Chromophore Interactions in Allophycocyanin†

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ABSTRACT: Allophycocyanin, which is normally isolated as a trimer ($\alpha_3\beta_3$), has now been successfully dissociated into a monomer ($\alpha\beta$) with strikingly different spectroscopic properties. In particular, upon dissociation the characteristic 650-nm absorption and 661-nm fluorescence emission bands of the trimer are completely lost and its fluorescence polarization properties are sharply altered. The spectroscopic characteristics of allophycocyanin monomers are much closer to those of C-phycocyanin than to trimeric allophycocyanin. A model for trimeric allophycocyanin is presented in which the appearance of the 650-nm absorption band is induced by a particular kind of chromophore–chromophore interaction. Similar results are found for both allophycocyanin II and III.

Allophycocyanins, C-phycocyanin, and phycoerythrins are chromoproteins with covalently attached linear tetrapyrrole groups that function as accessory pigments in the photosynthesis of blue-green and red algae. Allophycocyanin is characterized by its absorption spectrum (A_{max} = 650 nm) and its fluorescence emission (660 nm). It is composed of two polypeptide chains (α and β) which are present in equimolar amounts (Brown et al., 1975; Brown & Troxler, 1977; Gysi & Zuber, 1974, 1976). Both C-phycocyanin and allophycocyanin possess chemically identical chromophores (Chapman et al., 1967), but allophycocyanin has one attached to each α and β subunit while C-phycocyanin has two on each β and one on each α subunit (Glazer & Fang, 1973). Allophycocyanin is isolated between pH 6.0 and 7.0 as a trimer, $\alpha_3\beta_3$ (Kim et al., 1978; Cohen-Bazire et al., 1977), and is associated in vivo with the phycobilisomes (Gantt & Lipschultz, 1974; Gantt et al., 1976, 1979).

Three spectroscopic properties of allophycocyanin are not readily understood. (1) Although it has only two subunits and one type of chromophore, the absorption spectrum in the orange-red region shows at least three distinct transitions: 600, 625, and 650 nm (Gantt & Lipschultz, 1974; Bennett & Bogorad, 1971). (2) Its fluorescence polarization ($\alpha_3\beta_3$) is substantially lower than that of C-phycocyanin ($\alpha_3\beta_3$ or $\alpha_6\beta_6$) (MacColl et al., 1978). (3) Its fluorescence polarization spectrum between 610 and 640 nm (Cohen-Bazire et al., 1977; Grabowski & Gantt, 1978) does not exhibit the different regions that are associated with sensitizing and fluorescing chromophores in C-phycocyanin (Dale & Teale, 1970; Teale & Dale, 1970).

To understand these phenomena, we have developed the first methods to dissociate trimeric allophycocyanin into monomers ($\alpha\beta$). Spectroscopic study to compare allophycocyanin monomers with its trimers, as well as with C-phycocyanin monomers and polymers, has produced some understanding of these processes.

Experimental Section

Allophycocyanin was isolated and purified from the blue-green alga *Phormidium luridum*. For purification the protein soluble between 35 and 50% saturated ammonium sulfate at pH 6.0 was placed on a hydroxylapatite column. Allophycocyanin II was selectively purified on the hydroxylapatite column by a modification of the techniques of Zilinskas et al. (1978). A mixture of C-phycocyanin and allophycocyanin was placed on the column of 0.001 M potassium phosphate and 0.10 M sodium chloride. The C-phycocyanin was removed by elution with 0.040 M phosphate. The column was then eluted with 0.070 M phosphate and fractions were collected. As expected, allophycocyanin II (A_{620}/A_{653} = 0.62) eluted first followed by mixtures of types II and III. Isoelectric focusing of this allophycocyanin II showed a single band of protein. Only minute amounts of other bands were visible; these bands were discarded. The fractions containing both II and III were also run on the isoelectric column which separated the two, and very pure allophycocyanin III (A_{620}/A_{653} = 0.45) was thus obtained. Allophycocyanin II was used in most of the following experiments. The hydroxylapatite column also removed the allophycocyanin with relatively high absorbance at 680 nm (Ley et al., 1977; Glazer & Bryant, 1975; Zilinskas et al., 1978). The purest fractions were precipitated with ammonium sulfate and stored at 4 °C. Sodium dodecyl sulfate gel electrophoresis of this material showed no impurities.

