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A M_r 95,000 polypeptide in *Porphyridium cruentum* phycobilisomes and thylakoids: Possible function in linkage of phycobilisomes to thylakoids and in energy transfer

(red algae/phycobiliproteins/chlorophyll-binding protein)

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ABSTRACT **Two pigmented polypeptides with the same molecular weight (M_r 95,000) were isolated from the photosynthetic apparatus of *Porphyridium cruentum* by sodium dodecyl sulfate/polyacrylamide gel electrophoresis. A blue polypeptide from phycobilisomes had absorption and fluorescence emission spectra similar to those of allophycocyanin. A green-pigmented polypeptide from photosynthetic membranes (free of phycobilisomes) contained chlorophyll *a*. Several properties were common to the M_r 95,000 polypeptides from both sources: (*i*) identical molecular weights, (*ii*) identical gel electrophoresis patterns after limited protease digestion, and (*iii*) immunological crossreactivity with an IgG fraction directed against the M_r 95,000 polypeptide from phycobilisomes. On the basis of this evidence, a common polypeptide exists in phycobilisomes and thylakoids, and it probably anchors the phycobilisome to the thylakoid membrane. The fluorescence emission overlap of the blue and green polypeptides suggests that they are involved in the transfer of energy from phycobilisomes to thylakoids.**

Phycobilisomes are organelles on the photosynthetic membrane of red and blue-green algae (cyanobacteria) that function in absorbing light in the wavelength region where chlorophyll absorption is low. Such an extension of the absorption region especially benefits organisms growing under light-limiting conditions. Light absorbed by the phycobiliproteins (phycoerythrin, phycocyanin, and allophycocyanin) is funnelled through a long-wavelength-emitting allophycocyanin to chlorophyll *a* (reviewed in ref. 1). In cells of the red alga *Porphyridium cruentum*, Duysens (2) and French and Young (3) demonstrated energy transfer from phycoerythrin to chlorophyll. Because the transfer efficiencies from phycobiliproteins to chlorophyll are high (80–90%), it is expected that phycobilisomes have a functional attachment site in close proximity to photosynthetic reaction centers (4). Although phycobilisome attachment molecule(s) have not yet been found, such molecules have been identified for another thylakoid binding complex, the coupling factor (5). Assuming that there are specific attachment molecule(s) in phycobilisomes, we have undertaken a comparison of the polypeptide compositions of isolated phycobilisomes and of thylakoids (free of phycobilisomes) to determine which components are likely to be involved in phycobilisome/thylakoid attachment and energy transfer.

The phycobilisome protein composition of *P. cruentum* (synonymous with *P. purpureum*) has been reported (6). As with many other algae (7–9), the phycobilisomes were found to consist of many polypeptides, some of which appeared colored and others noncolored after $NaDodSO_4$/polyacrylamide gel electrophoresis. A pigmented polypeptide (F680) was reported to have a high molecular weight (95,000) and to constitute approximately 1.3% of the phycobilisome protein on the basis of Coomassie blue staining (6). As already reported in a preliminary communication (10), a polypeptide with the same molecular weight and a similar protease digestion pattern has been isolated from thylakoids (free of phycobilisomes) and has been suggested to be involved in anchoring the phycobilisome to the thylakoid.

In blue-green algae, Tandeau de Marsac and Cohen-Bazire (7) suggested that a M_r 75,000 polypeptide may be involved in connecting phycobilisomes to thylakoids. Their suggestion was based on observing a M_r 75,000 polypeptide in both phycobilisomes and thylakoid fractions. Recently a M_r 75,000 polypeptide derived from phycobilisomes of blue-green algae was designated as a new phycobiliprotein, and on the basis of its spectral properties it has been proposed as the terminal pigment in phycobilisomes (11).

We show here from a comparison of pigment and protein characteristics that isolated M_r 95,000 polypeptides from the phycobilisomes and from thylakoids are highly similar. Evidence presented is consistent with the notion that the polypeptide of M_r 95,000 is involved in attachment and energy transfer between phycobilisomes and thylakoids.

METHODS

Algal Culturing. *P. cruentum* was grown at 20°C in artificial seawater medium (12), under continuous fluorescent light illumination with shaking and aeration with 5% CO_2/95% air (13).

