across the monomer−monomer interface for phycocyanin 645 and phycoerythrin

Table 2: Some Properties of Phycocyanin 612

property	method	value
hydrodynamic radius (nm)	Stokes−Einstein equation	3.23
diffusion coefficient ($D_{20,W}$) (cm^2/s)	dynamic light scattering	6.62×10^{-7}
sedimentation coefficient ($s_{20,w}$) (S)	analytical ultracentrifugation	4.1
molecular mass (Da)	Svedberg equation	57000
frictional coefficient (f) (g cm^{-1} s^{-1})	Einstein−Sutherland equation	6.11×10^{-8}
f/f_o	f_o (frictional coefficent of a sphere)[a]	1.28

[a] $f_o = 6\pi\eta[3m\nu/(4\pi)]^{1/3}$.

Table 3: Ratio of Positive to Negative CD for the Bilins of Several Cryptomonad Biliproteins

Phycoerythrin 545	
dimers (pH 6.0, 2.5 g/L)	monomers (pH 4.5, 0.15 g/L)
1.50	2.07
Phycocyanin 645	
dimers (pH 6.0, 2.5 g/L)	monomers (pH 4.0, 0.15 g/L)
1.62	1.71
Phycocyanin 612	
dimers (pH 6.0, 2.5 g/L)	monomers (pH 4.3, 0.15 g/L)
4.01	3.89
Phycoerythrin 566	
dimers (pH 6.0, 0.15 g/L)	
1.51	
Phycocyanin 630	
dimers (pH 6.0, 0.15 g/L)	
3.36	

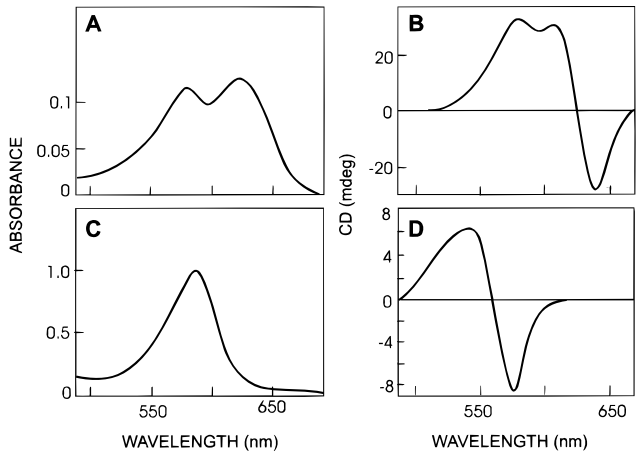

FIGURE 4: Absorption and CD spectra of phycocyanin 630 (A and B) and phycoerythrin 566 (C and D). The buffer was at pH 6.0, and the light path was 5 mm long.

545 (*5*−*7*). This proposed second intermonomer pair gives a weaker CD spectrum than the pairs within the monomers, and is therefore more challenging to verify. For phycoerythrin 545, the problem is augmented because the CD spectrum of the proposed second bilin pair lies underneath the spectrum of the more intense first pair. It is observed by obtaining a difference spectrum between dimers and monomers (*7*). To study this further, dimers of phycoerythrin 545 were treated with $KMnO_4$ ($0.95-2.28 \times 10^{-6}$ M), following earlier protocols (*34*) used for phycocyanin 645, and the CD spectra obtained (data not shown). Difference spectra between untreated and permanganate-treated protein showed the characteristics of exciton coupling. The difference spectra were similar to that found by subtracting the CD of monomers from that of dimers (*7*). A positive band was observed at 545 nm and a negative band at 562 nm. This difference spectrum is unlike the CD spectrum of the protein in permanganate, and appears to be the CD spectrum of the bilin pair across the monomer−monomer interface.

The situation for phycocyanin 645 was more favorable. The proposed second bilin pair appeared to produce a negative CD band on the blue edge of the spectrum (Figure 1). This second negative band vanishes when monomers were produced (*6*, *36*). A coupled pair of bilins across the protein monomer−monomer interface would split the spectrum of the bilins into higher- and lower-energy bands. A single band in a protein monomer would then be two bands in a protein dimer. One of these two bands would be negative and the other positive if there is exciton splitting.

