Bilin Chromophores as Reporters of Unique Protein Conformations of Phycocyanin 645

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ABSTRACT: At 45 °C, phycocyanin 645 maximally undergoes a reversible and stable conformational change. The change is observed in the visible (chromophore) region of the absorption and circular dichroism (CD) spectra. In the absorption spectrum, the absorbance is lower at 45 °C but remains much closer to the normal spectrum than to a strongly denatured spectrum. In the CD, a similar situation exists except that a negative band on the blue edge of the spectrum is much more strongly affected at 45 °C than the other bands. On returning to 20 °C, all these changes are restored to the original states. The protein is an $\alpha_2\beta_2$ dimer at both 20 and 45 °C, and CD in the far-UV shows the identical protein secondary structures at both 20 and 45 °C. Fluorescence studies show that energy transfer occurs at both temperatures. At 50 °C the results are saliently different as the secondary structure changes and the spectral changes are mostly irreversible. At 50 °C, some monomers ($\alpha\beta$) are produced, and these monomers are very unstable at that temperature, resulting in the formation of some fully denatured polypeptides. Stable monomers can be produced at 20 °C and have visible absorption and CD spectra identical to the dimer at 45 °C. Therefore, the chromophores are reporting a tertiary conformational change at 45 °C, in which the two halves of the dimer each assume a monomer-like conformation prior to dissociating. These results are compared with a hypothesis for the chromophore topography, and the CD change at the blue edge of the spectra may result from the separation at 45 °C of a chromophore pair.

Phycocyanin 645 is a chromoprotein that functions as a light-harvesting pigment in photosystem II. The protein is an $\alpha_2\beta_2$ dimer, which has a total of eight chromophores. This biliprotein is found in the flagellated cryptomonads; other biliproteins occur in cyanobacteria and red algae. A hypothesis on the chromophore topography of phycocyanin 645 suggests that there are two different pairs of chromophores whereas each half of the dimer has one more isolated chromophore (MacColl *et al*., 1994, 1995). The properties of biliproteins have been reviewed (Gantt, 1979; Holzwarth, 1991; MacColl & Guard-Friar, 1987; Scheer, 1981; Troxler, 1986; Zuber, 1987).

Proteins may respond to stimuli by changes in aggregation or in secondary or tertiary structures. Here the first two properties are measured directly and the chromophores are used as reporting groups for any tertiary changes. Absorption, CD, fluorescence spectroscopy, and molecular-weight determinations are employed to study the effects of temperature from 10 to 80 °C on the dimeric protein. A novel conformational state at 45 °C is studied extensively. Some temperature experiments are also carried out on monomers ($\alpha\beta$).

EXPERIMENTAL PROCEDURES

The protein was extracted from *Chroomonas* species, which was grown in the laboratory, and purified by ammonium sulfate precipitation and gel-filtration column chromatography (MacColl & Guard-Friar, 1983). The purified dimer was prepared in pH 6.0, 0.1 ionic strength, sodium phosphate buffer, and monomers were prepared in 0.1 M, sodium acetate, pH 4.0 buffer (MacColl *et al*., 1995).

CD (JASCO J-720 spectropolarimeter), UV/vis absorption (Beckman DU640), fluorescence (Perkin-Elmer Model LS50B), and spectral deconvolutions (Jandel PeakFit software) were performed as described previously (MacColl *et al.*, 1994). Temperature was controlled for the CD experiments by installing a remote thermal sensor near the sample cuvette.

Gel-filtration column chromatography was carried out using a Waters 625 LC system equipped with a Waters 996 photodiode array detector. A Shodex KW-803 column was used. An oven was used to maintain 45 °C or 50 °C in the column. Samples were heated to temperature in a water bath, placed in an autosampler carousel at the highest obtainable temperature of 40 °C, and then injected onto the column at 45 or 50 °C. Ambient temperature experiments were used as controls. The samples were at 40 °C in the carousel for less the 3 min. Controls using CD demonstrated that the short time at 40 °C did not affect the conformational state of the protein significantly.

Spectroscopic reversibility studies were performed by allowing the samples to remain in the spectrophotometer as the temperature was brought down from 45 or 50 °C to 20 °C. The time at 20 °C was recorded for each spectrum. All experiments, except fluorescence, were performed at 0.15 g/L protein, and fluorescence experiments were carried out at 0.015 g/L.

