Molecular Architecture of a Light-harvesting Antenna

ISOLATION AND CHARACTERIZATION OF PHYCOBILISOME SUBASSEMBLY PARTICLES*

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In addition to chlorophyll a, cyanobacteria and red algal chloroplasts contain high concentrations of light-harvesting proteins called biliproteins (1). In vivo, biliproteins are organized into high molecular weight complexes, phycobilisomes, which form regular arrays on the outer surface of the photosynthetic lamellae (2). In cyanobacteria (blue-green algae), the blue chromoproteins, C-phycocyanin (λmax 620 nm) and allophycocyanin (λmax 650 nm), are universally present as major biliproteins, whereas the red biliproteins, phycoerythrin (λmax 665 nm) and phycocyanin (λmax 585 nm), are variable components (1). In addition to the above, a minor biliprotein, allophycocyanin B (λmax 671 nm) has been well characterized (3–5). The highest molecular weight "linker" polypeptide (see below) in the phycobilisome is also a biliprotein with fluorescence emission properties similar to those of allophycocyanin B (6, 7). These components are believed to function as the terminal energy acceptors of the phycobilisome and mediate the transfer of energy from this particle to the protein–chlorophyll a complexes within the thylakoid membrane.

The general arrangement of the major light-harvesting components within phycobilisomes was established by Gantt and her co-workers (8–10), by kinetic studies of partial dissociation of phycobilisomes, with analysis by fluorescence emission spectroscopy and immunoelectron microscopy. These studies, with the unicellular red alga Porphyridium cruentum, led to a phycobilisome model in which a core of allophycocyanin was surrounded in succession by concentric layers of phycocyanin and phycoerythrin (8–10). Absorbed light energy was shown to be transferred in a linear pathway (11, 12):

phycoerythrin → phycoerythrin → allophycocyanin → chlorophyll a

A more detailed understanding of phycobilisome organization has come from studies of structurally simpler phycobilisomes, those of the red alga Rhodella violacea (13) and of numerous cyanobacteria (14, 15). Examination of these phycobilisomes by electron microscopy has shown them to have two morphologically differing substructures when seen in “face view.” In the most common type, there is a core of three contiguous objects, disc-like in the face view projection and arrayed in an equilateral triangle. Six rods, each composed of several stacked discs, radiate from the core in a hemisidiscoidal arrangement. In a second, simpler type, represented by the phycobilisomes of Synechococcus 6301 (15, 16), the core consists of only two contiguous objects surrounded by rods in an arrangement similar to that described for the first type.

The assembly of biliproteins into phycobilisomes is me-
diated by the specific interaction with several polypeptides first described by Tandeau de Marsac and Cohen-Bazire in 1977 (17). These polypeptides serve the dual function of assembling monomers of each individual biliprotein into a higher aggregate, trimmer, or hexamer, and linking these together to form substructures of the phycobilisome (18–20). Hence, they have been named "linker" polypeptides (18). A high molecular weight linker polypeptide functions in the organization of the phycobilisome core (7) and in its attachment to the membrane (17, 21).

The general features of the assembly and organization of the rod elements are now reasonably well understood (16, 18, 19). Much remains to be learned about the mode of attachment of the rods to the core and the structure of the core itself. It is evident that useful information may come from partial dissociation experiments on phycobilisomes. For example, fractionation of partially dissociated phycobilisomes of Nostoc sp. has led to the isolation of several spectroscopically distinguishable "forms" of allophycocyanin, designated allophycocyanin I, II, and III, respectively (26). Allophycocyanin I, a complex of trimeric allophycocyanin with a 37,000-dalton polypeptide, has been reported to have significantly lower absorbance at 650 nm relative to allophycocyanin and red-shifted fluorescence emission maximum (22, 24, 25). The molecular basis of the differences between allophycocyanins II and III is not known (24, 25). Chromatography of biliproteins from the filamentous cyanobacterium Mastigocladus laminosus led to the isolation of two allophycocyanins which appeared to be different conformers of the same protein (26, 27).

Since allophycocyanin of only one spectroscopic type is obtained upon extensive fractionation of cyanobacterial phycobilisomes (1), it is evident that partial dissociation leads to release of allophycocyanin components in complexes and in conformations which retain in part the organizational features of this biliprotein exhibited in the intact core.

In this report, we describe studies of the products of partial dissociation of the phycobilisomes of Synechococcus 6301 mutant AN112. The rods of the phycobilisomes of this mutant are only one disc in length; hence these phycobilisomes are greatly enriched in core components (16). The AN112 phycobilisome contains phycocyanin and allophycocyanin in a molar ratio of ~1.5:1. Its other components are a 27,000-dalton linker polypeptide, a large domain of which is known to be associated with phycocyanin (18), allophycocyanin B, and a 75,000-dalton linker polypeptide recently shown to carry a phycocyanobilin chromophore (7). The results of this investigation have led to a partial description of the organization of the phycobilisome core.

**EXPERIMENTAL PROCEDURES**

**Materials**—Density gradient column sucrose and Triton X-100 were obtained from Calbiochem, Lysozyme (egg white), agarose, and polyethylene glycol (average M~ = 4000) were from Sigma, Trypsin was from Boehringer Mannheim and sodium dodecyl sulfate (specially pure) was from BDH Chemicals, Ltd. Miranol S2M-SF was a gift of from Boehringer Mannheim and sodium dodecyl sulfate (specially Bio-Rad.

