A Terminal Energy Acceptor of the Phycobilisome: The 75,000-dalton Polypeptide of *Synechococcus* 6301 Phycobilisomes—A New Biliprotein

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ABSTRACT A rapid procedure is described for the isolation of "linker" polypeptides (Lundell, D. J., R. C. Williams, and A. N. Glazer. 1981. *J. Biol. Chem.* 256:3580–3592) of cyanobacterial phycobilisomes. The 75,000-dalton component of the core of *Synechococcus* 6301 phycobilisomes isolated by this procedure has been shown to carry a bilin similar in spectroscopic properties to phycocyanobilin. "Renatured" 75,000-dalton polypeptide has absorption maxima at 610 and 665 nm and a fluorescence emission maximum at 676 nm, similar to that of intact phycobilisomes. A complex of allophycocyanin and a 40,000-dalton bilin-carrying fragment of the 75,000-dalton polypeptide, obtained by limited tryptic digestion, is described. This complex, which lacks allophycocyanin B, shows a fluorescence emission maximum at 676 nm. The above data indicate that the 75,000-dalton polypeptide functions as a terminal energy acceptor in the phycobilisome.

In cyanobacteria and red algae, light in the visible region of the spectrum is absorbed and transferred to the photosynthetic reaction centers by a chain of chromoproteins, the phycobiliproteins—phycoerythrin, phycocyanin, allophycocyanin, and allophycocyanin B (1). The phycobiliproteins carry covalently attached, open-chain tetrapyrrole groups (2). The conformation and environment of these chromophores within the native proteins intensify their strong absorption bands between 500 and 700 nm. The phycobiliproteins are located in large particles of complex structure (phycobilisomes) that are attached to the outer surface of the photosynthetic lamellae (3).

We have chosen the phycobilisomes of the unicellular cyanobacterium *Synechococcus* 6301 for detailed study because these phycobilisomes are simpler in ultrastructure (4, 5) and contain fewer components than those of other organisms (6). Moreover, mutants which produce incomplete phycobilisomes have been well characterized (5, 7).

Ultrastructural studies show that *Synechococcus* 6301 phycobilisomes consist of a bicylindrical core surrounded by a hemidiscoidal array of up to six rods made up of stacked discs (4, 5). Details of the ultrastructure of wild-type and mutant particles have been presented recently (4, 5). Various in vivo and in vitro studies have shown that the core contains allophycocyanin (λ_{max} 650 nm), allophycocyanin B (λ_{max} 670 nm), and a 75,000-dalton polypeptide, whereas the rods are made up of disc-shaped phycocyanin hexamers stabilized by interaction with specific "linker" polypeptides of 33,000, 30,000, and 27,000 daltons (5, 7, 8). Thus, the disc proximal to the core has the structure $(\alpha\beta)_6$-27,000 daltons, whereas those distal to the core are phycocyanin $(\alpha\beta)_6$-33,000-, and $(\alpha\beta)_6$-30,000-dalton complexes (5, 7, 8).

The fluorescence emission maxima of pure phycocyanin, allophycocyanin, and allophycocyanin B are at ~650, 660, and 680 nm, respectively. The emission of intact phycobilisomes, upon excitation at 620 nm, is at ~676 nm, and can be shown to be made up of a strong 680-nm and a weaker 660-nm component (6). From these observations, it has been inferred that the path of energy transfer in these phycobilisomes is phycocyanin (λ_{max} ~620 nm) → allophycocyanin → allophycocyanin B.

We have reported a procedure for the purification of the 75,000-, 33,000-, 30,000-, and 27,000-dalton linker polypeptides. This procedure involved lengthy ion-exchange chromatography in concentrated urea solutions at pH 5 (8). The polypeptides purified in this manner showed no absorbance between 350 and 700 nm, and hence we concluded that they did not carry bilin prosthetic groups. However, in a previous study, we had shown that purification of the subunits of the biliprotein b-phycoerythrin under the same conditions led to extensive bleaching of phycoerythrobilin chromophores (9). Such bleaching was minimized when the purification was performed at ≤pH 3 (9). In view of these observations, we have developed a rapid isolation procedure for the linker polypeptides in which a strongly acidic pH is maintained throughout. Spectroscopic studies of the 27,000-, 30,000-, and 33,000-dalton polypeptides purified by the new procedure indicate the ab-

THE JOURNAL OF CELL BIOLOGY · VOLUME 91 OCTOBER 1981 315-319
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sence of bilins; but the 75,000-dalton polypeptide was shown to carry a bilin very similar to phycocyanobilin. Redlinger and Gantt (10) have recently reported that the phycobilisomes of the unicellular red alga *Porphyridium cruentum* contain a bilin-carrying 95,000-dalton polypeptide.

