Rod Substructure in Cyanobacterial Phycobilisomes: Phycoerythrin Assembly in Synechocystis 6701 Phycobilisomes

JEFFREY C. GINGRICH, ROBLEY C. WILLIAMS, and ALEXANDER N. GLAZER
Departments of Microbiology and Immunology, and of Molecular Biology, University of California, Berkeley, California 94720

ABSTRACT *Synechocystis* 6701 phycobilisomes consist of a core of three cylindrical elements in an equilateral array from which extend in a fanlike manner six rods, each made up of three to four stacked disks. Previous studies (see Gingrich, J. C., L. K. Blaha, and A. N. Glazer, 1982. *J. Cell Biol.* 92:261–268) have shown that the rods consist of four disk-shaped complexes of biliproteins with "linker" polypeptides of 27-, 33.5-, 31.5-, and 30.5-kdaltons, listed in order starting with the disk proximal to the core: phycocyanin $(\alpha\beta)_6$-27 kdalton, phycocyanin $(\alpha\beta)_6$-33.5 kdalton, phycoerythrin $(\alpha\beta)_6$-31.5 kdalton, phycoerythrin $(\alpha\beta)_6$-30.5 kdalton, where $\alpha\beta$ is the monomer of the biliprotein.

Phycoerythrin complexes of the 31.5- and 30.5-kdalton polypeptides were isolated in low salt. In 0.05 M K-phosphate-1 mM EDTA at pH 7.0, these complexes had the average composition $(\alpha\beta)_2$-31.5 and $(\alpha\beta)$-30.5 kdalton polypeptide, respectively. Peptide mapping of purified 31.5- and 30.5-kdalton polypeptides showed that they differed significantly in primary structure. In 0.65 M Na-K-phosphate at pH 8, these phycoerythrin complexes formed rods of stacked disks of composition $(\alpha\beta)_6$-31.5 or $(\alpha\beta)_6$-30.5 kdaltons. For the $(\alpha\beta)$-30.5 kdalton complex, the yield of rod assemblies was variable and the self-association of free phycoerythrin to smaller aggregates was an important competing reaction.

Complementation experiments were performed with incomplete phycobilisomes from *Synechocystis* 6701 mutant strain CM25. These phycobilisomes are totally lacking in phycoerythrin and the 31.5- and 30.5-kdalton polypeptides, but have no other apparent structural defects. In high phosphate at pH 8, the phycoerythrin-31.5-kdalton complex formed disk assemblies at the end of the rod substructures of CM25 phycobilisomes whereas no interaction with the phycoerythrin-30.5 kdalton complex was detected. In mixtures of both the phycoerythrin-31.5 and -30.5 kdalton complexes with CM25 phycobilisomes, both complexes were incorporated at the distal ends of the rod substructures. The efficiency of energy transfer from the added phycoerythrin in complemented phycobilisomes was ~96%. The results show that the ordered assembly of phycoerythrin complexes seen in phycobilisomes is reproduced in the in vitro assembly process.

The outer surfaces of the photosynthetic lamellae of cyanobacteria and of the red-algal chloroplast are studded with a regular array of large particles, phycobilisomes, which serve as light-harvesting components of the photosynthetic apparatus in these organisms (1). On a quantitative basis, the major constituents of the phycobilisome are the biliproteins, phycoerythrin (λ_{max} ~565 nm), phycocyanin (λ_{max} ~620 nm), and allophycocyanin (λ_{max} ~650 nm), which carry covalently attached open-chain tetrapyrrole prosthetic groups (2). In addition, these particles contain "linker" polypeptides in smaller amounts (3–5). Certain of the linker polypeptides are also biliproteins. As the name implies, the linker polypeptides direct the assembly of the phycobilisome and its attachment to the thylakoid membrane.

THE JOURNAL OF CELL BIOLOGY · VOLUME 95 OCTOBER 1982 170–178
© The Rockefeller University Press · 0021-9525/82/10/0170/09 $1.00

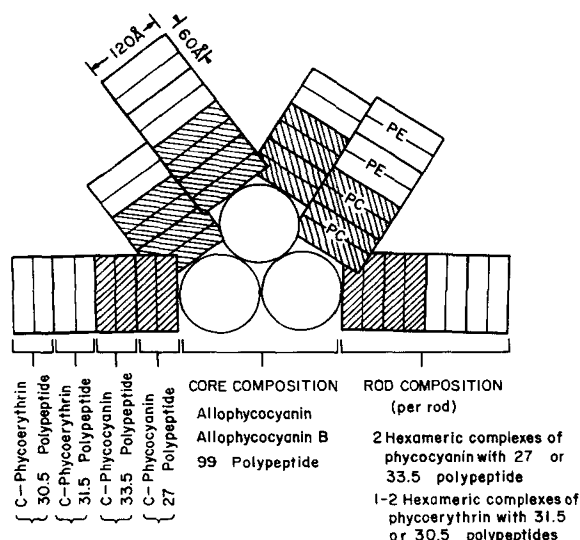

FIGURE 1 Schematic representation of the *Synechocystis* 6701 phycobilisome in "face" view. The model is based on electron-microscopic and biochemical studies of wild-type phycobilisomes and of incomplete phycobilisomes from mutants. The abbreviations are *PC*, phycocyanin; *PE*, phycoerythrin. (From reference 8.) Polypeptide numbers are mol wt $\times 10^{-3}$.

The morphology of the phycobilisomes of the unicellular cyanobacterium *Synechocystis* 6701 (Fig. 1, references 6–8) is common to the phycobilisomes of numerous other cyanobacteria (6, 9, 10) and those of the red alga *Rhodella violacea* (11). The rod substructures of *Synechocystis* 6701 phycobilisomes are made up of stacked disks, ~60 × 120Å. C-Phycocyanin is contained within the two disk elements of each rod proximal to the core whereas C-phycoerythrin is contained within the distal disks (6–8). Each C-phycocyanin and C-phycoerythrin-containing disk is an $(\alpha\beta)_6$ oligomer of the biliprotein (where $\alpha\beta$ is the monomer) held together by the interaction with a linker polypeptide. Analytical studies indicate that the composition of a disk is $(\alpha\beta)_6$ X_1, where X represents a linker polypeptide (8; see also references 5 and 12). Studies of numerous mutants of *Synechocystis* 6701, which form incomplete phycobilisomes, have indicated the arrangement of disk complexes within the rod substructures shown in Fig. 1.

The phycoerythrin-30.5-kdalton polypeptide complex occupies a terminal position in the rod substructure, whereas the phycoerythrin-31.5 kdalton polypeptide complex is adjacent to the phycocyanin portion of the rod. This assignment is based on two lines of evidence. When grown in red light, *Synechocystis* 6701 produces only 20% of the white-light level of phycoerythrin but an unaltered amount of phycocyanin (8, 13). In red-light phycobilisomes, the rods are only two to three disks in length (6, 7) and the 30.5-kdalton polypeptide is absent (8). Among mutants of *Synechocystis* 6701 that produce incomplete phycobilisomes, several have been isolated that lack the 30.5-kdalton polypeptide and have much decreased amounts of phycoerythrin; the 31.5-kdalton polypeptide is present in all such mutants (7, 8). Mutant phycobilisomes containing the 30.5-kdalton polypeptide but lacking the 31.5-kdalton polypeptide have not been detected.

This study examines the behavior of phycoerythrin-30.5 kdalton and phycoerythrin-31.5 kdalton complexes in in vitro assembly experiments. *Inter alia*, we show that the relative positioning of these complexes in structures assembled in vitro corresponds to that deduced from the earlier studies outlined above.