C-Phycocyanin (*P. luridum*) was purified by ammonium sulfate fractionation and isoelectric focusing (LKB Model 8101) in a pH 4–6 gradient.

Absorption spectra were taken on a Cary 118 spectrophotometer. Fluorescence emission and excitation spectra were obtained on a Perkin-Elmer MPF-44A fluorescence spectrophotometer equipped with an R777 HTV photomultiplier in the ratio mode. The degree of polarization was calculated as $p = (I_{vv} - GI_{vh})/(I_{vv} + GI_{vh})$, where $G = I_{hv}/I_{hh}$, a correction factor for the polarization due to the optics in the instrument. Protein concentrations were chosen so that reabsorption of emission was negligible. The fluorescence excitation spectra were corrected for the spectral radiance of exciting light. The emission spectra were not corrected, but these spectra were only used on a comparative basis. The photomultiplier response in the region considered was linear. Both the excitation and observation half-bandwidths were set at 6 nm.

Samples were prepared by exhaustively dialyzing aliquots of purified stock protein overnight into the appropriate buffer: sodium phosphate, pH 6.0 or 7.0; sodium acetate, pH 4.7 or 3.9. All buffers were 0.10 ionic strength. Protein concentrations were estimated from the specific extinction coefficient of 6.35 at 650 nm and pH 7.0 (Brown & Troxler, 1977).

Sedimentation equilibrium experiments were carried out as described previously (Kim et al., 1978) with 0.1 mg of allo-

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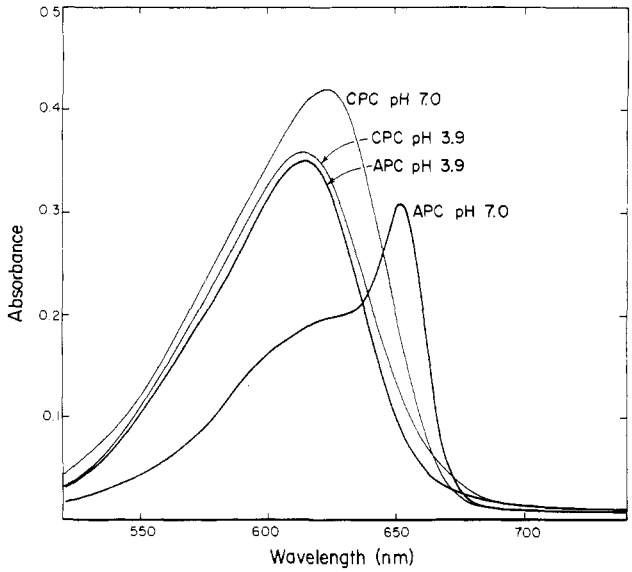

FIGURE 1: Absorption spectra of allophycocyanin (APC) and C-phycocyanin (CPC) at pH 3.9 and 7.0.

phycocyanin per mL at pH 3.9. The rotor speed was 34 000 rpm and the temperature 21 °C. The partial specific volume used in the calculations was 0.738 mL/g. Sedimentation velocity experiments were performed at 60 000 rpm with the photoelectric scanner of the Model E analytical ultracentrifuge.

Results

At pH 3.9 the sedimentation equilibrium results for allophycocyanin showed a single species (i.e., a linear plot of d ln c vs. dr^2) with a molecular weight of 32 400. Each data set had a precision of better than 0.5%. Since the subunit molecular weights are 15 000–18 000, the pH 3.9 material was considered to be a monomer. C-Phycocyanin, which has a molecular weight of 35 000 at pH 3.9, is also monomeric. Sedimentation velocity experiments on allophycocyanin at pH 3.9 showed a single boundary with $s_{20,w}$ of 2.7 S, which is a typical value for a globular protein of 32 400 molecular weight.

