of hydration or the degree of hydrogen bonding between adjacent sphingomyelin molecules. In any case, changes in these or similar interactions during the gel–liquid-crystalline transitions must account for the differences in ΔH and ΔS for the two forms.

Brandts et al. (1978) have observed several irreversible transitions above 50 °C in human erythrocyte ghosts, at least some of which involve phospholipid. It is possible that some of these transitions may appear to be irreversible because they are transitions of the sort observed with stearoylsphingomyelin. This possibility is strengthened by the fact that the content of C_{18}-sphingolipids in this membrane is quite high.

References

Albon, N., & Sturtevant, J. M. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 2258.

Barenholz, Y., Suurkuusk, J., Mountcastle, D., Thompson, T. E., & Biltonen, R. L. (1976) *Biochemistry 15*, 2441.

Brandts, J. F., Taverna, R. D., Sadasivan, E., & Lysko, K. A. (1978) *Biochim. Biophys. Acta 512*, 566.

Calhoun, W. I., & Shipley, G. G. (1979a) *Biochim. Biophys. Acta 555*, 436.

Calhoun, W. I., & Shipley, G. G. (1979b) *Biochemistry 18*, 1717.

Carter, H. E., & Gaver, R. C. (1967) *J. Lipid Res. 8*, 391.

Demel, R. A., Jansen, J. W. C. M., Van Dijck, P. W. M., & Van Deenen, L. L. M. (1977) *Biochim. Biophys. Acta 465*, 1.

Elliot, A. (1965) *J. Sci. Instrum. 42*, 312.

Estep, T. N., Mountcastle, D. B., Biltonen, R. L., & Thompson, T. E. (1978) *Biochemistry 17*, 1984.

Estep, T. N., Mountcastle, D. B., Barenholz, Y., Biltonen, R. L., & Thompson, T. E. (1979) *Biochemistry 18*, 2112.

Franks, A. (1958) *Br. J. Appl. Phys. 9*, 349.

Gaver, R. C., & Sweeley, C. C. (1965) *J. Am. Oil Chem. Soc. 42*, 294.

Lentz, B. R., Barenholz, Y., & Thompson, T. E. (1976) *Biochemistry 15*, 4521.

Lentz, B. R., Freire, E., & Biltonen, R. L. (1978) *Biochemistry 17*, 4475.

Loomis, C. R., Shipley, G. G., & Small, D. M. (1979) *J. Lipid Res. 20*, 525.

Oldfield, E., & Chapman, D. (1972) *FEBS Lett. 21*, 303.

Perron, R., Petit, J., & Mathieu, A. (1969) *Chem. Phys. Lipids 3*, 11.

Phillips, M. C., Williams, R. M., & Chapman, D. (1969) *Chem. Phys. Lipids 3*, 234.

Schmidt, C. R., Barenholz, Y., & Thompson, T. E. (1977) *Biochemistry 16*, 2649.

Shipley, G. G., Avecilla, L. S., & Small, D. M. (1974) *J. Lipid Res. 15*, 124.

Small, D. M. (1970) *Adv. Exp. Med. Biol. 7*, 55.

Suurkuusk, J., Lentz, B. R., Barenholz, Y., Biltonen, R. L., & Thompson, T. E. (1976) *Biochemistry 15*, 1393.

Untracht, S. H., & Shipley, G. G. (1977) *J. Biol. Chem. 252*, 4449.

Vaskovsky, V. E., & Kostetsky, E. Y. (1968) *J. Lipid Res. 9*, 396.

Molecular Topography of the Phycocyanin Photoreceptor from *Chroomonas* Species†

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ABSTRACT: Molecular topology of a light-harvesting phycobiliprotein, phycocyanin from *Chroomonas* species of cryptomonad algae, was investigated by using spectroscopic techniques. The phycocyanin consists of four subunits of the form $\alpha\alpha'\beta_2$. The two small subunits, α and α', cannot be differentiated by their molecular weights as determined by conventional methods such as sodium dodecyl sulfate gel electrophoresis and gel chromatography; the large subunit contains two chromophore molecules, one of which is phycocyanobilin. A dipole–dipole exciton coupling model was applied to interpret spectral properties of *Chroomonas* phycocyanin. Two main circular dichroic extrema, one with a strong negative band and the other with a positive band, are probably due to the result of exciton coupling of two phycocyanobilin molecules contained in the two β-subunit polypeptides. The visible absorption spectrum of *Chroomonas* phycocyanin with two main peaks at 645 and 583 nm was analyzed to yield five spectral components with their maxima at 573, 592, 617, 636, and 652 nm. The 592- and 652-nm bands are the split bands of phycocyanobilins in the β subunit due to exciton coupling, while the other three bands correspond to three types of noninteracting chromophores, with the 573-nm band assigned to an unknown bilin chromophore in the β subunit, the 617-nm band assigned to phycocyanobilin in the α subunit, and the 636-nm band assigned to the chromophore in the α' subunit. On the basis of the proposed exciton coupling model and the fluorescence excitation polarization spectrum, a tentative molecular topography of *Chroomonas* phycocyanin is described in terms of spectroscopic parameters.

On the basis of their color, there are two types of phycobilin photosynthetic accessory pigments. The red-colored phycoerythrins absorb light in the 550–600-nm region, and the blue-colored phycocyanins, including allophycocyanin, show maximum absorption in the 600–650-nm region (O'Carra & O'hEocha, 1976; Bogorad, 1975; Glazer, 1976).

Of all the phycobiliproteins, the cryptomonad biliprotein has been known to display some properties distinctive from the rest. The most important distinctions are its location and aggregation in cells (Dodge, 1969; Gantt et al., 1971; Faust & Gantt, 1973). The biliproteins from blue-green and red

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algae are located on the stomal side of the photosynthetic lamellae in the form of large, discrete aggregates called phycobilisomes (Gantt & Conti, 1966; Edward & Gantt, 1971; Wildman & Bowen, 1974). On the other hand, cryptomonad phycobiliprotein is contained within the intrathylakoidal spaces, and there is no evidence of phycobilisomes.

In general, physical and immunological properties and amino acid composition of cryptomonad phycobiliprotein have been found to be different from those of the other algal pigments, especially those from the procaryotic blue-green algae (MacColl et al., 1973, 1976). The subunit composition (MacColl et al., 1973; Mörshel & Wehrmeyer, 1976) and the existence of a second chromophore in the cryptomonad phycocyanin (Chapman et al., 1968; O'Carra et al., 1964; Killilea & O'Carra, 1968; Cole, 1967; Glazer & Cohen-Bazire, 1975) are not definitely established. Is it $\alpha_2\beta_2$ (MacColl et al., 1973) or $\alpha\alpha'\beta_2$ (Mörshel & Wehrmeyer, 1976)? The chromophore distribution on its polypeptide subunits also remains obscure.

This work was undertaken to resolve some of these questions, using the cryptophycean phycocyanin (a type I phycocyanin) of *Chroomonas* sp. as a representative of the cryptomonad biliproteins. In this paper, our results are also compared with conclusions reached by the earlier work of Glazer & Cohen-Bazire (1975) on another type I phycocyanin (Millport 64).

Materials and Methods

Materials

The *Chroomonas* phycocyanin (PC) was isolated and purified as follows. Frozen cells (1.5 kg) of *Chroomonas* sp. were the generous gift of Dr. H. W. Siegelman. Unless otherwise stated, all work with these cells and subsequent isolation procedures were carried out at 3 °C.