Phycocyanin 612 in the dimeric oligomer showed a CD (Table 3) that had a much greater positive to negative band ratio than those of phycocyanin 645 and phycocyanin 545. In addition for monomers, the ratio of positive to negative CD increased for phycocyanin 645 and phycoerythrin 545, but not for phycocyanin 612. A speculative reason for the increased ratio upon monomer formation would be the separation of the intermonomeric bilin pair with the newly formed isolated bilins having a positive CD. There was a need, therefore, to further study the bilin properties of phycocyanin 612.

Phycocyanin 612. One possible conclusion from the CD studies on phycocyanin 612 is that there is only the one pair of coupled bilins within the monomeric unit, and no pair of bilins across the monomer−monomer protein interface. Since two different pairs of bilins were proposed for both phycocyanin 645 and phycoerythrin 545, the absence of one pair in phycocyanin 612 would require substantial evidence. These three biliproteins are all $\alpha_2\beta_2$ oligomeric proteins having eight bilins. Previous studies with phycocyanin 612 did not consider the possibility of a pair of bilins across the monomer−monomer interface (*27*, *33*). The best evidence for the intermonomer pair of bilins is a difference CD spectrum of monomers subtracted from dimers. If a band is lost upon monomer formation, the difference spectrum would be qualitatively not like the monomer spectrum. The difference spectrum would contain the band lacking in monomers (Figure 5). The dimer, monomer, and difference spectra have the same bands (Figure 5A). An intermonomer pair of bilins, therefore, is apparently not present for dimers of phycocanin 612.

Phycocyanin 645 undergoes a change in tertiary structure at elevated temperatures, while the secondary protein structure is maintained (*5*). A result of this change is that the coupled pair of bilins across the monomer−monomer interface moves apart or changes in orientation, and the exciton splitting is lost. The negative CD band of the dimer on the blue edge disappears at elevated temperatures. The change is reversible (*5*). We heated phycocyanin 612 to look

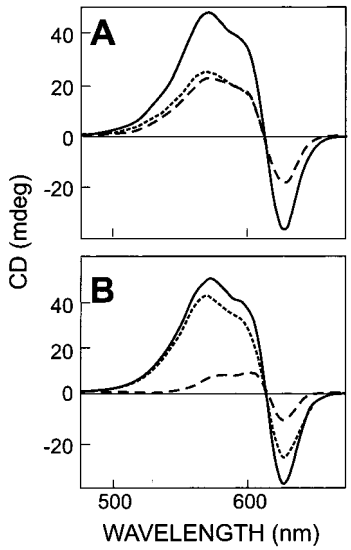

FIGURE 5: CD of phycocyanin 612. Dimer (—), monomer (···), and difference spectra (– – –) (A) and dimers at 20 °C (—) and 40 °C (···), and this difference spectrum (– – –) (B).

for a similar change. Although the CD spectrum was changed, there was no suggestion of the loss of any dimer band (Figure 5B), and again, this evidence is not consistent with the intermonomer pair of bilins.

Previously, the effect of temperature on the CD of phycocyanin 612 monomers was analyzed for bilin pairing (Figure 3A,B). The same experiment was carried out with phycocyanin 612 dimers (Figure 3C). The results were identical for dimers and monomers, and one pair of bands behaved as if they were produced by a coupled pair of bilins. The behavior of the other two bands indicated that they belonged to more isolated bilins.

These results taken together strongly support a novel model for the bilin topography of phycocyanin 612, in which there is only a coupled pair of bilins within a monomeric protein unit observed by CD spectroscopy. The bilins across the monomer−monomer interface are either farther apart or at a different orientation compared with those found for phycocyanin 645 and phycoerythrin 545 (*37*).