Secondary structure estimations were done on the CD data in the far-UV using Selcon (Sreerama & Woody, 1993). The protein weight was reduced by 11% to account for the chromophores and residual water in the lyophilized preparations. A mean residue molecular weight of 105.4 was used for phycocyanin 645. A 0.2-mm light path was used in the far-UV, and a 10-mm light path was used in the visible for CD measurements.

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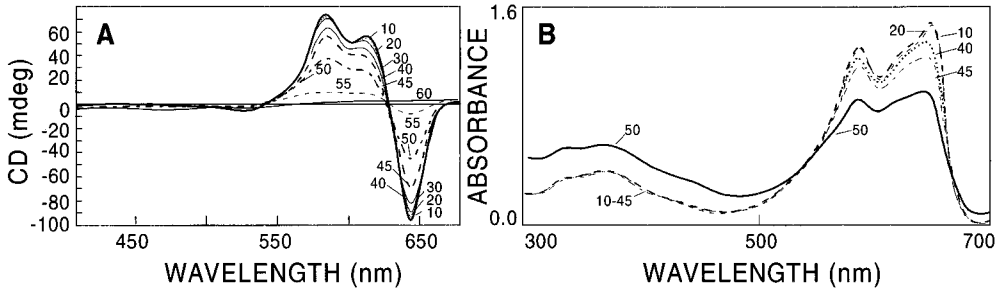

FIGURE 1: Temperature dependence of phycocyanin 645 visible absorption and CD spectra.

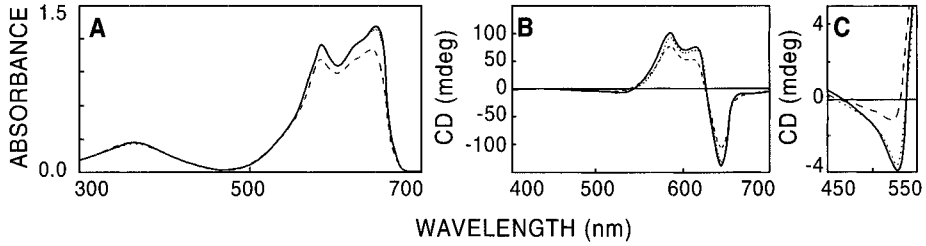

FIGURE 2: Visible CD and absorption spectra of phycocyanin 645 at 20 and 45 °C. Panel A, absorption; panel B, CD; and panel C, an expanded blue-edge region of the CD. Each panel shows the 20 °C spectrum of a sample previously at 45 °C for 10 min: 20 °C (solid line), 45 °C (dash), and 45 °C back to 20 °C (dots).

RESULTS AND DISCUSSION

Effects at 45 °C. When examined from 10 to 80 °C, phycocyanin 645 is mostly unchanged from 10 to 40 °C and completely denatured above 60 °C (Figure 1). This was the first temperature study on the properties of any cryptomonad biliprotein. Between these extremes, there appeared to be an intermediate situation. The visible CD was studied degree by degree from 44 to 50 °C (data not shown). The lowest temperature at which the CD changes at the blue edge of the spectrum were apparently maximal was 45 °C. For the absorption spectra, the 50 °C spectrum has a higher absorbance at 370 nm than the spectra at lower temperatures (Figure 1B). This suggests that at 50 °C the bilins are more cyclic (MacColl & Guard-Friar, 1987).

At 45 °C, the visible absorption spectrum showed less absorbance than at 20 °C (Figure 2A). It takes less than 10 min to obtain a completed effect. The effect was totally reversible, and when the sample returned to 20 °C, the spectrum was indistinguishable from the original (Figure 2A). This process could be repeated with the same sample with exactly the same result. At 45 °C, the visible CD spectrum differed from that at 20 °C (Figure 2B). At the blue edge of the spectrum, the change was considerably more dramatic (Figure 2C). The small negative band was completely lost at 45 °C. These effects were restored when the samples were returned to 20 °C (Figure 2B,C). The protein at 45 °C is stable, and little or no spectral change occurs for hours after the initial change at 10 min.