MATERIALS AND METHODS

Organisms and Culture Conditions

Synechocystis 6701 used in this study is maintained in the American Type Culture Collection (Rockville, MD; ATCC 27170) and in the culture collection of the Unité de Physiologie Microbienne, Institut Pasteur, Paris [PCC 6701; (14)]. Strain CM25 also used in this study is a *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine-induced mutant of *Synechocystis* 6701 similar to strain CM70 described previously (8). Strain CM25 was used in preference to CM70 because of its faster growth rate. The phycobilisomes of strain CM25 lack phycoerythrin and the 30.5- and 31.5-kdalton polypeptides, but in all other respects are equivalent to those of wild-type cells. Cells were grown in medium BG11, containing double the usual amount of carbonate (14), in warm white fluorescent light at 30°C. Cultures were supplied with an atmosphere of 4% CO_2–96% N_2, and were harvested when cell density reached 0.5–1.0 g wet weight/l.

Preparation of Phycobilisomes

Phycobilisomes were prepared as previously described (7) with Triton X-100 as the membrane solubilization reagent. To minimize proteolysis, 1 mM phenylmethylsulfonyl fluoride and 1 mM EDTA were included in the breakage buffer. A Sorvall TV850 vertical rotor (Dupont Instruments-Sorvall Biomedical Div., Dupont Co., Newtown, CT) was used for some preparations instead of a Spinco SW41 rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, CA). For such preparations, sucrose step gradients consisted of 3, 7, 9, 5, and 5 ml of 2.0, 1.0, 0.75, 0.5, and 0.25 M sucrose, respectively, in 0.75 M NaH_2PO_4-K_2HPO_4 at pH 8.0. Centrifugation was performed at 190,000 g_{max} for 2.5 h at 18°C.

Fractionation of Phycobilisome Components by Chromatography on Hydroxylapatite

Gradient fractions containing phycobilisomes from wild-type cells were passed through a Sephadex G-25 column equilibrated with 1 mM K-phosphate-0.1 M NaCl, pH 7.0. The entire colored eluate was applied to a column of hydroxylapatite (15) equilibrated with the same buffer. 2–4 mg of protein were adsorbed per ml of hydroxylapatite. Stepwise elution with increasing phosphate concentrations was performed with buffers containing 0.1 M NaCl at pH 7. For rapid isolation of the phycoerythrin fraction, the column was first eluted with 0.015 M K-phosphate-0.1 M NaCl, pH 7, and the phycoerythrin eluted as a concentrated solution with buffer 30 mM in K-phosphate.

Separation of Phycoerythrin Complexes by Sucrose Density Gradient Centrifugation

Immediately after elution from hydroxylapatite, phycoerythrin fractions were applied directly to 5–15% (wt/vol) linear sucrose density gradients in 50 mM K-phosphate-1 mM EDTA at pH 7.0. Centrifugation was performed in a Spinco SW41 rotor (Beckman Instruments, Inc., Spinco Div.) at 260,000 g_{max} for 19 h at 10°C, or in a Sorvall TV850 rotor (Dupont Instruments-Sorvall Biomedical Div., Dupont Co.) for 6 h at 190,000 g_{max} at 10°C. After centrifugation, the gradients were fractionated and corresponding fractions from each set of gradients were pooled.

Assembly Behavior of Phycoerythrin Complexes at High Phosphate Concentrations

Fractions containing phycoerythrin, the 30.5-kdalton polypeptide and/or the 31.5-kdalton polypeptide were obtained from the sucrose-density gradients described above. Aliquots (1.0 ml) of these fractions were passed through a Sephadex G-25 column (5.0 ml) equilibrated in and eluted with 0.65 M Na-K-phosphate, pH 8.0. The colored eluate was immediately applied to 5–15% (wt/vol) linear sucrose-density gradients, layered on a 1 ml cushion of 2 M sucrose, in 0.65 M Na-K-phosphate, pH 8.0. Centrifugation was at 200,000 g_{max} for 18 h at 10°C in a Spinco SW41 rotor (Beckman Instruments, Inc., Spinco Div.).

Assembly of Phycoerythrin Complexes with Mutant Phycobilisomes

Fractions containing phycoerythrin, the 30.5-kdalton polypeptide and/or the 31.5-kdalton polypeptide were obtained from the 50 mM K-phosphate sucrose density gradients (see above), transferred into 1 mM K-phosphate-0.1 M NaCl, pH 7.0, by gel filtration on Sephadex G-25, and then adsorbed to hydroxylapatite at 1 mg of phycoerythrin per ml of adsorbent. A concentrated protein solution was then obtained by elution with 0.1 M Na-K-phosphate, pH 8.0.

In a typical experiment, 1 vol of phycoerythrin complex containing solution (at a phycoerythrin concentration of ~0.4 mg/ml) was mixed with 2 vol of a solution of strain CM25 phycobilisomes (at a phycocyanin concentration of 0.22 mg/ml) in 0.75 M Na-K-phosphate, pH 8.0, to give an approximate molar ratio of phycoerythrin to phycocyanin of 1:1. The mixture was immediately passed through a Sephadex G-25 column equilibrated and eluted with 0.75 M Na-K-phosphate, pH 8.0. One ml of the colored eluate was applied to a 0.25–1.0 M linear sucrose-density gradient in 0.75 M Na-K-phosphate, pH 8.0, with a 1 ml cushion of 2 M sucrose in the same buffer at the bottom. Control solutions were prepared by adding one volume of 0.1 M Na-K-phosphate buffer, pH 8.0, to 2 vol of strain CM25 phycobilisome solution, and two volumes of 0.75 M Na-K-phosphate buffer, pH 8.0, to 1 vol of phycoerythrin complex-containing solution, respectively. These control solutions were treated in the same manner as the mixture described above. Centrifugation was performed in a Spinco SW41 rotor (Beckman Instruments, Inc., Spinco Div.) at 90,000 g_{max} for 15 h at 18°C.

Spectroscopic Measurements

Absorption spectra were measured on a Beckman Model 25 recording spectrophotometer (Beckman Instruments, Inc., Fullerton, CA). Corrected fluorescence emission spectra were obtained with a Perkin-Elmer (Norwalk, CT) MPF-44B recording spectrofluorometer, equipped with a DCSU-2 differential corrected spectra unit and a Hamamatsu R928 phototube (Hamamatsu Corp., Middlesex, NJ). Sample absorbance ranged from 0.05 to 0.1 cm^{-1} at λ_{max}.

Electron Microscopy

Preparations of biliprotein aggregates and phycobilisomes, negatively stained with 1% (wt/vol) uranyl formate, were examined in the electron microscope as described previously (7, 9). Electron micrographs were obtained under conditions of minimal beam-exposure (16).

SDS PAGE

Slab gel electrophoresis was carried out on 0.9-mm thick 14% polyacrylamide gels (30:0.8 wt/wt acrylamide:methylenebisacrylamide) as previously described (17). Gels stained with Coomassie Brilliant Blue were scanned with a Helena Laboratories (Beaumont, TX) Quick Scan densitometer operated at slow speed with narrow beam.

Purification of the 30.5 and 31.5-kdalton polypeptides on a preparative scale was performed by slab gel electrophoresis on 3-mm thick 14% polyacrylamide gels. After an abbreviated, 30 min, staining-destaining procedure, the 30.5- and 31.5-kdalton polypeptide bands were excised from the gel and electrophoretically eluted into dialysis bags. The contents of the bags were then exhaustively dialyzed against distilled water and lyophilized.

Peptide Maps of the 30.5- and 31.5-kdalton Linker Polypeptides

For tryptic digestion, lyophilized polypeptides were dissolved in 0.1 M Tris-Cl-0.5% SDS-10% (vol/vol) glycerol, pH 8.0, at a protein concentration of 2 mg/ml. Trypsin (1–10%, wt/wt) was then added and digestion allowed to proceed at 23°C for 3 min. The reaction was stopped by placing the reaction mixtures in boiling water for 3 min. An aliquot (30 μg of protein) was examined by SDS PAGE on 0.9-mm thick 15% polyacrylamide gels.