The PC was extracted from the flat, dark cells by repeated freezing and thawing 3 times with dry ice and 1 mM potassium phosphate buffer (pH 6.6), 0.2 M in NaCl. The intense blue-black liquid was first suction filtered through Celite and cheesecloth and then centrifuged at 15 000 rpm for 20 min to remove cell debris. The liquid was then gently concentrated 10-fold by vacuum dialysis. Subsequent work was done in pH 6.0, 0.01 M phosphate buffer and 0.2 M NaCl. The purity of material was judged by the ratio of A_{646} to A_{275} (or A_{280}). This ratio was about 1.25:1.0 for the crude extract. Initial purification was carried out by gel chromatography on a 2.5 × 95 cm Sephadex G-100 column. The peak fractions had a ratio of 4.7:1.0. Fractions which had ratios greater than 3.0 were pooled and then concentrated using an Amicon PM-10 membrane. The concentrated material had a ratio of 3.5 and gave an absorbance of 90.0 at 646 nm.

Further purification was done by running the sample on a 2.5 × 95 cm column packed with Sephacryl S-200 at a flow rate of 45 mL/h. Samples of 8 mL were applied and 4.4-mL fractions collected. Seventy percent of the absorbance at 646 nm was recovered with a ratio (A_{646}/A_{280}) of greater than 5.0, with the peak fractions as high as 5.8.

The *Chroomonas* phycocyanin isolated was kept in 0.01 M phosphate buffer of pH 6.0 containing 0.2 M NaCl and stored at 0–4 °C in the dark (~1 week) until used. It was chromatographically pure, showing a single component by Sephadex G-200 (M_r ~47 000 ± 1 500) and Sephacryl S-200 column chromatography.

Methods

Absorption Spectra. Absorption spectra were recorded at room temperature on a Cary 118C spectrophotometer.

Circular Dichroic (CD) Spectra. CD spectra were recorded at room temperature on a JASCO J-20 CD–ORD spectropolarimeter which was modified to enhance the signal-to-noise ratio by replacing the Pockels cell and associated circuitry with a Morvue photoelastic modulator (PEM-3) and a lock in amplifier/phase detector (Model PAR 121).

Fluorescence Excitation and Emission Spectra. A Perkin-Elmer Model MPF-3 fluorescence spectrophotometer equipped with a R106 type photomultiplier and a 150-W xenon lamp was used for recording luminescence spectra. A high-resolution spectrofluorometer assembled in this laboratory (Moore, 1975) with two Jarrell–Ash exciting monochromators, an SSR 1120 amplifier/discrimator, a 1105 data converter, a 1106 power supply console, a scanning emission monochromator, an EMI 9558 QM photomultiplier, and 150-W xenon arc lamp was also used to obtain the corrected emission spectrum of the native phycocyanin.

Fluorescence Excitation Polarization Spectrum of Chroomonas Phycocyanin. The high-resolution spectrofluorometer mentioned above was used to obtain the fluorescence excitation polarization spectrum of phycocyanin, as described previously (Song et al., 1976).

Fluorescence Lifetime. The fluorescence lifetimes were measured at room temperature with an SLM Model 480 phase-modulation spectrofluorometer, utilizing continuous light that had been sinusoidally modulated as described previously (Song et al., 1976). A picosecond streak camera was also used (with the help of Professor G. W. Robinson).

Subunit Isolation of Chroomonas Phycocyanin. (a) Subunit Isolation by a Cation-Exchange Chromatography. Cation-exchange chromatography with Bio-Rex 70, which has been successful for the subunit separation of C-phycocyanin (Glazer & Cohen-Bazire, 1975; Troxler et al., 1975) and C-phycoerythrin (Takemoto & Bogorad, 1975), was employed with minor modification.

Three milliliters of a phycocyanin sample in phosphate buffer (~0.5 mg/mL) was dialyzed against water at 5 °C for 2 h and then against 12% formic acid–0.01 M 2-mercaptoethanol solution for 5 h at 4 °C. The dialyzed solution was applied to a column, 0.7 × 15 cm, of Bio-Rex 70, which was preequilibrated with 12% formic acid–0.01 M 2-mercaptoethanol. The column was developed by linear gradient elution with a urea–HCl solution containing 0.01 M 2-mercaptoethanol. The initial concentration of urea was 4 M, and its final concentration was 10 M. Elution was monitored by absorbance at 660 nm. The pure fractions of the eluted components were located by preparing a profile of A_{690}/A_{600} and A_{600}/A_{660} (and A_{646}/A_{280}) changes as a function of the fraction number (Figure 1). The fractions containing pure components were pooled and dialyzed against a 9 M urea–HCl, pH 2.0, solution. Each fraction was collected and immediately put in ice. All the work was done under dim blue safety light.

(b) Subunit Isolation by a Gel Filtration Method. A method developed by Fish et al. (1969) for the determination of a subunit molecular weight was employed without modification. A phycocyanin sample in phosphate buffer was dialyzed against 6 M guanidine hydrochloride, pH 8.5, containing 0.1 M 2-mercaptoethanol at 38 °C for 5 h and then against 6 M guanidine hydrochloride, pH 6.5, containing 0.1 M 2-mercaptoethanol at room temperature for 4 h. This pretreated sample was applied to a column, 1.2 × 40 cm, of Sepharose 6B equilibrated with 6 M guanidine hydrochloride and 0.1 M 2-mercaptoethanol. Elution was monitored by absorbance at 400 nm. The flow rate was 3 mL/h, and fractions of ~2.5 mL were collected. Fraction D from the Bio-Rex 70 columns was also subjected to a Sepharose 6B–6 M guanidine hydro-

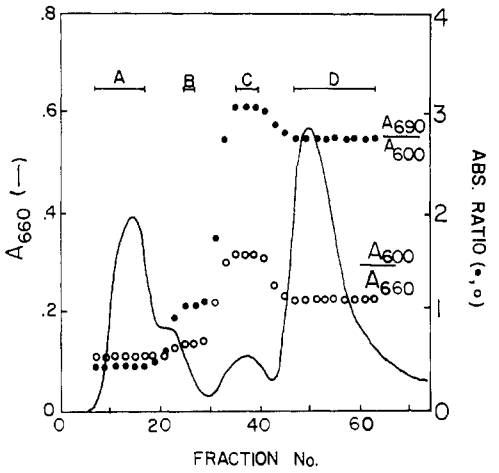

FIGURE 1: Bio-Rex 70 cation-exchange column chromatogram of *Chroomonas* phycocyanin. The elution profile was also monitored in terms of absorbance ratios shown in order to resolve distinct fractions (A, B, C, and D) of various components. (See text for details.)

chloride column operation. The chromatographic procedure and materials were identical with those described above, except that fraction D in 9 M urea–HCl medium was dialyzed against water before being dialyzed against guanidine hydrochloride–2-mercaptoethanol medium.

Renaturation of the Denatured Phycocyanin. The phycocyanin denatured and kept in 9 M urea–HCl, pH 2.0, was subjected to a stepwise dialysis at 4 °C by changing the dialysis medium from 5 M urea–HCl and 0.01 M 2-mercaptoethanol, pH 3.0, to a final dialysis buffer of 0.01 M phosphate and 0.2 M NaCl, pH 6.0.

Renaturation was followed by measurements of optical properties such as CD, absorption, and luminescence spectra. The renatured phycocyanin sample contained a considerable amount of precipitate, but it did not affect to a great extent the spectral measurements in the visible region. The four components of phycocyanin (obtained from the Bio-Rex 70 column) and their mixtures were treated by using the procedure described above.

