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SOME PROPERTIES OF ALLOPHYCOCYANIN FROM A THERMOPHILIC BLUE-GREEN ALGA

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Allophycocyanin was purified from the extremely thermophilic blue-green alga *Synechococcus lividus*. It was shown to be more stable to thermal or urea denaturation than allophycocyanin from a mesophilic organisms. Its amino acid composition and spectroscopic response to pH were investigated.

An analysis was made of the relatively low fluorescence polarization of allophycocyanin compared to that of a comparable sized aggregate of the biliprotein, C-phycocyanin. A rather speculative conclusion was reached that suggests that the lower polarization of allophycocyanin may be caused by orientations or positioning of the chromophores that are more favorable for intra-protein energy transfer.

1. Introduction

Allophycocyanin, a chromoprotein containing covalently bound tetrapyrrole groups, is located in the phycobilisomes of blue-green and red algae [1]. This protein participates in the transfer of excitation energy to the chlorophyll in the thylakoid membranes [2]. A structural model of the phycobilisome has been developed which places allophycocyanin in the core, adjacent to the membrane [3]. It has not been found in cryptomonads, the third class of algae which contains biliproteins [4].

Previous investigations of protein from thermophilic algae have focused on C-phycocyanin. In this paper we report for the first time the properties of allophycocyanin from a blue-green alga, *Synechococcus lividus*, which grows at temperatures as high as 73°C [5,6,7].

Allophycocyanin is composed of two different polypeptides in a 1:1 molar ratio as demonstrated by the sequencing studies of Brown et al. [8] and Brown and Troxler [9] and the subunit separation experiments of Gysi and Zuber [10,11]. The isolated protein was found by sedimentation equilibrium studies to have a molecular weight of about 103000 [12,13]; and each subunit molecular weight, obtained by electrophoresis in sodium dodecyl sulfate gels, proved to be about 15–18000. Thus a major in vitro form of allophycocyanin isolated from both mesophiles and thermophiles has an $\alpha_3\beta_3$ structure. In the present study we probed the relative stability of allophycocyanin obtained from each type of organism. In addition, the fluorescence polarization spectrum of allophycocyanin was examined.

2. Materials and methods

2.1. Extraction of allophycocyanin

Ten grams of cells were suspended in 100 ml of phosphate buffer, ionic strength (I) 0.1, pH 6.0, containing 10 g of mannitol as an osmotic stabilizer. Lysozyme (Sigma, 25000 units/mg) was added to a final concentration of 0.1%. The mixture was stirred at 37°C for 2 h and then at 4°C for 16 h. The cells were centrifuged at 1000g for 20 min and ruptured by resuspending the pellet in phosphate buffer without mannitol. The suspension was stirred at 4°C and centrifuged at 17000 g for 20 min. The buffer extraction and centrifugation were repeated twice daily until the supernatant was no longer blue. The extracts were stored in 50% saturated ammonium sulfate and cleaned of cellular debris by repeated centrifugation at 23000g for 30 min.

2.2. Purification of allophycocyanin

The absorbance maximum for the lowest energy-excited state of *S. lividus* allophycocyanin is 653 nm. Our primary criteria for protein purity were $A_{653}/A_{280} > 4.0$ and $A_{653}/A_{620} > 2.0$ in sodium phosphate buffer, pH 6.0, and 0.10 *I*.

Samples were placed on discontinuous sucrose gradients (2.0, 1.5, 1.0, 0.8, and 0.6 M sucrose) in phosphate buffer and centrifuged for 20 h at 140000g and 5°C in a Beckman type 40 rotor. Fractions enriched in A_{653} were pooled and precipitated with ammonium sulfate. This material was pelleted and dissolved in phosphate buffer for subsequent purification by isoelectric focusing.