For cyanogen-bromide cleavage, lyophilized polypeptides were redissolved at 1 mg/ml in 70% (vol/vol) formic acid and solid CNBr added to 1 mg/ml. Control polypeptide samples were incubated in 70% formic acid. After 24 h at 23°C, the samples were dried under vacuum. The residue was redissolved in SDS PAGE solubilization buffer as described previously (17), and examined in the same manner as the tryptic digests.

RESULTS

Phycobilisome Components Obtained by Chromatography on Hydroxylapatite

Early research on the stability of phycobilisomes showed that these particles could be isolated intact in solutions of high phosphate concentration, but that they dissociated in solutions of low salt concentration (18, 19). These observations apply fully to *Synechocystis* 6701 phycobilisomes. In 0.75 M Na-K-phosphate at pH 7.0, excitation of these phycobilisomes with light of 565 or 620 nm, the wavelengths of maximum absorption of phycoerythrin and phycocyanin, respectively, leads to little emission from these biliproteins. The energy is transferred efficiently to allophycocyanin and the terminal energy acceptors of the phycobilisome (20–22), and appears as fluorescence with a maximum at ~676 nm (7). In contrast, the fluorescence emission spectra of *Synechocystis* 6701 phycobilisomes in 0.001 M K-phosphate–0.1 M NaCl, pH 7.0, show little residual energy transfer between the major biliproteins, phycoerythrin, phycocyanin, and allophycocyanin. Consistent with these spectroscopic results, chromatography of dissociated *Synechocystis* 6701 phycobilisomes on hydroxylapatite showed the presence of specific complexes of individual biliproteins with linker polypeptides as well as of free biliproteins.

The elution pattern seen on hydroxylapatite chromatography is shown in Fig. 2. Five major zones of distinct composition were obtained (the number of the peak fraction of each component is given in parentheses and refers to Fig. 2): phycocyanin with the 27- and 33.5-kdalton polypeptides (fraction 2); phycocyanin, a trace of phycoerythrin, and the 27-, 33.5-, and 31.5-kdalton polypeptides (fraction 6); phycoerythrin with the 30.5- and 31.5-kdalton polypeptides (fraction 11); free phycocyanin (fraction 17); and allophycocyanin with the 99-kdalton polypeptide (fraction 28). The contents of these peak fractions were then subjected to centrifugation on linear 5–15% (wt/vol) sucrose-density gradients. The buffer composition for each gradient corresponded to that at which the fraction was eluted from hydroxylapatite. For all fractions, the linker polypeptides were found to cosediment with the biliproteins.

The particular associations of linker polypeptides with phycoerythrin and phycocyanin, evident from the above chromatographic fractionation, are compatible with the arrangement of the linker polypeptides and biliproteins within the rods of *Synechocystis* 6701 phycobilisomes deduced from studies of incomplete phycobilisomes from numerous mutants (see references 7 and 8). In this report, we focus on the purification and assembly properties of the complexes of phycoerythrin with the 30.5- and 31.5-kdalton polypeptides.

Purification of Phycoerythrin Complexes with the 31.5- and 30.5-kdalton Polypeptides

Considerable difficulty was encountered in the separation of the complexes of phycoerythrin with the 31.5- and 30.5-kdalton polypeptides from each other. Successful fractionation was achieved finally by centrifugation on 5–15% wt/vol linear sucrose-density gradients in 50 mM K-phosphate–1 mM EDTA, pH 7.0. Under these conditions, the phycoerythrin–31.5-kdalton polypeptide complex sedimented faster than did the complex with 30.5-kdalton polypeptide (see Fig. 3). Calibration of such gradients with proteins of known molecular weight (R-phycoerythrin, 240,000; allophycocyanin, 110,000) indicated that fractions 2, 6, and 11 (Fig. 3) would contain components of ~115, ~75, and ~35 kdaltons, respectively, as calculated by the method of Martin and Ames (23). Given the molecular weight of 40,000 for the $\alpha\beta$ monomer of phycoerythrin (8), such molecular weights would correspond to $(\alpha\beta)_2$-31,500 (111.5 kdaltons), $\alpha\beta$-30,500 (70.5 kdaltons), and $\alpha\beta$ (40 kdaltons). However, the diffuse nature of the bands on the gradients and the results of quantification of the ratio of components present in the various fractions by densitometry of SDS-PAGE patterns, indicates the need for caution in the interpretation. For example, the molar ratio of phycoerythrin monomers:linker polypeptide(s) (see Fig. 3) is 4:1 for fraction 1, 3:1 for fraction 3, 5:1 for fraction 6, and the ratio is higher

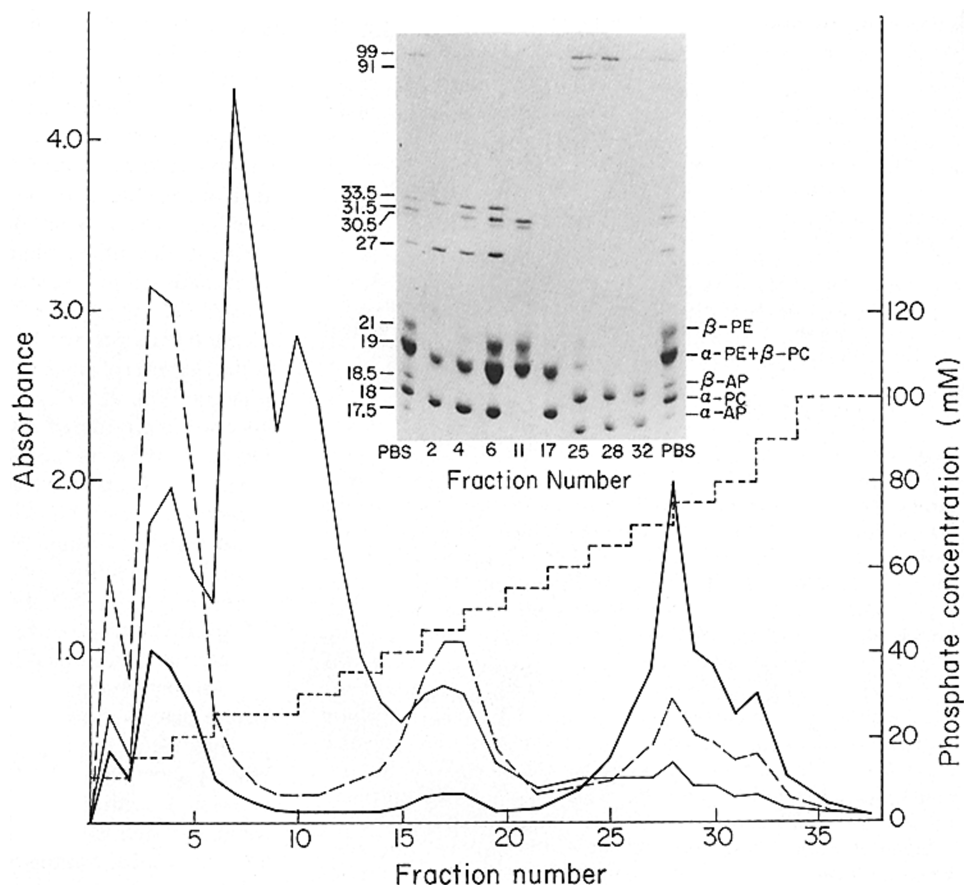

FIGURE 2 Chromatography of dissociated *Synechocystis* 6701 phycobilisomes on hydroxylapatite. A phycobilisome solution (~6 mg of total protein) in 1 mM K-phosphate-0.1 M NaCl, pH 7.0, was applied to a column of hydroxylapatite (1.2 × 2.2 cm). The column was developed stepwise, as indicated, with solutions of increasing phosphate concentration in 0.1 M NaCl, at a flowrate of ~60 ml/h. Absorbance of each fraction was monitored at 650 nm (▬), 620 nm (-----), and 565 nm (—). The *inset* shows SDS-PAGE polypeptide patterns of specific fractions. Abbreviations used are *PBS*, *Synechocystis* 6701 phycobilisomes; *α-AP, β-AP, α-PC, β-PC, α-PE, β-PE, α* and *β* subunits of allophycocyanin, phycocyanin, and phycoerythrin, respectively. Numbers on the ordinate axis of the gel are mol wt × 10^{-3}.