Chemical Treatment of Chroomonas Phycocyanin. (*a*) *Potassium Permanganate Treatment.* Potassium permanganate is a very efficient reagent for changing the optical properties of phycocyanin, most probably by modifying the chemical structures of chromophore molecules through oxidation.

One volume of phycocyanin stock solution (~0.5 mg/mL concentration in 0.01 M phosphate buffer and 0.2 M NaCl, pH 6.0) was mixed quickly with 9 volumes of a $KMnO_4$ solution (10^{-6}–10^{-5} M concentration in the same phosphate buffer) to make 2 mL of final volume containing (1–2) × 10^{-6} M phycocyanin. The mixture was kept in the dark for 30 min. At this low concentration of $KMnO_4$, the apoprotein was not affected, as monitored by UV absorption and CD.

(*b*) *Mercuric Acetate Treatment.* Another reagent which also significantly alters the spectral properties of phycocyanin, $Hg(OAc)_2$, was used for comparison with the effect of $KMnO_4$ at approximately the same concentration of the reagent.

Fluorescence Quenching Study. The dynamic fluorescence quenching of phycocyanin (A_{646} = 0.11 in phosphate buffer) was measured in terms of fluorescence lifetime as a function of KI concentrations. The possibility of an ionic strength effect on the protein structure was excluded, as an excess amount of KCl (>2 M) did not result in any significant change of optical properties of the protein. However, it is important that the solutions be kept under anaerobic conditions to prevent the formation of I_2 which reacts with the chromophore.

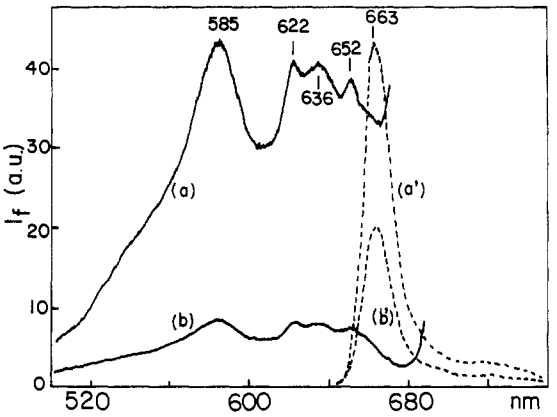

FIGURE 2: Fluorescence excitation (—) and emission (---) spectra of *Chroomonas* phycocyanin at 77 K. Solvent: 10% glycerol in 10 mM phosphate buffer and 0.2 M NaCl, pH 6.0. (a) λ_{em} ~670 nm; (b) λ_{em} ~690 nm; (a') λ_{ex} ~620 nm; (b') λ_{ex} ~540 nm.

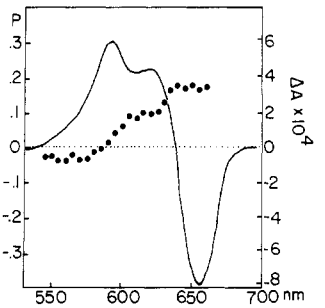

FIGURE 3: Polarized fluorescence excitation spectrum (●) of *Chroomonas* phycocyanin with respect to λ_{em} ~680 nm. The CD spectrum (—) is superimposed for comparison.

Results

The absorbance ratio A_{646}/A_{280} of *Chroomonas* phycocyanin used was 5.2, indicating that this biliprotein was in a very pure state (MacColl et al., 1976). The maxima of the absorption, CD, and fluorescence excitation spectra matched fairly well with one another, though not precise (spectra not shown). The two extrema of the CD spectrum in the visible region especially showed a substantial red shift from the absorption maxima. A poorly resolved shoulder around 550 nm in the fluorescence excitation spectrum and a weak negative CD band were seen in the 500–550-nm region.

The 77 K temperature fluorescence excitation spectrum (Figure 2) does not distinctly resolve the band around 550 nm. However, this spectrum shows a possible fifth band in the visible region at 636 nm. Figure 3 shows the fluorescence excitation polarization spectrum, superimposed on the CD spectrum for the visible region. Three CD bands with fluorescence polarization degrees of 0.2, 0.1, and ~0.03 are clearly resolved in the spectrum.

The fluorescence lifetime (τ_F) of *Chroomonas* phycocyanin was measured with various methods. The τ_F values in 0.01 M phosphate buffer (0.2 M NaCl, pH 6.0) at room temperature were 1.55 (by phase shift at 30 MHz), 1.42 (by modulation at 30 MHz), and 1.35 nsec (by picosecond streak camera). These values are somewhat shorter than those of C-phycocyanin [1.8 and 2.1 ns; Brody & Rabinowitch (1957) and Barber & Richards (1976)] and R-phycoerythrin [3.1, 3.5, and 7.1 ns; Barber & Richards (1976), MacDowall & Walker (1968), and Brody & Rabinowitch (1957)].

The elution profile of the phycocyanin subunits from a Bio-Rex 70 cation-exchange column shows two major peaks

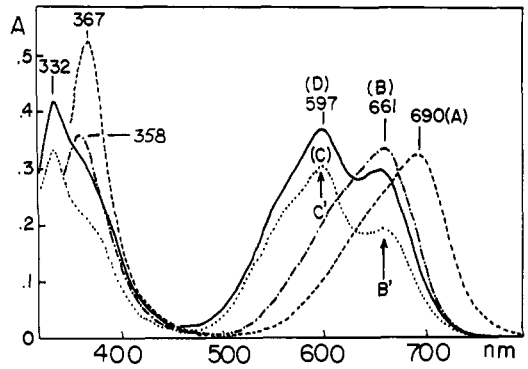

FIGURE 4: Absorption spectra of the four components of *Chroomonas* phycocyanin eluted from the Bio-Rex 70 column by acid urea. Spectra were taken after dialyzing against 9 M urea, pH 2.0. (---) Fraction A (fraction no. 14 from Figure 1); (-·-) fraction B (fraction no. 26 from Figure 1); (···) fraction C (fraction no. 37 from Figure 1); (—) fraction D (fraction no. 51 from Figure 1).

and two minor peaks (Figure 1). The minor components, designated B and C, are substantially overlapped with the major peaks, designated A and D, so that only a few fractions of B and C contain pure components on the basis of the absorbance ratio measured at various wavelengths as shown in Figure 1. *Chroomonas* phycocyanin has been reported to consist of two or three different types of subunits ($\alpha_2\beta_2$ or $\alpha\alpha'\beta_2$), and minor peaks B and C are of interest in this regard (vide infra). Similar elution patterns were obtained from the Bio-Rex 70 cation-exchange chromatograms of the phycocyanin samples prepared on different occasions.

Fractions A, B, C, and D were separately pooled. Their absorption spectra were determined (Figure 4). In contrast to fractions A and B, C and D show double-peaked visible spectra with the main absorption maximum at 597 nm and a second maximum at 661 nm (components C′ and B′, respectively; the latter chromophore is the same as that in fraction B, as will be discussed later). The only noticeable difference between C and D is the ratio of absorbances at 600 (chromophore C′) and 661 nm (chromophore B′); the ratio A_{600}/A_{661} is 1.57 for C and 1.22 for D. The same pattern can be observed in the UV region between the 332- and 358-nm shoulder, suggesting that these fractions are composed of aggregates of common subunits containing chromophores B′ and C′.

