Phycoerythrin 545: Monomers, Energy Migration, Bilin Topography, and Monomer/Dimer Equilibrium†

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ABSTRACT: Phycoerythrin 545 was isolated having an $\alpha_2\beta_2$ (dimer) protein structure at pH 6.0 and 2 g/L protein concentration with eight bilin chromophores. Monomers ($\alpha\beta$) were produced by lowering the protein concentration to 0.15 g/L and the pH to 4.5. Dimer dissociation was monitored by dynamic light scattering and gel-filtration column chromatography. Monomers were stable and had bilin optical spectra different from the $\alpha_2\beta_2$ dimers, although they have very similar protein secondary structures. The optical spectra of phycoerythrin 545 showed four types of behavior with temperature: 10−20 °C, dimers; 40−50 °C, dimers/monomers; 60 °C, nearly fully disordered; 70 °C, disordered α and β polypeptides. At 40 °C, the protein dissociated partially to monomer, which could be totally reversed to dimers at 20−25 °C. The visible circular dichroism difference spectrum for the protein dimers minus monomers exhibited positive and negative bands—such spectra may indicate exciton splitting between closely-spaced bilins. Circular dichroism also revealed a spectrum suggesting exciton coupling for the second excited state of the bilins. Ultrafast fluorescence using a two-photon method showed the fastest time for protein dimers to be 2.4 ps and monomers had a 39-ps lifetime. Phycocyanin 645 was found to have a 550-fs lifetime.

Light-harvesting pigments function by absorbing solar energy and having the energy migrate efficiently through the light-harvesting apparatus to a photosynthetic reaction center. Biliproteins are light-harvesting pigments found in cyanobacteria, red algae, and cryptomonads, and are normally coupled to photosystem II. The efficient operation of the light-harvesting systems depends on the optical properties and relative positioning of the chromophores. For biliproteins, the chromophores are bilins, open-chain tetrapyrroles, which are covalently attached to the apoproteins. The apoprotein maintains the bilins in a linear conformation (*1*−*8*).

Phycoerythrin 545 is a cryptomonad biliprotein, and several of its properties have been examined in this study. This biliprotein has an $\alpha_2\beta_2$ protein structure that has been shown to be stable from 2.0 to 10.8 g/L at pH 6.0 (*9*). Another cryptomonad biliprotein, phycocyanin 645, has been studied by optical spectroscopy (*10*, *11*). The circular dichroism (CD) spectrum of dimers of phycocyanin 645 exhibits two negative bands, one on the blue edge and the other on the red edge. These two features provided clues for development of a model for bilin topography. Such a model for a cryptomonad phycoerythrin would be of interest. Phycoerythrin 545 lacks the blue-edge negative band, and new approaches were necessary. Temperature−change experiments also revealed salient differences between the behaviors of the two biliproteins. Previously, this laboratory had studied the optical spectra of phycoerythrin 545 including the properties of its separated α and β polypeptides (*12*) and the effects of ferric ion on the protein (*13*).

In this study, protein monomers ($\alpha\beta$) were produced for the cryptomonad phycoerythrin. Dynamic light scattering and gel-filtration column chromatography were used to monitor molecular mass changes. Temperature was used to study phycoerythrin 545 using its bilins as reporter groups. A detailed study of the protein at 40 °C was carried out. A study was made concerning the properties of the second excited state for the bilins of this cryptomonad biliprotein. Two-photon ultrafast fluorescence was used to determine the energy-transfer times among the chromophores of both phycoerythrin 545 and phycocyanin 645. These results are used to discuss a possible model for bilin topography. Some analysis is made of the bilin-binding sites.

EXPERIMENTAL SECTION

Phycoerythrin 545 was extracted from laboratory harvests of *Rhodomonas lens* by two cycles of freeze−thawing. The protein was purified by ammonium sulfate fractionation and two types of gel-filtration column chromatography (*10*). Purified protein had an A_{545}/A_{280} greater than 6.8 and a single band on gel-filtration column chromatography. The protein was dialyzed into water, lyophilized, and stored in a freezer.

Absorption, circular dichroism (CD), fluorescence, and gel-filtration column chromatography were performed as described previously (*10*). Measurements were usually carried

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out at 0.15 and 2.5 g/L protein, except for fluorescence (0.015 g/L). Dimers were in pH 6.0, 0.1 ionic strength, sodium phosphate buffer. For temperature experiments, samples were kept at temperature for 10 min before spectra were taken. Visible and near-UV CD spectra were measured with a 0.5-cm light path, and CD spectra in the ultraviolet (UV) from 180 to 260 nm were done using a 0.01-cm light path for 0.15 g/L solutions. For 2.5 g/L protein solutions, a 0.001-cm light path was used for CD from 180 to 260 nm and a 0.02-cm light path from 260 to 700 nm. Protein secondary structure was obtained from CD spectra using the program Selcon (*14*). The visible CD was calibrated using the absorption maximum of neodymium glass at 586 nm. The fluorescence emission spectra were obtained using 5-nm slits and exciting the bilins at 500 nm. Absorption measurements were carried out in a 1-cm light path at 0.15 g/L and a 0.01-cm light path at 2.5 g/L.