**Preparation of Spheroplasts**—Fresh cells were harvested by centrifugation, washed once in 30 mm Tris-HCl, pH 6.8, and then suspended in 450 mm mannitol, 10 mm EDTA, 30 mm Tris-HCl, pH 6.8, containing 5 mg/ml of lysozyme. Following 3 h of incubation at 37 °C, the resulting spheroplasts were washed twice in 500 mm mannitol, 30 mm Tris-HCl, pH 6.8, and resuspended to 0.14 g wet cell weight per ml in an appropriate lysis buffer.

**Isolation of Phycobilisomes**—Phycobilisomes were isolated from spheroplasts or French-pressed cells (30,000 p.s.i.) as described (30, 31) except that the phosphate buffers were replaced with 800 mm Na2SO₄, 50 mm Tris-HCl, 1 mm 2-mercaptoethanol, pH 8.0.

**Spectrophotometric Measurements**—Absorption spectra were recorded on a Beckman 25 or a Cary 14 recording spectrophotometer. Corrected fluorescence emission spectra were determined with a Perkin-Elmer MFP-44B spectrofluorometer equipped with a DCSU-2 differential corrected spectra unit. Second derivative spectra were obtained through use of the derivative function of the DCSU-2 at a derivative increment of 5 nm. All samples for fluorescence emission were analyzed with a sample absorbance of 0.105 cm⁻¹ at λₘₐₓ, with excitation and emission slits of 4 nm. Circular dichroism spectra were recorded on an instrument described by Sutherland et al. (32) at a sample absorbance of 0.7 cm⁻¹ at λₘₐₓ.

**Sedimentation Studies**—Sedimentation coefficients were determined with a Spinco model E analytical ultracentrifuge equipped with absorption optics as described (33). Samples were prepared by overnight dialysis against the reference buffer (50 mm Tricine, 1 mm CaCl₂, pH 7.8) and analyzed at 360 nm at a protein concentration of 0.2-0.3 mg/ml. Centrifugation was performed in an AN-D rotor at 48,000 or 60,000 rpm.

**Polyacrylamide Gel Electrophoresis**—Samples were precipitated in 10% trichloracetic acid and resuspended in a solubilization buffer. For SDS-gel electrophoresis, samples were resuspended to 0.7 mg/ml in a buffer containing 200 mm Tris-HCl, pH 8.8, 2% SDS, 1 mm 2-mercaptoethanol, 20% (w/v) glycerol, 0.01% bromphenol blue, and 0.0025% phenol blue, then dissolved to 1 mg/ml in a solution of 5.5 M urea, 2% amphotol (pH 7-9), 1 mm 2-mercaptoethanol. Electrophoresis in a single dimension was performed on 0.9-mm-thick slab gels containing 0.1% SDS in the discontinuous buffer system of Laemmli (33). Both the 5% stacking gel and the 14% separating gel were prepared from a stock acrylamide solution (37.5:1, acrylamide:N,N'-methylenebisacrylamide), previously treated with activated charcoal as described by Chua (34). The concentration of Tris-HCl, pH 8.8, in the separating gel was increased from 0.375% (33) to 0.40% for optimal resolution of the biliprotein bands. Samples of 10 μl were applied to gels and electrophoresis was performed at 30 mA just until the bromphenol blue tracking dye ran off the gel. Following the run, gels were fixed for 1 h in 25% 2-propanol, 10% acetic acid, then stained for at least 2 h in 0.01% Coomassie brilliant blue R-250 in 10% acetic acid. Gels were destained in 10% acetic acid.

Two-dimensional mapping was performed essentially as described by O'Farrell (35). First dimension isoelectric focusing gels were cast in 3-mm (inner diameter) tubes and contained 2% amphotol (4:1 mixture of pH 4-6: pH 3-10 amphotole), along with 0.4% Triton X-100, 9.2 M urea, 3.8% acrylamide, and 0.2% N,N'-methylenebisacrylamide. Gels were prerun for 1 h prior to sample application (20 μg of protein/gel) and focused for 12 h at 450 V and 1 h at 500 V. Upon completion of isoelectric focusing, gels were equilibrated for 1 h in 10 ml of equilibration buffer, applied to a second dimension slab gel, and sealed with a 1% agarose solution (35). SDS was present at 2% in the equilibration buffer, but only 0.5% in the agarose solution. Bromphenol blue was present at 0.0025% in the equilibration buffer. Second dimension gel electrophoresis was identical with the procedure described above for SDS-gel electrophoresis.