The protein secondary structure in pH 6.0 buffer was determined at 20 and 45 °C (Figure 3). The spectra were very similar and demonstrated that no significant changes in secondary structure occurred at 45 °C. The predicted secondary structures from several determinations at each temperature were as follows: for 20 °C, 42.6% α helix, 12.8% β sheet, 24.4% β turn, and 20.2% other; and for 45 °C, 43.9% α helix, 12.9% β sheet, 23.4% β turn, and 19.8% other.

Gel-filtration column chromatography showed that the protein at 45 °C eluted at 12.2 min, which was precisely the same elution time as at ambient temperature, about 21 °C (Figure 4). The molecular weight and, therefore, the dimeric structure were retained at 45 °C.

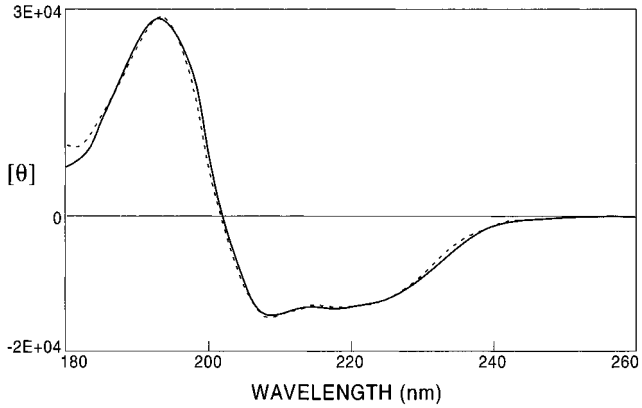

FIGURE 3: CD in far-UV for phycocyanin 645 at 20 °C (dash) and 45 °C (solid).

Fluorescence spectroscopy was performed by exciting the protein at 550 nm and measuring the emission spectrum. The results at both 20 and 45 °C showed that the emission maxima were at 662 nm (data not shown). Since 550 nm excited the highest-energy chromophores and 662-nm emission came from the lowest-energy chromophores, the energy-migration properties of the protein were maintained at 45 °C.

Effects at 50 °C. The effects of 50 °C on the visible absorption and CD spectra of phycocyanin 645 resembled those at 45 °C, but they were not completely reversible (Figure 5). In addition, the increase in absorption at 370 nm (MacColl & Guard-Friar, 1987) at 50 °C demonstrated that the chromophores are becoming more cyclic (Figure 5), and at 45 °C no change at 370 nm shows that the chromophores remained linear (Figure 2). Gel-filtration column chromatography showed that, although some dimers were still present, some dissociation occurred at 50 °C (Figure 4C). The small, slower band is a dissociation product of the dimers. The photodiode array detector of the chromatographic system showed that the small band at 50

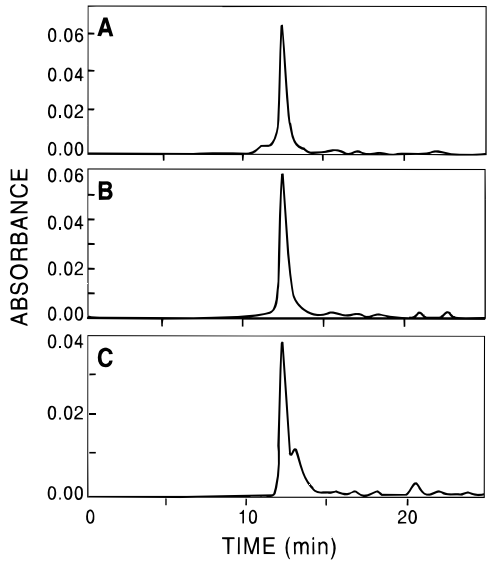

FIGURE 4: Gel-filtration column chromatography of phycocyanin 645 at 20 °C (A), 45 °C (B), and 50 °C (C). The same concentration and volume of a phycocyanin 645 solution were used in each case. In panel C, the second band of lower molecular weight is just to the right of the main band.

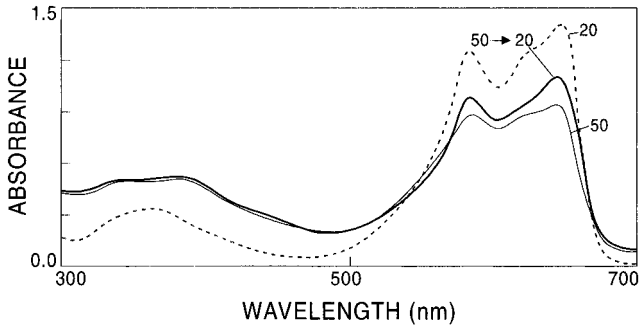

FIGURE 5: Visible absorption spectra of phycocyanin 645 at 50, 20, and 50 °C back to 20 °C.