In the higher-temperature experiments, a Shodex KW-803 column was used, and for other studies a 300SW Protein Pak column was selected for gel-filtration experiments. In the temperature studies, both the column and autosampler were maintained at 38−45 °C. The lower temperature experiments were at 25 °C. Samples were kept at each temperature for at least 10 min before injection onto the column.

The gel-filtration experiments were performed by running phycoerythrin 545 at 25 °C first in a series of three or more injections followed on the same day by a series of three or more injections at 38, 40, or 45 °C. Pairs of temperatures 25 and 38, 25 and 40, or 25 and 45 °C were run on the same day to have the chromatographic column in as much the same condition as possible for the comparison between 25 °C and elevated temperatures.

Dynamic light scattering measurements were made using a DP-801 instrument from Protein Solutions. The samples were illuminated at 780 nm using a solid-state laser. The dynamic light scattering, or quasi-elastic light scattering, experiment was used to obtain the diffusion coefficient (D). The hydrodynamic radius (R_H) can be calculated from the Stokes−Einstein equation (eq 1)

$$R_H = \frac{6\pi\eta D}{kT} \quad (1)$$

where η, k, and T are viscosity, the Boltzmann contant, and absolute temperture, respectively. The frictional coefficient (f) was obtained from eq 2 (the Einstein−Sutherland equation):

$$D = kT/f \quad (2)$$

The diffusion coefficient was corrected to the value at 20 °C and water (eq 3), where η_T was 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 temperture as the buffer:

$$D_{20,w} = D\frac{293}{T}(\eta_T/\eta_{20})(\eta_B/\eta_w) \quad (3)$$

In addition, molecular masses can be obtained by this method.

Ultrafast fluorescence was performed at the Center for Fluorescence Studies in Baltimore by methods described previously (*15*−*20*). A mode-locked Ti:sapphire laser was used for the two-photon experiments. The laser provided 80-fs pulses (measured by an autocorrelator) with 10-nm spectral distribution at 80-MHz pulse repetition. Excitation was at 870 nm for phycoerythrin 545 and phycocyanin 645. The solutions had 0.2 absorbance at the maxima and were in pH 6.0 or 4.5 buffer. The filters used were 600-nm interference and 720-nm short-wavelength glass. The choice of excitation at 870 nm avoided unwanted one-photon excitation. The phase angle and modulation of the emission were related to the intensity decay parameters, α and τ_i, and modulation frequency by equations described previously (*20*), where α_i are pre-exponential factors and τ_i are the decay times in the intensity decay.

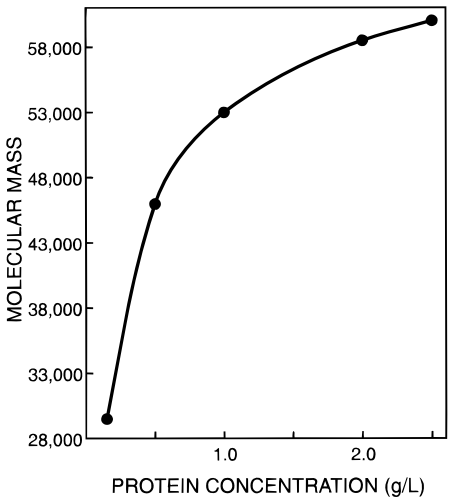

FIGURE 1: Molecular mass versus protein concentration for phycoerythrin 545 at pH 4.5. Dynamic light scattering data were 0.5−2.5 g/L, and gel-filtration column chromatography was 0.15 g/L.

RESULTS AND DISCUSSION

Dimers and Dimer Dissociation. At pH 6.0 and 2.5 g/L, dynamic light scattering gave a molecular mass for phycoerythrin 545 of 58 400 ± 3400 Da. The dimer should be in the range of 55 000−60 000 Da (*4*). In order to investigate the organization of the bilins, dissociation of the protein dimers was of interest. Many biliproteins tend to dissociate at slightly acidic pH values (*4*). Dynamic light scattering was employed at pH 4.5 between 0.5 and 2.5 g/L (Figure 1). At 1.0 and 0.5 g/L, the dimers began to partially dissociate. Measurements below 0.5 g/L for these samples were not possible with our instrumentation. More complete dissociation was monitored at 0.15 g/L by gel-filtration column chromatography at pH 4.5 (see below).

