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Characterization of Phycobilisome Glycoproteins in the Cyanobacterium *Anacystis nidulans* R2

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Concanavalin A-reactive linker and anchor subunits of phycobilisomes from *Anacystis nidulans* R2 (H. C. Riethman, T. P. Mawhinney, and L. A. Sherman, FEBS Lett. 215:209–214, 1987) were purified electrophoretically and analyzed for carbohydrate composition and quantity. Different quantities of glucose and *N*-acetylgalactosamine were found on the concanavalin A-reactive subunits analyzed. Proteolytic analysis of the purified subunits suggested that small regions of the 33- and 27-kilodalton linker polypeptides previously shown to be important for in vitro phycobilisome assembly contained the concanavalin A-reactive carbohydrates present on these subunits. The linker and anchor subunits from the morphologically different phycobilisome of *Synechocystis* sp. strain PCC6714 were also shown to be concanavalin A reactive. Membranes from iron-starved *Anacystis nidulans*, which lack assembled phycobilisomes and are associated with glycogen deposits, were shown to be depleted of linker and anchor proteins and to accumulate very large quantities of a concanavalin A-reactive, extrinsic membrane glycoprotein. We suggest that this iron stress-induced glycoprotein is associated with the glycogen deposits on the thylakoid surface and that the glycosylation of phycobilisome linker and anchor subunits is involved in the physiological regulation of phycobilisome assembly and degradation.

Phycobilisomes are the main light-harvesting structures for photosystem II (PSII) of red algal chloroplasts and cyanobacteria. They are very large (4 to 20 megadalton [MDa]) pigment-protein complexes that reside on the outer surface of the thylakoid membrane and are physically connected to the chlorophyll (Chl) *a* pigments of PSII. Details of phycobilisome structure have been reported for several cyanobacterial species (reviewed in references 6, 14, and 43). In *Anacystis nidulans*, there are about 370 covalently attached bilin molecules per phycobilisome. The low-molecular-mass biliproteins (19 to 24 kDa) constitute about 85% of the mass of the phycobilisome (41) and are assembled into the macromolecular structure by mainly nonpigmented linker and anchor polypeptides. The *A. nidulans* phycobilisome has a central core which consists of two cylindrical, allophycocyanin-containing complexes, to which are attached six rods. The rods contain phycocyanin biliproteins and four nonpigmented linker polypeptides (33, 30, 27, and 9 kDa). The core has one small (about 8-kDa) nonpigmented polypeptide, two types of allophycocyanin biliproteins, and a large (75-kDa) polypeptide which has been postulated to "anchor" the phycobilisome to PSII. This anchor protein contains a covalently linked bilin (23). We have recently shown that the 75-, 33-, 30-, and 27-kDa phycobilisome subunits are concanavalin A (ConA)-reactive and are probably glycoproteins (28).

Phycobilisomes compose a large fraction (up to 45%) of the total soluble protein in cyanobacterial cells (3, 25), and their metabolism is likely to have a considerable impact on the physiology of these organisms. Evidence for such effects are seen in the responses of cyanobacteria to various kinds of environmental stresses; iron, sulfur, phosphorus, and nitrogen stress are all associated with a depletion of phycobilisomes (32, 34, 35, 38). The observation that glucose represents a significant percentage of the mass of the phycobilisome (28) added another perspective to the possible roles which phycobilisomes serve in maintaining the cell's viability in natural environments, where such stresses would occur often. When these types of stresses have been investigated in detail, phycobilisome degradation is accompanied by a massive accumulation of glycogen (32, 35, 38). Furthermore, the initial site of glycogen granule accumulation in cells undergoing stress is at the outer surface of the thylakoid membrane, a region vacated by degraded phycobilisomes. This correlation is striking and suggests possible interactions between glycogen storage granules and phycobilisomes which may be governed by global stress regulons similar to those found in *Escherichia coli* (26).

To confirm more directly our previous suggestion (28) that the phycobilisome linker and anchor proteins are glycosylated, we purified individual linker and anchor proteins under highly denaturing conditions and analyzed the individual, purified subunits for their carbohydrate content. We also used the ConA-binding properties of the purified subunits to localize putative carbohydrate-containing regions of these molecules by proteolytic mapping of ConA-reactive fragments. Finally, we initiated an investigation of the possible involvement of these membrane-associated glycoproteins in phycobilisome metabolism and glycogen deposition by documenting the disappearance of the phycobilisome linker and anchor subunits after iron stress and the concurrent accumulation of an extremely abundant membrane-associated glycoprotein in iron-starved cells.

MATERIALS AND METHODS

Strain. The characteristics and growth conditions of *A. nidulans* R2 and *Synechocystis* sp. strain PCC6714 grown in either nutrient-sufficient or iron-limiting medium have been reported previously (8, 32).

Preparation of membranes and TX114 phase partitioning.

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Cells were harvested by centrifugation and broken by passage through a chilled French pressure cell (27). Membranes were isolated from the broken cells by differential centrifugation as described previously (27). *A. nidulans* R2 membrane components were separated into hydrophobic and hydrophilic fractions by using the Triton X-114 (TX114) phase partitioning system of Bordier (4) as modified by Bricker and Sherman (5). A total of three repetitions of this procedure were done for each membrane sample.