In our study, we find that the absorption spectrum of the "renatured" 75,000-dalton polypeptide shows a peak at 665 nm and the fluorescence emission spectrum a maximum at 675 nm. We also report the isolation of an 18S complex, obtained by partial dissociation of phycobilisomes, which contains the 75,000-dalton polypeptide, lacks allophycocyanin B, but still emits at 680 nm. These observations raise the possibility that it is the 75,000-dalton polypeptide, rather than allophycocyanin B, that functions as the terminal energy acceptor in phycobilisomes.

MATERIALS AND METHODS

Rapid Isolation of Linker Polypeptides

Synechococcus 6301 wild-type cells and those of mutant AN112, which makes phycobilisomes lacking the 33,000- and 30,000-dalton polypeptides and is depleted in phycocyanin (5), were grown as previously described. Phycobilisomes were prepared by the procedure of Yamanaka et al. (6) and exchanged into 0.1 M Na-phosphate, pH 7, by gel filtration on a column of Sephadex G-25. The phycobilisome solution was adjusted to ~2 mg protein/ml, and solid NH_4SCN was added to 0.5 M. The solution became cloudy within a few minutes. After 30 min, the solution was centrifuged and the blue precipitate collected. With wild-type phycobilisomes, SDS PAGE showed that the precipitate contained ~80% of the 75,000-, 50% of the 27,000-, and 30–50% of the 33,000- and 30,000-dalton polypeptides, but <3% total of the biliproteins phycocyanin, allophycocyanin, and allophycocyanin B. The precipitate was dissolved in 6 M guanidine·HCl–1% formic acid (vol/vol) at 2–4 mg protein/ml, and the solution was applied to a Sephacryl S-200 gel filtration column equilibrated with the same solvent; 1.0 ml of sample was loaded on a 1.5 × 50-cm column. The elution profile was determined from the absorbance of the eluate at 280 and 660 nm.

Isolation of the "18S Complex"

A more detailed report of the isolation and characterization of this complex is in preparation (G. Yamanaka, D. J. Lundell, and A. N. Glazer. Manuscript in preparation.). Briefly, phycobilisomes of strain AN112 were exchanged into 50 mM Tricine–5 mM $CaCl_2$–10% (vol/vol) glycerol, pH 7.8, by gel filtration on Sephadex G-25. Partial dissociation of the phycobilisomes takes place under these conditions. The mixture was fractionated on linear sucrose density gradients in the same buffer. The fastest sedimenting band, 18S, was collected. This complex has an absorption maximum at 649 nm and a fluorescence emission spectrum very similar to that of intact phycobilisomes, with a maximum at 676 nm. It contains allophycocyanin, phycocyanin, 27,000-, and 75,000-dalton polypeptides in an approximate molar ratio of 6:6:2:1, as well as an additional, not yet characterized polypeptide of 18,300 daltons present in a molar ratio to the 75,000-dalton polypeptide of 1–2:1. The 18S complex does not contain allophycocyanin B.

Limited Tryptic Degradation of the 18S Complex

The 18S complex was transferred into 10 mM Na-phosphate–0.5 M NaCl–10% (vol/vol) glycerol, pH 7, by passage through a column of Sephadex G-25. We added trypsin to a final concentration of 3–5% (wt/wt) and allowed digestion to proceed for 15 min at 22°C. The reaction was stopped by adding an excess of soybean trypsin inhibitor. The solution was left standing at room temperature for ~2 h and was then applied to a hydroxylapatite column equilibrated with 0.001 M Na-phosphate–0.1 M NaCl–10% glycerol (vol/vol), pH 7 (1 ml settled bed volume per mg protein). The column was developed stepwise with increasing concentrations of Na-phosphate in 0.1 M NaCl–10% glycerol (vol/vol), at pH 7.