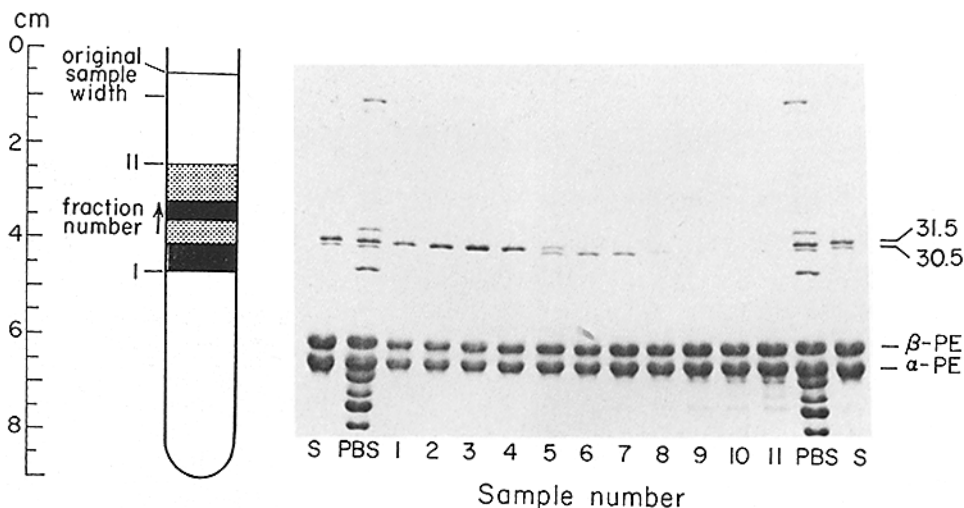

FIGURE 3 Separation of the 30.5- and 31.5-kdalton complexes of phycoerythrin. The phycoerythrin peak eluted from hydroxylapatite (Fig. 2, fraction 6) was applied to a 5–15% (wt/vol) linear sucrose-density gradient prepared in 50 mM K-phosphate-1 mM EDTA, pH 7.0. A volume of 0.75 ml of protein solution (0.75 mg/ml) was layered on top of a 12-ml gradient. After centrifugation in a Spinco SW41 rotor (see (Beckman Instruments, Inc., Spinco Div.) Materials and Methods), the colored zone was collected in 11 equal fractions. The polypeptide composition of each fraction, as resolved by SDS PAGE on a 14% polyacrylamide gel, is displayed in this figure. The abbreviations used are: *S*, sample of material applied to the gradient, *31.5* and *30.5*, positions of the 31.5- and 30.5-kilodalton linker polypeptides. Other abbreviations are defined in the legend to Fig. 2.

in fractions 7 and 8. It is clear from these results that, under the fractionation conditions used here, complex equilibria exist between the phycoerythrin complexes and free phycoerythrin.

Fractions rich in either of the two linker polypeptides have identical absorption spectra (λ_{max} 560 nm) and fluorescence emission spectra (λ^F_{max} 574 nm). This is in contrast to the distinctive spectra, dependent on the nature of the linker polypeptide, seen for the phycocyanin complexes with the 32.5- and 27-kdalton polypeptides in 0.05 M phosphate at pH 7.0, with *Anabaena variabilis* phycocyanin (12), and for the corresponding phycocyanin complexes with the 33.5- and 27-kdalton polypeptides from *Synechocystis* 6701 (J. C. Gingrich, unpublished data).

Behavior of Phycoerythrin-linker Polypeptide Complexes in High Phosphate

The phycoerythrin-linker polypeptide complexes were examined in 0.65 M Na-K-phosphate at pH 8.0. *Synechocystis* 6701 phycobilisomes are stable in this buffer. When fractions

containing phycoerythrin and 31.5 kdalton polypeptide in 0.65 M phosphate at pH 8.0 were subjected to analysis on sucrose-density gradients, most of the material was found in a sharp zone on the 2 M-sucrose cushion at the bottom of the gradients (Fig. 4*A*). Electron-microscopy examination of this material (Fig. 5*C*) showed that it consisted of rods of varying lengths made up of stacked disks. These rods were similar in appearance to the rod substructures of intact phycobilisomes. Analysis of the composition of these aggregates showed a ratio of phycoerythrin to the 31.5-kdalton polypeptide of 6:1, equivalent to that found in phycobilisomes (8). This indicates that each disk is an $(\alpha\beta)_6$-31.5 K complex.

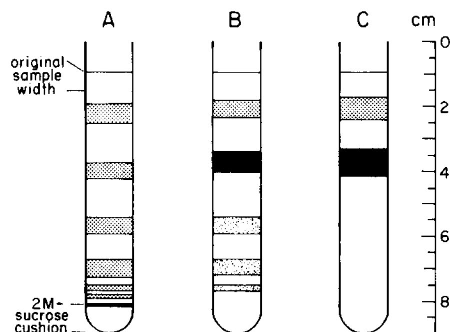

FIGURE 4 Behavior of phycoerythrin complexes with the 31.5- and 30.5-kilodalton polypeptides in high phosphate. Appropriate fractions from sucrose-density gradients in 50 mM K-phosphate, pH 7.0 (see Fig. 3) were passed through a column of Sephadex G-25 equilibrated and eluted with 0.65 M Na-K-phosphate, pH 8.0. The colored eluates were then applied to 5–15% (wt/vol) linear sucrose-density gradients formed on top of a 2 M-sucrose cushion in the same buffer. Gradient *A* was loaded with 0.14 mg of phycoerythrin-31.5 k-dalton polypeptide complex. The molar ratio of phycoerythrin to the 31.5-kdalton polypeptide in this fraction was 3:1. Gradient *B* was loaded with 0.18 mg of phycoerythrin complexes with the 30.5- and 31.5-kdalton polypeptides. The molar ratio of phycoerythrin to the 30.5- and 31.5-kdalton polypeptides in this fraction was 4:0.5:0.5. Gradient *C* was loaded with 0.18 mg of the phycoerythrin-30.5-kdalton polypeptide complex. The molar ratio of phycoerythrin to the 30.5-kdalton in this fraction was 5.5:1. Sample volume applied to the gradient was 0.75 ml in each instance. The relative amounts of the various components resolved by centrifugation are indicated by the degree of stippling or shading.

Under the same conditions, fractions containing phycoerythrin and the 30.5-kdalton polypeptide gave a component of ~250 kdaltons as the major assembly product (Fig. 4*C*). By electron microscopy, this component was seen to consist of small particles of variable morphology (Fig. 5*A*). Occasionally, a streak of faster sedimenting material, containing as much as 40% of the phycoerythrin applied to the gradient, extended to the 2 M-sucrose cushion. Such material consisted of short rods (Fig. 5*B*) and showed a ratio of phycoerythrin to 30.5-kdalton polypeptide of 6:1. In the majority of experiments only very small amounts of high molecular-weight aggregates were detected. The reasons for these variable results are not clear.