Purification of phycobilisomes and fractionation of phycobilisome components. Intact phycobilisomes were isolated and purified by the procedure of Yamanaka et al. (41). Absorption and fluorescence spectra of phycobilisomes acquired following their purification by sucrose density gradient centrifugation in high-salt buffer (0.75 M sodium potassium phosphate, pH 8.0) (41) indicated that the phycobilisomes were intact and that Chl was absent. Intact phycobilisomes were concentrated by pelleting in high-salt buffer. The pellet was then dissolved in 20 mM sodium potassium phosphate (pH 7.1), and the solution was dialyzed extensively against the same buffer (at 4°C).

Dialyzed phycobilisome material was separated into linker-enriched and linker-depleted fractions by the method of Lundell et al. (23). The phycobilisome material (10 ml, 2 mg of protein per ml) was brought to 0.5 M NH_4SCN by addition of solid reagent, and this mixture was incubated for 30 min at room temperature. The cloudy suspension was then centrifuged (8,000 × *g* for 10 min in a Beckman JA-20 rotor), and the deep-blue supernatant was separated from the pale blue-white pellet. The pellet was suspended to 10 ml in 20 mM sodium potassium phosphate (pH 7.1), and the procedure was repeated. The final pellet was suspended in a small volume of 20 mM sodium potassium phosphate (pH 7.1) and was stored frozen.

Individual subunits of phycobilisomes were separated by preparative lithium dodecyl sulfate (LDS)-polyacrylamide gel electrophoresis (PAGE) and then electroeluted from the gel matrix. From 1 to 1.5 mg of purified phycobilisome material was solubilized at 70°C for 10 min in electrophoresis sample buffer (15), layered onto a 10 to 20% acrylamide gradient gel, and then subjected to PAGE under standard conditions. After PAGE, the gel slab was stained for 10 min with Coomassie blue and then destained for 10 min. The bands of interest were excised, washed with water, incubated in Tris buffer (pH 8.0) to bring the pH to neutral, and stored at −20°C. Pooled bands from several gels were processed, and the protein was electroeluted as described previously (18). Prior to carbohydrate analysis of the phycobilisome fractions, samples were treated with the chloroform-methanol-aqueous extraction procedure of Wessel and Flügge (39) to remove salts, detergents, and dyes; this procedure also concentrated the material into a dry pellet.

Carbohydrate analysis and protein quantitation. Samples analyzed for carbohydrate content were hydrolyzed in 4 N HCl for 4 h at 100°C, and the released sugar monomers were derivatized (both as their per-*O*-trimethylsilyl-*O*-methoximes and as their alditol acetates) and quantitated as described previously (24). All carbohydrate standards were obtained from Pfanstiehl Laboratories (Waukegan, Ill.). Protein concentrations of the phycobilisome fractions were determined prior to the chloroform-methanol-aqueous extraction step; the samples were subjected to LDS-PAGE, and the gel was scanned after staining with Coomassie blue. Purified phycobilisomes were used as a standard, since the concentration of protein in intact phycobilisomes can easily be estimated from the extinction coefficient of phycocyanin, and the protein ratio of *A. nidulans* phycobilisome subunits has been determined (by comparison of the density of Coomassie-stained bands [41]). The ratio of phycobilisome subunits obtained from purified phycobilisomes was nearly identical by our method to the ratio determined previously (41). This method of protein quantitation has been used routinely for phycobilisome subunit analysis (1, 20), although it has possible shortcomings (see Discussion).

PAGE and protein blotting. LDS- and sodium dodecyl sulfate (SDS)-PAGE conditions have been described previously (15). The method of Towbin et al. (37) was used to transfer proteinaceous components (separated by LDS- or SDS-PAGE) to nitrocellulose. The postblot gels were stained with Coomassie blue, photographed, and then stained with silver by the method of Wray et al. (40).

Total transferred proteins were detected by an amido black staining procedure (29). When pieces of nitrocellulose were to be stained immunologically, the filter was first placed in blocking solution (3% [wt/vol] bovine serum albumin in 10 mM Tris [pH 7.4], 0.9% [wt/vol] NaCl, 0.5% [vol/vol] Tween-20) for 1 to 3 h at 37°C. After blocking, the filter was incubated at room temperature for 6 to 8 h in Tween-TTS (10 mM Tris, pH 7.4, 0.9% [wt/vol] NaCl, 0.5% [vol/vol] Tween 20) containing the primary antibody. After washing with Tween-TTS (three times for 15 min each with gentle shaking), the filters were incubated at room temperature for 3 to 5 h in a secondary antibody solution containing immunoglobulin G (IgG) directed against the F_c portion of the primary antiserum (horseradish peroxidase [HRP]-conjugated goat anti-rabbit IgG, 40,000:1 dilution in Tween-TTS; Cappel Laboratories). The filters were then washed as described above. HRP-labeled bands were detected by incubation of filters in a solution containing 0.0025% (wt/vol) *o*-dianisidine (Sigma Chemical Co.; base form) and 0.01% (vol/vol) H_2O_2 in Tween-TTS. These immunostaining procedures were adapted from Towbin et al. (37), Taylor et al. (36), and Smith and Fisher (33).