Phycobilisome Isolation. Cells were harvested at a cell density of 4–6 g (wet wt)/liter and disrupted with a French pressure cell [10,000 pounds/inch^2 (69 MPa)] in 0.5 M sodium phosphate buffer (pH 7.0). The homogenate was incubated in 1% (vol/vol) Triton X-100 and agitated 30 min at 4°C. After centrifugation (40,000 × *g*, 30 min) the supernatant was layered on a sucrose step gradient (0.25–2.00 M sucrose/0.1% Triton X-100/0.5 M sodium phosphate buffer, pH 7.0) and phycobilisomes were recovered from the 1–2 M sucrose interface after 3-hr centrifugation at 142,000 × *g* (13). This fraction was diluted (1:3, vol/vol) with 0.5 M sodium phosphate buffer (pH 7.0) and centrifuged at 40,000 × *g* to remove aggregated particles. Phycobilisomes were sedimented by centrifugation at 362,000 × *g* for 2 hr. Chlorophyll content in phycobilisome preparations was low. The ratio A_{663} (acetone extracts)/A_{545} (aqueous suspension) was <0.0005.

Thylakoid Isolation. Cells (4 g/liter) were disrupted with a French pressure cell (5,000 pounds/inch^2) in 0.3 M sucrose/25 mM Hepes–KOH, pH 7.5/1 mM $MgCl_2$. Low salt concentrations result in dissociation of phycobilisomes. The homogenate was centrifuged at 10,000 × *g* for 10 min (5°C) to remove

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the phycobiliproteins and other soluble components. The remaining green pellet was washed twice with 0.3 M sucrose/5 mM Hepes–KOH, pH 7.5/10 mM EDTA to further remove any remaining phycobiliprotein. The washed thylakoids were purified by flotation centrifugation according to Chua and Bennoun (14). Such thylakoids were free of phycobilisomes by electron microscope examination and were essentially free of phycobiliproteins as determined by A_{540}/A_{675} of <0.05.

Isolation of M_r 95,000 Polypeptides by NaDodSO$_4$/Polyacrylamide Gel Electrophoresis. The M_r 95,000 polypeptide was isolated from pelleted phycobilisomes that were solubilized (1 mg/ml) in 1% (wt/vol) NaDodSO$_4$/50 mM Tris·HCl, pH 6.8/100 mM 2-mercaptoethanol/10% (wt/vol) sucrose and either heated for 1 min at 100°C (for antigen preparation) or held at 12°C for 1 min (for pigment analysis). The solubilized phycobilisome preparation was electrophoresed on a 3-mm-thick 7.5–15% gradient acrylamide gel (6). The blue band at M_r 95,000 was lyophilized, reduced to a powder, and extracted in 50 mM sodium phosphate buffer (pH 7.0) (15). Reelectrophoresis of the isolated polypeptide at 200 μg of protein per 10-mm lane did not reveal any low molecular weight contamination. The system can detect as little as 2–4 μg of protein.

A green band of M_r 95,000 was isolated from thylakoid preparations. Thylakoids (1 mg/ml) were solubilized as above at 12°C. Polypeptides were separated by electrophoresis as described above. The M_r 95,000 polypeptide was one of three green bands observable before Coomassie blue staining (10). It was eluted from the gel as above, heated to 100°C, and reelectrophoresed. The green color was lost, but the M_r 95,000 polypeptide, as well as a minor band at *ca.* M_r 52,000, was detectable after staining with Coomassie blue.

Antibody Preparation. The M_r 95,000 polypeptide purified from phycobilisomes was solubilized in buffer (50 mM sodium phosphate, pH 7.0/100 mM NaCl/0.1% NaDodSO$_4$) and emulsified in Freund's complete adjuvant. For production of antisera, New Zealand White rabbits were subcutaneously injected at multiple sites with 1 mg of antigen. Booster shots (0.5 mg of antigen in Freund's adjuvant) were given after 3- and 6-week intervals. Ten days after the last injection, the rabbits were sacrificed, and the serum was fractionated with 50% saturated $(NH_4)_2SO_4$ as described by Kabat and Mayer (16). The IgG-containing fraction was obtained by chromatography on a 2.5 × 10 cm Affi-blue dextran column (Bio-Rad). Rabbit preimmune serum was negative when tested against the antigen.