The change in CD of phycocyanin 612 produced at 40 °C and pH 6.0 was totally reversible upon cooling the solution to 20 °C, while the change at 50 °C was only partially reversible (data not shown). The change at 40 °C was, therefore, probably not a result of the protein becoming denatured. Gel filtration column chromatography was used to study the protein at 20 and 40 °C by using a heated sample carousel and column heater. The elution time changed slightly at 40 °C, much less than would be expected for a complete dissociation to monomer (data not shown). In addition, the CD of monomers was qualitatively different from that at 40 °C and pH 6.0 (Figure 5), which nicely proves that dissociation is not the cause of the CD change between 20 and 40 °C. The blue edge of the spectra differed in the two cases. The secondary structure of phycocyanin 612 was obtained by CD for the protein at 40 °C. The 20 and 40 °C secondary structures were similar (Table 1). The change in the protein at 40 °C was therefore one in which tertiary structure is the principal factor, and the bilins act as naturally occurring reporter groups for this process. Monomers have

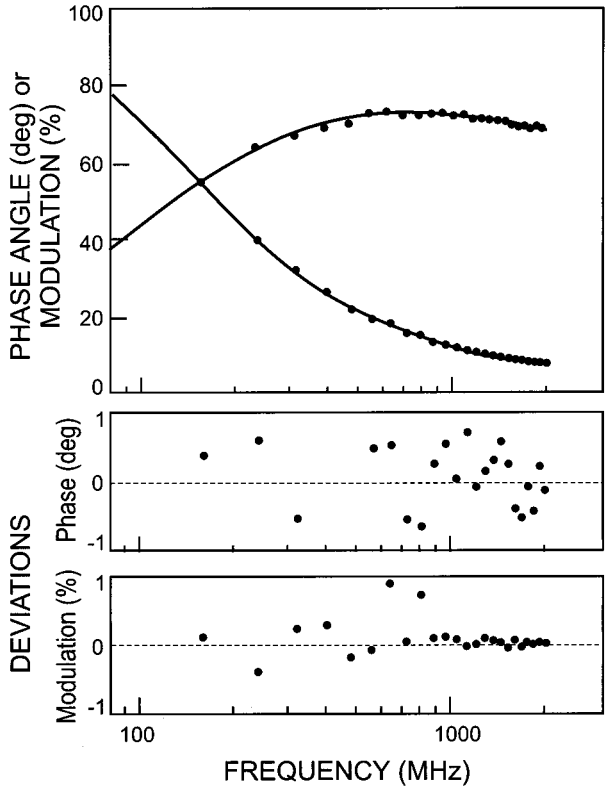

FIGURE 6: Time-resolved fluorescence using two-photon excitation of phycocyanin 612 dimers in the frequency domain. Excitation was at 800 nm. Protein was in pH 6.0 buffer.

a changed CD spectrum at 40 °C, but unlike that for protein dimers, the change is irreversible (data not shown).

The function of the topography of bilins is efficient and directional energy migration. Using two-photon excitation, a measure of some aspect of this energy migration was obtained. It was found that decay times of 37.4 and 1532 ps were obtained for phycocyanin 612 dimers by these methods (Figure 6). The 1.5 ns is the radiative lifetime of the excited state of the lowest-energy bilin for the protein and agrees with the 1−2 ns lifetimes obtained for many biliproteins (*16*). The 37.4 ps decay time probably reflects radiationless Förster resonance transfer in the weak coupling limit from one of the isolated bilins. The standard errors were 5% for the 37.4 ps and 0.7% for the 1.5 ns lifetime.

The lifetimes for phycocyanin 612 dimers were calculated from $I(t) = \Sigma_i \alpha_i e^{-t/\tau_i}$, where the α's are the pre-exponential factors (*24*). A rise time was not observed. The α's for the lifetimes of 37.4 and 1532 ps were 0.500 and 0.427, respectively.

