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Phycobilisome-associated glycoproteins in the cyanobacterium *Anacystis nidulans* R 2

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Four phycobilisome-associated proteins were found to be specifically reactive with the lectin concanavalin A after subunits of isolated *Anacystis nidulans* R 2 phycobilisomes were separated on polyacrylamide gels and transferred onto nitrocellulose. The concanavalin A-reactive phycobilisome components have proposed functions related to the orientation, assembly, and membrane attachment of the phycobilisome. Chemical analysis of the total isolated phycobilisome material indicated the presence of glucose (approx. 1.5% by wt) and *N*-acetylgalactosamine (0.15% by wt), consistent with our proposal that the concanavalin A-reactive polypeptides contain covalently linked, glucose-containing polysaccharides.

Phycobilisome; Glycoprotein; Concanavalin A; Cyanobacteria; (*Anacystis nidulans* R 2)

1. INTRODUCTION

Cyanobacterial PS II receives most of its light energy through an extrinsic, multisubunit pigment-protein complex, the phycobilisome. Most of the phycobilin pigments are covalently linked to several low-molecular-mass polypeptide subunits (14–24 kDa), which together comprise approx. 85% of the mass of the phycobilisome [1,2]. The remaining components of the phycobilisome are mainly non-pigmented, proteinaceous molecules known to be important in the association of the pigmented subunits into rods and discs (e.g. the 33, 30 and 27 kDa 'linkers') and in the attachment of the assembled phycobilisome to the thylakoid membrane (e.g. the 71 kDa 'anchor' polypeptide) [1,3,4]. The anchor polypeptide contains an attached bilin, and has been implicated as the functional link between the phycobilisome and PS II [5].

Although thylakoid membranes have generally been considered to lack glycoproteins [6], there have been conflicting reports concerning the association of sugars with specific thylakoid proteins [7,8]. Most of these studies have dealt only with thylakoids from higher plant chloroplasts, which, although remarkably similar in many respects to cyanobacterial thylakoids, differ significantly with respect to light-harvesting structures and membrane stacking. Here, we use the sensitive glycoprotein-staining technique of Clegg [9] to show that there are only a few Con A-reactive components associated with *Anacystis nidulans* R 2 membranes, and that four of these Con A-reactive components correspond to phycobilisome linker and anchor subunits. To our knowledge, this is the first report of cyanobacterial membrane-associated glycoproteins; the large

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Abbreviations: Con A, concanavalin A; FITC, fluorescein isothiocyanate; HRP, horseradish peroxidase; LDS, lithium dodecyl sulfate; α-methyl mannoside, methyl-α-D-mannopyranoside; PAGE, polyacrylamide gel electrophoresis; PS, photosystem

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amount of glucose associated with the phycobilisomes as well as the recognition of a conserved, polysaccharide-containing region on each of the functionally related linker and anchor subunits may have important implications with regard to phycobilisome energy transduction, its association with PS II, and its metabolism in response to various stress conditions.

2. MATERIALS AND METHODS

A. nidulans R 2 was grown in BG11 medium with constant illumination and aeration in 15 l carboys as described [10]. Membranes were prepared by differential centrifugation after disruption of cells by French press treatment [11]. Intact phycobilisomes were prepared by the procedure of Yamanaka et al. [2], and were dialyzed extensively against 10 mM Tris (pH 7.0) prior to chemical analysis. Phycocyanin and protein concentrations were determined as in [12,13]. Triton X-114 phase fractionation of *A. nidulans* R 2 membranes, electrophoresis, and immunoblotting procedures were also performed as described previously [11,14].

The glycoprotein detection procedure of Clegg [9] was followed, except that 0.5% (v/v) Tween 20 was included in all staining and washing solutions and *o*-dianisidine was used as the chromogenic substrate. A modification of the Clegg procedure was also used, in which FITC-conjugated Con A was used to probe the nitrocellulose filters directly. The FITC-stained bands were subsequently visualized by their green fluorescence upon illumination by long-wavelength ultraviolet light.

Carbohydrate analysis was performed on the phycobilisomes, following their hydrolysis in 0.2 ml of 4.0 N HCl for 4 h under nitrogen atmosphere, both as their per-*O*-trimethylsilyl *O*-methoxime derivatives and as their alditol acetates as described in [15]. All carbohydrate standards were obtained from Pfanstiehl Laboratories (Waukegan, IL).

