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THE ELECTROPHORETIC ISOLATION AND PARTIAL CHARACTERIZATION OF THREE CHLOROPHYLL-PROTEIN COMPLEXES FROM BLUE-GREEN ALGAE

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Summary

Three chlorophyll-protein complexes have been resolved from blue-green algae using an improved procedure for membrane solubilization and electrophoretic fractionation. One complex has a red absorbance maximum of 676 nm and a molecular weight equivalency of 255 000 ± 15 000. A second complex has an absorbance maximum of 676 nm, a molecular weight equivalency of 118 000 ± 8000, and resembles the previously described *P*-700-chlorophyll *a*-protein (CPI) of higher plants and algae. The third chlorophyll-protein has a red absorbance maximum of 671 nm and a molecular weight equivalency of 58 000 ± 5000. Blue-green algal membrane fractions enriched in Photosystem I and heterocyst cells do not contain this third chlorophyll-protein, whereas Photosystem II-enriched membrane fractions and vegetative cells do. A component of the same spectral characteristics and molecular weight equivalency was also observed in chlorophyll *b*-deficient mutants of barley and maize. It is hypothesized that this third complex is involved in some manner with Photosystem II.

Introduction

The function and composition of the photosynthetic apparatus of prokaryotic blue-green algae closely resembles that of higher plants [1]. Both con-

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Abbreviation: CPI, *P*-700 chlorophyll *a*-protein.

tain two photosystems acting in series and have essentially the same photosynthetic electron transport components. Structurally blue-green algae have a simple lamellar system in contrast to the stacked thylakoid system of higher plant chloroplasts. Both classes of organisms contain chlorophyll *a*; carotenoids and phycobiliproteins function as accessory pigments in blue-green algae whereas carotenoids and chlorophyll *b* serve the equivalent light-harvesting function in higher plants. The phycobiliproteins are easily extracted from the algal membranes with aqueous solvents. The aqueously washed membranes are a simpler system than the thylakoid membranes of higher plants to study the molecular organization of photosynthetic pigments in membranes since they contain only chlorophyll *a* and carotenoids.

Much of our present knowledge of such organization has come from the analysis of material yielded by chromatographic and/or electrophoretic fractionation of extracts of detergent-solubilized membranes. Green plants have been extensively studied in this manner but less has been done with the algal classes. In the case of the blue-green algae one chlorophyll-protein complex, CPI or the *P*-700-chlorophyll *a*-protein, has been isolated and characterized [2]. This complex accounts for only 30% of the total chlorophyll, contains the reaction center of Photosystem I, *P*-700, has a molecular weight equivalency of 110 000, and is thought to be ubiquitous in algae and higher plants. The remaining chlorophyll (70%) is no longer associated with protein after electrophoretic fractionation. Recently, milder procedures for the solubilization and fractionation of higher plant chloroplast membranes have resulted in the resolution of previously unobserved chlorophyll-protein complexes [3—7] and in a substantial reduction in the amount of chlorophyll not complexed with protein after fractionation [5—7]. It was therefore timely to reinvestigate the chlorophyll-protein content of the simpler blue-green algal membranes using these new procedures to determine if more could be learned about the organization of chlorophyll *a* and carotenoids in vivo.

This paper reports the resolution from the blue-green algal membranes of three chlorophyll-protein complexes, one of which appears to be analogous to the previously observed CPI. A second complex is spectrally similar to CPI but of a much higher molecular weight (cf. Ref. 21). The third complex closely resembles in size and spectrum the higher plant pigment-protein believed to be associated with Photosystem II [3,5,6,8,9].

Methods

Algal strains and culture method. Phormidium luridum var. *olivaceae* UTEX 426 and *Anabaena cylindrica* Lemmerman UTEX 377 cells were grown axenically in large scale cultures. The nutrient solution of Allen and Arnon [10] was supplemented with a trace element solution [11] but with twice the concentration of chelated iron (Chapman, D.J., personal communication). The cultures, grown at 22—25°C, were kept in suspension by magnetic stirring and vigorous aeration, and continuously illuminated with 'cool-white' fluorescent tubes at an intensity of 2.35 W/m^2. The cells were grown to late log phase and pelleted in a Sorvall RC-2B centrifuge using the Szent-Györgyi-Blum KSB continuous flow rotor. Centrifugation was at 10 000 × *g* at 5°C. The pelleted cells

were suspended in 50 mM Tris-HCl (pH 8.0), immediately frozen and stored at −18°C.

Heterocyst cells were induced in cultures of *A. cylindrica* by omitting the nitrogen source from the medium. Isolation of heterocyst cells was by the method of Stewart [12].