Gel Electrophoresis

We performed SDS PAGE, staining, and densitometric analyses as previously described (6, 7).

Spectroscopic Measurements

Absorption spectra were determined with a Beckman model 25 recording spectrophotometer (Beckman Instruments, Inc., Scientific Instruments Div., Irvine, Calif.). Corrected fluorescence emission spectra were determined as previously described (5). Relative quantum yields were determined from corrected emission spectra, recorded over the range 600–800 nm, and corrected for baseline.

RESULTS AND DISCUSSION

Isolation and Characterization of the 75,000-dalton Polypeptide

Previously, we had not detected bilins on the linker polypeptides from *Synechococcus* 6301 phycobilisomes (8). Because the conditions we had used for the isolation of these polypeptides could cause bleaching of covalently bound bilins, we have reinvestigated this point by developing a more rapid isolation procedure and minimizing the exposure of unfolded polypeptides to near neutral pH. We have found that 0.5 M NH_4SCN causes a rapid precipitation of the linker polypeptides, with almost no precipitation of phycobiliproteins. In neutral 0.5 M NH_4SCN, the absorption maxima of phycocyanin and allophycocyanin shift to 610–615 nm, and the absorption spectra are similar to those of the $\alpha\beta$ monomers of these proteins (11, 12). Presumably, dissociation of the biliprotein-linker polypeptide complexes in NH_4SCN permits aggregation of the linker polypeptides. As we have reported previously, linker polypeptides are very insoluble in aqueous buffer and neutral pH (8).

The gel filtration profile of the components of AN112 phycobilisomes which precipitate in 0.5 NH_4SCN is shown in Fig. 1, and an SDS polyacrylamide gel of selected fractions in Fig. 2. Material in the first peak (Fig. 1) absorbs strongly at 660 nm and 280 nm and is seen to consist of the 75,000-dalton polypeptide and ~5% of the 27,000-dalton polypeptide. It is important to note that subunits of phycocyanin and allophycocyanin (molecular weights in the range of 16,000- to 19,000-daltons [6, 13]) are absent from this fraction. The second peak shows 280-nm absorbance and elutes between two 660-nm absorbing peaks. This peak contains primarily the 27,000-dalton polypeptide and a small amount of the 75,000-dalton polypeptide (Fig. 2). The third peak contains material absorbing at 660 and 280 nm and contains the trailing portion of the 27,000-dalton polypeptide peak as well as phycobiliprotein subunits. It is clear from inspection of Figs. 1 and 2 that the 75,000-dalton

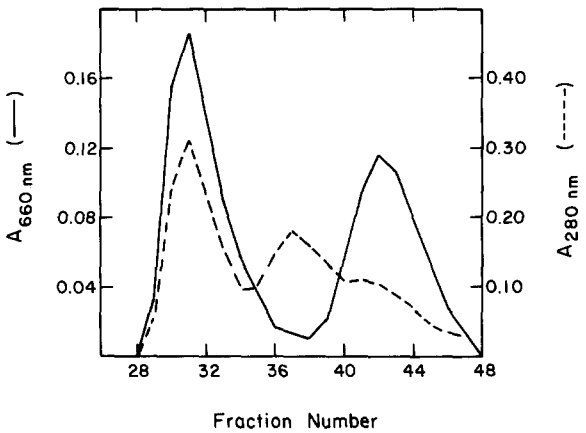

FIGURE 1 Gel filtration of linker polypeptides. The precipitate formed upon dissociation of strain AN112 phycobilisomes with 0.5 M NH_4SCN was dissolved at 2–4 mg protein/ml in 6 M guanidine HCl–1% formic acid and 1.0 ml applied to a 1.5 × 50-cm column of Sephacryl S-200 in the same buffer. Fractions of 1.0 ml were collected.

polypeptide carries a chromophore absorbing at 660 nm, whereas the 27,000-dalton polypeptide does not. When the above procedure is repeated with material derived from wild-type phycobilisomes, the 660-nm elution profile is the same as that shown in Fig. 1, but in the 280-nm profile the second peak is much higher and this peak contains the 33,000- and 30,000-dalton polypeptides in addition to the 27,000-dalton polypeptide. These observations confirm the conclusion that the 30,000- and 33,000-dalton polypeptides do not carry bilins.