°C had the visible spectrum of phycocyanin 645 (data not shown). Unlike the results at 45 °C, secondary structure estimations showed a change from those at 20 °C to 36.3% α helix, 17.1% β sheet, 25.3% β turn, and 22.4% other. The gel-filtration results suggested partial dissociation of dimers to monomers at 50 °C, but previous studies (MacColl *et al.*, 1995) demonstrated that at 23 °C dimers and monomers had identical secondary structures. Monomers were, therefore, examined at 45 and 50 °C.

Monomers. Monomers, which are stable, can be produced by bringing the dimers to pH 4.0 at ambient temperature (MacColl *et al.*, 1995). When monomers at brought to either 45 or 50 °C, they are very unstable and are denatured in a fairly short time (Figure 6). This process consists in the dissociation of monomers to α and β polypeptides, unfolding of the polypeptides, and aggregation of these polypeptides. Therefore, any dissociation of dimers to monomers at 50 °C would quickly produce changes in the overall secondary structure and cause the change in the protein state to be irreversible. At 45 °C, monomers do not form and the protein retains full reversibility.

The CD spectrum of monomers at 20 °C was compared with the dimer spectrum at 45 °C (Figure 7). The protein exhibits identical spectra under these two conditions. To better compare the spectrum of the dimer at 45 °C with that of the monomer at 20 °C, deconvolutions of the CD spectra

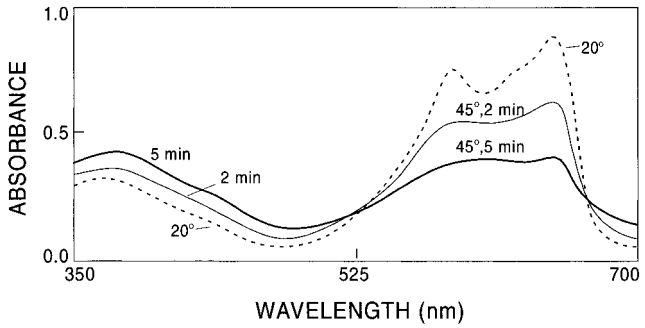

FIGURE 6: Visible absorption spectra with time of phycocyanin 645 monomers at 45 °C. Spectrum at 20 °C shown for comparison.

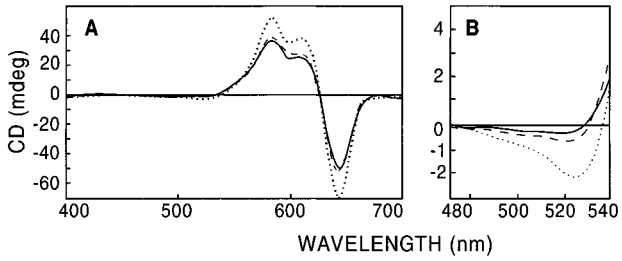

FIGURE 7: The CD spectra of phycocyanin 645 of monomers (20 °C) and dimers (45 °C). Dimers (20 °C) are shown for comparison. The lines are monomers, 20 °C (solid), dimers, 45 °C (dash), and dimers, 20 °C (dots). Panel B shows an expanded view of data from panel A between 480 and 540 nm.

Table 1: Deconvolution of the Visible CD Spectra of Dimers at 45 °C and Monomers at 20 °C

type of component	wavelength maxima (nm)	
	dimers (45 °C)	monomers
Gaussian[a]	643[b] (−)	644 (−)
Gaussian	612 (+)	613 (+)
Gaussian	584[b] (+)	585 (+)
Gaussian	559 (+)	561 (+)

[a] The fitting was done with the spectra in wave numbers (cm^{-1}). The four Gaussian fit had an r^2 of 0.9998. [b] This pair of bands may be produced by exciton splitting (MacColl *et al.*, 1994) from a pair of chromophores situated within an $\alpha\beta$ unit (monomer) of the dimer. The positive band at 559−561 nm formed when the pair of chromophores in the dimer at 20 °C across the monomer−monomer interface are separated either by monomer formation or by a conformational change at 45 °C in the dimer. The band at 612−613 nm is produced by the isolated chromophore on each $\alpha\beta$ unit.

were obtained (Table 1). A set of three components could not fit either spectrum very well (data not shown). Both spectra were best fitted by a set of four Gaussian components, and the wavelength maxima of the four components were very close for monomers at 20 °C and dimers at 45 °C. The protein, therefore, under these two seemingly very different conditions has very similar conformations.