Preparation of SDS extracts of blue-green algal membranes. Harvested algal cells in 50 mM Tris-HCl (pH 8.0) were broken by three freeze-thaw cycles, by sonication, or by passage through a French pressure cell at 700 kg/cm^2. Heterocyst cells were broken by three passes through a French pressure cell at 1120 kg/cm^2. An equal volume of 50 mM Tris-HCl (pH 8.0)/1 mM EDTA was added to the broken cells contained in a Kontes tissue grinder, the suspension homogenized, and then centrifuged at 19 000 × *g* for 10 min. The supernatant was discarded and the pellet rehomogenized in 50 mM Tris-HCl (pH 8.0)/1 mM EDTA. This last step was repeated at least twice or until there was no phycobiliprotein color in the supernatant. The quantity of chlorophyll in the pelleted membranes was determined and the membranes were then homogenized in 6.2 mM Tris/48 mM glycine/1% SDS (at 20°C) at a SDS to chlorophyll ratio (w/w) of 10 : 1 [7]. The homogenate was immediately centrifuged at 40 000 × *g* for 10 min. The SDS extracted material (supernatant) was made 10% (v/v) glycerol and aliquots containing 10—30 μg chlorophyll were immediately used for gel electrophoresis.

Preparation of SDS extracts of higher plant chloroplast membranes. Young leaves were obtained from greenhouse-grown maize (*Zea mays* L.) and from the chlorina-f2 mutant of barley (*Hordeum vulgare* L.). A chlorotic maize mutant ($Oy^{yg/+}$) was grown under high light intensity (2500 ft-candles) for approx. 10 days before the leaves were harvested. Seeds of this mutant were kindly provided by Dr. C.D. Miles (University of Missouri, Columbia, MO). Chloroplast membranes were isolated as previously described [7] and solubilized by the same procedure used for the blue-green algal membranes.

Gel electrophoresis. Electrophoresis of the SDS-extracted material was carried out as described previously [7]. Slab gels, varying in thickness but 11.5 cm in length, as well as tube gels, 0.7 cm × 10 cm, were used. Electrophoresis of the sample was for 30 min at 100 V. Molecular weights of the pigmented bands were estimated by comparing their electrophoretic mobilities with those of denatured proteins of known molecular weight [13].

The polypeptide composition of zone A-2 (see Fig. 1B) was determined by excising the band from fifteen cylindrical gels after electrophoresis. The gel slices were suspended in 50 mM NH_4HCO_3 at 4°C overnight, then dispersed by forcing them through 35 μM nylon mesh. The slurry was filtered through sintered glass and the polyacrylamide gel particles washed with 50 mM NH_4HCO_3. The green eluate was lyophilized and the dry powder stored at −18°C. The lyophilized sample was suspended in 0.15 ml of buffer containing 62.5 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 5% 2-mercaptoethanol and 0.001% bromophenol blue [14] and a tube containing the mixture immersed in a boiling water bath for 5 min. Proteins of known molecular weight were dissociated by the same procedure. The denature samples were electrophoresed using a modified system of Laemmli [14] in which the 3% stacking gel and the 10% separating gel contained 8 M urea. Proteins were located on gels by

staining with 0.1% Coomassie brilliant blue R in 30% isopropanol and 10% acetic acid followed by destaining in 5% methanol and 7% acetic acid. The marker proteins used and their subunit molecular weights were: hemocyanin, 290 000; β-galactosidase, 130 000 and 40 000; urease, 83 000; catalase, 60 000; ovalbumin, 43 000; alcohol dehydrogenase, 37 000; carbonic anhydrase, 29 000; chymotrypsinogen, 25 700; lysozyme, 14 300.

The proportion of the total chlorophyll in each chlorophyll-containing zone after electrophoretic fractionation was measured (cf. Ref. 7) by scanning the unstained gels at 672 nm using a Beckman ACTA CIII spectrophotometer gel scanning attachment. The absorption spectrum of chlorophyll-containing zones was recorded using an Aminco DW2 spectrophotometer into which a cuvette holding a slice of gel containing the pigmented component was placed.

Preparation of Photosysten I- and II-enriched fractions of P. luridum membranes. These membrane fractions were prepared as described previously [15]. Washed, pelleted membrane fragments were adjusted to a concentration of 0.3 mM chlorophyll *a* by adding 10 mM Tris-HCl (pH 7.5). An equal volume of 0.4% Triton X-100 in 10 mM Tris-HCl (pH 7.5) was added and the mixture was kept for 1 h in an ice bath. The solution was then centrifuged for 6 h at 125 000 × *g* in a Beckman SW-27 rotor through a discontinuous gradient of nine layers (5—70%) of sucrose dissolved in 10 mM Tris-HCl (pH 7.5). A Pasteur pipet was used to remove the fractions enriched in Photosystems I and II (cf. Ref. 15) from the gradient.