**Determination of Poly peptide Stoichiometry**—Two methods were employed to estimate the relative molar amounts of each polypeptide present in a sample. In the first method, Coomassie brilliant blue-stained SDS gels were scanned on a Quick Scan densitometer (Helena Laboratories, Beaumont, TX) and quantitated as described (28). In a second method, two-dimensional gels were analyzed. Coomassie brilliant blue-stained spots were cut out with a razor blade and dried overnight in test tubes (12 x 100 mm) in a 50 °C oven. SDS (2% w/v, 1.0 ml) was added to rehydrate each gel piece and the dye was eluted over an 18-h period on a rotary shaker (50 rpm) in the dark. The absorbance of eluates at 595 nm was then determined. A baseline value was obtained by incubating a gel piece from a region of the gel devoid of protein. Molar ratios were obtained by dividing the corrected absorbances of polypeptide spots by their respective molecular 

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1 The abbreviations used are: Tricine, N-(tris(hydroxymethyl))-methylglycine; 27K, 75K, etc., designations used to specify polypeptides of 27,000 and 75,000 daltons, etc., respectively; SDS, sodium dodecyl sulfate.
weights. The dye elution technique is compared with the gel scan method in Fig. 1, in the analysis of the same one-dimensional SDS gel containing different amounts of AN112 phycobilisome polypeptides. There is excellent agreement over a protein concentration range of 3.5-14 μg of phycobilisome/gel lane (actual amounts of protein/gel band are much less), these values differing by an average of 2%. The one exception is seen upon analysis of the 75K band, for which the gel scanning method consistently produces a value 35% lower than the dye elution technique. This difference appears small in Fig. 1 because the amounts of the different polypeptides are plotted on a molar basis. Such a difference is perhaps attributable to: 1) an abnormally narrow banding of this high molecular weight protein in a high percentage acrylamide (14%) gel or 2) metachromatic staining which causes a shift in the absorbance maximum of the dye bond to specific types of polypeptides (36). This latter effect would be absent for free dye eluted into 2% SDS.

Limited Tryptic Digestion—Phycobiliprotein particles, 11S and 18S, were digested with trypsin and examined by SDS-gel electrophoresis and absorption spectroscopy. For gel electrophoresis, samples were diluted to 100 μg/ml and equilibrated at 0 °C, and the reaction was started by the addition of trypsin to a final concentration of 5 μg/ml. Aliquots were removed at 1, 5, and 30 min, pipetted into ice-cold 10% trichloroacetic acid, and analyzed by SDS-gel electrophoresis as described above. For spectrophotometry, a sample was diluted to 150 μg/ml at room temperature and scanned from 700 to 500 nm at 60 min in a Cary 14 spectrophotometer. Trypsin was added to a final concentration of 5 μg/ml and the spectrum was redetermined at intervals up to 1 h.

RESULTS

The present work began as a study of the interaction between phycobilisomes and the photosynthetic membrane in which attempts were made to isolate biliprotein complexes specifically associated with chlorophyll a (and chlorophyll proteins). In these experiments, lysates were prepared by disruption of Synechococcus 6301 spheroplasts with mild detergents in buffers of low ionic strength and were examined by sucrose density gradient centrifugation. The density gradient fractionation was severely complicated by the presence of large amounts of phycoerythrin aggregates, varying widely in size. Consequently, a mutant of Synechococcus 6301, strain AN112, was chosen for further study. Strain AN112 contains only 35% of the wild type level of phycoerythrin, but more importantly, is unable to polymerize phycoerythrin into multi-disc rod-like structures because it lacks the 33,000- and 30,000-dalton linker polypeptides (16, 28).

AN112 spheroplasts were disrupted by brief sonication in a low ionic strength buffer (buffer A: 50 mM Tricine, 5 mM CaCl2, 10% (w/v) glycerol, pH 7.8), then incubated for 30 min under very mild solubilizing conditions (0.3% (v/v) Miranol S2M-SF). The lysate was clarified by centrifugation at 31,000 gmax for 30 min at 18 °C and 5-ml aliquots were layered onto 0.2-1.0 M sucrose gradients prepared in buffer A. Sedimentation was in a Spinco SW27 rotor at 26,000 rpm (122,000 × gmax) for 19 h at 18 °C. After centrifugation, it was noted that phycobilisomes were completely absent, but smaller biliprotein complexes were seen as bands in the region 1.5-5 cm from the top of the tube. Of particular interest was the fastest sedimenting of the biliprotein-containing components, which appeared as a homogenous turquoise band. SDS-polyacrylamide gel electrophoresis indicated that polypeptides contained in phycobilisomes represented ~80% of the protein in this band. The turquoise band exhibited an allophycocyanin-like absorption spectrum (λmax 649 nm), with a fluorescence emission maximum of 676 nm (excitation at 580 nm). Some chlorophyll a was present in the region of the sucrose density gradient just below this material, but did not appear to be associated with the biliprotein complex.

Fractionation of Partly Dissociated Phycobilisomes

The results described above suggested that the complex enriched in allophycocyanin was derived entirely from phycobilisomes. To obtain this component completely free of other cellular proteins, we attempted isolation from purified phycobilisomes as starting material. The standard buffer used in our laboratory for the purification of phycobilisomes, 0.75 M NaK-phosphate, pH 8.0 (30), would be incompatible with the Ca2+ in buffer A. Consequently, AN112 phycobilisomes were isolated in 0.8 M Na2SO4 buffered with 50 mM Tris-HCl, pH 8.0. The phycobilisomes obtained in this manner had identical properties with those purified in 0.75 M phosphate, pH 8.0 (30) and were completely free of chlorophyll a.

Phycobilisomes were precipitated by the addition of 2 volumes of 40% (w/v) aqueous polyethylene glycol (average M, = 4000), a step that did not affect the integrity of these particles, then centrifuged for 20 min at 17,000 × gmax at 5 °C. After the supernatant was thoroughly drained from the pellet, phycobilisomes were dissociated by reassociation in buffer A to a protein concentration of 1 mg/ml. After 1 h at room temperature, 0.5-ml aliquots were applied to 0.2-1.0 M sucrose density gradients prepared in buffer A and sedimented in a Spinco SW41 rotor at 29,000 rpm (143,000 × gmax) for 17 h at 18 °C. Centrifugation led to the separation of three bands of blue material (Fig. 2, inset).