Isoelectric focusing was done in a 110-ml LKB Ampholine column (8101) with a sucrose gradient and a pH 4–6 ampholyte. Allophycocyanin focused in two major bands and a third, thinner band all so close together that they could not be collected in separate fractions. The absorbance of each fraction was measured on a Gilford model 2400 spectrophotometer. Two runs through the column were necessary to obtain material with an A_{653}/A_{620} ratio of 2.1 to 2.2 (Cary 14 spectrophotometer). Centrifugation at 140000g for 30 min was used to remove insoluble material and produced an A_{653}/A_{280} ratio of 4.0 to 4.5. Protein was shown pure by sodium dodecyl sulfate gel electrophoresis.

The purified allophycocyanin was stored at 4°C under 50% saturated ammonium sulfate. For use, aliquots were pelleted by centrifugation, dissolved in pH 6.0 buffer, and dialyzed overnight at 4°C against the same buffer.

Small amounts of protein with unique spectral characteristics have been found associated with some allophycocyanin preparations [14–16]. These proteins have a higher absorbance at 680 nm relative to A_{653} than the major allophycocyanin form. Therefore the absorbance at 680 nm of fractions taken in isoelectric focusing experiments was measured, and any fraction with an elevated reading was not included in the purified preparation.

2.3. Denaturation experiments

Thermal denaturation experiments were performed on an Acta II spectrophotometer (1-cm light path). The temperature in the sample chamber was varied by water circulating from a Haake bath (model F). The thermal characteristics of the system were obtained by comparing the Haake bath temperature with the temperature of buffer in a test cuvette in the Acta II, measured with a copper-constantan thermocouple and a millivolt potentiometer (8690, Leeds and Northrup). The rate of heating was 40°C in 8 h. At each temperature the absorption was measured until a constant value was obtained.

Denaturation of the protein by urea was monitored on a Gilford spectrophotometer (1-cm light path). Since absorbances of samples treated with various concentrations of urea at ambient temperature (23°C) were constant after 4 h, all absorbances are reported as of that time. The urea (ultra-pure grade of Schwarz-Mann Co.) was purified by eluting a urea solution through a mixed ion exchange resin (AG-501-X8 analytic grade, Bio-Rad Laboratories). The purified urea was then evaporated to dryness.

Although data on urea and thermal denaturation of mesophilic allophycocyanins are available [8,9,17], no attempt was made to duplicate the experimental protocols of these investigators. Instead, our experiments with purified allophycocyanin from *Phormidium luridum* were performed under experimental conditions that exactly matched those for *S. lividus* allophycocyanin.

The effect of pH on the absorption spectrum of allophycocyanin was followed by first dialyzing the protein into pH 6.0, sodium phosphate buffer to remove residual ammonium sulfate. Equal volumes of this solution were then pipetted into dialysis sacs and the aliquots dialyzed overnight at 4°C into buffers of various pH values. The sacs were tightly closed to avoid dilution problems. The experiments were performed at room temperature.

2.4. Amino acid analysis

Samples for amino analysis were hydrolyzed in 6 N HCl for 24, 48 and 72 h under vacuum at 113°C. For cysteic acid analysis the procedure of Spencer and Wold [18] was used.

2.5. Fluorescence experiments

The absorbance of the sample used for fluorescence

studies was 0.12 at 653 nm. Fluorescence polarization experiments were performed on a Perkin-Elmer MPF-44A fluorescence spectrophotometer equipped with an R777 HTV photomultiplier. Emission was set at 700 or 720 nm for these measurements, and the instrument was operated in the ratio mode with excitation slits of 6 nm and emission slits at 10 nm.

Polarizations were calculated by

$$p = (I_{vv} - GI_{vh})/(I_{vv} + GI_{vh}) \quad (1)$$

where I is the intensity of light at a given wavelength, G is I_{hv}/I_{hh}, and v and h are vertically and horizontally polarized light, the first subscript referring to the exciting light and the second to the fluorescence emitted.

Fluorescence emission spectra were obtained using an RCA 4832 photomultiplier, which has a flat spectral response int the region of interest.