Fraction D was passed through a Sepharose 6B–guanidinium hydrochloride (6 M) column in order to resolve fraction D into its components, which were not resolved by Bio-Rex 70. We compared the elution profile of fraction D with that of the phycocyanin. Although fraction D was resolved into the same subunits as those of phycocyanin, the second peak was substantially lower than that of phycocyanin.

Fractions B and C are small (α) and large (β) subunits of phycocyanin, respectively, according to sodium dodecyl sulfate ($NaDodSO_4$) gel electrophoresis. Fraction C shows two absorption and emission maxima corresponding to chromophores B′ and C′ (Figure 4 and 5), suggesting that fraction C contains two different types of chromophore (597 and 661 nm for C′ and B′, respectively) bound to the β-subunit proteins of equal molecular weight as judged by $NaDodSO_4$ gel electrophoresis (data not shown). In $NaDodSO_4$ gel electrophoresis of fractions B and C, the former showed a trace amount of protein which corresponded to the latter;[1] however, fraction C showed no contamination by B.

[1] Visible only after an exhaustive staining and destaining cycle for the gel.

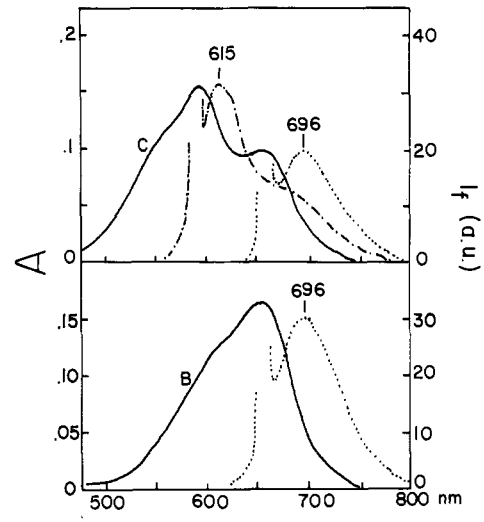

FIGURE 5: Absorption (—) and fluorescence spectra of B and C components of *Chroomonas* phycocyanin in 9 M urea, pH 2.0. Upper panel: spectrum C (-·-·-) with λ_{ex} 590 nm and (···) with λ_{ex} 660 nm. Lower panel: spectrum B, λ_{ex} 660 nm. The discontinuity in the fluorescence spectra is due to scattering of the exciting beam.

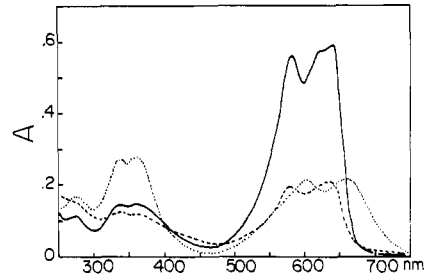

FIGURE 6: Absorption spectra of *Chroomonas* phycocyanin. (—) Native form in 10 mM phosphate buffer and 0.2 M NaCl, pH 2.0; (···) denatured phycocyanin, 9 M urea, pH 2.0; (---) renatured phycocyanin.

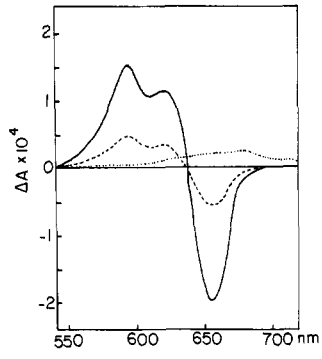

FIGURE 7: CD spectra of the native (—), denatured (···), and renatured (---) phycocyanin in media as used for Figure 5.

The optical spectra of denatured and renatured phycocyanin are shown in Figures 6–8. The renaturation was achieved by a stepwise dialysis method as described under Methods. From Figures 6–8, ~30% renaturation or reconstitution is achieved. This is of the same degree of renaturation as in C-phycocyanin [24%; Glazer et al. (1973)].

The urea-denatured phycocyanin shows drastically different spectral properties from those of the native phycocyanin. The absorption maxima are red shifted (Figure 6); the absorbances at the maxima are reduced to ~37% of the native protein; its CD spectrum has a broad positive band in the visible region with a maximum at 680–685 nm with a CD intensity less than 10% of the native protein (Figure 7). The 662-nm emission of native phycocyanin disappears completely; instead, three different emissions corresponding to chromophores of λ_{max}

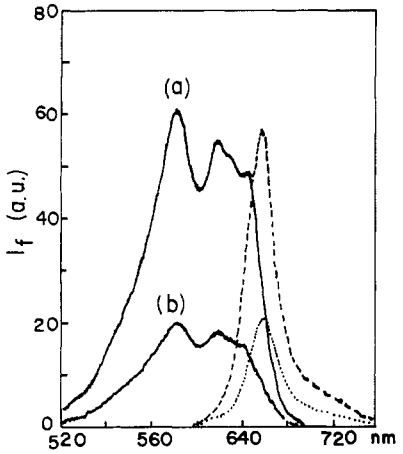

FIGURE 8: Fluorescence emission and excitation spectra of the native and renatured phycocyanin in 20 mM phosphate buffer and 0.2 M NaCl, pH 6.0, λ_{ex} 580 nm and λ_{em} 680 nm. (a) Native phycocyanin (fluorescence in broken line); (b) renatured phycocyanin (fluorescence in dotted line).

~597 and 661 nm (C′ and B′, respectively, of fraction C) and 690 nm (fraction A) appear with fluorescence intensities less than 1% of the native phycocyanin emission (Figure 8).

The renaturation of individual fractions A–D from Figure 4 was not successful. We compared the absorption spectra of A in the denatured and renatured states; the latter showed a substantial loss of protein (spectra not shown). The fluorescence emission and excitation spectra of the renatured D were also recorded. The renatured D gave excitation and emission spectra nearly identical with those of the native phycocyanin (cf. Figure 2), suggesting that D contained the fluorescence-emitting chromophores of phycocyanin and that the interchromophore energy transfer was almost as efficient in the renatured D as in the native phycocyanin. However, the absorption spectrum of renatured D did not fully match its corrected excitation spectrum (intensity ratio I_{617}/I_{573} = 1:1.15), suggesting that a portion of the renatured D protein remained in the denatured state.

The effect of fractions A and B on the renaturation of fraction D was examined. A mixture of 2 mL of fraction D (A_{600} = 0.305) and 1 mL of fraction A (A_{660} = 0.165) was treated in the renaturation process, and its fluorescence emission and excitation spectra were taken. Another mixture of 2 mL of fraction D (A_{600} = 0.305) and 1 mL of fraction B (A_{660} = 0.104) was treated by using the same procedure. The emission spectra of the renatured mixtures were identical with those of the native phycocyanin and the renatured D. The excitation spectra were very similar to the former (Figure 9). The overall fluorescence excitation intensity of the renatured mixtures increased substantially over that of D alone. In addition to the two major peaks (arrows) at ~570 and ~620 nm of the renatured D, the two peaks at 590 and 650 nm, which were almost completely buried in the spectrum of the renatured D, became conspicuous in the spectra of renatured mixtures. The effects of A and B on the renaturation of D were identical.

