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SPECTRAL ANALYSIS OF ALLOPHYCOCYANIN I, II, III AND B FROM *NOSTOC* SP. PHYCOBILISOMES *

BARBARA A. ZILINSKAS, LINDA S. GREENWALD, CINDY L. BAILEY and PETER C. KAHN

Department of Biochemistry and Microbiology, Cook College, Rutgers, The State University of New Jersey, New Brunswick, NJ 08903 (U.S.A.)

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Summary

Low temperature (—196°C) and room temperature (25°C) absorption spectra of a family of allophycocyanin spectral forms isolated from *Nostoc* sp. phycobilisomes as well as of the phycobilisomes themselves have been analyzed by Gaussian curve-fitting. Allophycocyanin I and B share long wavelength components at 668 and 679 nm, bands that are absent from allophycocyanin II and III. These long wavelength absorption components are apparently responsible for the 20 nm difference between the 680 nm fluorescence emission maximum of allophycocyanin I and B and the 660 nm maximum of II and III. This indicates that allophycocyanin I and B are the final acceptors of excitation energy in the phycobilisome and the excitation energy transfer bridge linking the phycobilisome with the chlorophyll-containing thylakoid membranes. These Gaussian components are also found in resolved spectra of phycobilisomes, arguing against this family of allophycocyanin molecules being artifactual products of protein purification procedures.

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* Supplementary data to this article are deposited with, and can be obtained from, Elsevier/North-Holland Biomedical Press B.V., BBA Data Deposition, P.O. Box 1345, 1000 BH Amsterdam, The Netherlands. Reference should be made to No. BBA/DD/152/47908/592 (1980) 267—276.
The supplementary information includes: Gaussian curve resolution of 25°C and —196°C absorption spectra of allophycocyanin II, allophycocyanin III and *Nostoc* sp. phycobilisomes; five-component Gaussian curve resolution of the —196°C absorption spectrum of allophycocyanin B.

Introduction

The phycobiliproteins are water-soluble, light-harvesting pigment proteins which are housed in multiprotein aggregates called phycobilisomes in the blue-green and red algae [1]. There are three major groups of biliproteins, phycoerythrin, phycocyanin and allophycocyanin, the chromophores of which are linear tetrapyrroles attached covalently to the apoproteins [2]. There are only two chromophores, phycoerythrobilin and phycocyanobilin, with either or both found in individual biliproteins. Since only two chromophoric moieties are found, the environment conferred on them by the apoproteins as well as chromophore-chromophore interactions accounts for the wide variety of spectral forms of these pigment proteins.

Recently a family of allophycocyanin spectral forms has been isolated from *Nostoc* sp. phycobilisomes [3,4]. Among these, allophycocyanin B, first identified by Glazer and coworkers [5,6], has been isolated from phycobilisomes and studied in some detail [4,7]. The four allophycocyanin species isolated from *Nostoc* sp. each contain only the phycocyanobilin chromophore. Allophycocyanin I, II and III have two subunit types, designated α and β, of 16 900 and 18 400 daltons as determined by SDS-polyacrylamide gel electrophoresis on a modified Weber and Osborn gel system [3,7]. In addition, allophycocyanin I has a γ subunit of 35 000 daltons. Allophycocyanin B has two subunits, one of a size similar to that of the 16 900-dalton subunit of allophycocyanin I, II, III, and the other of 15 300 daltons. The native allophycocyanin molecules are made up of a minimum of 3 α and 3 β subunits. Allophycocyanin II and III, which are trimers of 3 α and 3 β subunits, differ spectrally in the A_{620}/A_{650} ratio, but both have maximum fluorescence emission at 660 nm. Allophycocyanin I and B have fluorescence emission maxima at 680 nm, and absorption maxima at 620 and 650 nm (654 nm in allophycocyanin I). In addition, they also exhibit a distinct absorption peak (allophycocyanin B) or a poorly defined shoulder (allophycocyanin I) at 670 nm.

Intact phycobilisomes removed from the chlorophyll-containing membranes fluoresce at 680 nm [8,9]. Excitation energy is, therefore, transferred to allophycocyanin I and/or B from the other biliproteins and from these special allophycocyanin forms to chlorophyll *a* in the thylakoids. As allophycocyanin I and B are, therefore, the key biliproteins, we have investigated the relationship between them as well as the relationship between allophycocyanin II and III by measurement of low temperature (−196°C) absorption spectra and by computer-assisted Gaussian curve resolution of room and low temperature absorption spectra.