The diffusion coefficient (D) for dimers at pH 6.0 and 2.5 g/L was 6.69 ± 0.15 × 10^{-7} cm^2 s^{-1}. The diffusion coefficient at 20 °C in water (eq 3) was 6.22 × 10^{-7} cm^2 s^{-1}. A frictional coefficient (f) was calculated (eq 2) to be 6.50 × 10^{-8} g cm^{-1} s^{-1}. A frictional coefficient for a sphere, f_o, was also determined for a 59 000-Da protein having a partial specific volume of 0.73 to be 4.9 × 10^{-8} g cm^{-1} s^{-1} (eq 4). The value of m is molecular mass/Avodagro's number.

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

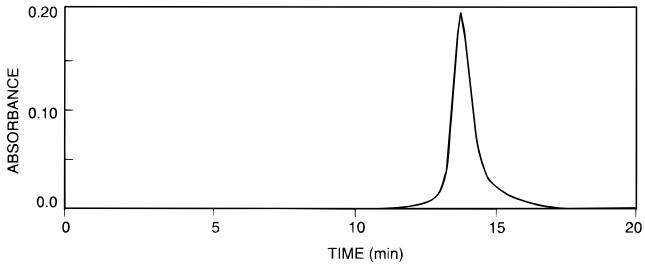

FIGURE 2: Gel-filtration column chromatography of phycoerythrin 545 at pH 4.5. Elution was monitored at 545 nm.

Table 1: Dynamic Light Scattering Studies on Phycoerythrin 545

	results	
properties	2.5 g/L, pH 6.0	2.5 g/L, pH 4.5
molecular mass (kDa)	58.4	60.3
$D_{20,w}$ (cm^2 s^{-1})	6.22×10^{-7}	6.18×10^{-7}
R_H (nm)	3.47	3.52
f (g cm^{-1} s^{-1})	6.50×10^{-8}	6.54×10^{-8}
f/f_o	1.33	1.34

The f/f_o was calculated to be 1.33 for dimers. This ratio suggests that dimeric phycoerythrin 545 is a nonspherical molecule (*21*). This general shape is of interest because neither X-ray crystallographic nor electron microscopy results are available for any cryptomonad biliprotein. The hydrodynamic radius was calculated from the Stokes−Einstein equation (eq 1) to be 3.47 nm for dimers. Results were also obtained for a pH 4.5 solution (Table 1), and the protein behaved very similarly at both pH values. The protein was still predominately dimer at pH 4.3 as dynamic light scattering of a 2.5 g/L phycoerythrin 545 solution yielded a molecular mass of 54 700 ± 4400 Da.

The dynamic light scattering results should yield a highly reliable diffusion coefficient. The sedimentation coefficient (s) of phycoerythrin 545 dimers is already known, $s_{20,w} = 4.3 \times 10^{-13}$ s (*9*). The Svedberg equation (eq 5) will allow a check of the molecular mass (M) from the diffusion and sedimentation coefficients:

$$M = \frac{sRT}{D(1 - \nu\rho)} \qquad (5)$$

R is the ideal gas contant, 8.31×10^7 erg mol^{-1} deg^{-1}, and ρ is the solution density. For phycoerythrin 545, the molecular mass is calculated to be 63 000 Da. This molecular mass is close to the expected dimer value and close to the dynamic light scattering finding (Table 1), and this molecular mass confirmed that phycoerythrin 545 is a homogenous dimer at pH 6.0 and 2.5 g/L.

Monomers. Phycoerythrin 545 is purified at pH 6.0 as an $\alpha_2\beta_2$ protein dimer (see above), in which there are more than one type of α polypeptide but all α's have the identical chromophore (*4*). There are three phycoerythrobilins on the β polypeptide and one cryptobilin 562 on the α (*22*−*24*, *12*). At pH 4.5, 0.1 M sodium acetate, gel-filtration column chromatography (Figure 2) on a 0.15 g/L protein solution showed a molecular mass of about 29 000 Da, which is one-half that of the dimer, indicating monomer ($\alpha\beta$) formation. The retention times were constant for at least 4 h after preparation of the pH 4.5 samples.