CD spectra of phycocyanin treated with $KMnO_4$ (Figure 10) indicated that all the chromophores are exposed to the oxidizing agent. However, there are differential reactivities or varying degrees of chromophore exposure, as revealed from the CD changes per [$KMnO_4$], $\Delta A/\Delta A_0$, at different wavelengths, where ΔA and ΔA_0 are CD intensities with and without $KMnO_4$, respectively (Figure 10b). In fact, from the plot of $\Delta A/\Delta A_0$ as a function of λ (data taken from Figure 10a), three prominent (592, 620, and 657 nm) and two less

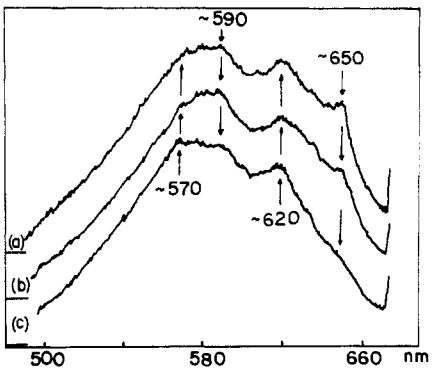

FIGURE 9: Excitation spectra of the renatured mixtures of component D with A (D + A) and with B (D + B), compared with that of the renatured component D alone, λ_{em} 680 nm. (a) D + A, (b) D + B, and (c) D, with their respective base lines indicated on the left ordinate. For the spectrum of the renatured D (c), the fluorescence intensity scale was expanded 1.5 times.

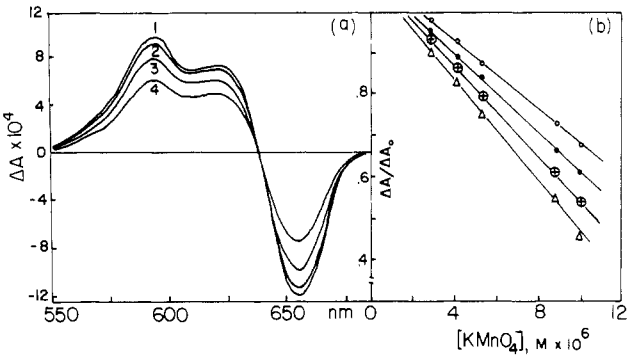

FIGURE 10: (a) CD spectra of *Chroomonas* phycocyanin treated with $KMnO_4$ at various concentrations. Concentration of phycocyanin was ~0.1 mg/mL. [$KMnO_4$]: (1) 0; (2) 2.9 μM; (3) 5.3 μM; (4) 8.8 μM. (b) Ratio of CD activity of the $KMnO_4$-treated phycocyanin to that of the native phycocyanin as a function of [$KMnO_4$] at different wavelengths. (○) 645 nm (relative slope ratio = 1.0); (●) 620 nm (1.24); (⊕) 592 and 660 nm (1.42); (△) 575 nm (1.60).

conspicuous (in the regions of 550–580 and 625–650 nm) CD transitions can be resolved, showing the five discrete $\Delta A/\Delta A_0$ regions at 550–585, 585–605, 605–630, 625–650, and 650–680 nm. Among the five CD bands, the 592- and 657-nm extrema show strikingly similar values of $\Delta A/\Delta A_0$ (Figure 10b; ⊕ symbol marks points from both curves because they fall so close together), suggesting that the two main CD bands arise from structurally and environmentally identical chromophores.

CD spectra and the plot of $\Delta A/\Delta A_0$ as a function of $Hg(OAc)_2$ showed results similar to those obtained from the $KMnO_4$-treated phycocyanin shown in Figure 10, confirming that there are at least five distinct CD bands in the visible region for *Chroomonas* phycocyanin.

In addition to the chemical treatments of phycocyanin with $KMnO_4$ and $Hg(OAc)_2$, a series of fluorescence quenching studies of the phycocyanin emission spectra by KI have been carried out in order to ascertain whether the chromophores are buried. From the slope of Stern–Volmer plots, the quenching rate constant was evaluated as 5.9×10^8 M^{-1} s^{-1}. This value suggests that the emitting chromophore is substantially exposed, since a steric factor correction for the protein and hydrated KI would certainly raise this value closer to the diffusion limit.

Discussion

Spectral Properties. Various spectra (Figures 2 and 3) of *Chroomonas* phycocyanin taken at room temperature in this work agree well with those reported for *Chroomonas* by other

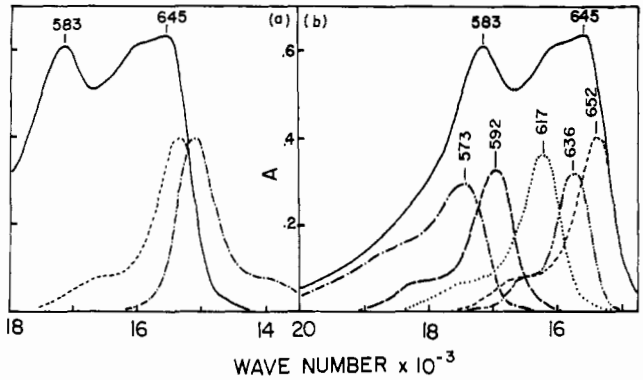

FIGURE 11: (a) Corrected emission spectra of *Chroomonas* phycocyanin and its mirror image absorbance band, which matches the red side of the absorption spectrum. (—·—) Corrected emission spectrum; (---) mirror image of corrected emission spectrum; (—) absorption spectrum. (b) Spectral resolution of the composite visible absorption of the native *Chroomonas* phycocyanin in terms of five distinct bands.

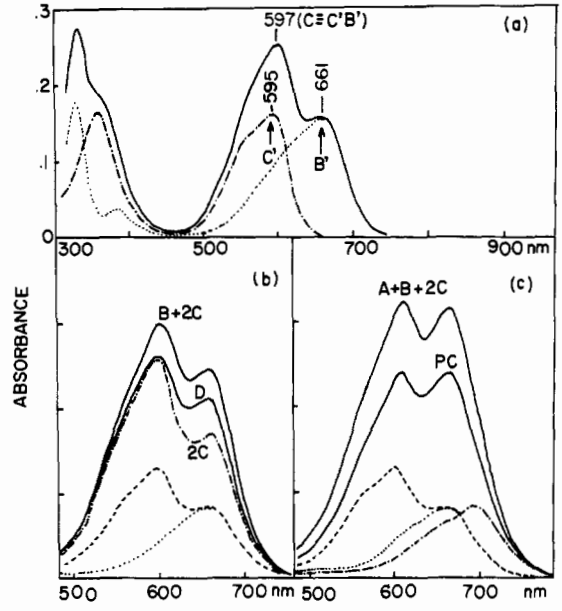

FIGURE 12: (a) Resolution of the absorption spectrum (—) of fraction C in 9 M urea, pH 2.0, into two fractions, C (—·—) and B (···), for the 600- and 661-nm peaks, respectively. (b) Comparison of the absorption spectrum (D) of fraction D with the composite spectra B + 2C and 2C computed from the spectra of two fractions, B (···) and C (---). See the text for an explanation. (c) Comparison of the absorption spectrum of the urea-treated phycocyanin (PC) with the composite spectrum A + B + 2C computed from A (—·—), B (···), and C (---). See the text for an explanation.

investigators (MacColl et al., 1973; Mörshel & Wehrmeyer, 1976; Chapman et al., 1968). We will compare the native *Chroomonas* spectra with native blue-green algal phycocyanin (C-phycocyanin) and denatured *Chroomonas* spectra.

The asymmetric visible peak of C-phycocyanin has been analyzed in terms of two tetrapyrrole chromophores, differing in either chemical structure or, more probably, in protein environment. The shorter wavelength absorber was denoted "sensitizing" ("s", α subunit) and the longer wavelength one "fluorescing" ("f", β subunit) by Teale & Dale (1970).[2] The spectrum of *Chroomonas* phycocyanin has likewise been discussed in terms of this model (MacColl et al., 1973; Mörshel & Wehrmeyer, 1976; Chapman et al., 1968; MacColl & Berns, 1978). However, the spectral properties of *Chroomonas* phycocyanin do not appear to be as simple as those of C-phycocyanin.