Decay times comparable to 37.4 ps have been frequently reported for other biliproteins where Förster resonance transfers were expected (*38*). For cyanobacterial allophycocyanin, very much faster time constants were obtained for its closely spaced pair of bilins (*39*). For this pair of bilins in trimers of allophycocyanin, there is a debate over whether delocalization occurs (*39*, *40*). The 37.4 ps lifetime found for dimers of phycocyanin 612 (Figure 6) is then most probably a Förster resonance transfer between more separated bilins. It is very likely that using instrumentation having a faster time resolution much faster times will also be discovered for cryptomonad biliproteins. The two-photon

Table 4: Tentative Assigments of Particular Bilins to the Bilin Models

biliprotein	polypeptide	bilin[a]	no. of conjugated double bonds	assignment[b]
phycocyanin 645	α	mesobiliverdin	9	coupled within monomer (α*β*)
	β	phycocyanobilin	8	coupled within monomer
		phycocyanobilin	8	not coupled
		15,16-dihydrobiliverdin	7	coupled between monomers
phycocyanin 612	α	phycocyanobilin	8	coupled within monomer
	β	phycocyanobilin	8	coupled within monomer
		phycocyanobilin	8	not coupled
		15,16-dihydrobiliverdin	7	not coupled
phycoerythrin 545	α	15,16-dihydrobiliverdin	7	coupled within monomer
	β	phycoerythrobilin	6	coupled within monomer
		phycoerythrobilin	6	not coupled
		phycoerythrobilin	6	coupled between monomers

[a] From Wedemayer et al. (*14*). [b] Tentative assigments of bilins assuming the *β*-polypeptide of the cryptomonad biliproteins resemble the three-dimensional structure the *β*-polypeptide of B-phycoerythrin. For phycocyanin 645, the mesobiliverdin and one of the phycocyanobilins are coupled within monomers, and two 15,16-dihydrobiliverdins are coupled between monomers.

method applied to dimeric phycocianin 645 and phycoerythrin 545 yielded decays of 0.55 and 2.4 ps, respectively (*7*).

Bilin Models. Monomers of phycocyanins 612 and 645 and phycoerythrin 545 are proposed to have identical bilin organizations with one coupled pair of bilins and two more isolated bilins. Dimers differ, and for phycocyanin 645 and phycoerythrin 545, a second coupled pair of bilins is proposed across the monomer−monomer interface; however, for phycocyanin 612 dimers, a second bilin pair is not detected in these experiments.

For phycoerythrin 545, a possible bilin model is proposed (Table 4). The existence of a pair of bilins across the monomer−monomer interface is now better supported. It is contained underneath the CD envelope of the proposed bilin pair within the monomeric protein as these two types of pairs have similar properties (*7*).

For phycocyanin 645, a bilin model was previously suggested (*5*, *6*). The evidence for a significantly modified proposal is now much stronger (Figure 7 and Table 4). A pair of coupled bilins across the monomer−monomer interface produces the bands on the high-energy side of the spectrum, and this is where 15,16-dihydrobiliverdins absorb (Table 4). They are found on the *β*-polypeptide of the protein. Wedemayer et al. (*14*) presented the structures of various cryptomonad bilins (Table 4).

For phycocyanin 612, the *β* polypeptide has the same bilins as phycocyanin 645. If the 15,16-dihydrobiliverdins were coupled, a negative CD band on the blue edge of the spectrum would be expected, and none is observed (Figure 1). The proposed bilin model for protein dimer is, therefore, unlike that for the other two cryptomonad biliproteins and shows one pair of bilins within each monomeric unit of the dimer and two uncoupled bilins within these monomeric units (Figure 7). The 15,16-dihydrobiliverdins could transfer energy by the Förster resonance method in the weak coupling limit to the isolated phycocyanobilin, and energy from this bilin could similarly be transferred to the bilin pair. This proposed sequence is supported by earlier studies (*27*, *33*).