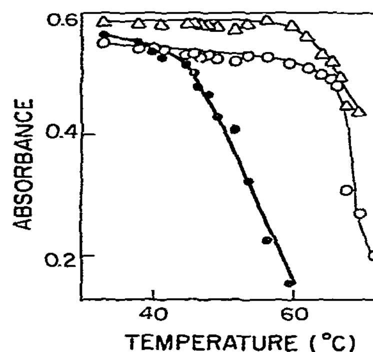


Fig. 1. Effect of temperature on absorbance of *S. lividus* allophycocyanin (○), *P. luridum* allophycocyanin (●), and *S. lividus* C-phycocyanin (△). The absorbances of the allophycocyanins were monitored at 652 nm and those of C-phycocyanin at 620 nm. Results of a single typical experiment are shown.

3. Results and discussion

The allophycocyanin from *S. lividus* has spectroscopic and subunit properties very similar to those of allophycocyanins from mesophilic organisms. The structural arrangement of the biliproteins in this thermophile is also generally the same as in various mesophilic blue-green or red algae [1,5,19–23]. However, *S. lividus* allophycocyanin is stable at much higher temperatures (fig. 1). Its C-phycocyanin is also stable (fig. 1). Similarly, the thermophilic protein is much more resistant to denaturation by urea than is the mesophile (fig. 2). However, only one mesophile allophycocyanin was compared to only one thermophile allophycocyanin, so these results are not necessarily definitive. The results in fig. 2 show the change in A_{653}. The A_{620} for the thermophilic protein was essentially unchanged even after 4 h at 8.0 M urea; the A_{620} of mesophilic allophycocyanin bleached considerably at much shorter times. In both cases, however, the loss of A_{653} was more rapid than that at 620 nm. Our observation of the relative resistance to denaturation of thermophilic versus mesophilic allophycocyanin agree with those obtained for a variety of proteins from other organisms [24–27]. Changes in absorption upon denaturation have long been noted [28] with biliproteins.

pH had about the same effect on both mesophilic and thermophilic allophycocyanin between pH 5 to 8.

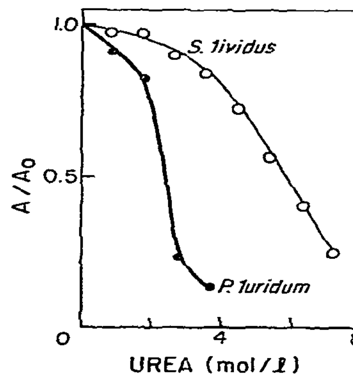
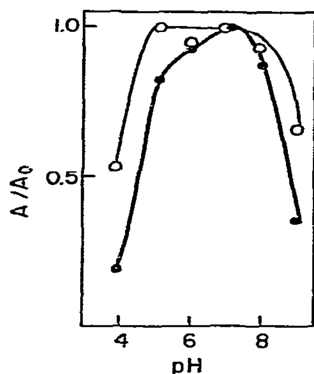


Fig. 2. Effect of urea on absorbance (653 nm) of *S. lividus* (○) and *P. luridum* (●) allophycocyanins. A_0 is absorbance at 653 nm (0.7) in the absence of urea.

Absorbance was slightly higher than the thermophilic protein than with the mesophilic at the pH extremes (fig. 3).

With C-phycocyanin a variety of aggregates form in invitro solutions, depending on the solvent, temperature, and protein concentration [28], and the aggregation properties of C-phycocyanins from mesophiles and thermophiles differ dramatically [7]. In contrast, *S. lividus* allophycocyanin aggregates are no larger at protein concentrations as high as 10 g/l (fig. 4) than at



ig. 3. Effect of pH on the absorbance at 653 nm of *S. lividus* ⟩) and *P. luridum* (•) allophycocyanins. A_0 is maximum ab- ›rbance for any pH.