Fractions 1 and 2 were most clearly resolved on sucrose density gradients when the initial protein concentration was kept to 1 mg/ml or lower; at much higher concentrations, they appear as a single blue band. The most rapidly sedimenting component (fraction 3) was well resolved and showed no evidence of dissociation to slower sedimenting particles.

Sedimentation Studies

The three biliprotein fractions (Fig. 2) were collected and fractions 2 and 3 were examined in the analytical ultracentrifuge after dialysis against 50 mM Tricine, 5 mM CaCl2, pH 7.8.

![Fig. 1. Comparison of two methods used to quantitate the amounts of polypeptide present in SDS-polyacrylamide gel bands.](image-url)
a molar ratio of 1:1 (1). For the purpose of comparison, all values were normalized to the α subunit of allophycocyanin. AN112 phycobilisomes had a phycocyanin:allophycocyanin ratio of ~1.4 (see also Ref. 28), fraction 1 of ~1.56, while

![Absorption spectra of fractions separated upon centrifugation of partially dissociated AN112 phycobilisomes on sucrose density gradients (see inset). Fraction 1, ---; Fraction 2, - - - -; Fraction 3, - - - -.

Over half of the material in fraction 2 sedimented with an 11 S boundary, the remainder was heterogeneous and sedimented more slowly. Fraction 3, examined at 0.3 mg/ml, sedimented as a single boundary of 18 S with no evidence of dissociation.

**Polypeptide Composition**

The SDS-polyacrylamide gel profiles of AN112 phycobilisomes and of fractions 1, 2, and 3 are compared in Fig. 3. Previous studies have shown that AN112 phycobilisomes contain two major linker polypeptides of 27,000 and 75,000 daltons in addition to the α and β subunits of the phycobiliproteins (Fig. 3A) (16). The 75K polypeptide was shown to be an integral constituent of the allophycocyanin-containing core, and the 27K polypeptide to be associated with the adjacent phycocyanin-containing discs (16, 18). Two minor polypeptide components of 10,500 and 45,000 daltons, evident in Fig. 3A, were also described (16, 28). The amount of the 10,500-dalton polypeptide appeared to be variable. However, more recently we have found that it loses Coomassie stain upon extensive destaining. At present, little is known concerning the 10,500- and 45,000-dalton polypeptides. However, both appear to be enriched relative to phycocyanin in phycobilisomes containing a higher than normal percentage of core components, i.e. in phycobilisomes of mutants with levels of phycocyanin lower than in those of wild type (28).

An important observation (Fig. 3) was that only the 18 S complex (fraction 3) contained the 75K polypeptide, whereas both fractions 2 and 3 contained the 27K polypeptide. Fraction 1 lacked both the 75K and 27K polypeptides, whereas the 10,500-dalton polypeptide was present mainly on this fraction, to a minor extent in fraction 2, but was absent from fraction 3. The 45,000-dalton polypeptide was present exclusively in fraction 2.

Table I presents the relative molar amounts of each polypeptide in intact phycobilisomes and in fractions 1, 2, and 3. In examining the data, it should be recalled that phycocyanin and allophycocyanin each consist of two subunits, α and β, in

![SDS-polyacrylamide gel electrophoresis profiles of AN112 phycobilisomes (A) and fractions obtained from partially dissociated phycobilisomes (B). Numbers at the bottom of each gel lane correspond to fractions 1, 2, and 3 from the sucrose density gradient shown in Fig. 2, inset. PBS, phycobilisome.

**TABLE I**

Quantitative analysis of the polypeptides present in AN112 phycobilisomes and in fractions obtained upon partial dissociation of the phycobilisomes

Stained gels (Fig. 4) of fractions 1–3 (Fig. 2) were scanned with a Quick Scan densitometer and the peak areas were integrated with a Numptionics Corp. electronic graphics calculator. The values obtained were divided by the appropriate molecular weights to obtain molar ratios. All values were normalized to the α subunit of allophycocyanin.

<table>
<thead>
<tr>
<th>Mr × 10^3</th>
<th>Identification of polypeptide bands^a</th>
<th>PBS</th>
<th>Fraction 1^b</th>
<th>Fraction 2</th>
<th>Fraction 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>10,500</td>
<td>AP-β and PC-α subunits</td>
<td>0.13</td>
<td>0.24</td>
<td>0.21</td>
<td>Abs</td>
</tr>
<tr>
<td>17,700</td>
<td>PC-α subunit</td>
<td>2.11</td>
<td>2.70</td>
<td>3.98</td>
<td>1.74</td>
</tr>
<tr>
<td>18,200</td>
<td>AP-β subunit</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>19,000</td>
<td>PC-β subunit</td>
<td>1.40</td>
<td>1.56</td>
<td>2.80</td>
<td>0.98</td>
</tr>
<tr>
<td>27,000</td>
<td></td>
<td>0.29</td>
<td>0.01</td>
<td>0.34</td>
<td>0.36</td>
</tr>
<tr>
<td>45,000</td>
<td></td>
<td>0.02</td>
<td>Abs</td>
<td>0.01</td>
<td>Abs</td>
</tr>
<tr>
<td>55,000</td>
<td></td>
<td>0.02</td>
<td>Abs</td>
<td>Abs</td>
<td>0.03</td>
</tr>
<tr>
<td>75,000</td>
<td></td>
<td>0.06</td>
<td>Abs</td>
<td>Abs</td>
<td>0.11</td>
</tr>
<tr>
<td>10,500`</td>
<td></td>
<td>0.06</td>
<td>Abs</td>
<td>Abs</td>
<td>0.11</td>
</tr>
</tbody>
</table>

^a The abbreviations used are: PC, phycocyanin; AP, allophycocyanin; PBS, phycobilisome; Abs, polypeptide absent.