3. RESULTS

The initial characterization of Con A-reactive *A. nidulans* R 2 membrane components is shown in fig.1. Total membrane material (T) was separated into hydrophobic detergent (D), hydrophilic aqueous (A), and detergent-insoluble

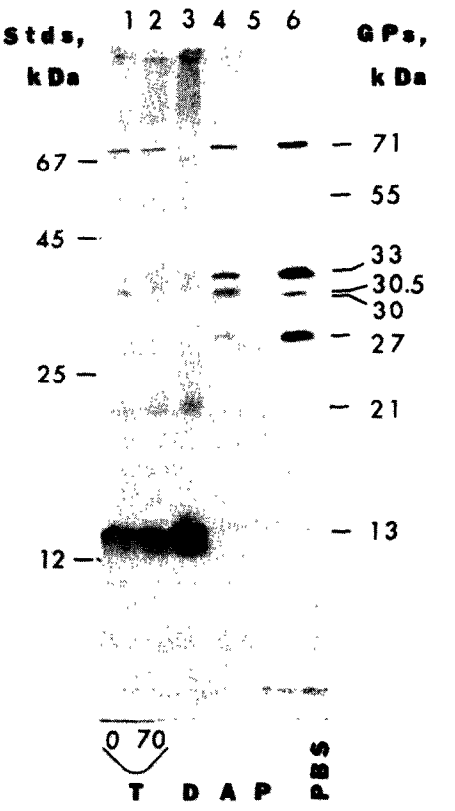

Fig.1. Con A-reactive membrane components from *A. nidulans* R 2. The material in each lane was separated on LDS-PAGE, transferred to a nitrocellulose filter, and stained for Con A-reactive components. Lanes: 1,2, total membrane material (T) solubilized in sample buffer at 0 and 70°C, respectively; 3,4,5, detergent (D), aqueous (A), and Triton X-114-insoluble pellet (P) fractions after phase partitioning, respectively; 6, purified phycobilisomes.

pellet (P) fractions using Triton X-114 phase partitioning [14]. Each fraction was separated on LDS-PAGE and the components were transferred onto a nitrocellulose filter; the filter was then stained for Con A-reactive glycoproteins as described in section 2. The most prominent Con A-reactive band detected in total membranes migrated at 13 kDa; a second, more diffuse band migrated at 21 kDa. Both of these bands represent hydrophobic molecules, since they partition exclusively into the detergent phase (fig.1, lane 3). Prominent aqueous phase components migrated at 71, 33, 30.5 and 27 kDa, whereas weakly reactive

aqueous components could be detected at 55 and 30 kDa (fig.1, lane 4). The detergent-insoluble pellet fraction (fig.1, lane 5) contained no Con A-reactive species. All of the Con A-reactive bands in both the detergent and the aqueous phases could be detected in total membranes, and the reactivity was similar whether the membranes were solubilized at 0 or 70°C prior to electrophoresis. However, solubilization of membranes at 70°C somewhat obscured the staining pattern in the 30–33 kDa range (fig.1, lane 2). Four of the aqueous phase, Con A-reactive components corresponded to Con A-reactive bands from isolated phycobilisomes (fig.1, lane 6).

The Con A-reactive phycobilisome components were characterized further by subjecting purified phycobilisomes to treatments designed to release loosely bound material. The boiling of phycobilisome samples for 5 min in sample buffer containing LDS and 2-mercaptoethanol (fig.2A, lanes