The fluorescence excitation polarization spectrum has three distinctive bands (Figure 3) with a polarization degree (P value) of +0.1 at 620 nm and ca. +0.2 at 645 nm. This suggests that there are at least two electronic transitions corresponding to one negative and one positive (shoulder) CD band in the region of 620–680 nm.

The spectral uniqueness of *Chroomonas* phycocyanin is also demonstrated in the fluorescence excitation spectra taken at 77 K (Figure 2), showing a more complicated spectrum than expected from the polarization spectrum. It reveals four well-defined spectral maxima and a shoulder in the visible region. It is especially noteworthy that peaks at 652, 636, and 622 nm are resolved for the 645-nm absorption band. Thus, the results shown in Figure 2 strongly suggest that the visible spectrum of *Chroomonas* phycocyanin is a composite of five, or at least four, different electronic transitions corresponding to the structurally or environmentally different chromophores.

We analyze the visible spectrum of native *Chroomonas* phycocyanin in terms of five electronic transitions. For this purpose, we make the following reasonable assumptions. (a) The chromophore of phycocyanin, phycocyanobilin, shows a single allowed transition in the visible region [i.e., $f_{Q_y} \gg f_{Q_x}$ and the Q_x–Q_y split is small; see Song (1978) for notation]. (b) The peaks at 652, 636, 622, and 585 nm (Figure 2) represent four of these electronic transitions, while the fifth band may be located in the 550–585-nm region (Figure 10). (c) The longest wavelength maximum at 652 nm at 77 K is the emitting state, exhibiting a mirror image to the fluorescence band (Figure 2b), as illustrated in Figure 11a. (d) The longest wavelength and 585-nm bands (592-nm CD maximum) arise from structurally and/or environmentally identical chromophores (cf. Figure 10; vide infra), which are most likely phycocyanobilins with identical fluorescence lifetimes. (e) The excitation peak at 622 nm (CD at 620 nm) originates from phycocyanobilin, as all C-phycocyanobilins containing only the phycocyanobilin chromophore show λ_{max} ~620 nm.

Figure 11b shows the resolution of the visible absorption band, showing five λ_{max} values. The above spectral resolution is consistent with the results of the chemical treatment of phycocyanin (Figure 10). The calculated oscillator strengths of the four components (652, 636, 617, and 592 nm) are ~1, while the 573-nm component possesses a value of ~2 which may be due to a contribution by two identical chromophores.

In contrast to the five-component chromophore analysis for the native phycocyanin spectrum, the urea-denatured phycocyanin reveals only three distinct bands (Figure 4), showing chromophore band maxima at 690 (= A), 661 (= B′ ≡ B), and 597 nm (C′). It is possible to analyze the spectrum of denatured phycocyanin in terms of individual contributions of chromophores bound to various subunit protein components.[3] The 661-nm absorption and the 696-nm fluorescence bands of chromophore C′ coincide with those of B (= B′) (Figure 5). Since $NaDodSO_4$ gel electrophoresis of fractions B and C, vide supra, shows them to be two different subunits, (α and β, respectively), the results shown in Figure 4 suggest that one (i.e., B′) of the chromophores present in C is identical with the B chromophore with an absorption maximum at 661 nm. The spectrum of C itself can thus be resolved in terms of two chromophores, B′ and C′, in a 1:1 ratio (Figure 12). The absorption spectrum of D can be resolved into B and C spectra in a 1:2 ratio, contributed by five chromophores ($B'_3C'_2$). The denatured phycocyanin spectrum is then

[2] Alternatively, s and f chromophores have been assigned to β and α subunits, respectively (Glazer, 1976).

[3] Unprimed letters refer to fractions from the Bio-Rex 70 column, while primed letters refer to their individual chromophores.

matched by the composite (A + B + 2C) or 1:3:2 ratio of the 690-, 661-, and 597-nm chromophores, exhibiting the resulting λ_{max} at 602 and 662 nm which agrees well with the absorption maxima of 602 and 664 nm, respectively, for *Chroomonas* phycocyanin in 9 M urea, pH 2.0. What can we infer about these three different chromophores?

The most abundant chromophore in the denatured *Chroomonas* phycocyanin is the 661-nm form. Its absorption spectrum matches well with those of C-phycocyanin and allophycocyanin in acidic urea (Glazer & Fang, 1973; Troxler et al., 1975) in spectral shape, λ_{max}, and the absorbance ratio of the visible maximum to the UV maximum. This indicates that the 661-nm chromophore is phycocyanobilin, as C-phycocyanin and allophycocyanin contain only phycocyanobilin as their chromophores. Thus, the present observation agrees in part with Chapman et al. (1968), who were able to detect phycocyanobilin by methanolic hydrolysis of phycocyanin. It is also clear that the binding environments of this common chromophore are different in the native states of these proteins (see later).

In contrast to the 661-nm chromophore, there are no references in the literature to which the 690-nm chromophore spectrum can be compared. However, the absorption spectra of the two chromophores are strikingly similar, except for a 29-nm (659 cm^{-1}) shift of the visible band (Figure 4). A shift of the same magnitude is seen for the UV (685 cm^{-1}) and fluorescence (662 cm^{-1}) bands. Thus, it can be suggested that the 690-nm chromophore is a protein-perturbed (e.g., different linkage) form of the 661-nm chromophore.

The 597-nm chromophore (cf. Figures 4 and 12) is not phycocyanobilin on the grounds that the Q_y band energy is too high when compared with that of phycocyanobilin examined under different conditions (O'hEocha et al., 1964; O'hEocha, 1963, 1965; Chapman, 1973). Phycoerythrobilin always exhibits a near-UV band at 380 nm (Bennett & Bogorad, 1971; Takemoto & Bogorad, 1975; O'hEocha, 1963, 1965; Chapman & Siegelman, 1967). Thus, the 597-nm chromophore possesses spectral resemblance to phycoerythrobilin, but its chemical identity awaits further elucidation. Nonetheless, it is clear that there exists a second chromophore distinct from phycocyanobilin in *Chroomonas* phycocyanin.

At this point, it is useful to compare our analysis of the spectral data described above with the previous analysis on another type I phycocyanin of Millport 64 (Glazer & Cohen-Bazire, 1975). On the basis of the absorption spectra of subunits (Figure 2B in their paper) which show three distinct absorption maxima, it was concluded that β subunit carries two bilin chromophores with λ_{max} of 600 and 660 nm while α subunit carries a hitherto undescribed chromophore with λ_{max} of 694 nm in acid urea. Our analysis substantially confirms this prediction.