A slight modification of the method of Clegg (9) was used to detect ConA-reactive glycoproteins after their separation by LDS-PAGE and their transfer to nitrocellulose. The nitrocellulose filter containing the transferred proteins was incubated for 1 h in a blocking solution which contained 2.5% (wt/vol) bovine serum albumin in Tween-TTS as well as 10 μM concentrations of the divalent cations Mg^{2+}, Ca^{2+}, and Mn^{2+}. The filter was then transferred to a solution containing 16 μg of ConA (Sigma) per ml of Tween-TTS plus cations for 1 h, washed five times for 5 min each with Tween-TTS plus cations, and then incubated for 1 h in a solution containing HRP (Sigma, type VI; 50 μg/ml in Tween-TTS plus cations). The filter was again washed five times for 5 min each with Tween-TTS plus cations and finally stained for HRP-reactive bands with *o*-dianisidine as described above.

Proteolytic mapping of proteins. A minor modification of the methods of Cleveland et al. (10) and Hames (17) was used to digest partially the electrophoretically purified proteins with proteases and then analyze the digestion products. All procedures were carried out at room temperature. Coomassie-stained bands from preparative gels were excised, rinsed with water, and then incubated in a solution containing 0.125 M Tris (pH 6.8) and 0.1% (wt/vol) SDS for 15 min at room temperature. Each band was then sliced into small (approximately 3-mm long) pieces. A gel was prepared (25-cm-long 10 to 20% resolving gel, with a 5-cm-long stack), and the wells of the stack were filled with 0.125 M Tris (pH 6.8)–0.1% SDS. The gel pieces were placed in the wells with

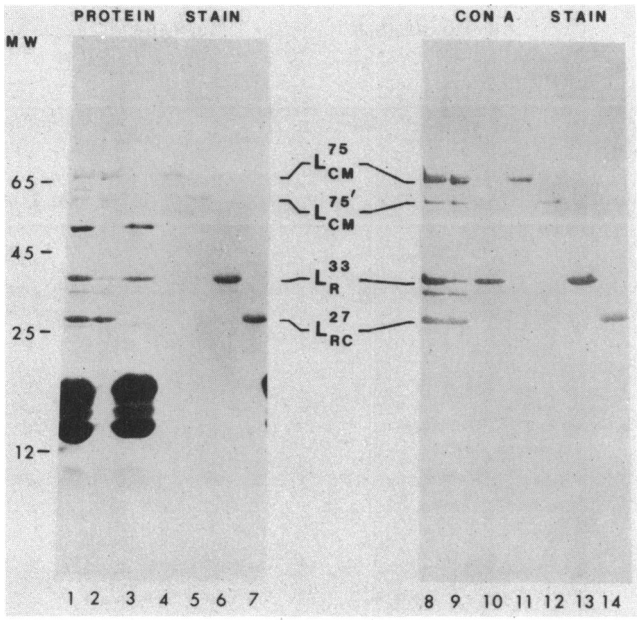

FIG. 1. ConA reactivity of *A. nidulans* R2 phycobilisome fractions. Purified phycobilisomes (lanes 1 and 8) were separated into a linker-enriched fraction (lanes 2 and 9) and the cognate linker-depleted fraction (lanes 3 and 10). The following subunits of phycobilisomes were electrophoretically separated on and subsequently eluted from polyacrylamide gels: L_{CM}^{75} (lanes 4 and 11), $L_{CM}^{75'}$ (lanes 5 and 12), L_{R}^{33} (lanes 6 and 13), and L_{RC}^{27} (lanes 7 and 14). Each fraction was subjected to LDS-PAGE, and the separated components were transferred to a nitrocellulose filter and stained either for total proteins with amido black (lanes 1 to 7) or for ConA-reactive components (lanes 8 to 14). MW, Protein standards: bovine serum albumin (65 kDa), ovalbumin (45 kDa), α-chymotrypsinogen A (25 kDa), and cytochrome *c* (12 kDa).

a spatula and overlaid with the above solution containing 20% (vol/vol) glycerol; a protease solution in 0.125 M Tris (pH 6.8) plus 1% SDS, 0.001% (wt/vol) bromophenol blue, and 10% (vol/vol) glycerol was then layered over the samples in the individual wells. The amount of protein in each well was adjusted by adding or subtracting gel slices, and optimal loading of both protein and protease was determined empirically. Electrophoresis was carried out at 1.5 W until the material migrated about halfway through the stacking gel, and then power was shut off for 20 min to allow digestion of the stacked proteins by the costacked protease. Power was then reapplied, and electrophoresis was completed.

RESULTS

Chemical analysis of phycobilisome fractions. The properties of the ConA binding to the phycobilisome linker and anchor components strongly suggested the presence of similar, covalently bound carbohydrate structures on each of these molecules (28). To confirm these results more directly, we attempted to determine the identity and quantity of the constituent sugars present in the phycobilisome and to investigate the subunit distribution of these sugars. Purified phycobilisomes were biochemically separated into linker-enriched and linker-depleted fractions (23), and several of the ConA-reactive subunits were electrophoretically purified. The subunit composition and the ConA reactivity of these phycobilisome fractions are shown in Fig. 1. Purified phycobilisomes often contain a breakdown product of L_{CM}^{75} (termed $L_{CM}^{75'}$), which migrates at 60 kDa. $L_{CM}^{75'}$ was immunoreactive with antianchor antibody (data not shown) and was ConA reactive (Fig. 1). The linker-enriched fraction contained nearly all of the L_{CM}^{75}, $L_{CM}^{75'}$, L_{R}^{30}, and L_{RC}^{27} present in this phycobilisome preparation (lanes 2 and 9), whereas the cognate linker-depleted fraction contained most of L_{R}^{33} as well as virtually all of the biliproteins and the 52-kDa protein of unknown function (lanes 3 and 10). Electrophoretically isolated L_{CM}^{75}, $L_{CM}^{75'}$, L_{R}^{33}, and L_{RC}^{27} were all highly pure (lanes 4 to 7), and each retained ConA reactivity after electroelution from the preparative gel bands (lanes 11 to 14).