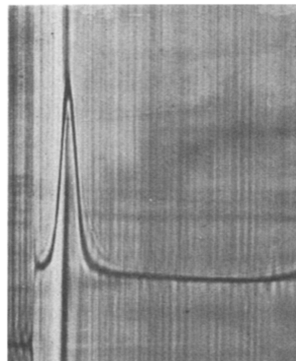


Fig. 4. Sedimentation profile of *S. lividus* allophycocyanin (10 g/l, pH 6.0). Sedimentation is from left to right at 60000 rpm after 16 min at full speed. $S_{20,w}$ = 5.56S.

›wer concentrations (0.06–0.6 g/l), where both meso- hilic and thermophilic protein are α_3 β_3. Thus quater- ary structure is not involved in the greater stability of . *lividus* allophycocyanin. Bryant [29] has suggested ıat C-phycocyanin can form larger aggregates than al- ›phycocyanin because it has an additional segment of mino acids on the C-terminal end of its β subunit.

We have looked for differences in amino acid com- osition between thermophilic and other allophyco- yanins (table 1). The proteins were generally similar, though there were some differences, especially in $\frac{1}{2}$- ysteine content. The amino acid compositions of ıesophilic and thermophilic C-phycocyanins are also ›ry similar [7,30], but the thermal stabilities are quite ifferent [28]. This phenomenon suggests that slight terations in amino acids can produce salient differ- ıces in ability to resist denaturation.

The tendency of C-phycocyanin to aggregate af- ›cts both its polarization of fluorescence and its en- ·gy transfer properties. Goedheer and Birnie [31] rst noted that as C-phycocyanin aggregates became rger their fluorescence polarization became lower, resumably because energy transfer was more exten- ve on larger aggregates having more chromophores ith more varied orientations. This relationship was ystematically studied by Teale and Dale [32] and ernotte [33]. Vernotte separated three C-phycocya- in aggregates and determined their respective fluores- ›nce polarizations at 620–630 nm excitation wave- ngths: 3S, 0.39–0.41; 6S, 0.29–0.31 [33]; and 11S, 0.09–0.10 (Vernotte, personal communication). Kessel et al. [34] studied even larger C-phycocyanin aggregates and found that for the 19S species p = 0.00. These po- larizations are taken from the long wavelength plateau, and values from the lower wavelength region are some- what less.

Since the chromophores of both allophycocyanin and C-phycocyanin are chemically identical [35], their fluorescence polarizations should be comparable, and their p_0 are assumed to be equal. The fluorescence po- larization spectrum of allophycocyanin from *S. lividus* (α_3 β_3, $S_{20,w}$ = 5.5S) is shown in fig. 5. Throughout the region of the first excited state from 550 to 650 nm, p = about +0.05 which is very much lower than the po-

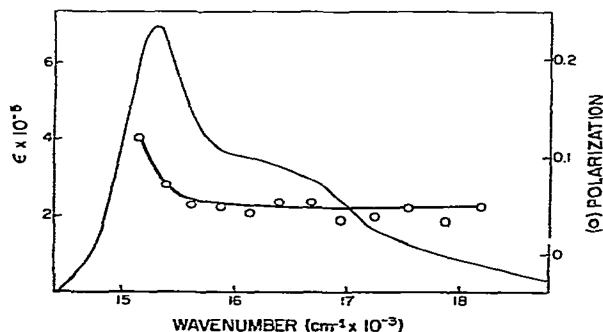


Fig. 5. Absorbance and fluorescence polarization spectra of *S. lividus* allophycocyanin. Extinction coefficient based on 103000 molecular weight. The standard deviation for each polarization value is 0.01.