Table 2: Secondary Structure Estimates for Phycoerythrin 545

		percentages			
protein	conditions	α helix	β sheet	β turn	other
dimer[a]	0.15 g/L, 20 °C, pH 6.0	49.8	11.9	21.6	17.6
monomer	0.15 g/L, 20 °C, pH 4.5	45.1	12.5	23.1	19.7
dimer/ monomer	0.15 g/L, 40 °C, pH 6.0	45.4	11.8	21.1	20.1
dimer	2.5 g/L, 20 °C, pH 6.0	56.0	7.8	20.0	17.3
dimer/ monomer[b]	2.5 g/L, 40 °C, pH 6.0	56.8	8.0	21.1	16.3

[a] At 0.15 g/L, 20 °C, and pH 6.0, the results suggest that some dimers may have dissociated to monomers. Monomers have slightly less α helix than dimers. [b] At pH 6.0 and 2.5 g/L, the dissociation at 40 °C to monomers is very small. This suggestion was confirmed by the gel-filtration column chromatography results, which showed a smaller change in retention time at 40 °C for the 2.5 g/L solution compared with the 0.15 g/L solution of phycoerythrin 545.

The visible absorption from the lowest-energy bilins and the bilin CD spectrum of protein monomers differed from those of the protein dimers, both at 0.15 g/L (Figure 3). A difference CD in the visible region showed a pH 6.0 minus pH 4.5 spectrum with extrema at 564 (−) and 538 (+) nm (Figure 3C).

The CD in the UV starting at 260 nm was nearly the same for both monomers and dimers (Figure 4A), and the secondary structures were reasonably similar. The monomer CD spectra were cutoff at about 190 nm because of the acetate absorption. Monomers have 45.1 ± 2.7% α helix, 12.5 ± 0.5% β sheet, 23.1 ± 2.6% β turn, and 19.7 ± 0.5% other, and dimers are 49.8 ± 3.4% α helix, 11.9 ± 1.6% β sheet, 21.6 ± 1.1% β turn, and 17.6 ± 0.7% other. The agreement between monomers and dimers in protein secondary structures suggested that these states were similar, but monomers might have a slightly smaller α-helix content (Table 2). It is not known if the bilins make a contribution to the CD at 180−260 nm, but the important observation is that dimers and monomers have similar secondary structures. The lack of similarity in the bilin spectra was shown by a CD difference spectrum (Figure 3C) and a shift in fluores-

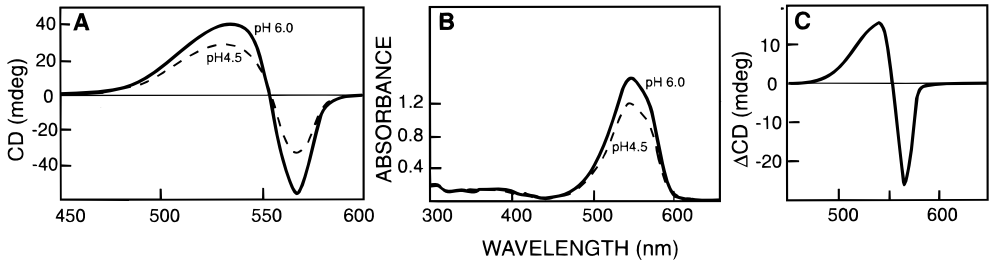

FIGURE 3: CD (A) and absorption (B) bilin spectra of phycoerythrin 545 at pH 6.0 and 4.5. A difference CD spectrum (C) is given for the pH 6.0 spectrum minus the pH 4.5 spectrum. All protein solutions were 0.15 g/L. Solutions were at 20 °C.

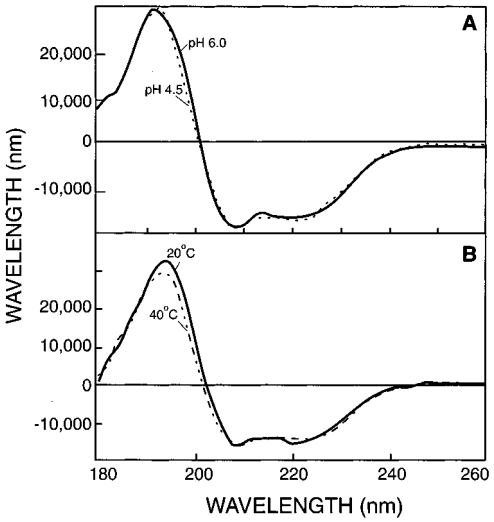

FIGURE 4: CD in the UV for phycoerythrin 545 at 20 °C in pH 6.0 and 4.5 (A) and in pH 6.0 at 20 and 40 °C (B). Protein concentration was 0.15 g/L. Scans were average for several runs and were noise-reduced. Baseline of buffer was subtracted from solution CD. The spectra at 2.5 g/L were nearly identical at 20 and 40 °C for phycoerythrin 545.

cence emission maxima (data not shown) from 584−585 nm (dimers) to 581 nm (monomers).

Since there are at least two different α polypeptides in phycoerythrin 545 (*9*), there will be a mixed population of $\alpha\beta$ and $\alpha'\beta$ monomers. Estimates of 9400 and 10 500 Da was made for two α polypeptides.