Materials and Methods

Nostoc sp. was grown in a New Brunswick Scientific fermentor on medium CG-10 according to the method of Rusckowski and Zilinskas [10]. Phycobilisomes were isolated as described by Zilinskas et al. [3], following a modification of the procedure of Gray and Gantt [11]. Allophycocyanin I, II and III were isolated from dissociated phycobilisomes by brushite chromatography as described previously [3]. Allophycocyanin B was separated from the other allophycocyanin spectral forms obtained from phycobilisomes according to the

protocol of Greenwald [4]. Allophycocyanin B eluting from the brushite column between allophycocyanins I and II was concentrated (pooled fractions) with an Amicon PM-10 ultrafilter and dialyzed against 0.04 M potassium phosphate buffer, pH 7.0. This material was then chromatographed on a small brushite column (1 cm × 15 cm) that had been equilibrated with the same buffer. The column was developed with equilibration buffer (100 ml) and 1-ml fractions in which the A_{670}/A_{650} ratio was greater than 0.6 were pooled and used for spectral analyses. All allophycocyanin samples were stored in concentrated form (1—5 mg/ml) in 100 mM potassium phosphate buffer, pH 7.0, containing 0.02% NaN_3, at 4°C. Generally, absorption spectra were measured within 24 h after isolation from the phycobilisomes. Absorption spectra were measured in a Cary 17D spectrophotometer. For low temperature measurements, samples in 100 mM potassium phosphate, pH 7.0, were mixed with glycerol to a concentration of 45% buffer, 55% glycerol. Glycerol itself had no obvious effect on the room temperature spectra of the biliproteins. The cuvette was then immersed quickly in an Oxford DN704 cryostat containing liquid nitrogen, and spectra were taken at −196° C. As the samples were cracked glasses, scattering was subtracted by a reference cuvette containing a suspension of $BaSO_4$ diluted with H_2O so that the absorbance of the frozen sample at 720 nm equaled the absorbance at 450 nm. These two wavelengths were selected because at these wavelengths there was minimal absorbance in all samples when measured at room temperature. $BaSO_4$ was prepared by mixing aqueous solutions of 1 M $MgSO_4$ and 1 M $BaCl_2$ in equal proportions.

For some of the low temperature samples, the subtraction of light scattering by the $BaSO_4$ suspensions was not perfect. The small overcorrections and undercorrections that occurred occasionally were removed by using a modification of the method of Leach and Scheraga [12]. They treat the scattered absorbance (A_s) at any wavelength as an exponential in the wavelength:

$$A_s(\lambda) = C\lambda^p \tag{1}$$

in which the constants C and p are parameters to be determined experimentally.

For turbid specimens having zero absorption, the measured absorbance is due exclusively to scattering. If there is a continuous region of zero absorption, a log-log plot of scattered absorbance vs. wavelength will be linear with slope p and intercept C. Our $BaSO_4$ suspensions, when run as sample against a water reference, do, in fact, follow a function of the form of Eqn. 1.

If the $BaSO_4$ correction is imperfect, the discrepancy will also be a function of the form of Eqn. 1. The additional correction is determined by using the fact that the absorbance is zero at both ends of the scan, which yields two wavelengths at which absorbance is due exclusively to scattering. Eqn. 1, having two unknowns, is solved for C and p, and the scattering contribution to the absorbance is then computed at all wavelengths and subtracted from the data. Of the low temperature spectra presented here, only those of allophycocyanin I and II were corrected for scatter in this way.

The Cary 17D was interfaced to a minicomputer system that has been described by Kahn [13]. Absorption spectra were recorded simultaneously on the spectrophotometer's strip chart and in the computer's memory, from which

the data were transferred at the end of the scan to a magnetic disc [14]. The spectra in the present work were recorded at 0.2 nm intervals as single scans; signal averaging was not employed. The data presented here, moreover, are representative scans of typical individual protein preparations. The spectral characteristics of the purified proteins do vary slightly from preparation to preparation, the origin and extent of the variations being discussed elsewhere [3,4]. These differences are small and do not affect the principal conclusions to be drawn.

Data were generally recorded from 450 to 720 nm, but only the 1005 data points occurring between 500 and 700 nm had significant absorbance and are shown in the figures. Room temperature spectra of buffer vs. buffer were flat, making baseline corrections unnecessary.