Table 1
Comparison of the amino acid compositions of several allophycocyanins and C-phycocyanin (S. lividus)

Biliprotein and source	Amino acid (residues per unit molecular weight) [a]																
	Asp	Thr	Ser	Glu	Pro	Gly	Ala	Val	Met	Ile	Leu	Tyr	Phe	Lys	His	Arg	½-Cys
Allophycocyanin																	
S. lividus [this study]	29.9	17.1 [b]	22.7 [b]	33.3	13.3	25.7	43.6	24.3	10.8	18.6	30.4	17.2	8.3	15.4	0.8	22.0	5.7 [c]
P. luridum [8]	29.5	28.6	21.2	28.6	13.5	27.6	35.9	22.5	8.3	13.8	27.3	20.2	6.7	15.1	1.6	18.0	2.6
Synechococcus sp. 6312 [13]	30.5	20.0	25.2	31.5	9.5	28.4	41.0	28.4	8.4	18.9	29.4	17.7	6.3	16.8	0	21.0	1.2
Synechococcus sp. 6301 [13]	28.6	19.8	24.2	30.8	7.7	28.6	38.5	27.5	5.5	22.0	30.8	19.8	4.4	12.1	0	22.0	N.D. [d]
Synechocystis [13]	28.0	17.9	31.4	25.8	6.7	30.2	41.4	26.9	11.2	20.2	26.9	19.0	5.6	14.6	0	17.9	1.5
M. laminosus [10]	32.6	20.0	25.2	31.5	12.6	26.3	48.3	27.3	7.4	25.2	29.4	20.0	5.3	16.8	0	18.9	3.2
M. laminosus [11]	32.6	21.0	26.3	31.5	9.5	28.4	46.2	28.4	7.4	25.2	29.4	18.9	5.3	16.8	0	18.9	N.D.
C. caldarium [9]	31.3	17.9	24.4	31.9	10.5	29.7	24.5	17.9	8.0	20.3	33.2	17.6	7.1	14.8	1.4	17.6	3.7
C-phycocyanin [6]	32.4	16.8	20.4	36.0	13.2	25.2	49.2	19.2	7.2	18.0	31.2	14.4	10.8	12.0	3.6	24.0	7.2

a) Calculations based on a unit molecular weight of 36000.
b) Extrapolated to zero hydrolysis time.
c) Determined as cysteic acid.
d) N.D. = not determined.

larization of the comparable 6S unit of C-phycocyanin ($p = +0.27$) and, in fact, lower than the polarization of the much larger 11S aggregate. The fluorescence polarization of allophycocyanin is a particularly significant property because of the relationship between it and intramolecular energy transfer, and because of the special function this protein is viewed as serving in excitation energy transfer [2]. This phenomenon is general and not limited to thermophilic allophycocyanin since allophycocyanin from mesophilic algae produce very similar polarizations (unpublished results). Several possible explanations for this unexpected finding were examined in turn. One explanation might be that the lower polarization of allophycocyanin is caused by greater rotational freedom of its tetrapyrrole chromophores. As motion of a fluorescent entity becomes more rapid, its orientations become more random, and its polarization of fluorescence is lowered. Such motion could occur if the protein were partially denatured, as was shown with serum albumin complexed with fluorescent probes [36,37], or if the bond between chromophore and protein permitted it. However, the same fluorescence polarization of allophycocyanin was obtained in pH 6.0 buffer and pH 6.0 plus 40% sucrose. If greater rotational freedom were responsible for the observed inconsistency, the more viscous sucrose solvent should have reduced the molecular mobility and raised the fluorescence polarization. Since the polarizations in both solvents were identical within experimental error, this explanation is virtually eliminated.