Temperature. The CD and absorption spectra were monitored from 10 to 80 °C (Figure 5) using the bilins of the protein as naturally occurring reporter groups. Four different types of results were noted as follows: 10−20 °C, dimers; 40−50 °C, a different state—one much closer to native than to disordered; 60 °C, a CD spectrum close to fully disordered; 70 °C, fully disordered. The 30 °C spectrum was very close to that at 20 °C but showed a small amount of the 40−50 °C change.

Gel-filtration column chromatography was carried out at 38−45 °C by placing a steel column in a column heater (data not shown). Retention times were measured at 25, 38, 40, and 45 °C. The 38−45 °C retention times were slightly slower than the 25 °C ones. The results demonstrated that dimers were somewhat dissociated at higher temperatures and were reduced in molecular mass by roughly 6000 Da. The changes in retention times at higher temperature were much less than expected for a complete conversion to monomers.

The retention times were 10.427 ± 0.0217 min at 25 °C and 10.644 ± 0.031 min at 40 °C, for a difference of 0.217 min; for 25 and 38 °C, the retention time difference was 0.225 min; and for 25 and 45 °C, the difference was 0.252 min for 0.15 g/L protein. When a sample, which had been maintained at 40 °C for 1 h, was returned to 25 °C, there was a total return to the original retention time that was obtained prior to bringing the sample to 40 °C. These results differed sharply from those obtained for phycocyanin 645 where no dissociation was detected at these temperatures (*11*). The two proteins, therefore, required quite different interpretations for these temperature−change experiments. Gel-filtration column chromatography was also carried out on phycoerythrin 545 in pH 6.0 buffer at 2.5 g/L. The results of heating the samples to 40 °C and returning to 25 °C were analogous to the results at lower protein concentration. The 40 °C samples showed dissociation to some monomers, which was totally reversed on cooling. There was a major difference in quantitation, however. The results at 40 °C and 2.5 g/L showed a change in retention time of only about 0.087 min compared with a change of 0.217 min at 0.15 g/L. Therefore, as could be predicted, much less dissociation to monomer occurred at the higher protein concentration.

When samples at 40 °C were returned to 20 °C, the absorption and CD spectra reversed completely to the original 20 °C results and the fluorescence showed substantial recuperation (Figure 6). For the absorption spectra, this process of 20 to 40 to 20 °C was repeated a few times for

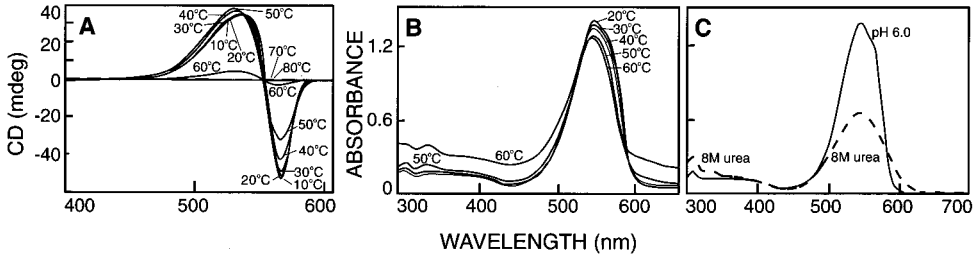

FIGURE 5: CD (A) and absorption (B) visible bilin spectra as a function of temperature for phycoerythrin 545. Solutions were at 0.15 g/L protein in pH 6.0 buffers. Samples were at each temperature for 10 min before spectra were taken. In panel C, the absorption spectra of the phycoerythrin 545 in pH 6.0, buffer and 8.0 M urea, pH 6.0 are shown.

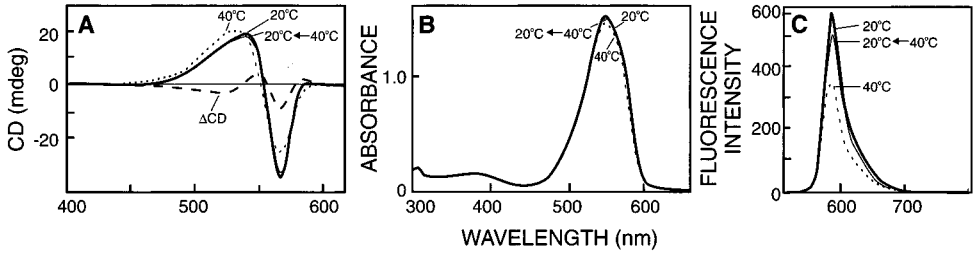

FIGURE 6: Reversibility of 40 °C bilin changes for phycoerythrin 545 using CD (A), absorption (B), and fluorescence (C). Protein concentration was 0.15 g/L for CD and absorption and 0.015 g/L for fluorescence. Each sample was maintained at 40 °C for 10 min before changing each back to 20 °C. Difference spectrum of 20 minus 40 °C is labeled ΔCD. Analogous results were obtained at 2.5 g/L for CD and absorption.