The absorption spectra of allophycocyanin and C-phycocyanin are shown in Figure 1. The two biliproteins differed saliently at the higher pH, where allophycocyanin is a trimer and C-phycocyanin is a mixture of aggregates, but they were virtually identical in the monomer state. Fluorescence excitation and emission spectra were obtained at different pH values (Figure 2). At pH 6.0 and 7.0 allophycocyanin was in the trimer state, with a fluorescence maximum at 661 nm. At pH 3.9 it was in the monomer state, with an emission maximum at 642 nm, and the absorption and fluorescence excitation spectra diminished concurrently in the 650-nm region. Dialysis of the pH 3.9 solution to pH 6.0 partially restored the 650-nm band. Brown & Troxler (1977) were able to regenerate a typical allophycocyanin spectrum from the denatured subunits.

To better understand the process of generating the 650-nm absorption band, we selected an intermediate pH of 4.7. A pH 4.7 solution was diluted serially and each dilution studied by absorption and fluorescence spectroscopy. At 1.5 h after dilution the fluorescence emission spectra (Figure 3) were consistent with a monomer–trimer equilibrium which is totally monomeric at the lowest concentrations. Both fluorescence excitation and absorption spectra showed a lowering of the A_{650}/A_{625} ratio with dilution (data not shown). These spectroscopic changes were also examined as a function of time (Figure 4). Again the results clearly suggested a monomer–trimer equilibrium with the 661-nm emission decreasing gradually and the 642-nm band simultaneously increasing.

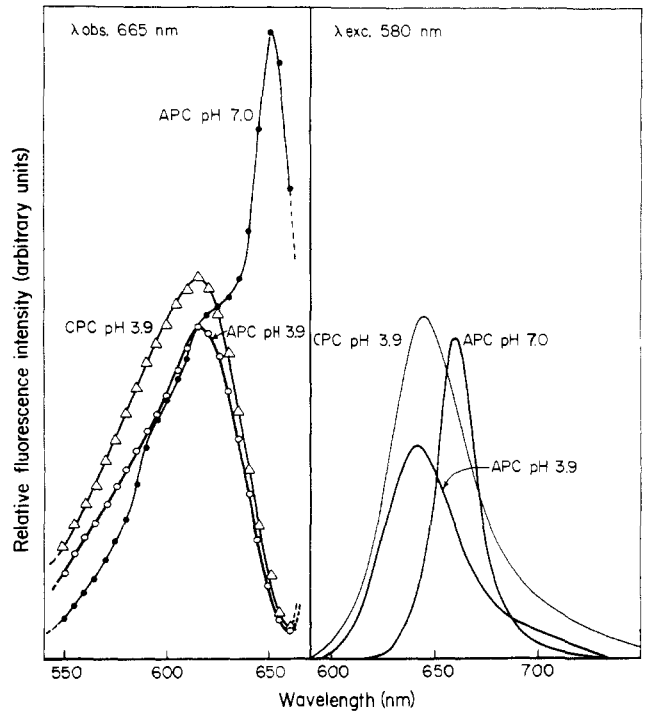

FIGURE 2: Fluorescence excitation and emission spectra of allophycocyanin at pH 3.9 and 7.0 and C-phycocyanin at pH 3.9.