Spectra were recorded on magnetic discs and then read back into the computer's memory for analysis by the interactive graphics-assisted procedures described by Kahn and Bailey [15]. The Gaussian bands are each defined by:

$$a(\lambda) = a_0 \exp[-(\lambda - \lambda_0)^2/\Delta^2] \quad (2)$$

where $a(\lambda)$ is the absorbance at wavelength λ, a_0 is the absorbance at the peak of the band, exp is the standard exponential, λ_0 is the wavelength at which the peak occurs, and Δ, the halfwidth, is half the band width at the points where the absorbance falls to exp(−1) times its maximum height, a_0. The choice of three parameters, a_0, λ_0, and Δ, thus determine the absorbance at any wavelength for a given band. The sum at each wavelength of the absorbance contribution of each band yielded the calculated spectra shown for the various samples in the figures. Resolution of a spectrum was carried out by choosing parameters to define a set of bands. A calculated spectrum was then constructed by the computer from the resulting bands and compared visually with the experimental spectrum by displaying the two curves together on the screen of a Tektronix 4010 high resolution graphics terminal [15]. If the match between experimental and calculated spectra was poor, one or more band parameters were altered, and a new curve was calculated. The procedure was repeated until the fit could not be improved further. The final result was then drawn on paper by the computer's digital XY plotter. The spectra and resolutions shown in this paper are photoreproductions of plotter output.

Although the curve fitting procedure was visual, the root mean square residual was obtained as a measure of the discrepancy between experimental and calculated spectra. The calculation was over all 1005 data points (500 nm to 700 nm) used in the resolution. For the spectra reported in Table I, the largest root mean square residual was 0.0009 absorbance unit in the case of allophycocyanin B at −196°C (Fig. 2b). This figure is 2.1% of the peak absorbance in the spectrum.

Because of the inherently poorer definition of the room temperature data, the spectra at −196°C were resolved first. The resulting bands were then used to resolve the corresponding room temperature curves. In fitting the low temperature bands to the room temperature data, every effort was made to hold the peak positions constant. Only the peak heights and halfwidths were varied freely. Bands were moved only if a reasonable fit to the room temperature curves could be obtained in no other way. The rationale behind this

procedure was that electronic transitions present at −196°C must also be present at room temperature even if they are not obvious upon inspection. The possible implications of the occasional departures of room temperature peak wavelengths from those at low temperature are discussed below.

Results and Discussion

Table I shows the band parameters for the curve resolutions of allophycocyanin I, II, III and B and the phycobilisomes from which these biliproteins are obtained. The resolved spectra of allophycocyanin I and B are shown in Figs. 1 and 2, respectively. The other spectra will be found in the BBA Data Bank.

Allophycocyanin I and B

Cooling allophycocyanin I to −196°C resolves the poorly defined broad tail near 670 nm into a peak at 668 nm and a definite shoulder near 680 nm (Fig. 1). The major room temperature absorption band at 653.3 nm is displaced to 649.6 nm at low temperature. Resolution of the low temperature spectrum indicates the presence of major components with peak positions at 679.3, 668.4, 650.5, 637.3, 600.5 and 557.0 nm. Resolution of the room temper-

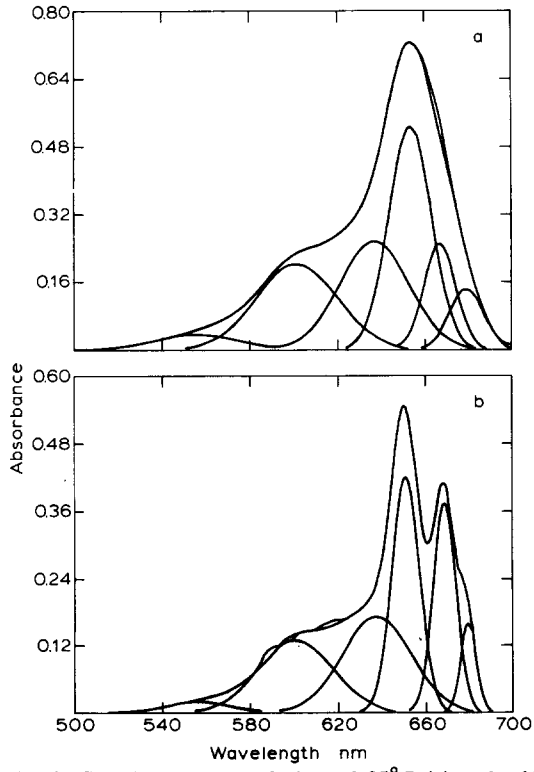

Fig. 1. Gaussian curve resolution of 25°C (a) and −196°C (b) absorption spectra of allophycocyanin I from *Nostoc* sp. phycobilisomes. Experimental and calculated spectra and the individual Gaussian components are plotted.