A second argument is that if allophycocyanin had more chromophores per weight of protein that C-phycocyanin, the probability for energy transfer would be increased, and fluorescence polarization would be reduced. In fact the opposite is true. By studying the absorption spectra of denatured allophycocyanin and C-phycocyanin, Glazer and Fang [38] proposed that allophycocyanin has fewer chromophores per unit weight (6 per $\alpha_3\beta_3$) than C-phycocyanin (9 per $\alpha_3\beta_3$). The chromophore content of native allophycocyanin has not been determined, but we have estimated it by a method devised by Brody and Brody [39]. In this method the number of chromophores, S, is calculated from the weight (m) of a particular protein aggregate divided by the weight (M) of protein which contains 1 mole of chromophore, so that $S = m/M$. The function M is obtained by relating specific absorption coefficient (ϵ_S) to the molar absorption coefficient (ϵ_M), since $\epsilon_M = M\epsilon_S$. This relationship is substituted in

$$\frac{1}{\tau} = \frac{8\pi n^2 c(\ln 10)\bar{\nu}^2_{\max}}{N \times 10^{-3}\,\phi} \int_0^\infty \epsilon_M(\bar{\nu})\,d\bar{\nu} \tag{2}$$

to give

$$M = \frac{\phi N \times 10^{-3}}{8\pi n^2 c(\ln 10)\bar{\nu}^2_{\max}\,\tau \int_0^\infty \epsilon_S(\bar{\nu})d\bar{\nu}}\,, \tag{3}$$

where ϕ is quantum yield, n is the refractive index, τ is fluorescence lifetime, c is the speed of light, N is Avogadro's number, and $\bar{\nu}_{\max}$ is the wave number of the maximum absorption.

From the absorption spectrum of *S. lividus* allophycocyanin (fig. 5) we determined its $\bar{\nu}^2_{\max}$ as 2.345×10^8 cm^{-2} and its $\int_0^\infty \epsilon_S d\bar{\nu}$ as 9979.3 cm$^{-2} \cdot \ell \cdot g^{-1}$. By averaging various literature results [8,9,14,40] we estimated that $\phi = 0.61$, $\tau = 2.7 \times 10^{-9}$, and $\epsilon_S = 6.79\ \ell \cdot cm^{-1} \cdot g^{-1}$. The refractive index is unknown, but values between 1.33 and 1.5 are used in the literature for various proteins. Since $m = 103000$ for *S. lividus* allophycocyanin [6,12] M calculated from eq. (3) is between 18940 and 14891 depending on n, and there are between 5.4 and 6.9 chromophores per $\alpha_3\beta_3$ structure. These results are quite close to those of Glazer and Fang [38], but the experimental errors in ϕ, τ, and ϵ_S plus uncertainty about n do not allow us to specify an exact number. However, these results, when taken with those of Glazer and Fang [38], do indicate that allophycocyanin probably does not have more tetrapyrrole groups than C-phycocyanin and that this suggestion cannot explain the lower fluorescence polarization.

A third approach to an explanation of the polarization results involves consideration of the excitation energy transfer process. We examined the mechanism by which both allophycocyanin and C-phycocyanin are thought to transfer energy, i.e., Förster's radiationless dipole-dipole interaction. The transfer rate from excited donor to acceptor [41] is derived as

$$k_T = 9(\ln 10)\,\phi_D\,K^2 J/128\pi^5 N\,n^4 \tau_D R^6 \tag{4}$$

where ϕ_D and τ_D are donor emission quantum yield and lifetime, in the absence of acceptor; K^2 is a di-

mensionless dipole-dipole orientation factor; J is a modified spectral overlap parameter; and R is the distance between donor and acceptor. The contribution of each to the relative difference in fluorescence polarization was estimated.

The quantum yields (ϕ) are in the same range, 0.53 to 0.68 for allophycocyanin [13,40] and 0.41 to 0.81 for C-phycocyanin [39,40,42–44]. Fluorescence lifetime (τ) is 2.7 nsec for allophycocyanin [40], slightly larger than the 1.8 to 2.2 ns reported for C-phycocyanin [39,40,42,43]. This difference is probably insignificant, but it should be noted that longer lifetimes favor energy transfer.