The visible absorption spectra of the 75,000-dalton polypeptide (Fig. 1, fraction 31) and *Synechococcus* 6301 phycocyanin, both in 6 M guanidine HCl–1% formic acid, are compared in the upper panel of Fig. 3. The spectra are similar but show a number of differences: the absorption spectrum of the 75,000-dalton polypeptide is slightly red-shifted relative to that of phycocyanin, particularly at shorter wavelengths; the ratios of the two maxima are somewhat different; the 75,000-dalton polypeptide spectrum has a shoulder in the 600- to 640-nm region. From the available data, it is clear that the bilin on the 75,000-dalton polypeptide is closely related to phycocyanobilin, the prosthetic group of phycocyanin (1, 2). It may in fact be phycocyanobilin and the observed differences in the absorption spectra in Fig. 3 may be due to residual three-dimensional structure in the 75,000-dalton polypeptide remaining even in 6 M guanidine HCl, or to the nature of the amino acid sequence about the bilin-attachment site, or to aggregation of the 75,000-dalton polypeptide.

Pepsin digestion of the 75,000-dalton polypeptide produces a blue-colored bilin peptide of 1,000–2,000 daltons with a λ_{max} of 650 nm in 30% (vol/vol) aqueous acetic acid. This suggests that the 75,000-dalton polypeptide carries one bilin. If it is assumed that this bilin has the spectroscopic properties of phycocyanobilin and that the absorbance of the 75,000-dalton polypeptide at 280 nm is 1.0 ml $mg^{-1}cm^{-1}$, it can likewise be estimated that there is one bilin per 75,000-dalton polypeptide.

"Renaturation" of the 75,000-dalton Polypeptide

When denaturing agents are removed by dialysis against neutral buffers, the 75,000-dalton polypeptide comes out of solution. However, if dialysis is performed against either 3 M urea–50 mM Na-phosphate, pH 7.0, or 20% (vol/vol) glycerol–0.5% Triton X-100–50 mM Na-phosphate, pH 7.0, solutions of 75,000-dalton polypeptide of as much as 1.0 absorbance/cm at 660 nm can be obtained. The spectra in the two solvents are qualitatively almost identical above 600 nm, with maxima at 665 and 610 nm, but differ grossly below 500 nm (see lower panel of Fig. 3). The ratio of the long wavelength to the short wavelength maximum changes from 0.9 in 6 M guanidine HCl–1% formic acid (Fig. 3, *top*) to 2.0 in 3 M urea, pH 7.0 (Fig. 3, *bottom*). For phycocyanin the corresponding ratios are 1 and ~3, respectively, and indicate significant refolding of phycocyanin in 3 M urea, pH 7.0. For the 75,000-dalton polypeptide, the ratio of $A_{360\,nm}/A_{280\,nm}$ is virtually the same in 6 M guanidine HCl–1% formic acid and 3 M urea at pH 7.0. The shorter wavelength portion of the spectrum of the 75,000-dalton polypeptide in 20% glycerol–0.5% Triton X-100 differs markedly from that seen in 3 M urea at pH 7. We do not know which, if either, of the two spectra shown in the lower panel of Fig. 3 resembles that of the 75,000-dalton polypeptide in its native environment within the phycobilisome. However, the fluorescence emission spectra in the two solvents are identical with maxima at 676 nm (Fig. 3). The quantum yield is 0.16 in 3 M urea relative to a value of 1.0 for native allophycocyanin in 0.01 M NH_4-acetate, pH 7. This low fluorescence quantum yield also suggests that the 75,000-dalton polypeptide is not in