Phycoerythrin, free of linker polypeptides, in 0.65 M Na-K-phosphate at pH 8.0, produces gradient patterns equivalent to that illustrated in Fig. 4*C*. Moreover, the major component of apparent molecular weight of ~250,000 formed in 0.65 M Na-K-phosphate solutions of phycoerythrin and the 30.5-kdalton polypeptide (Fig. 4*C*) contained a very small amount of the linker polypeptide. The 30.5-kdalton polypeptide was found in association with phycoerythrin fractions of lower molecular weight, as well as in the rod aggregates described above (Fig. 5*B*). These observations indicate that in 0.65 Na-K-phosphate, pH 8.0, two competing reactions take place: the aggregation of phycoerythrin monomers to assemblies of ill-defined morphology with molecular weight of ~250,000, and aggregation into disks and thence to rods dependent on the inclusion of the

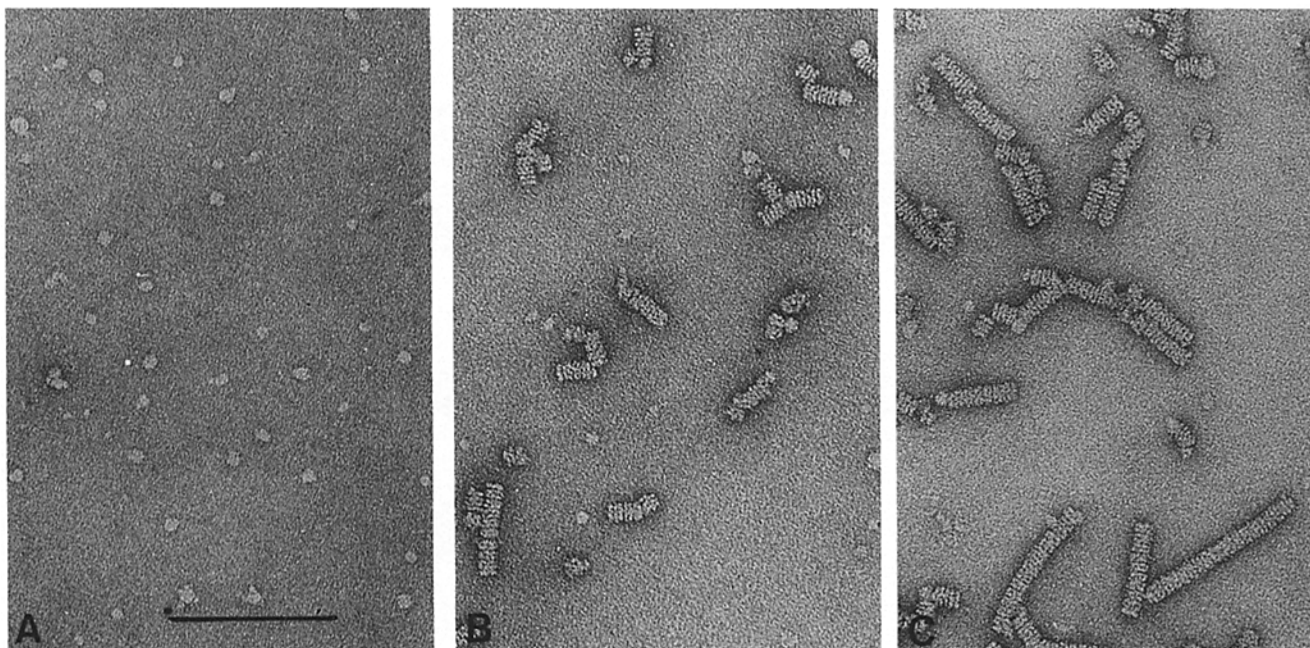

FIGURE 5 Electron micrographs of structures formed from fractions containing phycoerythrin and the 30.5- or 31.5-kdalton polypeptides in 0.65 M Na-K-phosphate at pH 8.0. *A*, major aggregate formed by the phycoerythrin-30.5-kdalton polypeptide fraction (see Fig. 4, gradient *C*); *B*, rods sedimenting in a diffuse zone below the major aggregate seen in Fig. 4, gradient *C* (see text); *C*, major aggregates, rods of variable length, formed by the phycoerythrin-31.5-kdalton polypeptide fraction, recovered from the top of the 2 M-sucrose cushion (Fig. 4, gradient *A*). Bar, 0.1 μm. × 225,000.

30.5-kdalton polypeptide in a molar ratio to phycoerythrin monomer of 1:6 within the highly ordered aggregates.

In 0.65 M Na-K-phosphate solutions containing phycoerythrin and both the 30.5- and 31.5-kdalton polypeptides rod aggregates are formed (Fig. 3 *B*). The average length of these rods is intermediate between those shown in Fig. 5 *B* and *C*. The rods contain both the 31.5- and 30.5-kdalton polypeptides in comparable amounts, with the 30.5-kdalton polypeptide predominating in the aggregates with the lower sedimentation-coefficient and the 31.5-kdalton in the faster sedimenting ones.

Assembly of Phycoerythrin Complexes with Strain CM25 Phycobilisomes

To prevent self-association of the phycoerythrin-linker polypeptide complexes before the addition of incomplete phycobilisomes, the solutions of these complexes were kept in 0.1 M Na-K-phosphate at pH 8.0 prior to mixing. Consequently, temporary exposure of CM25 phycobilisomes to a lowered phosphate concentration was unavoidable (see Materials and Methods). Under the conditions used CM25 phycobilisomes were exposed to 0.53 M phosphate for <2 min before transfer back into 0.75 M Na-K-phosphate, pH 8.0, by gel filtration. The sedimentation behavior, polypeptide composition (Fig. 6 *A*), morphology (Fig. 7 *A*), and spectroscopic properties of phycobilisomes treated in this manner were indistinguishable from those of control phycobilisomes maintained in 0.75 M phosphate throughout. The results of the mixed assembly experiments are summarized in Table I and illustrated in Figs. 6–8.

When a mixture containing eleven hexamer equivalents (i.e., eleven of the double disk rod elements) of phycoerythrin-31.5 kdalton polypeptide complex per CM25 phycobilisome was subjected to sucrose-density gradient centrifugation, the phycobilisome band sedimented faster than control phycobilisomes (compare Fig. 6 *A* and *B*) and contained a substantial amount of phycoerythrin. Electron microscopy (Fig. 7 *B*) indicated a substantial increase in average rod-length of the "complemented" phycobilisomes. From the absorption spectrum of the phycobilisomes, it was calculated (see reference 8) that an average of 6.5 phycoerythrin hexamers were attached per phycobilisome. The SDS-PAGE pattern (Fig. 6 *B*) showed the presence of 31.5-kdalton polypeptide in molar ratio to phycoerythrin of 1:6. The fluorescence spectrum of the complemented CM25 phycobilisomes showed ~96% efficient energy transfer from the added phycoerythrin to the terminal acceptors (Fig. 8, compare 580-nm emission of intact and dissociated phycobilisomes). From the variation in rod length in the complemented particles (see Fig. 7 *B*), it is evident that the in vitro addition of phycoerythrin is a random process. It should also be noted that gradient E of Fig. 6 showed that when the same concentration of phycoerythrin-31.5-kdalton polypeptide complex was subjected to the complementation procedure in absence of CM25 phycobilisomes, most of the material assembled into rods that sedimented to the top of the 2 M-sucrose cushion in the gradient (see Fig. 7 *D*). Such material was not present in gradient 7 *B*, suggesting that most of the phycoerythrin competent to form rod assemblies had attached to the CM25 phycobilisomes.