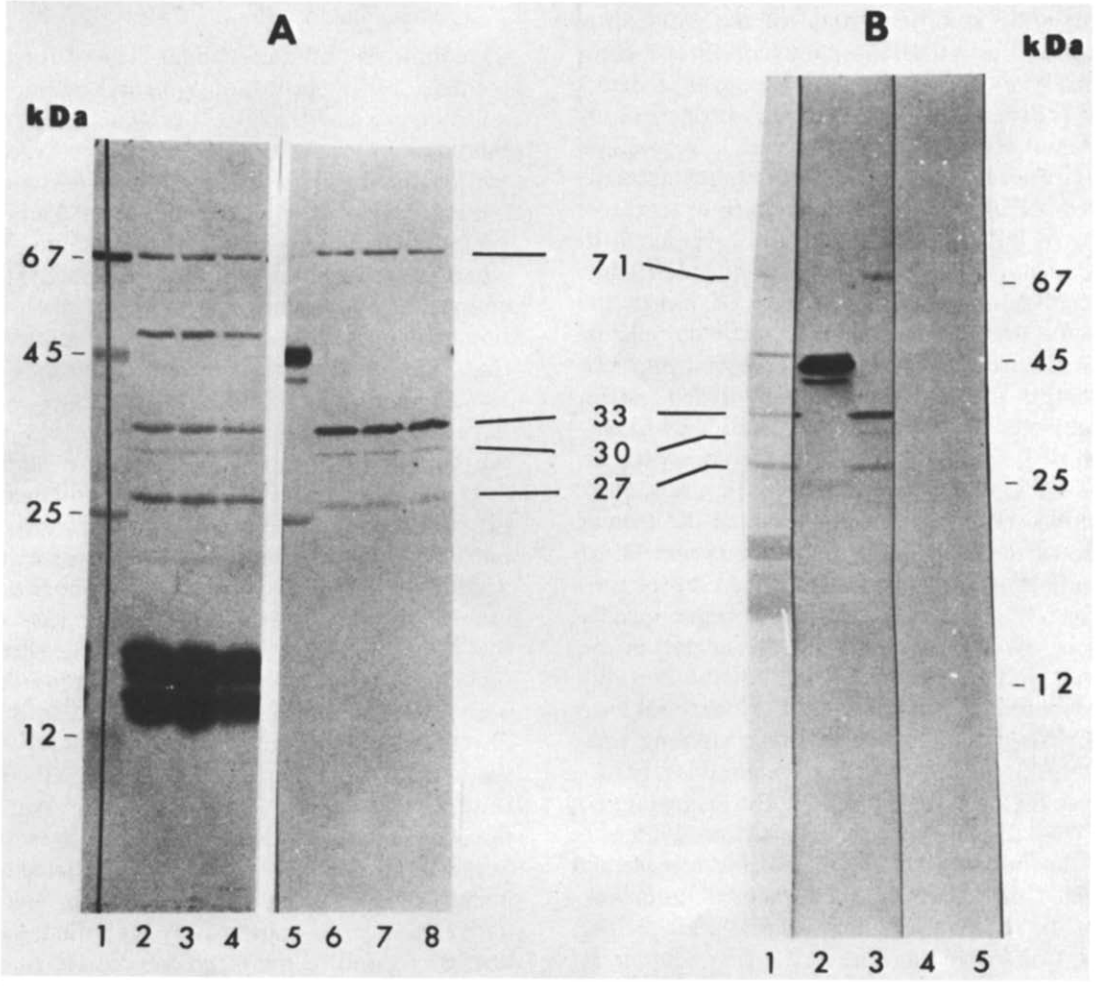

Fig.2. Specific staining of phycobilisome linker and anchor subunits with Con A. (A) Purified phycobilisomes were subjected to different denaturing conditions prior to electrophoresis, transferred to nitrocellulose, and then stained for either total protein using amido black (lanes 1–4) or Con A-reactive components (lanes 5–8). Lanes: 1,5, standard proteins – bovine serum albumin (65 kDa), ovalbumin (45 kDa), chymotrypsinogen A (25 kDa) and cytochrome *c* (12 kDa); 2–4, 6–8, isolated phycobilisomes that were heated in sample buffer at 70°C for 5 min (lanes 2,5), boiled in sample buffer for 5 min (lanes 3,6), or treated with 4:1 chloroform/methanol (lanes 4,8). (B) Standard proteins and phycobilisomes were electrophoresed and transferred to nitrocellulose, then stained for Con A-reactive components in the absence (lanes 2,3) or presence (lanes 4,5) of α-methyl mannoside. Lanes: 1, stained with amido black; 1,3,5, purified phycobilisomes; 2,4, standard proteins.

3,7), or the treatment of phycobilisomes with chloroform/methanol (4:1) prior to solubilization and electrophoresis (fig.2A, lanes 4,6) had little or no effect on either the total subunit composition of the phycobilisome material (fig.2A, lanes 1–4) or on the Con A reactivity of the subunits (fig.2A, lanes 5–8). The species responsible for Con A binding are thus very tightly associated with, and probably covalently linked to, the respective subunits yielding positive Con A staining.

Control experiments yielded further information regarding the specificity and strength of Con A binding to the immobilized phycobilisome components. The Clegg method of glycoprotein detection [9] depends on the binding properties of Con A and HRP as well as enzymatic peroxidase activity. Therefore, it was possible that the bands detected were not due to Con A, but to direct binding of HRP to immobilized components or to an endogenous peroxidase activity of specific immobilized components. Omission of either the Con A or the HRP incubation steps completely abolished chromogenic substrate deposition, confirming that the activity was totally dependent on the specificity of Con A binding (not shown). Inclusion of α-methyl mannoside (a competitive inhibitor of Con A sugar binding) in the Con A incubation solution abolished most of the Con A binding to the phycobilisome subunits and all of the binding to ovalbumin, a positive control protein (fig.2B). This indicated that the sugar-specific region of the Con A molecule participated in the observed binding. The binding affinity of Con A is dependent on pH and salt conditions, and when we varied these parameters during the staining procedure (using either the Clegg method or FITC-conjugated Con A), we observed the following: (i) at pH 7.8, the Con A binding was most intense, and inclusion of α-methyl mannoside completely inhibited Con A binding to both ovalbumin and each of the reactive phycobilisome subunits; (ii) at pH 6.8, Con A binding was very strong, and inclusion of α-methyl mannoside completely abrogated Con A binding to ovalbumin. However, Con A binding to each of the reactive phycobilisome subunits in the presence of α-methyl mannoside was still approx. 25–50% that of the pH 6.8 control; and (iii) the addition of 1 M NaCl to the normal buffer (pH 7.4) decreased Con A binding to both ovalbumin (slightly) and each of the reactive phycobilisome subunits (about 50% of the pH 7.4 control), but inclusion of α-methyl mannoside in addition to 1 M NaCl abrogated all Con A binding. In each of these experiments, the α-methyl mannoside inhibition of Con A-staining intensity affected the reactive phycobilisome subunits by approximately the same factor, whereas the staining intensity of the ovalbumin control was consistently more sensitive to inhibition by α-methyl mannoside. These results suggest that the reactive phycobilisome subunits share similar binding sites for Con A, and that these sites have properties which differ from the site present on the positive control glycoprotein, ovalbumin.