TABLE I

THE PEAK WAVELENGTHS, HALFWIDTHS AND HEIGHTS OF GAUSSIAN BANDS OF ALLOPHYCOCYANIN (APC) FORMS AND PHYCOBILISOMES (PBsomes) FROM *NOSTOC* SP.

Protein	Band No.	25°C Gaussian fit				−196°C Gaussian fit			
		Peak position (nm)	Half-width (nm)	Peak absorbance	Band area as % of total area	Peak position (nm)	Half-width (nm)	Peak absorbance	Band area as % of total area
APC I	1	679.0	11.2	0.144	6.8	679.3	5.1	0.160	5.5
	2	666.7	10.5	0.250	11.1	668.4	7.5	0.374	19.1
	3	652.9	14.0	0.525	31.1	650.5	9.0	0.420	25.7
	4	636.6	22.5	0.256	24.4	637.3	22.2	0.160	25.9
	5	601.2	26.0	0.203	22.4	600.5	24.0	0.129	21.0
	6	557.0	25.0	0.039	4.1	557.0	20.0	0.020	2.7
APC B	1	679.0	10.5	0.140	5.5	679.4	7.4	0.040	15.9
	2	670.8	10.7	0.255	10.3	666.6	7.7	0.012	5.0
	3	651.2	14.2	0.339	18.1	650.2	7.2	0.028	11.0
	4	623.0	24.0	0.435	39.3	628.2	19.8	0.040	43.5
	5	593.0	20.5	0.200	15.3	593.7	20.5	0.016	17.8
	6	562.8	25.0	0.121	11.4	562.0	25.0	0.005	6.8
APC II	1	678.5	5.0	0.010	0.1	679.2	9.0	0.020	0.7
	2	649.0	13.8	1.165	40.4	649.0	7.0	0.992	28.5
	3	632.0	10.0	0.270	6.8	634.9	12.0	0.270	13.3
	4	619.3	15.5	0.530	20.6	619.2	17.0	0.410	28.6
	5	598.0	16.0	0.400	16.1	598.0	7.8	0.125	4.0
	6	587.0	10.0	0.073	1.8	588.0	5.5	0.100	2.3
	7	574.0	18.0	0.190	8.6	581.0	25.0	0.189	19.4
	8	548.8	25.0	0.090	5.6	542.0	20.0	0.038	3.1
APC III	1	651.7	11.8	0.395	35.4	650.8	8.5	1.025	38.4
	2	635.0	14.0	0.215	22.9	631.0	19.3	0.357	30.4
	3	615.0	14.7	0.143	16.0	615.0	10.0	0.060	2.6
	4	597.2	14.0	0.085	9.0	597.2	15.0	0.225	14.9
	5	588.0	15.0	0.060	6.8	588.0	7.0	0.042	1.3
	6	571.0	18.0	0.050	6.8	571.5	20.0	0.110	9.7
	7	545.0	20.0	0.020	3.0	540.0	15.0	0.040	2.6
PBsomes	1	678.5	7.0	0.010	0.3	678.5	7.5	0.075	0.7
	2	666.1	11.0	0.037	1.6	666.1	8.0	0.160	1.5
	3	650.5	12.0	0.140	6.5	650.5	7.9	0.890	8.3
	4	633.7	19.8	0.188	14.4	631.5	11.4	1.260	17.0
	5	618.4	23.3	0.211	19.1	618.4	7.8	0.288	2.7
	6	595.2	22.0	0.208	17.7	598.7	22.3	1.012	26.7
	7	572.0	13.0	0.210	10.6	571.6	7.7	0.612	5.6
	8	550.6	19.2	0.257	19.1	553.8	21.8	1.060	27.3
	9	522.0	22.0	0.125	10.7	520.0	25.0	0.340	10.1

ature spectrum yields bands whose maxima are very close to those of the low temperature curve with small changes in half-band widths and larger changes in relative intensities of each band. This conformity of peak positions of Gaussian curves that are fit to 25°C and −196°C absorption spectra is expected and confirms that seen in spectral analyses of phycobilisomes and various chlorophyll-proteins by use of the fourth derivative [16].