Immunological Identification of Proteins Bound to Nitrocellulose Paper. Protein samples, resolved on NaDodSO$_4$/polyacrylamide gels, were electrophoretically transferred to nitrocellulose sheets. Transfer of the protein bands was ascertained by their presence on the nitrocellulose sheets upon staining with amido black (17).

Protein A from *Staphylococcus aureus* (Pharmacia) was iodinated by a modification of the procedure of Hubbard and Cohn (18) utilizing glucose oxidase and lactoperoxidase coimmobilized on an acrylic resin (New England Nuclear). One millicurie (3.7×10^7 becquerels) of carrier-free Na^{125}I was allowed to react with 100 μg of protein A. The iodinated protein A was collected in the void volume after passage through a Sephadex G-50 column (in a 1 ml tuberculin syringe). A specific activity of 1–10 μCi/μg was obtained.

Nitrocellulose sheets containing the electrophoretically transferred proteins were treated with a 3% (wt/vol) bovine serum albumin (50 mM NaCl/10 mM Tris·HCl, pH 7.4) solution, washed, and incubated with the anti-M_r 95,000 IgG at concentrations and times indicated by Towbin *et al.* (19). The immunological reaction sites were labeled by soaking washed nitrocellulose sheets in ^{125}I-labeled protein A [100 μg/20 ml in 3% (wt/vol) bovine serum albumin/150 mM NaCl/10 mM Tris·HCl, pH 7.4] for 2 hr at 22°C. The sheets were washed (150 mM NaCl/10 mM Tris·HCl, pH 7.4), dried between sheets of filter paper in a gel drier (Hoefer, San Francisco, CA) for 30 min at 50°C, and autoradiographed on Kodak X-Omat XAR film for 1–3 days.

Spectroscopic Measurements. Fluorescence spectra, emission and excitation, were made with an Aminco Bowman fluorometer (American Instrument, Urbana, IL) with a spectral correction attachment (13). Absorption spectra were measured on a Cary 17 spectrophotometer (Varian Instrument, Palo Alto, CA). Reference solutions for highly scattering samples, such as suspensions from pulverized lyophilized samples, contained $BaCO_3$ or pulverized gels without sample to duplicate the light-scattering properties of the sample.

RESULTS

A high molecular weight polypeptide (M_r 95,000) was present both in thylakoids from which phycobilisomes had been removed and in phycobilisomes (Fig. 1, lanes C and B, respectively). This result suggested that thylakoids and phycobilisomes might have a polypeptide component in common. To test this possibility the polypeptides from the two sources were isolated and examined further. When isolated from phycobilisomes this polypeptide was blue, and it is hereafter referred to as the blue polypeptide. Although it was one of nine colored polypeptides visible on unstained NaDodSO$_4$/polyacrylamide gels (6), it was clearly separated from any others by its large molecular weight. Other blue polypeptides were in the 19,000–13,000 M_r region and corresponded to the commonly recognized α and β polypeptides of phycobiliproteins (1, 21).

The M_r 95,000 polypeptide isolated from thylakoids was obtained from a green band after electrophoresis at 12°C and is hereafter referred to as the green polypeptide. Chromophore association with the green band occurred only under partially denaturing conditions (nonheated). After complete dissociation (heating at 100°C for 1 min) and reelectrophoresis of the green band, there was no green color detectable, but after staining

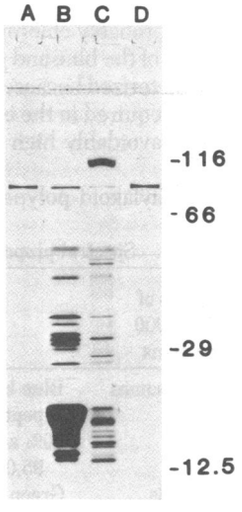

FIG. 1. NaDodSO$_4$/polyacrylamide gel electrophoretogram of *P. cruentum* thylakoids, phycobilisomes, and the M_r 95,000 polypeptides isolated from each. Electrophoresis was carried out as described (6, 20). All samples were solubilized by heating (100°C) for 1 min prior to electrophoresis. The gel was stained with Coomassie brilliant blue. Lanes A and D, M_r 95,000 polypeptides isolated from phycobilisomes and thylakoids, respectively; lane B, phycobilisomes; lane C, thylakoids. $M_r \times 10^{-3}$ is given on the right.