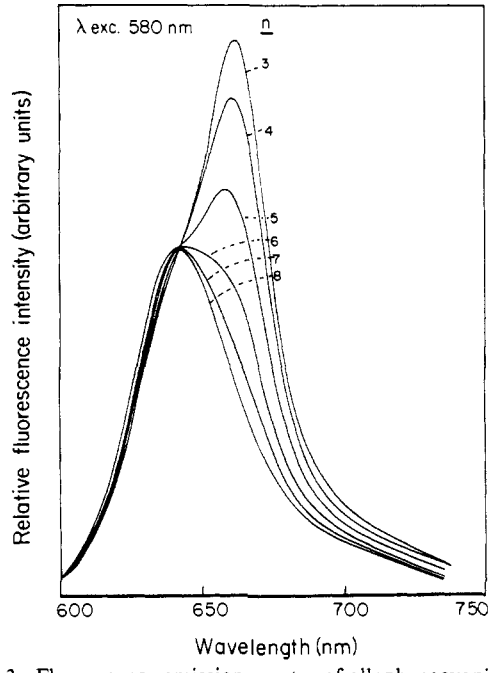

FIGURE 3: Fluorescence emission spectra of allophycocyanin at pH 4.7. The spectra were obtained 1.5 h after a solution of A_{650}^{1cm} = 2.0 had been diluted consecutively with additional pH 4.7 buffer by a factor of $(1/2)^n$, where n is given in the figure. At this time the absorption at 650 nm of the n = 3 dilution was ~0.1. The spectra were normalized at 642 nm.

Fluorescence polarization spectra have showed two major differences between C-phycocyanin and allophycocyanin (MacColl et al., 1978). Allophycocyanin trimer has a lower degree of polarization and lacks the stepwise increase in polarization in the wavelength region within bands. These differences are not found in the polarization spectra of monomeric allophycocyanin and C-phycocyanin (Figure 5). The

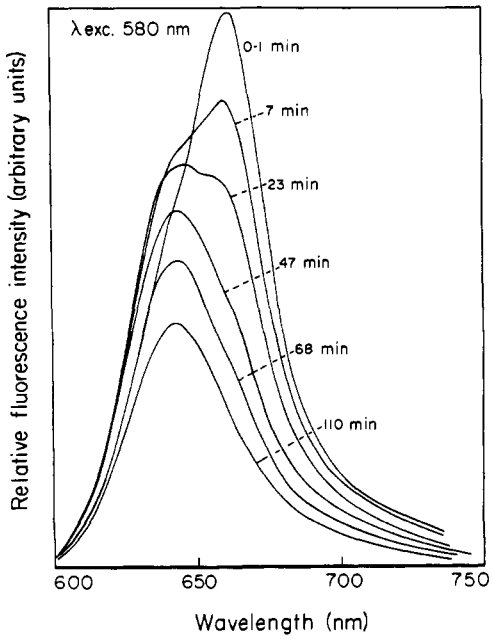

FIGURE 4: Fluorescence emission spectra of allophycocyanin at pH 4.7 diluted as described in Figure 3 for the most dilute solution. Spectra were recorded at the time intervals indicated in the figure. The same experiment at pH 7.0 yielded similar results, but dissociation was much smaller.

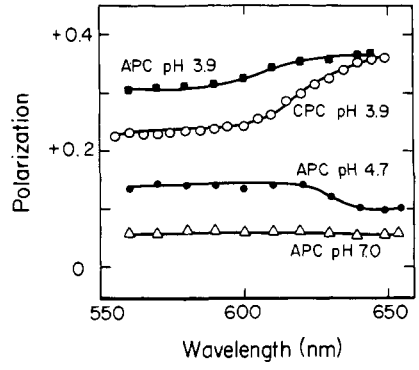

FIGURE 5: Fluorescence polarization spectra of C-phycocyanin and allophycocyanin. The observation wavelength was 700 nm; both excitation and observation half-bandwidths were set at 6 nm. The pH values of the samples are indicated in the figure. Similar results were obtained in all cases with a 670-nm observation wavelength.

allophycocyanin monomers manifest the two plateaus, and the degree of polarization is actually higher than that of C-phycocyanin in the low-wavelength region. Allophycocyanin polarization is higher in the lower wavelength region, suggesting better alignment between the chromophores. At pH 4.7 the spectrum apparently is a combination of values typical of trimers and monomers for an allophycocyanin II solution with an A_{650} of 0.1. The lower trimer polarization dominates near 650 nm, since the monomers have negligible absorbance in this region.