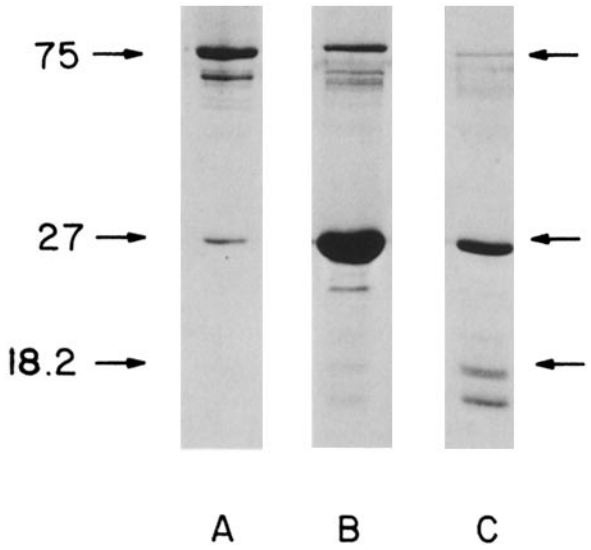

FIGURE 2 SDS PAGE of fractions from the Sephacryl S-200 column (Fig. 1). Lane *A*, fraction 30; lane *B*, pool of fractions 37 and 38; lane *C*, pool of fractions 40–45 inclusive. The light band below the 75,000-dalton polypeptide and that below the 27,000-dalton polypeptide (in lane *B*) are breakdown products formed on storage of phycobilisomes. The loads of protein applied to lanes *A*, *B*, and *C* were not equal.

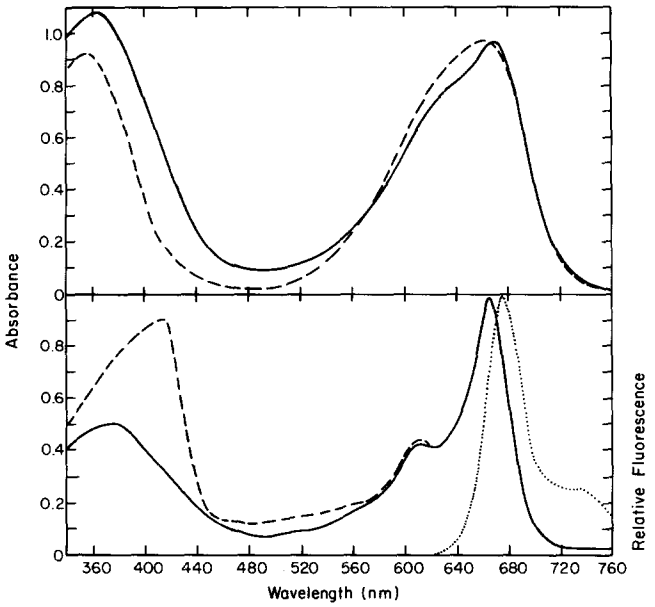

FIGURE 3 Absorption and fluorescence spectra of the 75,000-dalton polypeptide. The *upper panel* shows a comparison of the visible absorption spectra of the 75,000-dalton polypeptide (——), and of *Synechococcus* 6301 phycocyanin (-----), both in 6 M guanidine HCl–1% formic acid, matched to equal absorbance at their long wavelength maxima. The *lower panel* shows the absorption spectrum of the 75,000-dalton polypeptide in 3 M urea–50 mM Na-phosphate, pH 7 (——), and in 20% glycerol-0.5% Triton X-100–50 mM Na-phosphate, pH 7.0 (-----), matched to equal absorbance at 665 nm. The dotted line in the lower panel shows the fluorescence emission spectrum (λ^F_{max} = 676 nm) of both these samples in arbitrary units.

its native conformation. The excitation spectra for 676 nm emission for the 75,000-dalton polypeptide are similar to the absorption spectra.

Tryptic Degradation of the "18S Complex"

What is the function of the bilin covalently attached to the 75,000-dalton polypeptide? The absorption and fluorescence emission spectra of the "renatured" 75,000-dalton polypeptide suggest that it may function as a terminal energy acceptor in the phycobilisome. Since the 75,000-dalton polypeptide is associated with the core of the phycobilisome (5) and possibly with the thylakoid membrane (14, 15), such a role is plausible. However, as noted above, the spectroscopic properties of the isolated 75,000-dalton polypeptide may not correspond to those of its "native" conformer.