These samples were treated to remove salts, detergents, and dyes (39) and subjected to hydrolysis in 4 N HCl, and the released sugars were derivatized (24). Glucose (Glc) and *N*-acetylgalactosamine (GalNAc) had been positively identified in hydrolysates of total phycobilisome material (28); therefore, these two sugars were quantitated in the phycobilisome fractions (Table 1). Purified, whole phycobilisomes contained about 2.4% Glc by weight, which represents about 800 molecules of glucose per phycobilisome (assuming a molecular mass of 6 MDa per phycobilisome [14]). Sixty-five molecules of the amino sugar GalNAc were also present per phycobilisome, yielding a molar ratio of Glc to GalNAc of 12.5. The linker-enriched (NH_4SCN precipitate) fraction was analyzed as well, and the weight ratio of Glc to GalNAc indicated an approximately twofold enrichment of GalNAc in this fraction compared with total phycobilisomes. The L_{CM}^{75} anchor polypeptide contained the most Glc and GalNAc of the individual subunits, whereas the L_{CM}^{75} breakdown product $L_{CM}^{75'}$ contained the same amount of Glc but none of the GalNAc found in intact L_{CM}^{75}. Thus, all of the GalNAc was localized near the carboxy or amino terminus of L_{CM}^{75}.

L_{RC}^{27} contained Glc and GalNAc in about the same molar ratio as L_{CM}^{75} (3.7 versus 4.0), but contained only about 1/15th the molar quantity of each. By contrast, L_{R}^{33} possessed only Glc at approximately 16 molecules per subunit. Interestingly, the two subunits associated with the phycobilisome core substructure (L_{CM}^{75} and L_{RC}^{27}) both contained similar molar ratios of Glc to GalNAc, whereas the subunit associ-

TABLE 1. Carbohydrate content of *A. nidulans* R2 phycobilisome fractions

Fraction	% Glc (wt/wt)	% GalNAc (wt/wt)	Wt ratio (Glc/GalNAc)	Mol of Glc/mol of subunit[a]	Mol of GalNAc/mol of subunit[a]	Mol of Glc/mol of GalNAc
Total phycobilisome	2.44	0.24	10.17	812.4	65.2	12.5
NH_4SCN precipitate	4.20	0.72	5.83	—[b]	—	—
L_{CM}^{75}	14.40	4.40	3.27	59.9	14.9	4.0
$L_{C}^{75'}$	18.40	0	—	61.3	0	—
L_{R}^{33}	8.80	0	—	16.1	0	—
L_{RC}^{27}	2.45	0.79	3.10	3.7	1.0	3.7

[a] A phycobilisome molecular mass of 6.0 MDa was assumed in the calculations.
[b] —, Not determined.

ated with rods alone (L_R^{33}) contained only Glc. The ConA reactivity of these subunits was correlated with the presence of covalently linked carbohydrates (Table 1). It is extremely unlikely that non-covalently attached carbohydrates would have remained associated with the individual phycobilisome subunits after denaturing LDS-PAGE, electroelution in the presence of SDS, and several extractions with organic solvents. Since ConA is moderately reactive with branched, terminal, nonreducing α-D-glucopyranosyl residues (31), it is likely that such residues participate in the specific ConA binding observed.

Structural studies of ConA-reactive phycobilisome components. Previous investigations (13, 44) demonstrated that the anchor polypeptide of phycobilisomes from many species of cyanobacteria and red algae were immunologically related. Antibodies specific for this component cross-reacted with the analogous protein from other species but not with other phycobilisome components. Antibodies raised against the *Synechocystis* sp. strain PCC6714 anchor component displayed the same properties, reacting specifically with the anchor components of both *Synechocystis* sp. strain PCC6714 and *A. nidulans* R2 (data not shown). A structural analysis of the *A. nidulans* R2 anchor protein (L_{CM}^{75}) was undertaken to compare the immunoreactive regions of this molecule with ConA-reactive regions. The anchor protein was purified by preparative LDS-PAGE and digested with either chymotrypsin or pronase, and the digestion products were analyzed (Fig. 2). Amido black staining of the digestion products after electrophoresis and transfer to nitrocellulose (lanes 1 to 3, both panels) demonstrated that partial proteolysis occurred under these conditions and that a large number of digestion products were transferred to nitrocellulose in quantities sufficient for immunodetection or ConA detection. The postblot gels stained with Coomassie blue showed similar digestion patterns in each concentration series of the proteases, although slightly variable rates of digestion were seen for each particular well (due to slight variability in the protein content of the bands loaded into each well). ConA recognized L_{CM}^{75} fragments of ≥40 kDa (Fig. 2A and B, lanes 4 to 6). The abrupt cutoff of ConA-reactive bands at this size is striking, as is the consistency of this feature with both types of proteases. Very weakly ConA-reactive bands could sometimes be seen in the 20-kDa to 40-kDa range. Digestion products of L_{CM}^{75} immunodecorated with antianchor antibody displayed the same general features as the ConA-reactive fragments, except that fragments in the 20- to 40-kDa range were more immunoreactive than ConA reactive (Fig. 2, lanes 7 to 9, both panels). These results indicate that the strongly ConA-reactive region of L_{CM}^{75} is localized to less than half of the molecule and that this part of L_{CM}^{75} corresponds to the region most strongly immunoreactive with a heterologous, monospecific antibody. Since carbohydrate structures are often highly immunogenic, the ConA-binding sites may in fact represent parts of the binding sites for these antibodies.