Table 1

Carbohydrate content of purified *A. nidulans* R 2 phycobilisomes

Sugar	μg sugar/ mg protein	% sugar (w/w)	μg sugar/ mg phycocyanin
Glucose	14.7	1.47	26.6
N-Acetyl-galactosamine	1.43	0.14	2.5

To obtain an estimate of the types and quantities of sugars associated with the phycobilisomes, purified phycobilisomes were dialyzed extensively against 10 mM Tris (pH 7.4) to remove loosely bound material, hydrolyzed, and then assayed for the presence of sugar monomers. As shown in table 1, significant quantities of glucose and *N*-acetylgalactosamine could be detected in purified phycobilisome material. Since Con A has a moderate binding affinity for terminal glucosyl residues, it is likely that the Con A-reactive phycobilisome bands contain such residues. Several additional sugars were detected in hydrolysates of phycobilisome material, but these have not as yet been positively identified; they do not correspond to the more common sugars, and might be specifically modified hexoses.

4. DISCUSSION

We have used a combination of biochemical and chemical procedures to demonstrate that four phycobilisome-associated proteins are specifically glycosylated. Of the greater than 90 discrete

polypeptides discernible in isolated *A. nidulans* R 2 membranes [16], no more than eight are specifically reactive with the lectin Con A (fig.1), and four of these Con A-reactive components are the functionally related linker and anchor phycobilisome subunits. The Con A reactivity of these subunits is not appreciably affected by treatments which normally release non-covalently bound ligands (fig.2A).

The mechanisms by which the linker and anchor subunits orient and assemble the biliproteins into an efficient PS II light-harvesting structure are uncertain. The finding that these proteins are specifically glycosylated suggests a role for carbohydrates in these mechanisms. Since the Con A-binding affinity of each of these glycoproteins was affected by salt, pH, and the presence of α-methyl mannoside in very similar fashions, the chemical structures and microenvironments of the polysaccharide(s) responsible for the Con A interaction with the respective subunits must be very similar (or identical) [17]. Interestingly, the analogous phycobilisome subunits of *Synechocystis* PCC6714 are also Con A reactive (Riethman and Sherman, unpublished), even though the *Synechocystis* PCC6714 anchor polypeptide migrates at 95 kDa instead of 71 kDa. The polysaccharide structure and microenvironment recognized by Con A may be a specific feature of phycobilisome linker and anchor components, and could be crucial to the related functional properties of these subunits. This hypothesis will be testable from a variety of perspectives, including genetic and mutational analyses as well as biochemical/reconstitution studies.

When one notes the relative quantity of sugars (particularly glucose) associated with purified phycobilisomes (table 1) and the abundance of phycobilisomes in a normal cell, it becomes apparent that this light-harvesting structure is capable of storing significant energy reserves in the form of glucose or glucose-containing polysaccharides. This may be relevant to the observation that, under a variety of stress conditions (including iron, nitrogen, and phosphate stress), the phycobilisomes are rapidly degraded [18–20]. In the case of iron or nitrogen stress, the disappearance of phycobilisomes correlates with the appearance of intracellular polysaccharides containing large amounts of glucose [18,19]. This raises the possibility of an adaptive physiological switch involving phycobilisome degradation coupled to polysaccharide synthesis, and/or phycobilisome assembly coupled to polysaccharide degradation.

In summary, the *A. nidulans* R 2 phycobilisome subunits known to function in pigment orientation, phycobilisome assembly, and membrane attachment each share a very similar affinity for Con A. The presence of significant quantities of sugars (particularly glucose) in hydrolyzed samples of purified phycobilisomes leads us to conclude that the Con A-reactive polypeptides contain covalently linked polysaccharides composed largely of glucose.

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