Allophycocyanin III was likewise observed at both pH 3.9 and 6.0–7.0 by absorption and fluorescence spectroscopy. The same loss of 650-nm absorption noted above for allophycocyanin II was found together with the loss of 661-nm emission at pH 3.9 (not shown).

Discussion

Allophycocyanin monomers have both the α and β subunits that characterize the protein, but they lack the 650-nm absorption band and consequently the 661-nm emission band. In the trimers, however, we propose that chromophore–chromophore interaction between spatially close and properly oriented tetrapyrroles is responsible for the 650-nm absorption band and thus produces the functionally important 661-nm emission.

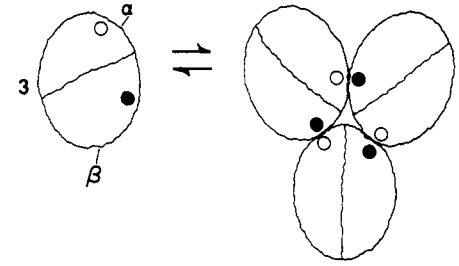

FIGURE 6: A simplified pictorial model for the unique chromophore–chromophore interaction in allophycocyanin (II and III) that may be responsible for the 650-nm absorption band. An alternative to the depicted intersubunit chromophore interaction which would achieve the same ultimate chromophore interaction would be a conformation change that occurs upon aggregation to trimers. This conformational change would result in close proximity of the two chromophores on the monomer subunit that produces the special chromophore pairs. It is not mandatory that all three pairs of chromophores be totally identical. Only one pair is required to produce the 650-nm band.

In contrast, C-phycocyanin experiences a blue shift (Figure 1) when pH 4.7–7.0 solutions, which are mostly hexamers or trimers, are adjusted to pH 3.9 (monomer), but no absorption bands appear or disappear. Nevertheless, recent picosecond studies on C-phycocyanin show that the larger aggregates have better chromophore–chromophore positioning for excitation energy transfer than monomers (Kobayashi et al., 1979). In the absence of allophycocyanin's special chromophore–chromophore interaction the two biliproteins are difficult to distinguish by absorption or emission spectroscopy (Figures 1 and 2). The major spectroscopic distinction between them is that when allophycocyanin monomers are assembled into trimers, a special interaction occurs between the chromophores, resulting in the 650-nm interaction band.

Allophycocyanin and C-phycocyanin differ also in the extent of polarization. Goedheer & Birnie (1965) demonstrated for C-phycocyanin that there is an inverse relationship between aggregate size and polarization. Vernotte determined that between 620 and 630 nm C-phycocyanin monomers have +0.39–0.41 polarization, trimers have +0.29–0.31 (Vernotte, 1971), and hexamers have +0.09–0.10 (C. Vernotte, personal communication). Allophycocyanin (*P. luridum*) in trimer form (Figure 5) has a polarization of +0.05, which is very low compared to a similar size of C-phycocyanin. This is even more remarkable because allophycocyanin has fewer chromophores per similarly sized unit.

Introduction of an entirely new chromophore arrangement for allophycocyanin would make the low polarization understandable. The usual partition into sensitizing and fluorescing chromophores could vanish, depending on the particular chromophore combination that produces the 650-nm band. We propose that an interaction occurs in the trimer within special chromophore pairs, formed when chromophores on the α subunits are brought into proximity with the chromophores on the β subunits (Figure 6). Förster very weak dipole–dipole coupling can occur between the pairs and thus allows for a possible lowering of the fluorescence polarization. The depolarization is dependent on the angles among the six tetrapyrroles.

The nature of the chromophore–chromophore interaction in these trimers is unknown. Many examples of excitation energy transfer involving either very weak or strong coupling

of dipoles are known for both photosynthetic and nonphotosynthetic pigments. In the Dale and Teale hypothesis for excitation energy transfer for C-phycocyanin the transfers are via very weak coupling (Förster, 1959). Such coupling, however, would not generate the 650-nm absorption band because this mechanism maintains the monomer's spectrum. Another prominent mechanism for energy transfer is strong dipole–dipole coupling. However, the circular dichroism spectrum of allophycocyanin (Brown et al., 1975; Brown & Troxler, 1977; Gysi & Zuber, 1979) excludes the presence of strongly coupled excitons, since it is not conservative with equal negative and positive bands (Tinoco, 1963).