The spectra at 25°C and −196°C and Gaussian curve fitting of allophyco-

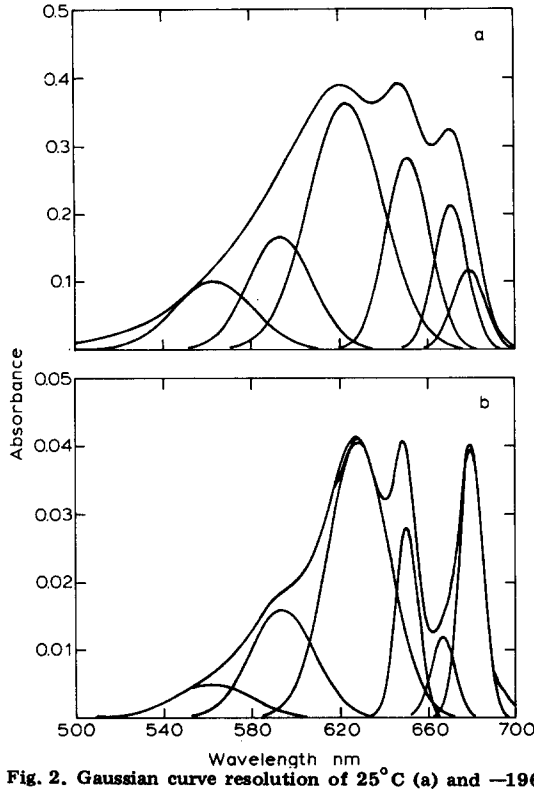

Fig. 2. Gaussian curve resolution of 25°C (a) and −196°C (b) absorption spectra of allophycocyanin B. Experimental and calculated spectra and the individual Gaussian components are plotted.

cyanin B are shown in Fig. 2. Room temperature absorption peaks occur at 670.8, 648.0 and 622.5 nm, and low temperature bands at 677.4, 648.2 and 627.0 nm. The peak positions (Table I) of the Gaussian components at 25°C are similar to those resolved from the low temperature data, except that the 670.8 and 628.2 nm components (−196°C) are blue-shifted, respectively, to 666.6 and 623.5 nm (25°C).

The room temperature absorption spectra of allophycocyanin I and B (Figs. 1 and 2) show that the ratio of 670 to 650 nm absorbance is similar for these two pigments. The 5.5 nm red shift of the allophycocyanin I absorption maximum at 653.5 nm (cf. allophycocyanin B at 648 nm) overlaps the 670 nm peak sufficiently to prevent resolution of the two peaks at room temperature, while, in contrast, allophycocyanin B has two well-separated peaks at 648 and 670.8 nm. At low temerature, allophycocyanin I and B show definite spectral similarities. A prominent shoulder at 679 nm in allophycocyanin I compares with the 677.4 nm band in allophycocyanin B; in addition, the former has a second long wavelength band at 668 nm that is not obvious in the latter. As with the 25°C spectra, the 196°C allophycocyanin B band at 678 nm (671.5 nm at 25°C) is of comparable magnitude to the bands of allophycocyanin B at 648 and 627 nm (622.5 nm at 25°C). In contrast, there are no intense absorbance bands in allophycocyanin I below 650 nm (Figs. 1 and 2). Gaussian curve resolution of these two pigments indicates close resemblance in the red region

of the spectrum. The peak positions of the Gaussian components at 650 nm and above at both 25°C and −196°C are essentially identical, and the half-band widths are also fairly close; only the relative heights of the bands differ.

Allophycocyanin II and III

The spectra of allophycocyanin II and III are strikingly similar (data bank). The major difference is the relative absorbance at 620 and 650 nm. While allophycocyanin II at 25°C has its major peak at 648.0 nm with shoulders at ≃590 and 620 nm, allophycocyanin III has its major band at 649.5 nm and only one prominent shoulder at ≃592 nm. Cooling the samples sharpens and resolves the shorter wavelength bands but does not significantly alter the positions of the major peaks.

The major significance of the Gaussian curve analysis of allophycocyanin II and III absorption spectra (Table I) is the absence of long wavelength components at 668 and 679 nm, which are characteristic of allophycocyanin I and B. These long wavelength components are apparently responsible for the 20 nm red-shifted fluorescence maximum of allophycocyanin I and B, which indicates that these pigments are the final acceptors of excitation energy in the phycobilisome and immediate donors to chlorophyll *a* in the membranes.