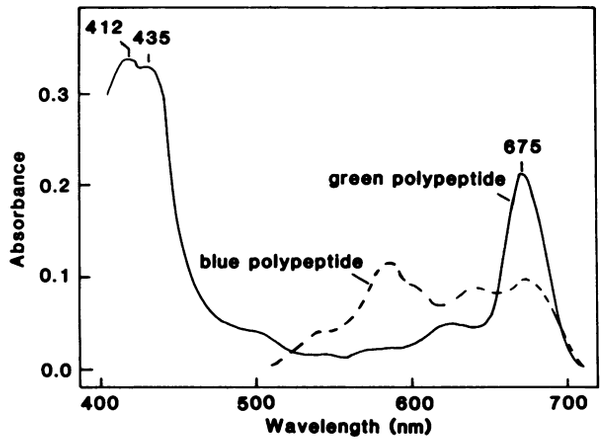

FIG. 2. Absorbance spectra (23°C) showing chlorophyll enrichment in the green polypeptide from thylakoids (——) when compared with the blue polypeptide from phycobilisomes (– – –). The M_r 95,000 bands were isolated from $NaDodSO_4$/polyacrylamide gels as described in the text, and the samples were dialyzed against 100 mM sodium phosphate buffer (pH 7.0) and adjusted to 100 μg of protein per ml of buffer for spectral measurements. The spectrum of the blue polypeptide solution was not resolvable below 500 nm because of high scatter.

with Coomassie blue there was a band that corresponded in molecular weight to the blue polypeptide (Fig. 1, lanes A and D).

The green band contained chlorophyll *a* as evidenced by its absorption and excitation characteristics (Fig. 2 and Table 1). In contrast, the blue polypeptide had its major absorption in the region of allophycocyanin and phycocyanin (*ca.* 650–610 and 585 nm). It also contained chlorophyll as suggested by the 675-nm absorbance peak, and the 685-nm emission peak when excited at 440 nm (chlorophyll). Interestingly, the blue polypeptide when excited at 580 nm produced an emission maximum (*ca.* 680 nm) separate from that (*ca.* 685 nm) when excited at 440 nm. This suggests the presence of two separately emitting chromophores which, however, are not energetically coupled in this isolated preparation. *In vivo*, both chromophores are probably energetically coupled, and transfer would proceed to the 685-nm emitting chromophore (probably chlorophyll). The absorption and emission properties of the blue and green polypeptides could be only partially characterized because of extensive fading after $NaDodSO_4$ treatment required in the isolation of the polypeptides and because of unavoidably high light scattering by samples.

The phycobilisome and thylakoid polypeptides (M_r 95,000) have the same apparent molecular weight as determined by $NaDodSO_4$ electrophoresis. They could be either the same protein with different chromophores or completely different proteins. To test whether these two polypeptides have additional common properties, they were analyzed by peptide mapping and immunological crossreactivity. According to their protease digestion patterns, the two polypeptides can be considered the same. A comparison of their peptide maps (Fig. 3) resulting from limited hydrolysis with protease V8 from *S. aureus*, showed that both the blue and the green polypeptides had the same number of bands of comparable densities and identical electrophoretic mobilities. None of the resulting protease digest bands was colored; they were detected only after staining.

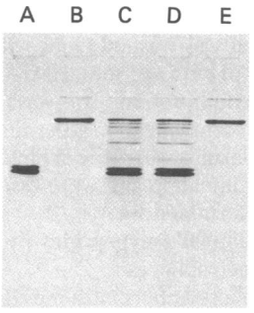

FIG. 3. $NaDodSO_4$/polyacrylamide gel electrophoresis of partially digested M_r 95,000 polypeptides isolated from phycobilisomes and thylakoids. Samples were treated with or without *S. aureus* protease V8 (Miles) at 25 μg/ml and incubated for 30 min at 37°C. See Cleveland *et al.* (22) for other details. The gel was stained with Coomassie brilliant blue. Lanes: A, protease V8; B, 95,000 M_r polypeptide from phycobilisomes without protease treatment; C, 95,000 M_r polypeptide from phycobilisomes, protease treated; D, 95,000 M_r polypeptide from thylakoids, protease treated; E, 95,000 M_r polypeptide from thylakoids without protease.