the same sample with identical results. Similar results were obtained at 0.15 and 2.5 g/L. These two concentrations have particular importance because at 0.15 g/L and pH 4.5 monomers were produced and at 2.5 g/L and pH 6.0 homogenous dimers were verified by dynamic light scattering (Table 1) and previous data (*9*). The 40 °C bilin absorption spectrum was stable over at least 2 h. The fluorescence maximum at 20 °C was 584−585 nm, and at 40 °C there was a blue shift to 581 nm. This shift indicated that at 40 °C the bilins that were fluorescing were directly changed. Biliproteins have lowest-energy bilins, which fluoresce, and higher-energy bilins, which transfer energy to the fluorescing bilins (*4*).

Studies at 50 °C yielded results for visible absorption, CD, and fluorescence emission identical to the findings at 40 °C (data not shown). The changes brought about at 50 °C were essentially completely reversed when the samples were returned to 20 °C. The fluorescence emission at 50 °C blue-shifted to 580 nm and returned to its expected maximum at 20 °C.

CD spectra in the UV were obtained from 180 to 260 nm for phycoerythrin 545 at 20 and 40 °C (Figure 4B). The spectra were virtually identical, and the secondary structures are therefore nearly identical at both temperatures (Table 2). At 40 °C, the secondary structure was 45.4% α helix, 11.8% β sheet, 21.1% β turn, and 20.1% other for the 0.15 g/L solutions. The secondary structure at 2.5 g/L, 20 °C, and pH 6.0 should be considered the best estimate of the conformation of the dimer since physical measurements verify it possesses a homogenous dimeric structure under these conditions (Table 2).

For C-phycocyanin, the phycocyanobilins have been studied under ordered and disordered conditions for the protein by Scheer and Kufer (*25*). It was found from theoretical calculation and model compounds that when the protein was disordered the phycocyanobilins showed a decrease in visible and an increase in near-UV absorbance. This change signals that the bilins are linear in the ordered protein and more cyclic in the disordered protein. For phycoerythrin 545, fully disordered protein was obtained in 8.0 M urea (Figure 5C). The visible absorbance was hypochromic in the disordered state, but the near-UV changed only slightly. There was no increase at 380 nm, but an increase was observed in the small band at 310 nm. Therefore, changes in bilin conformation in phycoerythrin 545 will be observed by decreased visible absorption and slightly increased A_{310}. The studies of temperature on phycoerythrin 545 demonstrated that the bilins had similar conformations from 10 to 40 °C (Figure 5). Therefore, the bilin conformation and protein secondary structure (Figure 4B) did not influence the changes in optical spectra observed at 40 °C. At 2.5 g/L protein, analogous absorption changes were observed at 20 and 40 °C (data not shown).

Difference spectra for 20 minus 40 °C spectra were obtained for the CD (Figure 6A) and absorption (data not shown) results. The results for absorption show a major difference band at 570 nm and shoulder at 544 nm, and the CD show difference extrema at 522 (−), 547 (+), 565 (−), and 582 (+) nm. The 40 °C CD spectrum had extrema at 564 (−) and 531 (+) nm, and the 20 °C spectrum had extrema at 564 (−) and 538 (+) nm.

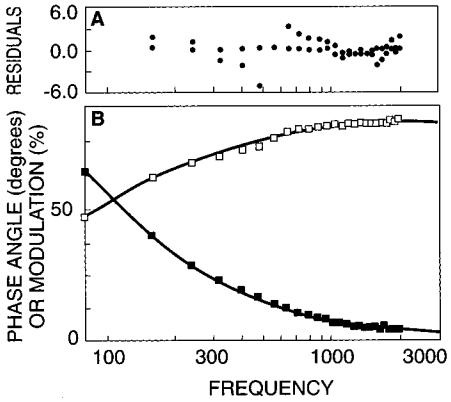

FIGURE 7: Frequency-domain fluorescence intensity decay measurements of phycoerythrin 545 for two-photon excitation at 870 nm.

Table 3: Ultrafast Fluorescence Studies, by a Two-Photon Method on Phycoerythrin 545 and Phycocyanin 645

protein	lifetimes (ps)
dimers, phycoerythrin 545	2.40, 483, 2505
dimers, phycocyanin 645	0.55, 108, 1468
monomers, phycoerythrin 545	39, 423, 2500

Monomer (pH 4.5) and purified β polypeptide (pH 6.0) did not show the same spectroscopic change as did dimers at 40 °C (data not shown). The β polypeptides were produced as described previously (*19*).