^b See sucrose density gradient profile in Fig. 1.

^c This polypeptide is a degradation product of the 75K polypeptide. This component was particularly evident in small phycobilisomes of mutants such as AN112, since these phycobilisomes did not separate as cleanly as those of wild type from other cellular components during sucrose density gradient centrifugation. The amount of the 55K polypeptide increased on prolonged storage at the expense of the 75K polypeptide.

^d Values in parentheses were calculated from the absorbance of eluates of gels (see "Experimental Procedures").
fraction 2 was higher in phycocyanin (phycocyanin:allophycocyanin = 2.6) and fraction 3 was enriched in allophycocyanin (phycocyanin:allophycocyanin = 0.98). The 75K polypeptide, seen exclusively in fraction 3 (Fig. 3B), was enriched 2-fold relative to allophycocyanin when compared to the corresponding ratio in phycobilisomes. Quantitation of the total amounts of phycocyanin and allophycocyanin in each of fractions 1–3 showed that the amount of phycocyanin in each fraction was nearly equal (30–35%), while nearly 50% of the allophycocyanin was found in fraction 3. A more detailed examination of the composition of fractions 1–3, by two-dimensional SDS-polyacrylamide gel electrophoresis, is presented below.

**Spectroscopic Studies**

The composition of fractions 1–3, the conformation of the biliproteins in these fractions, and the interactions among these biliproteins were examined by several spectroscopic techniques.

**Fraction 1**—The sedimentation rate of the material in this zone corresponded to that of a phycocyanin or allophycocyanin trimer (approximately 120,000 daltons), and both of these biliproteins were present in this fraction (see Fig. 3B, lane 1). The absorption spectrum of fraction 1 had a peak at 621 nm and a shoulder at 650 nm, consistent with the presence of both phycocyanin and allophycocyanin. On excitation at 580 nm, fraction 1 displayed a broad fluorescence maximum at 656 nm and an inflection at 680 nm. Three components, centered at 640, 661, and 680 nm, were seen in the second derivative of the emission spectrum (Fig. 4A), indicating that the 656 nm maximum was a composite of phycocyanin and allophycocyanin emissions. The 680 nm component reflected the presence of allophycocyanin B in this fraction (see below). The circular dichroism spectrum of fraction 1 (Fig. 5A) was similar to that reported for trimeric Synechococcus 6301 phycocyanin (37). The contribution from allophycocyanin to the circular dichroism, if any, was weak.

In sum, the above results indicate that fraction 1 was a mixture of free phycocyanin and allophycocyanin, and some allophycocyanin B.

**Fraction 2**—This fraction exhibited a broad absorption spectrum with a maximum at 628 nm (Fig. 2). A narrow emission spectrum with λ<sub>max</sub> at 655 nm was obtained upon 580 nm excitation. Second derivative analysis of this spectrum showed a major component at 654 nm and a very small component at 680 nm (Fig. 4A). Thus, phycocyanin made the quantitatively dominant contribution to the emission spectrum of fraction 2. The ratio of phycocyanin to allophycocyanin in this fraction was 2.6:1 (see Table 1), but the emission spectrum and its second derivative indicated that there was little energy transfer from phycocyanin to allophycocyanin in this polydisperse fraction.

**Fraction 3**—In contrast to the material in fractions 1 and 2, the material in fraction 3 exhibited the properties of a well-defined macromolecular complex. As noted above, this fraction was monodisperse in the analytical ultracentrifuge. The absorption spectrum of this fraction (Fig. 2) resembled that of allophycocyanin, with λ<sub>max</sub> at 649 nm and a prominent shoulder at 600 nm, but in addition showed a broad tail of absorption in the 670–690 nm region. As shown in Fig. 4B, the fluorescence emission spectrum of fraction 3 showed a near-identity to that of intact AN112 phycobilisomes, with components at 660 and 680 nm. The circular dichroism spectrum of this fraction (Fig. 5) was very similar to that of AN112 phycobilisomes in the region of allophycocyanin and allophycocyanin B maximal absorption (640–690 nm).

The fluorescence data, combined with the results of analytical ultracentrifugation, show that fraction 3 consisted of 18 S particles within which the biliproteins showed tightly coupled energy transfer and retained some of the structural features characteristic of intact phycobilisomes.