The overlap integral, J, is found [41] to be

$$J = \int_0^\infty \epsilon(\lambda) f(\lambda) \lambda^4 \, d\lambda \Big/ \int_0^\infty f(\lambda) \, d\lambda, \tag{5}$$

where ϵ is the molar extinction coefficient and f is the fluorescence emission. For *S. lividus* allophycocyanin we calculated J from its absorbance (fig. 5) and its fluorescence emission (not shown) by trapezoidal integration. We also obtained the absorbance and fluorescence emission spectra of C-phycocyanin of *S. lividus, Plectonema boryanum* and *P. luridum*, and calculated the C-phycocyanin-C-phycocyanin overlaps for each. The extinction coefficients per mole of chromophore (ϵ_M^c) were calculated from the literature reports of a single group [13,38,45] to make the allophycocyanin to C-phycocyanin comparison most meaningful. Thus if ϵ_M for the C-phycocyanin is 3.33×10^5 and if there are three chromophores per monomer, $\epsilon_M^c = 1.11 \times 10^5$. For allophycocyanin $\epsilon_M = 2.25 \times 10^5$, and since there are reported to be two chromophores per mole, $\epsilon_M^c = 1.13 \times 10^5 \text{ cm}^{-1} \text{ M}^{-1}$. We also used the data of Grabowski and Gantt [40] on both allophycocyanin and C-phycocyanin from *Nostoc* sp. in addition to those listed above to generate a generalized ratio of the overlaps of allophycocyanin's absorption and emission to C-phycocyanin's absorption and emission of 1.4. This superior spectral overlap of allophycocyanin implies a higher rate of intra-protein energy transfer and thus a lower fluorescence polarization.

Both R and K^2 may also contribute to higher energy transfer in allophycocyanin. Consider a simple speculative calculation: the ratio of k_T for allophycocyanin to that of C-phycocyanin can be represented as

$$(k_T)_A/(k_T)_C = [JK^2/\tau_D R^6]_A / [JK^2/\tau_D R^6]_C. \tag{6}$$

Substitution of the values of J and τ into eq. (6) yields

$$(k_T)_A/(k_T)_C = 1.1(K^2/R^6)_A/(K^2/R^6)_C. \tag{7}$$

Thus if K^2 and R^6 were negligible, the rate of energy transfer of allophycocyanin would be only slightly better than that of C-phycocyanin, even though its polarization is much lower. It is reasonable to suggest the possibility that, while J contributes to the lower polarization, the positions of tetrapyrrole groups in allophycocyanin are also more favorable for intraprotein energy transfer. Either the distance between chromophores or their dipole-dipole orientations may be arranged so that the transfer of excitation energy is facilitated. There are several ways that manipulation of chromophore arrangement could change the fluorescence polarization. One very specific way exists for the polarization results to be explained but strong evidence for this mechanism is not yet available. The fluorescence polarization spectra of the C-phycocyanin aggregates show a behavior [32,33] indicating that two types of chromophore are present. By segregating the chromophores into two types, the effective number of any one kind contributing to the lowering of the fluorescence polarization through energy transfer is reduced. If the chromophores of allophycocyanin are assembled so that all the chromophores contribute to the same energy transfer pool then its lower polarization value might be explained.

In conclusion, it was found at least for one pair of mesophilic versus thermophilic allophycocyanins that the protein from the high temperature alga denatured less readily. The denaturation of C-phycocyanin has recently been studied in detail by Chen et al. [46] and Scheer and Kufer [47]. In the future, application of their ideas to allophycocyanin should be very informative. In addition, new functional models for phycobilisomes as proposed by Bekasova and Evstigneev [48] will probably generate new experiments on this protein. The energy transfer mechanisms for these proteins are not yet known in great details, and our results suggest that subtle differences occur between allophycocyanin and other types of biliprotein. Energy transfer studies on the picosecond scale have recently appeared adding a new dimension to the understanding of these processes [49,50], and finally evidence

has appeared suggesting other biliproteins besides allophycocyanin may participate in direct excitation energy transfer to chlorophyll *a* [51].

Acknowledgement

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