When CM25 phycobilisomes were mixed with a solution containing both phycoerythrin-30.5 kdalton and phycoerythrin-31.5 kdalton polypeptide complexes, the results (see Figs. 6 *C* and 7 *C*, and Table I) were similar to those obtained for addition of the phycoerythrin-31.5 kdalton polypeptide complex, although a smaller average amount of phycoerythrin was attached per phycobilisome. In the experiment illustrated in

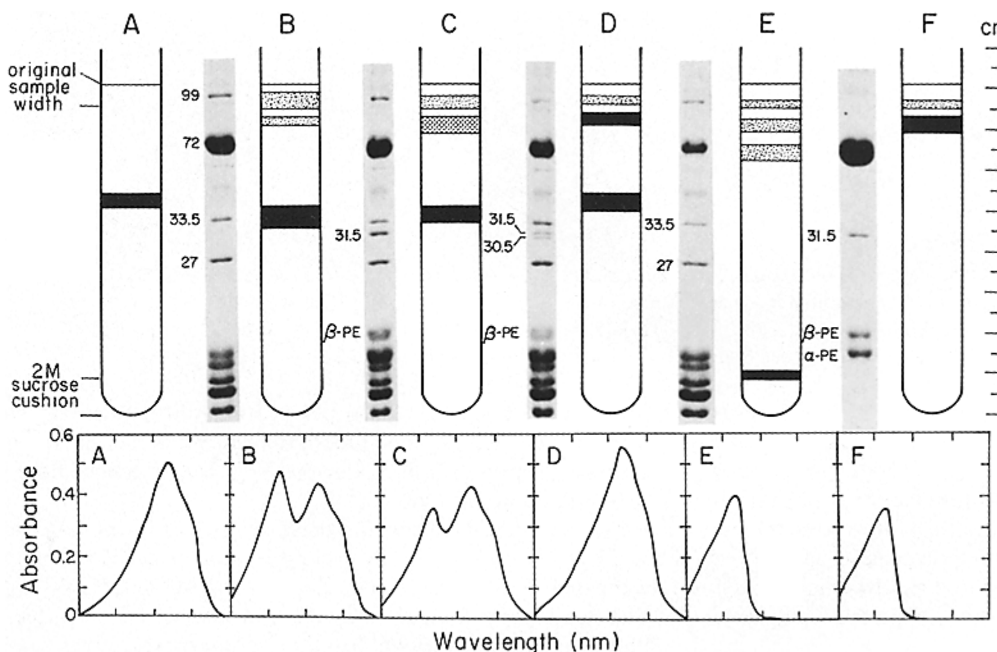

FIGURE 6 Interaction between strain CM25 phycobilisomes lacking phycoerythrin with phycoerythrin-31.5-kdalton and phycoerythrin-30.5-kdalton complexes. Appropriate mixtures (see text) in 0.65 M Na-K-phosphate, pH 8.0, were fractionated on 5–15% wt/vol linear sucrose-density gradients in the same buffer. *A*, CM25 PBS control; *B*, CM25 PBS + PE-31.5-kdalton polypeptide; *C*, CM25 PBS + PE-31.5-kdalton polypeptide + PE-30.5-kdalton polypeptide; *D*, CM25 PBS + PE-30.5-kdalton polypeptide; *E*, PE-31.5-kdalton polypeptide control; *F*, PE-30.5-kdalton polypeptide control. The polypeptide composition of the fastest sedimenting component, as determined by SDS PAGE, is shown to the right of each of gradients *A*–*E*, and the absorption spectra of these components for the range 500–700 nm are shown in the lower part of the figure. The abscissa is marked at 50-nm intervals. The 72-kilodalton component seen in the SDS-PAGE pattern *A*–*E* is bovine serum albumin added as carrier for trichloroacetic acid precipitation. For composition of the mixtures applied to gradients *A*–*D*, see Table I. The abbreviations *31.5* and *30.5* represent the 31.5- and 30.5 kilodalton polypeptides. Other abbreviations are defined in the legend to Fig. 2.

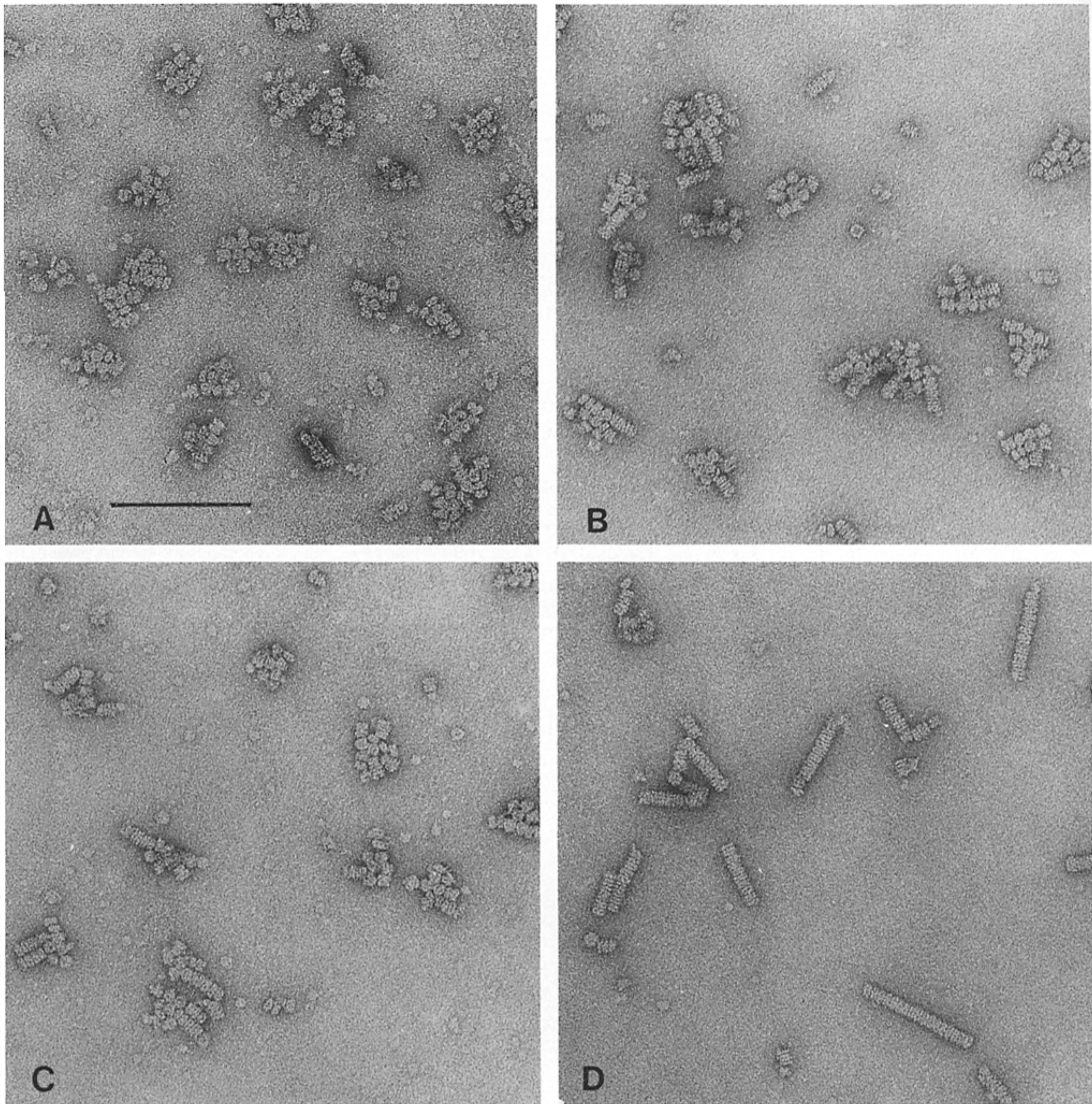

FIGURE 7 Electron micrographs of the fastest sedimenting components from gradients *A–C* and *E* (Fig. 6). *A*, CM25 PBS control; *B*, CM25 PBS + PE-31.5-kdalton polypeptide; *C*, CM25 PBS + PE-31.5-kdalton polypeptide + PE-30.5-kdalton polypeptide; *D*, PE-31.5-kdalton polypeptide control. For abbreviations, see legends to Figs. 2 and 6. Bar, 0.1 μm. × 225,000.

Fig. 6 *C*, the complemented phycobilisomes contained an average of four hexamers of phycoerythrin, 2.5 copies of the 31.5-kdalton polypeptide, and 1.5 copies of 30.5-kdalton polypeptide, i.e., one linker polypeptide per hexamer of phycoerythrin.

In mixtures of CM25 phycobilisomes with various amounts of the phycoerythrin–30.5-kdalton polypeptide complex no interaction was observed by spectroscopic techniques, SDS-PAGE analysis, or electron microscopy (Table I; Fig. 6 *D*).