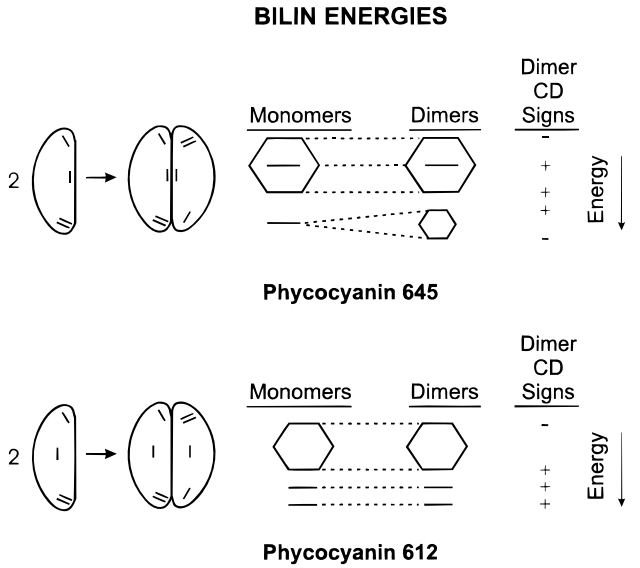

FIGURE 7: Models of bilins on monomers and dimers of phycocyanins 645 and 612 and energy states of the bilins. The energy levels connected by brackets are suggested to be exciton coupled. The arrow points to higher energies. On the left, the protein monomers and dimers are shown with the bilins drawn as lines. Monomers of the two proteins have identical bilin arrangements. Dimers of the two proteins differ, and only phycocyanin 645 apparently has pairing of bilins across the monomer−monomer interface. Phycoerythrin 545 dimers have analogus bilins pairing organization as dimers of phycocyanin 645, but the energy levels of the bilins for phycoerythrin 545 overlap much more extensively.

The role of the 15,16-dihydrobiliverdin is to extend light absorption to higher energies and transfer the energy toward the lowest-energy absorption band. Exciton splitting for this bilin would further extend the wavelength range of absorption and allow for better spectral overlap with the next bilin in the migration sequence. Phycocyanin 612 lacks these two benefits.

For several other noncryptomonad biliproteins, X-ray crystallography has provided important information concern-

ing the three-dimensional positioning of the bilins (*28*, *29*, *41*−*43*). No such data exist for any cryptomonad biliprotein, although there have been preparations of crystals (*44*, *45*). There are several studies on the primary structure of these proteins (*32*, *46*−*49*). Sidler et al. (*46*) have shown that for phycocyanin 645 the two β-polypeptide phycocyanobilins are attached to cysteines at positions 82 and 158 and the 15,16-dihydrobiliverdin is doubly attached at positions 50 and 61. Mesobiliverdin is situated at position 18 for both the α- and α'-polypeptides. This bilin is the lowest-energy bilin discovered thus far (Table 4).

Godovac-Zimmermann et al. (*49*) determined the sequence of the β-polypeptide of a cryptomonad phycoerythrin and found that the cysteines expected at positions 50 and 61 were replaced by valine and glutamate, respectively. They noted that this results in the absence of the doubly linked bilin. For the phycoerythrin 545 used in this study, spectroscopic measurement showed there were three phycoerythrobilins on the β-polypeptide (*37*).

Sidler et al. (*46*) developed the remarkable fact that there is strong sequence homology between the β-polypeptide of phycocyanin 645 and the β-polypeptides of red algal and cyanobacterial phycoerythrins. The X-ray structure of red algal B-phycoerythrin is known (*41*), and shows no sign of β-bilin pairing. The α-polypeptides appear to be unique (*9*, *46*) for cryptomonad biliproteins, and their phylogenetic origin is unresolved. If the three-dimensional structure for the β-polypeptide of the cryptomonad biliproteins resembles the β-polypeptide of B-phycoerythrin, then the proposed coupled pair of bilins within cryptomonad monomers ($\alpha\beta$) would have to be between a bilin on the β-polypeptide and the bilin on the α-polypeptide. In general, there are three possibilities for the two bilins forming the intramonomer pair: (*1*) two bilins on the β-polypeptide, (*2*) the β82 bilin and the bilin on the α-polypeptide, and (*3*) the β158 bilin and the bilin on the α-polypeptide.