Phycobilisomes

The room temperature absorption spectrum of *Nostoc* sp. phycobilisomes from which these allophycocyanins are obtained shows only a poorly defined shoulder at 650 nm, the major absorption band of allophycocyanin. However, the individual allophycocyanin forms can be detected in the −196°C spectrum of the phycobilisomes and its Gaussian curve resolution (data bank). The area under the long wavelength bands (at 666 and 678 nm) is small (Table I) as would be expected from the relatively small amounts of allophycocyanin I and B [4] in these large multiprotein aggregates. That the peak positions of these components in the phycobilisome are similar to those of the isolated allophycocyanin molecules argues against their simply being artifactual products of purification procedures. Others [17,18] have shown that the spectral properties of the isolated phycobilisome are essentially identical to those observed in vivo.

Curve resolution

Curve resolution of a single spectrum does not, in principle, yield a unique set of bands in all cases [19,20]. In the absence of other information, one should, therefore, use the minimum number of bands that will fit the data. The agreement between experimental and calculated spectra could be improved by adding extra bands, but the result would make little biological sense. As an example, the 25°C allophycocyanin B spectrum in Fig. 2a can be resolved equally well with only five bands. The two long wavelength bands can be merged into a single band with minor changes made elsewhere in the resolution. However, outside information is supplied by the requirement in the low temperature spectrum (Fig. 2b) of a sixth band. A suggestion of its presence can be seen in the asymmetry of the trough near 660 nm, and gross mismatch in the 660 nm trough is apparent in a five band fit of the −196°C spectrum (data bank). All attempts to eliminate the mismatch by broadening the bands

on either side of the trough resulted in very poor fits near the peaks.

While the allophycocyanin B data demonstrate the presence of at least six bands, they are not sufficient as yet to define with precision all of the band parameters. Thus, the position of the second band is seen in Table I to change by 4.2 nm between −196°C and 25°C, and that of band 5 changes by 5.2 nm. The position of the longest wavelength band, however, is firmly fixed near 679 nm for both allophycocyanin I and B, a result that is not affected by uniqueness questions elsewhere in the resolution and which is the basis of our principal conclusion.

Although such differences between low and room temperature peak positions as we find here can be explained in terms of the limitations of curve resolution, they have another possible source. The normal fluctuations that occur in protein structure [21] may give rise to a family of closely related conformational states having different spectral properties. Equilibration among such states is rapid [21] with respect to the time required for sample freezing, which is on the order of seconds. Since measured spectra are a weighted average over all states present, spectral changes could arise from a cooling induced shift in the equilibrium. The present data provide no basis for distinguishing between these two alternatives.

The application of statistical tests would provide quantitative justification for the best fitted resolution of a given spectrum, and a non-linear least squares regression is being developed for this purpose. The conclusions drawn here, however, depend on aspects of the visual analysis that would not be affected by the application of statistical methods. The existence and position of the 679 nm band of allophycocyanin I and B and the absence of such a band in allophycocyanin II and III are not in doubt. Also clear is the requirement for six bands in the −196°C resolution of the allophycocyanin B. Regression procedures would alter slightly many of the band parameters in Table I, but it is unlikely that the overall patterns would change substantially. The constraint on the visual resolution of fitting both 25°C and −196°C spectra of each sample is severe.

Whatever the details of particular band parameters, phycocyanobilin provides the allophycocyanins with overlapping electronic transitions covering the spectrum from 550 to 680 nm. Phycocyanin and phycoerythrin extend the range of the phycobilisome to approx. 500 nm, a total range that spans most of the visible spectrum, with light harvested at any wavelength transferred efficiently to the photochemical centers in the lamellae. The broadly variable response of the tetrapyrroles is most likely dictated by the protein environment. We suggest that this susceptibility to environmentally induced spectral variation arises from the open chain, flexible tetrapyrrole structure. Different protein environments could easily force considerable variation in tetrapyrrole conformation. The chromophores' spectral characteristics, in turn, are likely to depend sensitively upon conformation. Electron delocalization between adjacent pyrrole rings, for example, will decrease sharply as the pyrroles deviate from co-planarity due to interpyrrole twist imposed by the protein. Any alteration from a planar conformation, moreover, introduces asymmetry. The transitions will then become optically active and detectable, therefore, by circular dichroism, measurements of which will also aid in reducing the uniqueness

problem in curve resolution [22]. These experimental studies and a theoretical correlation of tetrapyrrole spectral properties with conformation are now in progress.

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