Similar proteolysis experiments were carried out with the L_R^{33}, L_R^{30}, and L_{RC}^{27} linkers to attempt to localize ConA-reactive regions on these molecules. Figure 3 is representative of the results found with both L_R^{33} and L_{RC}^{27}. None of the proteolytic fragments of L_R^{33} which were separated by LDS-PAGE and transferred to nitrocellulose (Fig. 3, lanes 1 to 3) were reactive with ConA (lanes 4 to 6). The existence of a 28-kDa proteolytic fragment (lane 1) which was not ConA reactive (lane 4) indicates that the ConA-reactive region of L_R^{33} is near either the carboxy or the amino terminus of the polypeptide. The postblot gel showed an

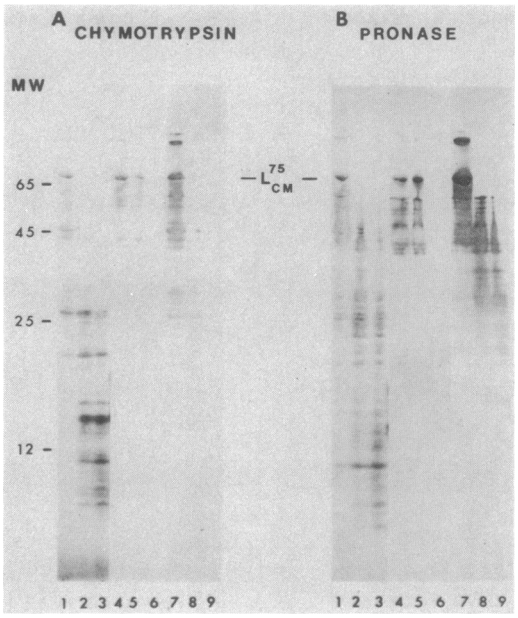

FIG. 2. Proteolytic mapping of ConA-reactive sites and antigenic sites on the *A. nidulans* R2 anchor protein L_{CM}^{75}. Gel-purified L_{CM}^{75} was treated with the proteases chymotrypsin (A) or pronase (B), and the digestion products were separated by LDS-PAGE. Separated components were transferred to nitrocellulose filters and stained with amido black to detect total transferred protein (lanes 1 to 3, both panels), with ConA to detect carbohydrate-containing fragments (lanes 4 to 6, both panels), and with monospecific antiserum reactive with L_{CM}^{75} to detect antigenic proteolytic fragments (lanes 7 to 9, both panels). The antiserum was generated against L_{CM}^{95} of *Synechocystis* sp. strain PCC6714 and exhibited specific cross-reactivity with L_{CM}^{75} of *A. nidulans* R2. (A) Either 0.1 μg (lanes 1, 4, and 7), 1.0 μg (lanes 2, 5, and 8), or 10 μg (lanes 3, 6, and 9) of chymotrypsin was added to the respective sample-containing wells of the gel. (B) Either 0.001 μg (lanes 1, 4, and 7), 0.01 μg (lanes 2, 5, and 8), or 0.1 μg (lanes 3, 6, and 9) of pronase was added to the respective sample-containing wells of the gel. MW, Protein standards (same as Fig. 1).

identical digestion pattern in both lanes 1 to 3 and lanes 4 to 6 (data not shown). All L_{RC}^{27} proteolytic fragments also lacked ConA reactivity, including a 24-kDa chymotrypsin fragment. By contrast, an L_R^{30} proteolytic fragment migrating at 15 kDa did retain the ability to bind ConA (data not shown). These results indicate a preferential localization of ConA-binding sites near the end(s) of L_R^{33} and L_{RC}^{27} but not necessarily L_R^{30}.

ConA reactivity of *Synechocystis* sp. strain PCC6714 phycobilisomes. The possibility that the ConA reactivity exhibited by the *A. nidulans* R2 anchor and linker polypeptides was limited only to this species of cyanobacteria was assessed (Fig. 4). Phycobilisomes were isolated from *Synechocystis* sp. strain PCC6714, a facultatively heterotrophic cyanobacterium which possesses phycobilisomes with a morphology significantly different from that of phycobilisomes found in *A. nidulans* R2 (14). An obvious biochemical difference between these phycobilisomes is that the anchor component of *Synechocystis* sp. strain PCC6714 migrates at 95 instead of 75 kDa (14). The purified phycobilisomes from this species were fractionated by LDS-PAGE, transferred to nitrocellulose, and stained either for total protein (Fig. 4, lane 1) or for ConA-reactive glycoproteins (lane 2). The linker and anchor components of phycobilisomes from this organism specifically bound ConA (Fig. 4, lane 2), suggest-