Energy Migration. The topography of the bilins in a biliprotein has the purpose of providing highly efficient, radiationless energy migration from the site of absorption to the next pigment in the photosynthetic chain. Ultrafast time-resolved fluorescence provides a method to measure these transfers.

Two-photon excitation has never before been applied to cryptomonad biliproteins. In this method using a Ti:sapphire laser, the high peak power of the laser pulses can produce a two-photon excitation, in which the bilin simultaneously absorbs two long-wavelength photons to give the excited state. The excitation wavelength was chosen so as not to excite the bilin by a one-photon event. The two-photon excitation spectrum was obtained from 775 to 910 nm. Its shape was similar to the one-photon spectrum (data not shown). The two-photon cross section for phycoerythrin 545 was estimated to be 2×10^{-49} cm^4/photon for 800-nm excitation. This cross section is considered to be high and comparable with the two-photon cross section of fluorescein. For phycoerythrin 545, lifetimes of 2.40 ± 0.77, 483 ± 1.5, and 2505 ± 10.6 ps were obtained (Figure 7) from $I(t) = \sum_i \alpha_i e^{-t/\tau_i}$ (*20*), were the α's are the pre-exponential factors. The fastest time of 2.4 ps may be the energy migration time among the chromophores. The 2505-ps time was the fluorescence decay from the lowest-energy bilins. A rise component was not observed.

For comparison, similar experiments were performed on another cryptomonad biliprotein, phycocyanin 645. The results were 0.55, 108 ± 1.3, and 1464 ± 9.3 ps, for a three-component fit. The lifetime of 550 fs was the fastest yet obtained for any cryptomonad biliprotein.

Phycoerythrin 545 at pH 4.5 was also examined (Table 3). The lifetimes were 39, 423, and 2500 ps. The results suggested that the energy-transfer process resulting in the

fastest time for dimers was affected by monomer formation. The 39-ps lifetime should still be considered very fast, however, and monomers were clearly functioning as energy migration systems.

Bilin Binding Regions. It would be interesting if the amino acid sequences around any of the four cysteines, which have bilins attached, were found in other non-biliprotein light-harvesting pigments. The sequence of the 10 amino acid residues before and after each of these four cysteines was investigated. No matches with data from a sequence bank were found to any non-biliprotein photosynthetic pigments. Only two sequence homologies greater than 50% were found for any protein. For cryptomonad biliproteins, the sequence starting at bilin-bearing cysteine 82 is CLRDAEIILRY. The region is highly conserved for several biliproteins (*26*−*28*). A search found that a protein, human α-fetoprotein (*29*), has a sequence CLEDGEKIMSY, which has 6 matches out of these 11 residues. Another match was found of 5/10 with glycolate oxidase (spinach).

Exciton Coupling. The CD spectrum of a group of chromophores is extremely sensitive to chromophore interaction. In general, the CD of a chromophore may be either positive or negative. However, there is a situation where the signs of chromophore bands are exactly determined. If the chromophores in a pair are close together and properly orientated, they can engage in exciton coupling. The CD of the monomeric chromophores then is split into two bands, one positive and one negative.

It may be a conservative spectrum with the sum of all rotational strengths being zero when calculated on an energy scale (*30*). Some pigments may not give conservative CD spectra for particular bands. An example is the nonconservative CD spectrum of the LH1 complex of bacteriochlorophyll dimers (*31*), while the LH2 complex has a conservative CD spectrum (*32*).

The visible CD difference spectrum between dimers and monomers resembled a spectrum produced by exciton coupling (Figure 3C). The positive difference band is at 538 nm and the negative is at 564 nm. To obtain this difference spectrum, the monomer spectrum was subtracted from the dimer spectrum. If dimers have a pair of bilins that become separated in monomers, the difference spectrum will be that of the exciton pair minus the spectra of the monomer bilins obtained from the separation of the pair. There are two possibilities if a pair of closely spaced bilins were present on dimers but absent on monomers. The pair could be within the monomeric unit but become separated or changed in orientation by a protein conformational change on monomer formation, or the pair could separate with one chromophore remaining on each protein monomer. The measurement of a similar secondary structure for dimers and monomers suggests, but does not prove, that the latter may be more likely. For phycocyanin 645 (*10*, *11*), the blue-edge negative CD band allowed elucidation of a possible bilin pair, and for phycoerythrin 545 this analogous negative band was apparently hidden under the more intense red-edge negative CD band.