**Polypeptide Composition of Fractions 1–3 as Revealed by Two-dimensional Mapping**

Although the gross polypeptide composition is adequately displayed by one-dimensional SDS-polyacrylamide gel elec-
trophoresis, complete resolution of the biliprotein subunits is not achieved in such a system. For example, the \( \alpha \) subunit of phycocyanin, the \( \beta \) subunit of allophycocyanin, and \( \alpha \) subunit of allophycocyanin B are not resolved from one another in the pattern shown in Fig. 3. However, as shown in Fig. 6, resolution of the low molecular weight components of the phycobilisome and of its partial dissociation products was achieved by isoelectrofocusing in the first dimension and SDS-polyacrylamide gel electrophoresis in the second.

Four major spots (1–4) were present in the phycobilisome map (Fig. 6A). These were shown to correspond to the \( \alpha \) and \( \beta \) subunits of phycocyanin and allophycocyanin by comparison with gels containing these purified proteins (data not shown). We had previously named the “smaller” subunit of allophycocyanin (spot 4) and “larger” (spot 3) as \( \alpha \) and \( \beta \), respectively (30). However, on the basis of sequence determination we have since found the reverse to be true (5). Thus, to conform with the nomenclature proposed by Glazer et al. (38) based on NH\(_2\)-terminal sequence homologies, the “larger” more acidic polypeptide (spot 3) is designated as \( \alpha \) \((M_r = 18,200)\) and the “smaller” and more basic one (spot 4) as \( \beta \) \((M_r = 17,600)\).

As shown in Fig. 6, B–D, phycocyanin (spots 1 and 2) was present as a major component in all three fractions. Allophycocyanin (spots 3 and 4) was a major component of fraction 1, was about equimolar with phycocyanin in fraction 3, but was very low in amounts in fraction 2—consistent with the data in Table I. More interesting, however, was the distribution and relative abundance of the three minor phycobilisome polypeptides (spots 5–7 in Fig. 6A). The most basic of these (spot 5) was recently identified as the \( \alpha \) subunit of allophycocyanin B (5). This biliprotein, first described in 1975, has absorption \((\lambda_{\text{max}} = 671 \text{ nm})\) and emission \((\lambda_{\text{em}} = 680 \text{ nm})\) maximum red-shifted relative to allophycocyanin and, like the latter, is made up of \( \alpha \) and \( \beta \) subunits in equal amounts (3, 4). We have recently demonstrated that allophycocyanin B is a hybrid protein in which the \( \alpha \) subunit is unique but the \( \beta \) subunit is identical with that of allophycocyanin (spot 4). The unique \( \alpha \) subunit was shown to determine whether the protein exhibited the spectroscopic properties of allophycocyanin or of allophycocyanin B (5). As shown in Fig. 6, the allophycocyanin B \( \alpha \) subunit (spot 5) was very abundant in fraction 1, was a minor component of fraction 2, and was completely absent in fraction 3 (Fig. 6D, open circle). However, in this latter particle, another polypeptide (spot 7) was present. This component was absent in both fractions 1 and 2 (Fig. 6, B and C, open circles). Based on densitometer scans of isoelectric focusing tube gels prior to staining, polypeptide 7 was found to carry a blue chromophore. The third minor polypeptide, spot 6, was present in all fractions, but always as a minor component. This unidentified polypeptide is present in two-dimensional gels of highly purified phycocyanin, but not in those of allophycocyanin (data not shown), and might be derived from phycocyanin.

An estimate of the molar percentage of each spot present in the 18 S particle (Fig. 6D) is shown in Table II. Although the dye elution method used in this analysis (see “Experimental Procedures”) gave accuracy that was comparable to gel scanning, the quantitation of two-dimensional gels suffered from the need to equilibrate first dimension isoelectric focusing gels.

### Table II

<table>
<thead>
<tr>
<th>Spot number</th>
<th>( M_r )</th>
<th>Identification of spot</th>
<th>Molar percentage of total biliprotein</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>17,700</td>
<td>PC-( \alpha ) subunit</td>
<td>29.1 ( \pm ) 1.2</td>
</tr>
<tr>
<td>2</td>
<td>19,000</td>
<td>PC-( \beta ) subunit</td>
<td>22.3 ( \pm ) 4.2</td>
</tr>
<tr>
<td>3</td>
<td>18,200</td>
<td>AP-( \alpha ) subunit</td>
<td>22.6 ( \pm ) 2.6</td>
</tr>
<tr>
<td>4</td>
<td>17,900</td>
<td>AP-( \beta ) subunit</td>
<td>16.7 ( \pm ) 4.6</td>
</tr>
<tr>
<td>5</td>
<td>17,900</td>
<td>AP B-( \alpha ) subunit</td>
<td>Absent</td>
</tr>
<tr>
<td>6</td>
<td>17,600</td>
<td>Unknown</td>
<td>2.1 ( \pm ) 0.4</td>
</tr>
<tr>
<td>7</td>
<td>18,300</td>
<td>Unknown</td>
<td>7.2 ( \pm ) 1.9</td>
</tr>
</tbody>
</table>

\( ^a \) See Fig. 5.

\( ^b \) The abbreviations used are: PC, phycocyanin; AP, allophycocyanin; AP B, allophycocyanin B.
Digestion with Trypsin (min)

in an SDS-containing buffer prior to electrophoresis in the second dimension. As a consequence, a variable loss of certain polypeptides occurred, resulting in significant fluctuation in the data, particularly for the phycocyanin and allophycocyanin β subunits. Nevertheless, phycocyanin and allophycocyanin were seen to account for approximately 90% of this particle, while the 18.3K polypeptide (spot 7) represented about 7%. Since the 18 S particles contained about 50% of the allophycocyanin, 33% of the phycocyanin, and apparently all of the 18.3K polypeptide present in the phycobilisome, the last component accounted for about 3% of the total biliprotein subunit population per phycobilisome—a value roughly equivalent to that for the α subunit of allophycocyanin B (Fig. 6, spot 5).