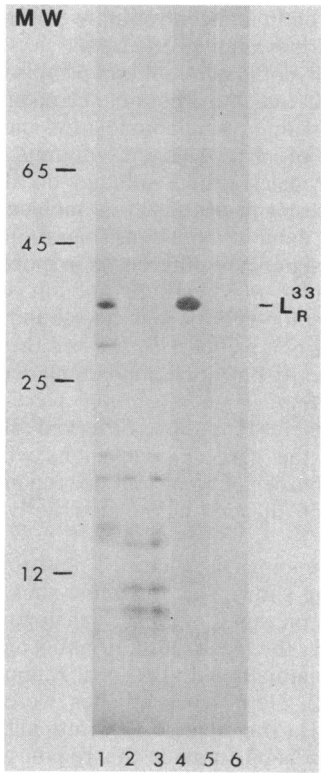

FIG. 3. Proteolytic mapping of ConA-reactive sites on L_R^{33}. Gel-purified L_R^{33} was treated with pronase, and the digestion products were separated by LDS-PAGE. The electrophoretically separated components were transferred to a nitrocellulose filter, and the filter was stained either for transferred protein with amido black (lanes 1 to 3) or for carbohydrate-containing proteolytic fragments with ConA (lanes 4 to 6). A total of 0.01 μg (lanes 1 and 4), 0.1 μg (lanes 2 and 5), or 1.0 μg (lanes 3 and 6) of pronase was added to the sample-containing wells of the gel. MW, Protein standards (same as Fig. 1).

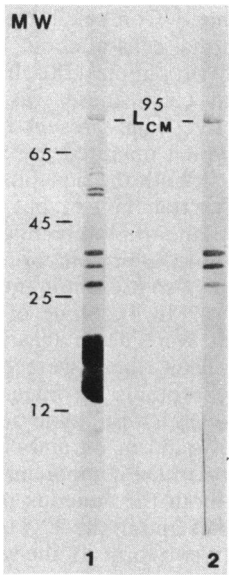

FIG. 4. ConA-reactive subunits of *Synechocystis* sp. strain PCC6714 phycobilisomes. Purified phycobilisomes from the cyanobacterium *Synechocystis* sp. strain PCC6714 were subjected to LDS-PAGE, and the separated subunits were transferred to nitrocellulose and stained for either total transferred proteins with amido black (lane 1) or for ConA-reactive components (lane 2). L_{CM}^{95} is the anchor polypeptide. MW, Protein standards (same as Fig. 1).

ing that carbohydrate-bearing structural components of these glycoproteins are similar in *Synechocystis* sp. strain PCC6714 and *A. nidulans* R2.

Glycoprotein composition of membranes from iron-stressed *A. nidulans* R2. The results described above indicated that the linker and anchor phycobilisome subunits were glucose-containing glycoproteins (Table 1). Since iron-stressed cells are depleted in phycobilisomes (with glycogen granules accumulating in the intermembrane spaces formerly occupied by phycobilisomes [32]), it was of interest to determine the complement of membrane-associated glycoproteins from iron-stressed cells and to compare them with membrane glycoproteins from normal cells.

Membranes from iron-stressed cells were separated into hydrophobic, hydrophilic, and insoluble fractions by phase partitioning (see Materials and Methods). Purified phycobilisomes and membranes from normally grown cells were included in this experiment for comparison. The sample components were separated by LDS-PAGE, and a protein blot of the gel was stained for ConA-reactive glycoproteins. Some of the phycobilisome material dissociated from membranes during isolation of membranes from normal cells, leaving relatively weak but still visible ConA-reactive bands which corresponded to the aforementioned phycobilisome anchor and linker subunits (Fig. 5, compare lanes 1 and 2). In

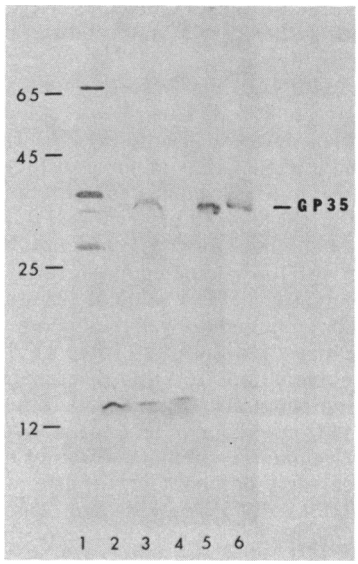

FIG. 5. Glycoprotein composition of iron-stressed membranes from *A. nidulans* R2. Sample components were separated by LDS-PAGE and then transferred to nitrocellulose and stained for ConA-reactive glycoproteins. Membranes from iron-deficient cells (lane 3) were separated into hydrophobic (lane 4), hydrophilic (lane 5), and detergent-insoluble pellet (lane 6) fractions by TX114 phase partitioning prior to electrophoresis. Lane 2 is a sample of membranes from normally grown cells, and lane 1 contains purified phycobilisomes for comparison. Lanes 2 to 6 were solubilized on ice prior to electrophoresis, whereas lane 1 was solubilized at 70°C for 5 min. Protein standards (in kilodaltons) are indicated at the left.