Conclusions. The denaturation of biliproteins is accompanied by a large loss of visible absorption caused by a change in the chromophores from a linear to cyclic conformation. The changes to dimers at 45 °C are much more subtle, and unlike total biliprotein denaturation, are fully reversible. What occurs is the formation of a novel conformational state. This new conformation resembles the other in oligomeric and secondary structure of the protein. The chromophores, however, must be in different environments. This spectroscopic change could be produced by altering the interaction of chromophores with amino acids, solvation of chromophores, or chromophore−chromophore interaction.

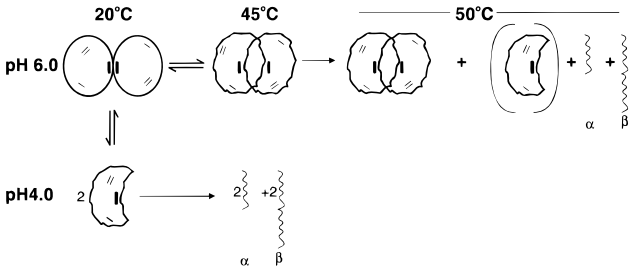

FIGURE 8: Summary of the effects of temperature and pH on phycocyanin 645. Chromophores are represented by lines in the protein. Note that, at 45 °C, a pair of chromophores is shown to separate, and these two chromophores are drawn more heavily for easy viewing.

The change at the blue edge of the spectrum may be produced differently and is discussed later. At 45 °C, the conformations of the chromophores in the dimer are similar to those at 20 °C since the characteristic increase in the 370-nm band for a linear to cyclic change (MacColl & Guard-Friar, 1987) is not observed (Figure 2A). The identity between the absorption and CD spectrum of monomers at 20 °C and those of dimers at 45 °C (Figure 7) suggests similar events have occurred, and that the dimers at 45 °C have each half ($\alpha\beta$) of the protein in a situation strongly resembling that found in monomers (Figure 8). The conformations of the chromophores in monomers and both types of dimer are the same.

Monomers are unstable at 45 °C (Figure 8), and this shows that the dimeric structure lends significant thermal stabilization to the protein. One obvious difference between monomers and dimers is the replacement of water in the monomers for protein−protein contacts. The partial reversibility that occurs when the protein is brought back to 20 °C from 50 °C is probably produced from the dimers still present at 50 °C, as the denatured polypeptides can self-assemble and may precipitate from solution.

The model for chromophore organization in protein dimers ($\alpha_2\beta_2$) at 20 °C (MacColl *et al.*, 1994) suggested speculation that the blue-edge negative CD band might be produced by exciton splitting between a closely-spaced pair of chromophores (Figure 8). When a pair of identical chomophores engage in exciton splitting, the number of absorption and CD bands increases to two, and CD shows these bands as one positive and one negative of equal rotational strength—a conservative spectrum. At 45 °C, the conformational change in the dimer may result in losing this exciton splitting spectrum by either the separation of the chromophore pair or a change in the orientation of the chromophores in the pair. For certain orientations of the chromophores in a dimer, the CD is zero. This change in geometry might be a preliminary stage before chromophore cyclization. At 45 °C, the two bands presumably caused by exciton splitting might be replaced by a single monomeric chromophore band from two chromophores. The large change in the CD at 45 °C in the region of the blue-edge negative band compared with the relatively smaller changes in the rest of the spectrum also indicated that exciton splitting might be responsible for the blue-edge negative in the protein dimer at 20 °C.

The assembly of phycocyanin 645 proceeds with the formation of monomers ($\alpha\beta$) from the newly synthesized α and β polypeptides. The next step would be dimerization of two monomers. These current results suggest that the initial product of this dimerization would be a tertiary conformational state exactly like that of the dimers at 45 °C. This state would rearrange to the structure of dimers found in solution at 20 °C. Since monomers at ambient temperature and both types of dimer have identical secondary structures, this structure is likely to be established very early in assembly.

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