Both M_r 95,000 polypeptides were found to specifically react with the IgG fraction developed against the blue polypeptide. Antibodies were not developed against the green polypeptide. An immunological reaction band in the M_r 95,000 region with the phycobilisome polypeptide (Fig. 4, lane B) and the thylakoid polypeptide (Fig. 4, lane C) was identified in both cases with a stained control (Fig. 4, lanes A and D). By this criterion, the two proteins appear to be the same.

To determine how universal the M_r 95,000 polypeptide is in Rhodophyceae, phycobilisomes from several other species were compared after $NaDodSO_4$/polyacrylamide gel electrophoresis (Fig. 5). In *P. cruentum*, *P. sordidum*, and *Centroceras clavulatum* a polypeptide of M_r 95,000 was quite prominent. Al-

Table 1. Spectral properties of isolated M_r 95,000 proteins from phycobilisomes and thylakoids

Source of M_r 95,000 proteins	Fraction*	Emission, nm (23°C) 440-nm excitation	Emission, nm (23°C) 580-nm excitation	Maximal-absorption regions, nm (23°C)
Phycobilisomes	Blue band M_r 95,000 polypeptide (in buffer)	685	680	675, 645–610,† 585
	90% acetone extract of M_r 95,000 blue band	—	—	—
Thylakoids	Green band M_r 95,000 polypeptide (in buffer)	688	—	675, 435, 412
	90% acetone extract of M_r 95,000 green band	680, 620	—	663, 435, 412

* Protein bands were isolated from the gel and dialyzed overnight against 100 mM sodium phosphate buffer (pH 7.0). Measurements in buffer were made at 100 μg of protein per ml of buffer except for measurements in acetone, which were at equivalents to 100 μg of protein per ml of acetone extract.

† The λ_{max} shifted within the range given, possibly due to variable residual $NaDodSO_4$ from the electrophoretic isolation. Results are from four different extractions.

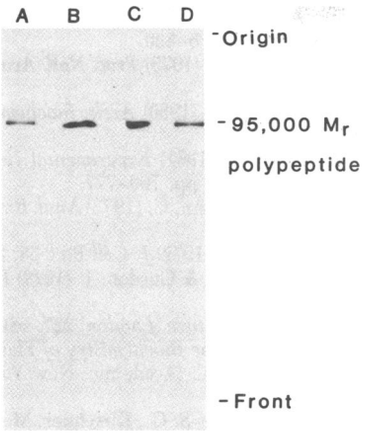

FIG. 4. Immunological crossreactivity of 95,000 M_r polypeptides from phycobilisomes and thylakoids. Isolated 95,000 M_r polypeptides from thylakoids and phycobilisomes were electrophoresed and transferred to nitrocellulose paper. The polypeptides were allowed to react with an IgG fraction against the phycobilisome 95,000 M_r polypeptide and the bound IgG was detected with ^{125}I-labeled protein A. Replicate lanes from the nitrocellulose sheet were stained with amido black. Lanes A and D, amido black-stained 95,000 M_r polypeptides isolated from phycobilisomes (lane A) and thylakoids (lane D). Lanes B and C, autoradiograph showing ^{125}I-labeled protein A bound to the IgG that had reacted with the 95,000 M_r polypeptide isolated from phycobilisomes (lane B) and thylakoids (lane C).

though *Griffithsia pacifica* had a faint band at 95,000, its major high molecular weight was at 75,000. Whether the 95,000 M_r polypeptide was partially degraded to the M_r 75,000 polypeptide is not known, but degradation is considered probable.