addition, a very strongly ConA-reactive band was found in membranes from normal cells at about 13 kDa; this protein was shown to be hydrophobic (28). In contrast to membranes from normal cells, there was a very abundant, strongly ConA-reactive band present in membranes from iron-stressed cells which migrated at 35 kDa (lane 3); this protein was present in both the aqueous phase (lane 5) and the insoluble pellet fraction (lane 6), but was absent from the hydrophobic phase (lane 4). Interestingly, the ConA-reactive, hydrophobic 13-kDa protein present in membranes from normal cells was also present in membranes from iron-deficient cells (lanes 2 to 4). None of the ConA-reactive phycobilisome bands were detectable in membranes from iron-stressed cells. Thus, there were only two detectable ConA-reactive glycoproteins in membranes from iron-stressed cells: a 13-kDa hydrophobic component (probably identical to the one found in normal cells), and a second, extremely abundant 35-kDa component (designated GP35). GP35 was found in both the aqueous phase of the TX114-partitioned membranes and in the TX114-insoluble fraction, possibly indicating (i) two forms of the same protein, (ii) two populations of the same protein having distinct associations with other macromolecular components of the cell, or (iii) (least likely) two distinct, unrelated, comigrating glycoproteins.

DISCUSSION

We have demonstrated directly the presence of significant quantities of carbohydrates on phycobilisome linker and anchor polypeptides that were purified to homogeneity under strongly denaturing conditions. Glc was present on each of these proteins and probably accounts for the ConA-binding properties of these subunits. The ConA-reactive regions of several of these molecules were localized to regions previously shown to be important in phycobilisome assembly and function.

The Glc and GalNAc distribution among the phycobilisome fractions we analyzed (Table 1) was consistent with the ConA reactivities of these fractions (Fig. 1). Particularly striking was the intense ConA staining of the small amount of L_{CM}^{75} (and $L_{CM}^{75'}$) in Fig. 1, consistent with the large amount of Glc found on these subunits (Table 1). The estimated stoichiometry of subunits for the *A. nidulans* phycobilisome (20), combined with the calculated molar ratios of Glc for the subunits we analyzed, yields a value of 225 molecules of Glc per phycobilisome, a value well below the 812 Glc per phycobilisome measured directly (Table 1). There are several possible explanations for this discrepancy. (i) Glc is present in large amounts on subunits other than those analyzed. L_R^{30} is ConA reactive and probably contains some Glc. The other remaining subunits were not ConA reactive but could conceivably contain Glc. (ii) The molecular mass of the *A. nidulans* R2 phycobilisome was not 6 MDa. Estimates ranging from 4.5 to 6.5 MDa have been reported, yielding a range of molar ratios of Glc per phycobilisome from about 600 to 880. (iii) Purified phycobilisomes were contaminated with cosedimenting glycogen granules. Originally considered highly unlikely, the recent evidence suggesting the presence of glycogen granules on the external surface of thylakoids in *Anabaena variabilis* (11) makes this a possibility. Glycogen contamination of the purified phycobilisome fraction would have led to an overestimate of Glc in the phycobilisome while not affecting the carbohydrate analysis of the purified subunits. A comparison of the two quantities would thus lead to the discrepancies seen. (iv) Estimates of protein concentration were inaccurate. We used densitometric scanning of Coomassie-stained gels (1, 20) to estimate phycobilisome subunit protein concentration. Lundell et al. (22) indicate a possible error of ±10% by this method, based on amino acid composition and the estimated molecular mass of phycobilisome subunits. (v) The estimated molecular mass of these subunits on SDS-PAGE was significantly different from the actual molecular mass. Glycoproteins often exhibit anomalous migration on polyacrylamide gels, and significant differences in mobility relative to the true molecular mass of the subunit will affect the calculated molar ratio of both sugar to subunit and subunit to phycobilisome. Lomax et al. (19) showed that the predicted molecular masses (from nucleotide sequence data) of two linkers from *Fremyella diplosiphon* were 17 and 19% smaller, respectively, than those observed after electrophoresis. Although the general conclusions will not change, these possible sources of error and discrepancies should be kept in mind when interpreting the quantitative data in Table 1.

Previous immunological studies of phycobilisome anchor components have shown that antigenic sites are conserved between species on this functionally analogous subunit (13, 44). Interestingly, the localization of antigenic determinants overlapped substantially with the localization of ConA sites on L_{CM}^{75} (Fig. 2). Both types of sites were preferentially found on a 30-kDa fragment of L_{CM}^{75} although the antibody also reacted with bands outside this region which were not reactive with ConA. Since the antibody used in this study was raised against the *Synechocystis* sp. strain PCC6714 anchor, the immunologically reactive regions of L_{CM}^{75} represent structurally conserved regions of this phycobilisome component. It is therefore significant that most of the structurally conserved regions of L_{CM}^{75} correspond to the region which is glycosylated; this highlights the probable importance of the Glc-containing sites in the function of the anchor polypeptide.

The ConA-reactive sites on phycobilisome anchor and linker subunits are not limited to *A. nidulans* R2. *Synechocystis* sp. strain PCC6714 phycobilisomes, which are morphologically and biochemically different from those found in *A. nidulans* R2 (14), nevertheless retained the ConA-binding sites on the analogous anchor and linker subunits (Fig. 4). The presence of carbohydrates on the analogous phycobilisome subunits of two evolutionarily disparate cyanobacterial species indicates a role for the carbohydrates in one or more of the functional aspects associated with phycobilisome anchor and linker polypeptides.