Allophycocyanin B has previously been assumed to be the terminal energy acceptor in phycobilisomes. However, recently we have isolated an 18S complex from strain AN112 phycobilisomes which contains no allophycocyanin B but still has a fluorescence emission maximum at ~676 nm (G. Yamanaka, D. J. Lundell, and A. N. Glazer, manuscript in preparation). This particle contains phycocyanin, allophycocyanin, 27,000-, and 75,000-dalton polypeptide in an approximate molar ratio of 6:6:2:1 and 1–2 mol of an uncharacterized 18,300-dalton polypeptide per 75,000-dalton polypeptide. Limited trypsin digestion of this complex (see Materials and Methods) results in a cleavage of the 75,000-dalton polypeptide to a 40,000-dalton polypeptide, seen to be blue-colored on SDS polyacrylamide gels, and the 27,000-dalton polypeptide to a series of products of 20,000–25,000 daltons. Chromatography of the trypsin-treated 18S complex on hydroxylapatite yields a phycocyanin fraction eluting at ~20 mM Na-phosphate, and two fractions containing similar amounts of allophycocyanin. One, eluting at 120–150 mM Na-phosphate, has the absorption spectrum of pure allophycocyanin (λ_{max} 650 nm) and contains only the α and β subunits of this protein (13). The second which elutes at 40–50 mM Na-phosphate contains the α and β subunits of allophycocyanin, the 40,000-dalton polypeptide, and the 18,300-dalton uncharacterized polypeptide. The ratio of the β subunit of allophycocyanin to the 40,000-dalton polypeptide in this fraction is ~3:1, as determined by densitometry of stained SDS polyacrylamide gels. This fraction has an absorption maximum at 657 nm and a fluorescence emission maximum at 676 nm. These observations suggest that the trypsin digestion of the 18S complex leads to partial dissociation, one of the products of which is a complex made up in the main of an allophycocyanin trimer, $(\alpha\beta)_3$, bound to a 40,000-dalton fragment of the 75,000-dalton polypeptide, which carries the bilin of the latter. Moreover, in this complex, the 40,000-dalton polypeptide appears to act as the terminal energy acceptor. This lends support to the view that the 75,000-dalton polypeptide acts as a terminal energy acceptor in the phycobilisome.

Although the molecular properties of allophycocyanin B and of the 75,000-dalton polypeptide are very different, the long wavelength absorption bands and fluorescence emission spectra of these proteins are very similar. It is not clear at this time whether the 75,000-dalton polypeptide acts as the sole terminal energy acceptor of the *Synechococcus* phycobilisome, or whether this polypeptide and allophycocyanin B function in parallel in the transfer of energy to the reaction centers in the thylakoid membrane.

The *Synechococcus* allophycocyanin–40,000-dalton polypeptide complex produced by limited proteolysis is strikingly similar to a component called allophycocyanin I (AP-I) isolated by Zilinskas et al. (16) from *Nostoc* sp. phycobilisomes. AP-I was reported to contain ~1 blue-colored 35,000-dalton polypeptide per trimer of allophycocyanin and to have a fluorescence emission maximum at 681 nm (17). Zilinskas et al. (16) and Troxler et al. (18) believe that the 35,000-dalton polypeptide of AP-I is a component of *Nostoc* sp. phycobilisomes, whereas in *Synechococcus* 6301 it is clear that such a component is seen only after limited proteolysis.

Occurrence of Biliproteins

The 75,000-dalton polypeptide joins a short list of proteins with covalently bound tetrapyrrole prosthetic groups. The phycobiliproteins are the largest group of these proteins, of which phycoerythrin and phycocyanin are the most abundant members. All of these proteins contain α and β subunits of <20,000 daltons that carry covalently bound bilins (1). Rhodophytan phycoerythrins also contain a γ-subunit of ~30,000 daltons with attached bilins (9, 19). A second type of biliprotein, widely distributed, is the plant photomorphogenetic chromoprotein, phytochrome, a single polypeptide of 120,000 daltons with a single covalently bound bilin (20). Curiously, the only occurrence of biliproteins in the animal kingdom reported to date is among the Labridae, a family of sea fishes related to the sea perches, in which these chromoproteins are found as coloring pigments in the skin (see reference 2 for a review).

This work was supported in part by National Science Foundation grant PCM 79-10996. D. J. Lundell was supported by a postdoctoral fellowship GM 07179-02 and G. Yamanaka by training grant T32 GM 07232-04 from the National Institute of General Medical Sciences, U. S. Public Health Service.

Received for publication 1 June 1981, and in revised form 10 July 1981.

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