The absorption spectrum of α subunit reported by Glazer & Cohen-Bazire (1975) is essentially identical with that of our fraction A (= α'), while their β subunit shows a strikingly similar spectrum to our partially aggregated fraction D (= $\alpha\beta_2$) (compare their spectrum Figure 2B with our Figure 4). Our subunit composition of fraction D was based on the spectral analyses (vide supra; Figures 5 and 12). Namely, D carries two 597-nm chromophores [the 600-nm chromophore of Glazer & Cohen-Bazire (1975)] and three 661-nm chromophores (the 660-nm chromophore of theirs). The same analysis was carried out on their "β subunit" fraction (after enlarging their Figure 2B), yielding the same composition as in our analysis. In other words, their β spectrum almost perfectly matched our composite spectrum computed from the spectra of two different chromophores, i.e., 661- and 595-nm chromophores with a 3:2 ratio. We also see similarity between the elution profiles from the cation-exchange chromatography of isolated subunits (compare their Figure 1 with our Figure 1). We obtained two poorly resolved minor components, B and C, in addition to major fractions A and D, while they did not see the former between the two major fractions. This difference may be due to either different acids used (formic acid vs. acetic acid) or, more likely, our linear gradient elution with HCl–urea. Mörshel & Wehrmeyer (1976) also found two small subunits (α and α') in *Chroomonas* phycocyanin. It is possible that the presence of α' subunit is species dependent.

Molecular Topography. Chroomonas phycocyanin consists of two small and two large polypeptide subunits, forming a tetrameric structure (see beginning of paper).

Mörshel & Wehrmeyer (1976) were able to detect two different small subunits having molecular weights of 9200 and 10400, respectively, by $NaDodSO_4$ gel electrophoresis and proposed the subunit structure $\alpha\alpha'\beta_2$. On the other hand, MacColl et al. (1973, 1976) observed only one band corresponding to a molecular weight size of 10000 for the small subunit in the $NaDodSO_4$ gel, thus proposing the $\alpha_2\beta_2$ structure for the biliprotein. The reported molecular sizes of the small subunits from the above two groups are practically identical and within the range of experimental error of gel electrophoresis.

However, they may be different in other physical and/or chemical properties which are not shown by the gel electrophoretic results. *Chroomonas* phycocyanin yields three (α, α', and β) subunits in our cation-exchange (Bio-Rex 70) procedure which depends on charges rather than molecular weight. Among the four chromatographic fractions eluted from the Bio-Rex 70 column by acidic urea, fractions A, B, and C (Figure 1) represent the three subunits α', α, and β, respectively, as they were not further separated into subcomponents on $NaDodSO_4$ gel electrophoresis. The present work supports the structure $\alpha\alpha'\beta_2$ suggested by Mörshel & Wehrmeyer (1976), since the absorption spectrum of the denatured phycocyanin is a result of the composite spectra $A(\alpha')\beta(\alpha)C(\beta)$ in the ratio of 1:1:2 or in terms of the chromophore ratio of 1:1:4 (Figure 12). A calculation shows that the number of phycocyanobilins per subunit is $\sim$1 [ranging from 0.7 to 1.4, depending on assumed ϵ_{645} values and using $\epsilon_{max} = 3.55 \times 10^4$ M^{-1} cm^{-1} per chromophore of C-phycocyanin in acidic urea (Glazer et al., 1973; Jung, 1978)]. Since the absorption spectrum of the denatured phycocyanin contains a contribution from the 1:1 $\alpha\alpha'$ (i.e., A and B in Figures 4 and 11) subunits, the chromophore content of α' should also be 1. The ratio of phycocyanobilin of the β subunit to that of the α subunit is 1:1 based on the spectral analysis; likewise, the ratio of phycocyanobilin to the second, 597-nm chromophore in the β subunit is also 1:1. Each β subunit contains one phycocyanobilin and one 573-nm chromophore.

The fact that the small and large subunits (α and β, respectively) contain one and two chromophores, respectively, is a common feature for the biliproteins from blue-green and red algae (O'Carra & O'hEocha, 1976; Glazer & Fang, 1973; Troxler et al., 1975). It is interesting that this generality is also applicable to the cryptomonad biliprotein. The denatured phycocyanin gives an absorption spectrum which consists of three spectral components corresponding to three different types of chromophores (Figure 4). On the other hand, the native protein shows a spectrum consisting of five spectral components (Figures 2, 3, and 10). The question then arises as to where the two extra bands in the native protein originate.

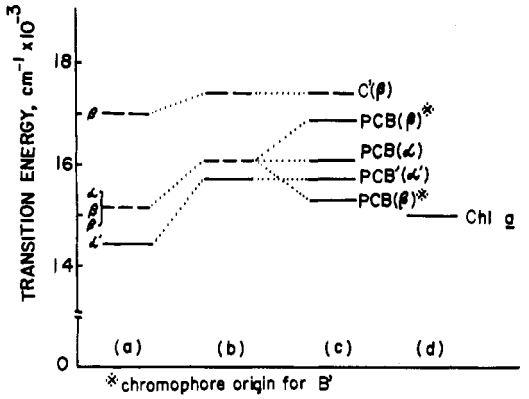

FIGURE 13: Energy level diagram for the *Chroomonas* phycocyanin. (a) In 9 M urea. The chromophores tend to assume circular conformations upon denaturation, as evidenced by a red shift of the Q_y band and hyperchromicity of the Soret band. (b) Hypothetical, protein-bound chromophores in the absence of dipole–dipole coupling of two PCB molecules. (c) The effect of exciton split between two adjacent PCB's. (d) The Q_y band of chlorophyll *a* is shown for comparison.

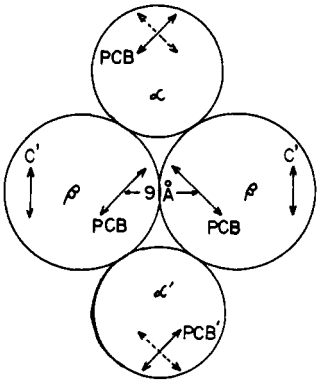

FIGURE 14: Possible chromophore arrangement for the *Chroomonas* phycocyanin. (PCB) Phycocyanobilin; (PCB') environmentally and/or covalently linking variant of PCB; (C') phycoerythrobilin-like chromophore. The molecular weights of α (and α') and β subunits are roughly 10 000 and 15 000, respectively. Note that the binding of the smaller subunits to the β subunits enforces the interaction among subunits (see text). The double arrows represent transition moments for the corresponding states shown in Figure 13. The dotted double arrows on α and α' represent alternative orientations.

Insight can be obtained from the chromophore–protein interaction. Phycocyanobilin chromophores in α and β subunits do not have a significant, conformationally dependent interaction with polypeptide chains in the presence of urea (Figure 7); thus, their spectra should be identical with each other, as observed. In the native state, however, phycocyanobilin chromophores in different subunits must experience different environments due to different conformational interaction with their respective polypeptide chains, resulting in distinctive optical properties (Figures 7 and 10).

The chromophore binding site of *Chroomonas* phycocyanin does not involve a Trp residue(s). Denaturation of phycocyanin with $NaDodSO_4$ did not result in an increase of the fluorescence intensity of a Trp residue(s), suggesting that there is no significant interaction between the aromatic amino acid and the chromophore in the phycocyanin. It should also be noted that the chromophores of the native phycocyanin are rigidly held at their binding sites, giving rise to large, induced CD, since the CD intensity of denatured protein is negligible (Figure 7).

However, the chromophore–protein interaction cannot completely resolve the problem, as at most only four absorption bands can be expected: one band from the α subunit and two from the β subunit. To account for the five-component composite spectrum of the native *Chroomonas* phycocyanin, we note that the CD spectrum (Figure 7) shows + (592 nm) and − (657 nm) extrema. We attribute this CD splitting to a dipole–dipole coupling (exciton) between the two interacting chromophore molecules. These CD bands have approximately perpendicularly oriented transition dipoles (Figure 3). During the renaturation process of the $\alpha\beta_2$ fraction (D) (Figure 1), addition of α or α' to the former simultaneously induced two excitation peaks at 590 and 650 nm (Figure 9).