In addition, the monomer CD spectrum (Figure 3A) still showed the residual characteristics of a spectrum exhibiting exciton coupling. Further speculation would be that a second distinct pair of bilins, which was not affected by protein monomer formation, may exist. For the difference spectrum between protein at pH 6.0 and 4.5, the extrema were at 538 (+) and 564 (−) nm, and for the CD spectrum at pH 4.5 the extrema were at 528 (+) nm and 565 (−) nm. Please note the caveat that, instead of a chromophore pair, there are other factors that could fortuitously yield a CD spectrum resembling that of a delocalized pair. If the CD was indicative of a delocalized pair in the region from 400 to 700 nm of the first excited state of the bilins, the CD for the second excited state of the bilins from 260 to 400 nm should also be suggestive of exciton coupling. CD in this region showed the combination of positive and negative bands expected for excition splitting (Figure 8). Certain non-cryptomonad biliproteins did not show this band shape in the near-UV, and had entirely negative CD bands from 260 to 400 nm (*33*).

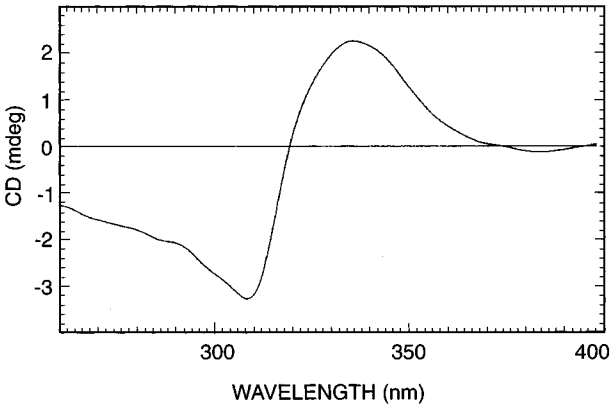

FIGURE 8: CD of phycoerythrin 545 in the near-UV. The protein concentration was 2.5 g/L, the buffer was pH 6.0 sodium phosphate, and the temperature was 20 °C.

The 40 °C state produced a visible CD difference spectrum (Figure 6A) when subtracted from the 20 °C spectrum with extrema at 522 (−), 547 (+), 565 (−), and 582 (+) nm. The CD difference spectrum for the pH 6.0 spectrum minus the pH 4.5 spectrum was simpler having only two extrema at 564 (−) and 538 (+) nm (Figure 3C). It is reasonable to assume that the 547 (+) and 565 (−) difference spectrum for the temperature−change experiment has the same origin as the 538 (+) and 564 (−) nm bands in the pH-change experiment. The agreement between the difference spectrum of dimers (pH 6.0) minus monomers (pH 4.5) and dimers at 20 °C minus dimers/monomers at 40 °C both at pH 6.0 suggests that the difference spectrum is not caused by effects of pH on the bilins. The other transitions observed for this difference spectrum (Figure 6A) when compared to that for a pH 6.0 minus a pH 4.5 solution (Figure 3C) could indicate a conformational change on top of the dissociation.

The 2.4- and 0.55-ps times observed for phycoerythrin 545 and phycocyanin 645, respectively, may be the transfer times for the movement of excitation energy. In addition, possibly there are still faster times yet hidden. If the chromophores on the biliprotein are isolated from each other, then these transfers will occur by a Förster resonance mechanism (*34*). If there are exciton-coupled dimers or larger groups of bilins, then the energy levels of the individual bilins can be split into high- and low-energy exciton states. The measured times may then be relaxations from the high- to low-energy states. A non-cryptomonad biliprotein, allophycocyanin, has been studied by photophysical methods, and these energy-transfer mechanisms have been considered (*35*−*38*). A

major difference between the studies on allophycocyanin and cryptomonad biliproteins is that an X-ray crystal structure is known for allophycocyanin (*39*), but not for any cryptomonad biliprotein. For other photosynthetic light-harvesting complexes, a combination of approaches, including spectroscopy and X-ray crystallography, have produced models for energy migration (*40*−*45*).

Bilin Topography. Without X-ray data, only a speculative model can be put forward for the bilin topography of phycoerythrin 545, which may serve as a paradigm for the study of this topic through optical spectroscopy. Studies on monomers (Figure 3A) and separated β polypeptides (*12*) suggest a coupled pair of bilins could be present within this polypeptide on protein monomers and dimers, and for the protein dimer this would account for four of the bilins. The difference spectra between dimers and monomers (Figures 3C and 6A) and supporting data (Figure 4) suggest that there may be a second coupled pair of bilins across the monomer−monomer interface. Additional experiments will be needed to determine if there are one or two pairs at this interface. If there is only one, then there will also be two uncoupled bilins on the dimer. Fluorescence results (Figure 6C) suggest that the pair of bilins closely spaced on adjacent monomer units could be the lowest-energy fluorescing bilins.

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