Limited Tryptic Digestion of 11 S and 18 S Complexes

In previous studies (Refs. 18 and 30 and Footnote 2), we found that native biliproteins are highly resistant to digestion by trypsin, whereas the susceptibility of the linker polypeptides depends upon the nature of the aggregate of which they form a part. In intact Synechococcus 6301 phycobilisomes, the 75- and 45-kilodalton polypeptides are rapidly degraded, whereas the 27-, 30-, and 33-kilodalton polypeptides are far more resistant to degradation (30). In contrast, in dissociated phycobilisomes, the 27K polypeptide is rapidly degraded by trypsin. From these observations, from in vitro reconstitution experiments (18), and from studies of mutants (16, 28), it appears that the 27K polypeptide participates in the binding of the phycocyanin-containing rods to the core units. The resistance of the 27K polypeptide to proteolysis is therefore a

*G. Yamanaka, D. J. Lundell, and A. N. Glazer, unpublished observations.

**Fig. 7.** Analysis of partial tryptic digests of the 18 S particles (18S Fr) and of fraction 2 (11S Fr). Samples were diluted to 100 μg of protein/ml with buffer A and cooled to 0 °C. Trypsin was added to a final concentration of 5 μg/ml. Aliquots were removed at 1, 5, and 30 min and pipetted into 10% (w/v) trichloroacetic acid. Controls (C) received no trypsin. T denotes the position of trypsin.

**Fig. 8.** Analysis of the effect of partial tryptic digestion of 18 S particles on their absorption spectrum. Sample was diluted to 150 μg of protein/ml with buffer A and scanned in a 10-mm path length cuvette at 60 nm/min (spectrum C). Trypsin was then added to a final concentration of 5 μg/ml and the spectrum was scanned at 1, 6, 12, 20, 40, and 60 min after enzyme addition, as shown.
\textbf{DISCUSSION}

A major problem in the determination of the macromolecular organization within phycobilisomes has been the difficulty in isolating, from partially dissociated particles, subassemblies representing specific phycobilisome domains. One reason for such difficulty has been the generation of a heterogeneous mixture of components from the rod substructures during phycobilisome dissociation. The rods have generally proven to be more stable at low ionic strength than the core substructures (e.g. Ref. 39). The rod length in intact phycobilisomes varies; in \textit{Synechococcus} 6301 particles, rod length ranges from one disc to seven discs on a single phycobilisome (see Refs. 15 and 16). On dissociation at low ionic strength, a heterogeneous mixture of complexes derived from the rods is obtained, and these components interfere with the analysis of products originating from the core substructures.

The \textit{Synechococcus} 6301 mutant AN112 used in this study offers the following advantages for the detailed study of phycobilisome structure: 1) a simple two-disc core as compared to the more complex three-disc cores of most other phycobilisomes; 2) only two major biliproteins (phycoerythrin is absent); 3) only two major linker polypeptides, as compared to four in the wild type and more in most other phycobilisomes (16); 4) the absence of rods of more than one disc in length (16, 28); 5) invariance in phycobilisome composition under a wide variety of culture conditions (28). Because of the relative simplicity of AN112 phycobilisomes, we were able to interpret the dissociation pattern of these particles.

The structure of AN112 phycobilisomes, as revealed by electron microscopy, is shown in Fig. 9A. A core substructure of eight allophycocyanin-containing trimers is surrounded by...
up to six phycocyanin-containing hexamers (16). This interpretation of the ultrastructure is supported by the analytical data in Table I.

Partial dissociation of the AN112 phycobilisome yielded a subassembly particle of unique composition—the 18 S particle. This particle contains all of the 75K and 18.3K polypeptides, half of the allophycocyanin, and none of the allophycocyanin B of the phycobilisome. The molar ratio of phycocyanin: allophycocyanin:27K:75K in the 18 S particle is approximately 6:6:2:1 (see Table I). From the molecular weights of monomeric phycocyanin (36,700) and allophycocyanin (35,800), and assuming one copy of 75K per particle, the above data lead to a molecular weight of 564,000, a value reasonably consistent with a sedimentation coefficient of 18 S.

In intact phycobilisomes, the ratio of phycocyanin monomer to the 27K polypeptide is 6:1 (Table I; also Ref. 16). However, since the ratio of phycocyanin to 27K in the 18 S particle is 1:3, we infer that the phycocyanin in this subassembly is organized into two (αβ)6,27K trimers. The remainder of the phycocyanin of the original hexamers is found in fraction 1. This interpretation is supported by the nature of the spectroscopic changes that accompany tryptic degradation of the 18 S particle (see “Results”).

Circular dichroism spectra (Fig. 5) of the 18 S particle and of intact AN112 phycobilisomes show similar long wavelength components centered at 655 and 665 nm. This supports the contention that the organization of allophycocyanin leading to strongly enhanced 650 nm absorbance is common to both the AN112 phycobilisome and the 18 S subassembly.