Excitation energy transfer by intermediate coupling, on the other hand, would split the monomer spectrum in a way consistent with allophycocyanin's electronic spectrum (Kasha, 1963). Cryptomonad phycocyanin has recently been discussed in terms of exciton interaction (Jung et al., 1980). The alternative to our proposed exciton splitting is that certain of the tetrapyrroles must have unique conformational states or environments. In addition, a complex ordering of the chromophore dipoles would be required to explain the fluorescence characteristics. The advantage of our model is that the single assumption of the special chromophore interaction explains both the 650-nm absorption and the fluorescence properties without the need for additional assumptions.

References

Bennett, A., & Bogorad, L. (1971) *Biochemistry 10*, 3625.

Brown, A. S., & Troxler, R. F. (1977) *Biochem. J. 163*, 571.

Brown, A. S., Foster, J. A., Voynow, P. V., Franzblau, C., & Troxler, R. F. (1975) *Biochemistry 14*, 3581.

Chapman, D. J., Cole, W. J., & Siegelman, H. W. (1967) *Biochem. J. 105*, 903.

Cohen-Bazire, G., Béguin, S., Rimon, S., Glazer, A. N., & Brown, D. M. (1977) *Arch. Microbiol. 111*, 225.

Dale, R. E., & Teale, F. W. J. (1970) *Photochem. Photobiol. 12*, 99.

Förster, T. (1959) *Discuss. Faraday Soc. 27*, 7.

Gantt, E., & Lipschultz, C. A. (1974) *Biochemistry 13*, 2960.

Gantt, E., Lipschultz, C. A., & Zilinskas, B. (1976) *Biochim. Biophys. Acta 430*, 375.

Gantt, E., Lipschultz, C. A., Grabowski, J., & Zimmerman, B. K. (1979) *Plant Physiol. 63*, 615.

Glazer, A. N., & Fang, S. (1973) *J. Biol. Chem. 248*, 659.

Glazer, A. N., & Bryant, D. A. (1975) *Arch. Microbiol. 104*, 15.

Goedheer, J. C., & Birnie, F. (1965) *Biochim. Biophys. Acta 94*, 579.

Grabowski, J., & Gantt, E. (1978) *Photochem. Photobiol. 28*, 39.

Gysi, J., & Zuber, H. (1974) *FEBS Lett. 48*, 209.

Gysi, J., & Zuber, H. (1976) *FEBS Lett. 68*, 49.

Gysi, J. R., & Zuber, H. (1979) *Biochem. J. 181*, 577.

Jung, J., Song, P.-S., Paxton, R. J., Edelstein, M. S., Swanson, R., & Hazen, E. E., Jr. (1980) *Biochemistry 19*, 24.

Kasha, M. (1963) *Radiat. Res. 20*, 55.

Kim, C. S., Moross, G., Moyer, W., Kaufman, G., MacColl, R., & Edwards, M. R. (1978) *Anal. Biochem. 86*, 371.

Kobayashi, T., Degenkolb, E. O., Bersohn, R., Rentzepis, P. M., MacColl, R., & Berns, D. S. (1979) *Biochemistry 18*, 5073.

Ley, A. C., Butler, W. L., Bryant, D. A., & Glazer, A. N. (1977) *Plant Physiol. 59*, 974.

MacColl, R., Edwards, M. R., & Haaksma, C. (1978) *Biophys. Chem. 8*, 369.

Teale, F. W. J., & Dale, R. E. (1970) *Biochem. J. 116*, 161.

Tinoco, I., Jr. (1963) *Radiation Res. 20*, 133.

Vernotte, C. (1971) *Photochem. Photobiol. 14*, 163.

Zilinskas, B. A., Zimmerman, B. K., & Gantt, E. (1978) *Photochem. Photobiol. 27*, 587.