Glycopeptides have not been reported in the limited linker protein sequencing work published to date (12). However, the partial amino acid sequences determined for the *Mastigocladus laminosus* $L_R^{34.5,PC}$ and $L_R^{34.5,PEC}$ linkers include a number of ambiguous residues identified only by amino acid analysis (rather than by direct Edman degradation). The hydrolysis conditions required for amino acid analysis would liberate covalently linked carbohydrates from these residues. Among the ambiguous residues are several serines and threonines, residues capable of mediating O-linkages to carbohydrates. A comparison of the *M. laminosus* partial linker sequence data (12) with the predicted amino acid sequences of linker proteins from other species (generated from nucleotide sequence analyses of cloned genes [2, 7, 19]) indicates several evolutionarily conserved regions among these polypeptides. The carboxyl-terminal region of these linker proteins is highly conserved, and this region has been

proposed to function in hexamer assembly in *M. laminosus* (12).

ConA-binding sites were preferentially localized near the termini of two of the linkers found in the rod substructures of *A. nidulans* phycobilisomes (Fig. 3). In the case of L_R^{33}, a 28-kDa pronase fragment was no longer ConA reactive (Fig. 3), whereas in the case of L_{RC}^{27}, a 24-kDa chymotrypsin fragment lacked ConA reactivity (data not shown). Using trypsin proteolysis of 18S core particles from *Synechococcus* sp. strain 6301 and phycobilisomes from the mutant strain AN112, Lundell and Glazer (20, 21) showed that cleavage of L_{RC}^{27} from 27 to 25 kDa correlated with dissociation of the 18S core particle and that cleavage of L_{RC}^{27} to 22 kDa released an intact $(\alpha^{PC}\beta^{PC})_6$. 22-kDa hexameric phycocyanin complex from the AN112 phycobilisome core. In limited trypsin digestions of reconstituted phycocyanin-linker polypeptide complexes from *Synechococcus* sp. strain 6301, Lundell et al. (22) found that 23 kDa of L_{RC}^{27} and 22 to 28 kDa of L_R^{33} were protected from trypsin by their interactions with phycocyanin, whereas L_R^{30} was degraded to fragments of less than 20 kDa. In *Nostoc* spp., cleavage of 6 kDa from a 29-kDa linker prevented attachment of the phycobilisome core to rods, although phycoerythrin and phycocyanin could still assemble into otherwise normal rods (43). The X-ray crystallographic structure of C-phycocyanin and the proteolysis experiments of Yu and Glazer (42) led Schirmer et al. (30) to suggest that most of both L_{RC}^{27} and L_R^{33} are buried in the central channel of the phycocyanin trimer, leaving only a small region of each exposed to protease (and, presumably, available for interaction with adjacent substructures). Füglistaller et al. (12) showed that the C-terminal ends of two rod linkers from *M. laminosus* were sensitive to proteases and that proteolysis of 5 kDa from the C-terminus of these linkers led to dissociation of hexamer biliprotein aggregates. Thus, the ends of L_{RC}^{27} and L_R^{33} (particularly the C-terminal end) appear to be crucial for the direction of biliprotein assembly by these linker components. The presence of carbohydrates at the end(s) of these molecules, exposed to possible enzyme action, implicates glycosylation and deglycosylation as possible mechanisms for the regulation of phycobilisome assembly and degradation.

The degradation of phycobilisomes is a common response of cyanobacterial cells to stress conditions (32, 34, 35, 38). In at least several types of nutrient stresses (including iron stress), the degradation of phycobilisomes is accompanied by the deposition of large amounts of glycogen granules on the thylakoid surface (32, 35, 38). The results shown in Fig. 5 demonstrate that an extrinsic membrane glycoprotein (GP35) accumulates in iron-stressed *A. nidulans* cells. In related experiments, we have found that GP35 is the single most abundant membrane-associated protein in iron-stressed cells. It may be identical to the 34-kDa protein found in the "heavy" sucrose density gradient fraction by Guikema and Sherman (16). GP35 has been purified to homogeneity (Riethman and Sherman, unpublished data), and future analysis of its structure should prove to be very interesting. This protein is very basic, extrinsic, and ConA reactive; all of these properties are shared with the phycobilisome linker and anchor proteins. Its migration is very dependent on gel conditions and the protein load; in some cases GP35 formed aggregates of high molecular mass, and its "normal" migration on LDS-PAGE was anywhere from 36 to 30 kDa. GP35 was present in the TX114-insoluble fraction of membranes as well as in the aqueous fraction (Fig. 5); this may be explained by either simple aggregation of GP35 into an insoluble form or its possible association with other cellular components, particularly glycogen.

A. nidulans cells subjected to iron stress accumulate a stress-induced extrinsic membrane glycoprotein in very large quantities. Since iron-starved cells lack assembled phycobilisomes and accumulate very large amounts of carbohydrates in the form of glycogen deposits on the thylakoid surface, we speculate that the iron stress-induced glycoprotein is involved in glycogen granule formation. Additionally, we speculate that the glycosylated regions of the phycobilisome linker and anchor proteins are key regulatory sites for phycobilisome assembly and degradation in response to iron stress. Further analyses of phycobilisome components and GP35 during iron stress and upon recovery from iron stress are required to test these contentions.

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