The structure of the 18 S particle deduced from the above data is shown in Fig. 9C. Since this particle has a molecular weight of ~560,000, and since the 18 S particle fraction (Fig. 2, fraction 3) contains ~50% of the total allophycocyanin and ~33% of the total phycocyanin of the AN112 phycobilisome, it is evident that two copies of this subassembly are released from each phycobilisome (Fig. 9, compare A and C).

The remainder of the AN112 phycobilisome is distributed between fractions 1 and 2. Fraction 1 contains phycocyanin, allophycocyanin, and allophycocyanin B, but no linker polypeptides (see Fig. 3B, lane 1 and Fig. 6B). On the basis of their sedimentation behavior, these proteins appear to be in their trimeric aggregation state and the fluorescence emission spectrum of fraction 1 (Fig. 4A) indicated little energy transfer between phycocyanin and the allophycocyanins. Fraction 2 contained predominantly phycocyanin and the 27K polypeptide, and small amounts of allophycocyanin and allophycocyanin B (see Fig. 3, lane 2 and Fig. 6C). Analytical ultracentrifugation showed this fraction to be heterogeneous. Its major 11 S component was probably the phycocyanin-27K linker polypeptide complex, (αβ)6,27K (18). The fluorescence emission spectrum of fraction 2 was a narrow band centered at 655 nm, with virtually no energy transfer to allophycocyanin or allophycocyanin B (Fig. 4A). This spectrum resembled that obtained for the (αβ)6,27K phycocyanin complex reconstituted in vitro from purified components (see Fig. 6 of Ref. 18). The absence of energy transfer from phycocyanin to allophycocyanin indicates that these biliproteins are present in separate aggregates which co-sediment in fraction 2.

The results discussed above place numerous constraints on the location of the various components of the phycobilisome. One model which incorporates these constraints is shown in Fig. 9, A and B. It is proposed that the AN112 phycobilisome consists of two types of subassemblies. Each subassembly is represented twice in the structure and contains portions of both core and rod domains. One type of subassembly gives rise to two copies of the 18 S particle and free phycocyanin. The second type of subassembly contains the remainder of the phycobilisome, including all of the allophycocyanin B. It readily dissociates into a mixture of complexes, the largest of which is the phycocyanin complex, (αβ)6,27K, found in fraction 2 (Fig. 2). It is noteworthy that the quantitative data presented in Tables I and II indicate that there are four allophycocyanin B α subunits and four 18.3K polypeptides in the AN112 phycobilisome. Ultrastructural studies (16) show that the core is made up of eight discs corresponding in dimensions to (αβ)6 trimers. If it is assumed that the allophycocyanin B α subunits and the 18.3K polypeptides are symmetrically distributed among the eight discs, each disc would have the composition αββX where α and β are the subunits of allophycocyanin and X is either an allophycocyanin B α subunit or an 18.3K polypeptide. This leads to the conclusion that the (αβ)6 trimer of purified allophycocyanin (40, 41) is produced in vitro by association of monomers during the purification procedure. We have reached the same conclusion concerning the origin of the (αβ)6 trimer of allophycocyanin B in an earlier study (5). In essence, extreme care has to be exercised in relating the structures of stable phycobiliprotein aggregates isolated in vitro to those originally present in the phycobilisome.

As shown in Fig. 4B, the fluorescence emission spectrum of the 18 S particle is made up of two components, with emission maxima at 660 and 680 nm. The 660 nm emission can be attributed to allophycocyanin. The 680 nm emission, characteristic of allophycocyanin B, cannot derive from this protein since it is absent from these particles (see Fig. 6D). Instead, the most probable candidate for the terminal energy acceptor of the 18 S particle appears to be the 75K bilin-bearing polypeptide, previously shown to exhibit an emission maximum near 680 nm as an isolated polypeptide in solution (7). Fractionation and reconstitution data indicate that the 18.3K polypeptide does not function as a 680 nm emitter.

The above observations pose interesting questions concerning the pathways of energy transfer within phycobilisomes. Previously, the energy transfer pathway was postulated to be

\[ \text{Phycocyanin} \rightarrow \text{allophycocyanin} \rightarrow \text{allophycocyanin} \rightarrow \text{allophycocyanin B} \]

\[ (\lambda_{\text{max}} 660 \text{ nm}) \rightarrow (\lambda_{\text{max}} 650 \text{ nm}) \rightarrow (\lambda_{\text{max}} 670 \text{ nm}) \]

The data presented above, as well as studies of the 75K polypeptide (7), indicate that this component emits at ~680 nm in the 18 S subassembly. A plausible scheme (see also Ref. 8) which includes the 75K polypeptide in the energy transfer sequence, is

\[ \text{Phycocyanin} \rightarrow \text{allophycocyanin} \rightarrow \text{allophycocyanin} \rightarrow \text{allophycocyanin B} \rightarrow 75 \text{K polypeptide} \]

This scheme assumes that of all the phycobilisome chromophores the bilin on 75K approaches most closely an energy acceptor within the thylakoid membrane. There is substantial evidence that the high molecular weight polypeptide of the phycobilisome is involved in attachment of the particle to the thylakoid membrane and may in fact be partly buried within the membrane (6, 17). Detailed kinetic spectroscopic studies on phycobilisomes and the various subcomponents are needed to establish the actual pathways of energy flow.

REFERENCES

4086 Phycobilisome Subassembly Particles

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