Furthermore, the rates of change in $\Delta A/\Delta A_0$ with $KMnO_4$ or $Hg(OAc)_2$ (Figure 10) are identical for the 592- and 657-nm regions. Thus, it is apparent that these bands are due to exciton-type coupling between the two interacting chromophores, resulting in the five-component composite spectrum (Figure 11). The exciton split, $\Delta E_{\pm}$, is calculated as 1671 cm^{-1} from the CD extrema (Figure 3). This corresponds to the chromophore–chromophore transition dipole distance of ~9 Å, using a simple dipole approximation (Kasha et al., 1975).

We assign the dipole-coupled chromophore pair to the β subunits of phycocyanobilin (PCB); the lower energy component (652 nm) of the exciton pair is the fluorescent state. The third PCB then accounts for the 617-nm band (Figure 11), which is associated with an α subunit. Figure 13 illustrates the energy states of chromophores in *Chroomonas* phycocyanin.

Figure 14 depicts a qualitative molecular topography of the *Chroomonas* phycocyanin photoreceptor. The two PCB dipoles are coupled, with perpendicular polarizations for the resulting transitions (Figure 3). The oscillators for α and α' subunits are neither perpendicular nor parallel, as the fluorescence polarization suggests (Figure 3). The fluorescence polarization of the emitting 657-nm excitation band is substantially lower than the theoretical maximum value of 0.5, even under conditions where no rotational depolarization is significant (Figure 3). We attribute this difference to the overlapping bands, particularly by the α'-subunit chromophore (PCB') with its transition dipole oriented at some angle to the fluorescence oscillator.

The two β subunits provide the site for exciton interactions. Such interactions are apparently strengthened by the addition of α and α' subunits as suggested by an enhanced fluorescence intensity (Figure 9). These β-bound chromophores are more exposed to the bulk medium than others (cf. Figure 10). The biological significance of exciton interactions in the phycocyanin can be described in terms of two consequences: (a) the visible absorption coverage is expanded, and (b) the energy transfer from phycocyanin to chlorophyll is enhanced due to the increased spectral overlap integral and the state degeneracy between Q_y states of the chromophores involved, as has been elucidated for dinoflagellate anntenna photoreceptors (Song et al., 1976; Koka & Song, 1977).

Alternatives to the proposed model shown in Figure 13 can be considered in terms of the α chromophore providing the fluorescent (f) state. For example, let us consider the following two cases.

Case I: No β–β Exciton Coupling and α as the Fluorescent Chromophore. This model is not compatible with the five-component absorption spectrum of type I phycocyanin (Figure 11). Since $\alpha\beta_2$ exhibits spectral characteristics of intact protein, α–α dipole–dipole coupling can be excluded. If $\alpha_2\beta_2$ were the subunit composition, no more than three spectral components would have arisen.

Case II: β–β Exciton Coupling but α as the Fluorescent Chromophore. The α subunit of C-phycocyanin from blue-

green and red algae absorbs at a wavelength longer than the β subunit (Glazer et al., 1973); this was considered a confirmation of the fluorescent (f) chromophore (α) (Teale & Dale, 1970). The five-component spectrum can be accounted for in terms of this model. However, results shown in Figures 9 and 10 are not compatible with this model.

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References

Barber, D. J. W., & Richards, J. T. (1976) *Photochem. Photobiol. 25*, 565.
Bennett, A., & Bogorad, J. L. (1971) *Biochemistry 10*, 3625.
Bogorad, L. (1975) *Annu. Rev. Plant Physiol. 26*, 369.
Brody, S. S., & Rabinowitch, E. (1957) *Science 125*, 555.
Chapman, D. J. (1973) in *The Biology of Blue-Green Algae* (Whitton, B. A., & Carr, N. G., Eds.) p 171, University of California Press, Berkeley, CA.
Chapman, D. J., & Siegelman, H. W. (1967) *J. Am. Chem. Soc. 89*, 5979.
Chapman, D. J., Cole, W. J., & Siegelman, H. W. (1968) *Phytochemistry 7*, 1831.
Cole, W. J. (1967) *Eur. J. Biochem. 3*, 202.
Dodge, J. A. (1969) *Arch. Mikrobiol. 69*, 266.
Edward, M. R., & Gantt, E. (1971) *J. Cell Biol. 50*, 896.
Faust, M. A., & Gantt, E. (1973) *J. Phycol. 9*, 489.
Fish, W. W., Mann, K. G., & Tanford, C. (1969) *J. Biol. Chem. 244*, 4989.
Gantt, E., & Conti, S. F. (1966) *Brookhaven Symp. Biol. 19*, 393.
Gantt, E., Edward, M. R., & Provasoli, L. (1971) *J. Cell Biol. 48*, 280.
Glazer, A. N. (1976) *Photochem. Photobiol. Rev. 1*, 70.
Glazer, A. N., & Fang, S. (1973) *J. Biol. Chem. 248*, 659.
Glazer, A. N., & Cohen-Bazire, G. (1975) *Arch. Microbiol. 104*, 29.
Glazer, A. N., Fang, S., & Brown, D. M. (1973) *J. Biol. Chem. 248*, 5679.
Jung, J. (1978) Ph.D. Dissertation, Texas Tech University, Lubbock, TX.
Kasha, M., Rawls, H., & El-Bayoumi (1965) *Pure Appl. Chem. 11*, 137.
Killilea, S. D., & O'Carra, P. (1968) *Biochem. J. 110*, 457.
Koka, P., & Song, P.-S. (1977) *Biochim. Biophys. Acta 495*, 220.
MacColl, R., & Berns, D. S. (1978) *Photochem. Photobiol. 26*, 246.
MacColl, R., Habig, W., & Berns, D. S. (1973) *J. Biol. Chem. 248*, 153.
MacColl, R., Berns, D. S., & Gibbons, O. (1976) *Arch. Biochem. Biophys. 177*, 266.
MacDowall, F., & Walker, M. (1968) *Photochem. Photobiol. 7*, 109.
Moore, T. A. (1975) Ph.D. Dissertation, Texas Tech University, Lubbock, TX.
Mörshel, E., & Wehrmeyer, W. (1976) *Arch. Microbiol. 105*, 153.
O'Carra, P., & O'hEocha, C. (1976) in *Chemistry and Biochemistry of Plant Pigments* (Goodwin, T. W., Ed.) p 328, Academic Press, New York.
O'Carra, P., O'hEocha, C., & Carroll, D. M. (1964) *Biochemistry 3*, 1943.
O'hEocha, C. (1963) *Biochemistry 2*, 375.
O'hEocha, C. (1965) *Annu. Rev. Plant Physiol. 16*, 415.
O'hEocha, C., O'Carra, P., & Mitchell, D. (1964) *Proc. R. Ir. Acad., Sect. B 63*, 191.
Song, P.-S. (1978) *Chem. Soc. Annu. Rep. 74*, 18–40.
Song, P.-S., Koka, P., Prézelin, B., & Haxo, F. (1976) *Biochemistry 15*, 4422.
Takemoto, A., & Bogorad, L. (1975) *Biochemistry 14*, 1211.
Teale, F. W. J., & Dale, R. E. (1970) *Biochem. J. 116*, 161.
Troxler, R. F., Foster, J. A., Brown, A. S., & Franzblau, C. (1975) *Biochemistry 14*, 268.
Wildman, R. B., & Bowen, C. O. (1974) *J. Bacteriol. 117*, 866.