Comparison of the 31.5- and 30.5-kdalton Linker Polypeptides by Peptide Mapping

The linker polypeptides were purified by preparative SDS PAGE. After this procedure, the polypeptides migrated as a closely spaced doublet on SDS gels (see Fig. 9). Purified 27-, 33.5-, and 99-kilodalton linker polypeptides migrated as single components after the same procedure, and we do not have an explanation for the behavior of the 31.5- and 30.5-kilodalton polypeptides at this time.

The peptides obtained from these two polypeptides by limited tryptic digestion and by cyanogen bromide cleavage were compared by SDS PAGE (see Fig. 9 *A* and 9 *B*, respectively). The results show that the 31.5- and 30.5-kdalton polypeptides differ from each other significantly in primary structure.

DISCUSSION

Studies of *Synechocystis* 6701 mutants that produce incomplete phycobilisomes have established the location of the various components within the rod substructures of the particles. The

Table I

Complementation of Strain CM25 Phycobilisomes with Phycoerythrin–Linker Polypeptide Complexes

Gradient*	CM25 PBS conc.‡§	PE-31.5K conc.‡§‖	PE-30.5K conc.‡§‖	Approximate number of PE hexamers per phycobilisome	
				Added	Incorporated
	mg/ml	*mg/ml*	*mg/ml*		
B	0.15	0.13 (5:1)	—	11	6.5
C	0.15	0.065 (24:1)	0.065 (24:1)	11	4.0 (1.25:1)¶
D	0.15	—	0.13 (18:1)	11	0
—	0.08	—	0.20 (12:1)	32	0

* See corresponding gradients in Fig. 6.

‡ Abbreviations used are *PBS*, phycobilisomes; *PE*, phycoerythrin; *31.5*, 31.5-kdalton polypeptide; *30.5*, 30.5-kdalton polypeptide.

§ The concentrations given are those in the complementation mixture before application to the gradient.

‖ The molar ratio of phycoerythrin to linker polypeptide is given in parentheses.

¶ The molar ratio of the 31.5- to the 30.5-kdalton polypeptide in the complemented phycobilisomes is given in parentheses.

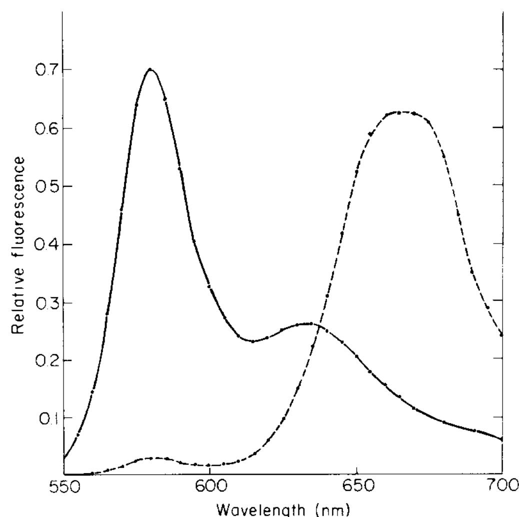

Figure 8 Fluorescence-emission spectra of intact and dissociated strain CM25 phycobilisomes reconstituted with the phycoerythrin-31.5-kilodalton complex. Fluorescence emission spectrum of reconstituted phycobilisomes in 0.75 M Na-K-phosphate, pH 8.0 (•----•) and after dilution to 0.15 M Na-K-phosphate, pH 8.0 (•—•) is that of particles such as those shown in Fig. 7 *B* (see also Fig. 6, gradient *B*). Excitation was at 540 nm with excitation and emission slits set at 4 nm and sample absorbance of 0.125/cm at 620 nm.

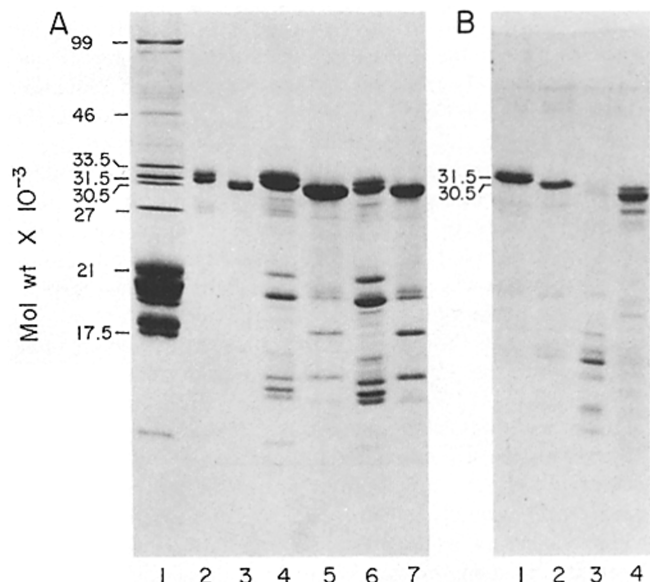

Figure 9 Peptide maps of the 31.5- and 30.5-kdalton polypeptides. *A.1*, *Synechocystis* 6701 PBS control; *2*, 31.5; *3*, 30.5; *4* and *6*, 31.5 after 3 min digestion with 1% (wt/wt) and 3% (wt/wt) trypsin, respectively; *5* and *7*, 30.5 after 3 min digestion with 1% (wt/wt) and 3% (wt/wt) trypsin, respectively. *B.1*, 31.5; *2*, 30.5; *3* and *4*, 31.5 and 30.5, respectively, after 24 h cleavage with CNBr at 23°C. For experimental details, see Materials and Methods. For abbreviations, see legends to Figs. 2 and 3.

average rod is made up of four disks, ~60 × 120 Å. Proceeding outward from the disk proximal to the core, the rods are made up of phycocyanin $(\alpha\beta)_6$-27 K and $(\alpha\beta)_6$-33.5 K complexes, then phycoerythrin $(\alpha\beta)_6$-31.5 K and $(\alpha\beta)_6$-30.5 K complexes, where 27 K, 33.5 K, 31.5 K, and 33.5 K, are linker polypeptides of 27-kdaltons, etc., and $\alpha\beta$ are monomers of the biliproteins (7, 8). It was not evident from the earlier studies whether sufficient structural information is present within the components of the rod substructure to assure correctly ordered assembly. This question is examined here for the two terminal disks—the complexes of phycoerythrin with the 31.5-kdalton and 30.5-kdalton polypeptides.

The purification procedure employed here led to the isolation of low molecular-weight complexes of the 31.5- and 30.5-kdalton polypeptides with phycoerythrin of average composition $(\alpha\beta)_2$-31.5 K and $(\alpha\beta)$-30.5 K, respectively. The behavior of these complexes on sucrose-density gradient centrifugation indicated that they existed in equilibrium with free phycoerythrin.

Upon transfer into solutions of high phosphate concentration, both complexes gave rise to stacked-disk aggregates within which the molar ratio of phycoerythrin monomer to linker polypeptide (6:1) was characteristic of that determined for intact phycobilisomes (8). For the phycoerythrin complex with the 31.5-kdalton polypeptide, rod formation is the dominant aggregation reaction in high phosphate, whereas for the 30.5-kdalton polypeptide complex, association of free phycoerythrin into ~250,000 dalton irregular aggregates represented a major competing reaction. When mixed together in 0.65 M phosphate at pH 8.0, the two phycoerythrin complexes coassembled into rods.