For phycocyanin 645, the highest-energy bilin on one monomer is presumed to be coupled across the monomer−monomer interface with the same bilin on the other monomeric unit of the protein dimer (Figure 7). The absorption maximum of a bilin will be determined by multiple factors, but foremost is the number of conjugated double bonds in its structure (Table 4); the fewer such bonds, the higher the energy. The 15,16-dihydrobiliverdin is, therefore, the bilin coupled across the adjacent monomers, and this bilin is doubly linked at β50 and β61. One of the remaining two phycocyanobilins may be isolated, and the second could be coupled within the protein monomer to the mesobiliverdin (Table 4). For phycoerythrin 545 and phycocyanin 612, the process of assignment is analogous (Table 4). For phycocyanin 645 and phycoerythrin 545, the proposed coupling of particular bilins replaces earlier models (*36*, *37*). These speculations await the determination of the three-dimensional structures by X-ray diffraction of appropriate cryptomonad biliprotein crystals.

For phycoerythrin 566 and phycocyanin 630, much more work should be employed to produce evidence for models of bilin topography. Nonetheless, from their dimer CD spectra alone, it is possible to speculate that phycocyanin 630 may resemble the bilin model of phycocyanin 612 (Figure 7), and phycoerythrin 566 could resemble the model for phycocyanin 645 (Figure 7).

The uniquely complex CD spectrum of dimers of phycocyanin 645 has afforded the best opportunity for the current progress. The resulting models suggest that the coupled pair of bilins within the monomers should be the lowest-energy chromophores, and they should be the vehicles for energy migration from one dimeric protein to the next pigment in the directed flow of energy.

Energy transfer from the uncoupled bilins to the lowest-energy coupled bilins will proceed by the Förster resonance mechanism in the weak coupling limit (*50*). Chromophores tend to retain their spectra during this energy transfer process. Likewise, energy transfer out of the high-energy coupled pair of 15,16-dihydrobiliverdins to the lower-energy coupled pair of phycocyanobilins will be by the Förster resonance mechanism. Within the proposed coupled pairs, the movement of energy will be by internal conversion from the high- to the low-energy states of the exciton split bands.

CONCLUSION

The development of models for the bilin topography of these three cryptomonad biliproteins was primarily based on a careful examination of the CD spectra of dimers versus monomers for the three proteins. The monomers and dimers of phycocyanin 612 have qualitatively the same CD, and therefore, exciton splitting across the monomer−monomer interface is not possible. Dimers of phycocyanin 645 clearly show two negative CD bands, and one of these negatives disappears upon monomer formation. For this biliprotein, a pair of coupled bilins is suggested across the monomer−monomer interface. For dimers of phycoerythrin 545, difference CD spectra reveal the analogous bilin pairing as found for phycocyanin 645. The monomer of all three biliproteins exhibits CD spectra that suggest that a pair of bilins exists within the monomeric units as well as two unpaired bilins. All the monomer CD spectra possessed a single negative band, and the bilin organization in monomers of these three cryptomonad biliproteins is perhaps identical (Figure 7).

The suggested role of a coupled pair of bilins within the monomeric units is special and five-fold. (1) The two bands of the intramonomer coupled pair present an excellent target for receiving energy from the other bilins within the dimer because of their wide energy range of absorption. (2) Once within the coupled pair, energy will move very rapidly from its high- to low-energy states. (3) From the low-energy state of the pair, the energy will move to the next chromoprotein in the energy migration chain. (4) The higher-energy exciton band may be situated so that back transfer by a Förster resonance mechanism to other bilins is significant; very rapid transfer to the low-energy exciton state would greatly reduce the chances of this possibility. (5) Like all bilins, the pair harvests photons. This coupled pair of bilins, thus, could provide key functioning of rapid and directional energy migration for these proteins.

The remaining bilins are either isolated or paired across the monomer−monomer interface. Their function is to harvest energy and transfer the energy to the coupled pair within the monomeric units. A wide range of light absorption wavelengths is particularly important to organisms living in underwater habitats where they have restricted access both to light intensity and to the full spectrum of light.

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