DISCUSSION

In an earlier paper (6), we reported that a blue M_r 95,000 polypeptide is present in isolated phycobilisomes. This polypeptide constituted approximately 1.3% of total stainable protein of the phycobilisome. Thus, in each *P. cruentum* phycobilisome with M_r *ca.* 20×10^6 (1) there would be 1–2 M_r 95,000 polypeptides. Thylakoid membranes (free of phycobilisomes) also contained this polypeptide, as judged on the basis of the following criteria: (*i*) the same apparent molecular weight, (*ii*) the same molecular weight peptides after protease digestion, and (*iii*) immunological crossreactivity. The major difference in the thylakoid M_r 95,000 polypeptide was its green color due to chlorophyll *a*. The variable pigmentation associated with the M_r 95,000 polypeptide raised the possibility that there may be two separate polypeptides—i.e., a green polypeptide from thylakoids and a blue one from phycobilisomes. Evidence presented in this paper supports the hypothesis that the M_r 95,000 polypeptides from thylakoids and phycobilisomes are the same except for chromophore associations, but it is recognized that complete identity has not been shown absolutely. On one hand, the chlorophyll phycobilin chromophores associated *in vitro* could have been lost during the isolation procedure; on the other hand, it is possible that the M_r 95,000 polypeptide coisolated with a chlorophyll polypeptide or with a phycobilin polypeptide of similar electrophoretic mobility.

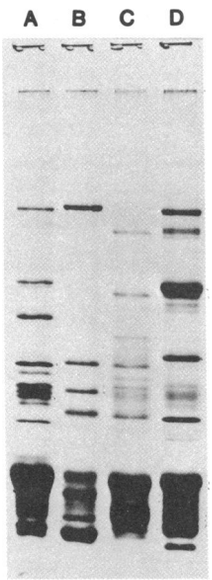

FIG. 5. Comparison of polypeptides of phycobilisomes of several rhodophyceaen species after NaDodSO$_4$/polyacrylamide gel electrophoresis. Electrophoresis and staining were carried out as in Fig. 1. The M_r 95,000 polypeptide of *P. cruentum* (lane A), *P. sordidum* (lane B), and *Centroceras clavulatum* (lane D) was blue before staining. The 95,000 M_r polypeptide of *Griffithsia pacifica* (lane C) was detected only after staining and only as a faint band.

Functionally, the blue 95,000 M_r polypeptide is probably involved in the terminal phase of energy transfer in *P. cruentum* phycobilisomes. Supporting evidence for this is the polypeptide's long-wavelength emission at 680 nm and 685 nm and its association with the phycobilisome core (6). The high molecular weight polypeptides of *Synechococcus* (11) and *P. cruentum* may be functionally closely related because both are blue with similar fluorescence emission characteristics (675–680 nm). It is of interest to note that large molecular weight polypeptides (M_r *ca.* 95,000) are found not only in red algae (Fig. 5) but also in at least three blue-green algae, namely *Pseudoanabaena* (23), *Nostoc* sp., and *Tolypothrix* (unpublished observations). It will be of interest to ascertain whether the M_r 75,000 and M_r 95,000 polypeptides from the various algae have common immunological reactive sites.

The M_r 95,000 polypeptide is the most likely candidate as a linker protein that could anchor phycobilisomes to thylakoid membranes. Because phycobilisomes are isolated in the presence of Triton X-100, which solubilizes lipophilic components, the M_r 95,000 polypeptides could be retained with the phycobilisomes. On the other hand, intact thylakoids, isolated in the absence of detergents, could retain the M_r 95,000 polypeptide(s) if they are at least partially embedded in the thylakoid membranes. Thus, the molecule(s) could be envisioned as a common component present in both thylakoids and phycobilisomes that anchors phycobilisomes to thylakoids.

The particular pigments associated with the M_r 95,000 polypeptide suggest that this polypeptide plays a principal role in energy transfer from phycobilisomes to thylakoids. Not only does the blue polypeptide appear to function as a terminal energy acceptor in phycobilisomes, but, as pointed out (Table 1), this polypeptide also binds some chlorophyll *a*. If the same polypeptide can bind both far-emitting phycobilins and chlorophyll *a* chromophores, then the dual function of phycobilisome/thylakoid attachment and energy transfer may be accomplished by the same molecule. Whereas from the spectral data of the 95,000 M_r polypeptide a definitive conclusion on the pigmentation was not possible, there is little doubt from the immunological results that a common high molecular weight component is present in both phycobilisomes and thylakoids.

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