The two complexes were examined for their ability to complement phycobilisomes from *Synechocystis* 6701 mutant strain CM25. These phycobilisomes totally lack phycoerythrin and the 31.5- and 30.5-kdalton polypeptides. By morphological and

spectroscopic criteria, as well as compositional analysis, CM25 phycobilisomes differ from wild-type particles solely in the absence of the phycoerythrin disks from the rods. The results of the complementation experiments were unambiguous. In high phosphate at pH 8.0, the phycoerythrin-31.5-kdalton polypeptide complex adds with high efficiency to CM25 phycobilisomes. Under the same conditions, no addition of the phycoerythrin-30.5-kdalton polypeptide complex takes place. When a mixture of the two phycoerythrin complexes is added together to CM25 phycobilisomes, both are incorporated into the rod substructures. Consequently, it can be concluded that in vitro only the phycoerythrin-31.5-kdalton polypeptide complex is competent to add to the distal phycocyanin disk of the CM25 phycoerythrin rod substructure. The presence of a phycoerythrin-31.5-kdalton polypeptide disk appears to be a prerequisite to the incorporation of the phycoerythrin-30.5-kdalton polypeptide complex onto the rod substructure. Thus, the order of the two distal disks seen in wild-type phycobilisomes is reproduced in the in vitro assembly process. Since peptide mapping shows the 31.5- and 30.5-kdalton polypeptides to differ significantly from each other in amino-acid sequence, the observed selectivity can reasonably be attributed to the distinctive structural properties of these linker polypeptides.

It is evident from this study that complementation of mutant phycobilisomes offers a useful approach to the study of details of the assembly of these structures. This approach to the analysis of the assembly of structures made up of many different components was first introduced in studies of the morphogenesis of bacteriophage T4 (24). The dependence of the incorporation of the phycoerythrin-30.5-kdalton polypeptide complex into the phycobilisome on the prior addition of the phycoerythrin-31.5-kdalton polypeptide complex has parallels in phage assembly where most of the steps are constrained to occur in a strict sequential order—a process of "self-regulated assembly" (25, 26). For example, in the T4 tail-assembly sequence (25), virtually none of the associations in the pathway will take place unless the preceding steps have been completed. If an intermediate protein is missing, all of the remaining components (with one exception) remain unassembled as stable soluble monomers.

We would like to thank Ms. Lauren Blaha for the isolation of the nitrosoguanidine-induced mutant strain CM25 of *Synechocystis* 6701.

This work was supported in part by National Science Foundation grants PCM79-10996 (A. N. Glazer) and PCM80-10650 (R. C. Williams). J. C. Gingrich was supported by a fellowship from the U. S. Public Health Service training grant T32-GM 07232-05 from the National Institute of General Medical Sciences.

Received for publication 19 April 1982, and in revised form 10 June 1982.

REFERENCES

1. Gantt, E. 1980. Structure and function of phycobilisomes: light-harvesting pigment complexes in red and blue-green algae. *Int. Rev. Cytol.* 66:45–80.
2. Glazer, A. N. 1981. Photosynthetic accessory proteins with bilin prosthetic groups. *In* The Biochemistry of Plants. Academic Press, New York 8:51–96.
3. Tandeau de Marsac, N., and G. Cohen-Bazire. 1977. Molecular composition of cyanobacterial phycobilisomes. *Proc. Natl. Acad. Sci. U. S. A.* 74:1635–1639.
4. Yamanaka, G., A. N. Glazer, and R. C. Williams. 1978. Cyanobacterial phycobilisomes. Characterization of the phycobilisomes of *Synechococcus* sp. 6301. *J. Biol. Chem.* 253:8303–8310.
5. Lundell, D. J., R. C. Williams, and A. N. Glazer. 1981. Molecular architecture of a light-harvesting antenna. In vitro assembly of the rod substructures of *Synechococcus* 6301 phycobilisomes. *J. Biol. Chem.* 256:3580–3592.
6. Bryant, D. A., G. Guglielmi, N. Tandeau de Marsac, A.-M. Castets, and G. Cohen-Bazire. 1979. The structure of cyanobacterial phycobilisomes. A model. *Arch. Microbiol.* 123:113–127.
7. Williams, R. C., J. C. Gingrich, and A. N. Glazer. 1980. Cyanobacterial phycobilisomes. Particles from *Synechocystis* 6701 and two pigment mutants. *J. Cell Biol.* 85:558–566.
8. Gingrich, J. C., L. K. Blaha, and A. N. Glazer. 1982. Rod substructure in cyanobacterial phycobilisomes: analysis of *Synechocystis* 6701 mutants low in phycoerythrin. *J. Cell Biol.* 92:261–268.
9. Glazer, A. N., R. C. Williams, G. Yamanaka, and H. K. Schachman. 1979. Characterization of cyanobacterial phycobilisomes in zwitterionic detergents. *Proc. Natl. Acad. Sci. U. S. A.* 76:6162–6166.
10. Rosinski, J., J. F. Hainfeld, M. Rigbi, and H. W. Siegelman. 1981. Phycobilisome ultrastructure and chromatic adaptation in *Fremyella diplosiphon. Ann. Bot (Lond.)*.47:1–12.
11. Morschel, E., K.-P. Koller, W. Wehrmeyer, and H. Schneider. 1977. Biliprotein assembly in the disc-shaped phycobilisomes of *Rhodella violacea.* I. Electron microscopy of phycobilisomes *in situ* and analysis of their architecture after isolation and negative staining. *Cytobiologie.* 16:118–129.
12. Yu, M.-H., A. N. Glazer, and R. C. Williams. 1981. Cyanobacterial phycobilisomes. Phycocyanin assembly in the rod substructures of *Anabaena variabilis* phycobilisomes. *J. Biol. Chem.* 256:13130–13136.
13. Tandeau de Marsac, N. 1977. Occurrence and nature of chromatic adaptation in cyanobacteria. *J. Bacteriol.* 130:82–91.
14. Rippka, R., J. Deruelles, J. B. Waterbury, M. Herdman, and R. Y. Stanier. 1979. Generic assignments, strain histories, and properties of pure cultures of cyanobacteria. *J. Gen. Microbiol.* 111:1–61.
15. Siegelman, H. W., G. A. Wieczorek, and B. C. Turner. 1965. Preparation of calcium phosphate for protein chromatography. *Anal. Biochem.* 13:402–404.
16. Williams, R. C., and H. W. Fisher. 1970. Electron microscopy of tobacco mosaic virus under conditions of minimal beam exposure. *J. Mol. Biol.* 52:121–123.
17. Yamanaka, G., and A. N. Glazer. 1980. Dynamic aspects of phycobilisome structure. Phycobilisome turnover during nitrogen starvation in *Synechococcus* sp. *Arch. Microbiol.* 124:39–47.
18. Gantt, E., and C. A. Lipschultz. 1972. Phycobilisomes of *Porphyridium cruentum.* I. Isolation. *J. Cell Biol.* 54:313–324.
19. Gantt, E., and C. A. Lipschultz. 1973. Energy transfer in phycobilisomes from phycoerythrin to allophycocyanin. *Biochim. Biophys. Acta.* 292:858–861.
20. Ley, A. C., W. L. Butler, D. A. Bryant, and A. N. Glazer. 1977. Isolation and function of allophycocyanin B of *Porphyridium cruentum. Plant Physiol. (Bethesda).* 59:974–980.
21. Gantt, E. 1981. Phycobilisomes. *Annu. Rev. Plant Physiol.* 32:327–347.
22. Lundell, D. J., G. Yamanaka, and A. N. Glazer. 1981. A terminal energy acceptor of the phycobilisome: The 75,000-dalton polypeptide of *Synechococcus* 6301 phycobilisomes—a new biliprotein. *J. Cell Biol.* 91:315–319.
23. Martin, R. G., and B. N. Ames. 1961. A method for determining the sedimentation behavior of enzymes: Application to protein mixtures. *J. Biol. Chem.* 236:1372–1379.
24. Edgar, R. S., and W. B. Wood. 1966. Morphogenesis of bacteriophage T4 in extracts of mutant-infected cells. *Proc. Natl. Acad. Sci. U. S. A.* 55:498–505.
25. Kikuchi, Y., and J. King. 1975. Genetic control of bacteriophage T4 base-plate morphogenesis. III. Formation of the central plug and overall assembly pathway. *J. Mol. Biol.* 99:695–716.
26. King, J., R. Griffin-Shea, and M. T. Fuller. 1980. Scaffolding proteins and the genetic control of virus shell assembly. *Q. Rev. Biol.* 55:369–393.