Chlorophyll determination. The concentration of chlorophyll in samples extracted in 80% (v/v) acetone was determined spectrophotometrically using Arnon's equations [16].

P-700 determination. The concentration of *P*-700 was determined from chemical difference spectra of ferricyanide-oxidized against sodium ascorbate-reduced samples. A differential extinction coefficient for *P*-700 of 64 $mM^{-1} \cdot cm^{-1}$ at 697 nm was used [17].

Source of chemicals. Acrylamide (enzyme grade) and *N,N'*-methylene bisacrylamide were purchased from Eastman Kodak Co., Rochester, NY; $(NH_4)_2SO_5$ from the J.T. Baker Chemical Co., Phillipsburg, PA; and Tris, glycine, SDS, *N,N,N',N'*-tetraethylmethylenediamine and other chemicals from Sigma Chemical Co., St. Louis, MO.

Results

Thylakoid membranes of higher plant chloroplasts solubilized in SDS and fractionated by the electrophoretic system of Markwell et al. [7] are resolved into five chlorophyll-containing zones. This is shown for maize thylakoid membranes in Fig. 1A. Band A-1, which contains chlorophyll *a* and protein, is probably analogous to CPI (cf. Ref. 7). Bands AB-1, AB-2, AB-3 are pigment-protein complexes containing chlorophylls *a* and *b*. The F zone is composed of chlorophyll not complexed with protein. When membranes of the blue-green algae. *P. luridum* and *A. cylindrica* are solubilized and fractionated using an identical procedure, four pigmented zones, three of which are coincident with protein stain, are observed (Fig. 1B). In this report these bands are termed A, A-1, A-2, and F in order of increasing electrophoretic mobilities.

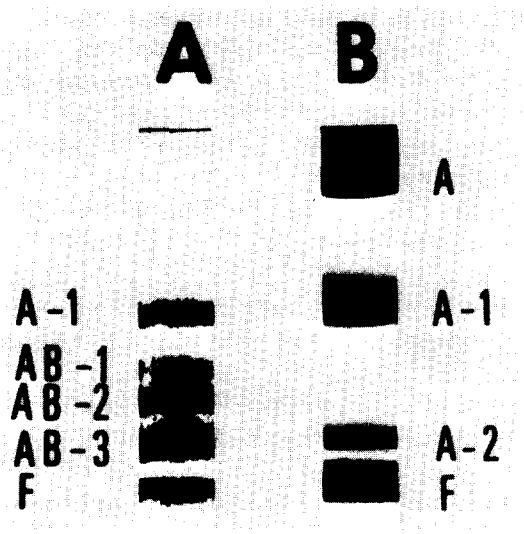

Fig. 1. Electrophoresis of SDS-extracted material from photosynthetic membranes of (A) *Zea mays*, and (B) *P. luridum* before staining for proteins. The electrophoretic system is that of Markwell et al. [7]. *Zea mays* was electrophoresed for 60 min, *P. luridum* for 30 min.

The A-1 band of *P. luridum* and *A. cylindrica* has a similar absorption spectrum, electrophoretic mobility, and function to the A-1 band of higher plants and to the CPI band observed in the electrophoretic system of Thornber and Highkin [18]. The spectrum of band A-1 of these algae shows a red maximum at 676 nm compared with values of 673—676 nm for CPI [18,19] and band A-1 [7] of higher plants and of 677 nm for CPI of blue-green algae [19]. The molecular weight equivalency of this band is 118 ± 8 kilodaltons which lies well within the range of published values, 100—130 kilodaltons, for higher plant A-1 and CPI [2,4,7]. Analysis of the complex extracted from the gel revealed the presence of *P*-700.

The spectrum of band A, red maximum at 676 nm, resembles that of the A-1 band (Fig. 2). This band has a molecular weight equivalency of 255 ± 15 kilodaltons and contains *P*-700. Betweeen 80 and 85% of the total chlorophyll loaded onto the gel is accounted for in bands A and A-1 at the end of electrophoresis.

The F zone has a red maximum at 669 nm (Fig. 2). This band has essentially the same spectral and electrophoretic characteristics as the F zone of higher plants and is therefore believed to be composed of chlorophyll that is not complexed with protein. The upper boundary of this zone does stain for protein; however, this does not necessarily indicate a chlorophyll-protein is present since all low molecular weight polypeptides of the membrane would run in this area of the gel. Only 8—12% of the total chlorophyll is contained in this zone, a value much lower than the 70% reported earlier [19].

The absorption spectrum of band A-2 (Fig. 3A) from the blue-green algal membranes has a maximum at 671 nm. A chemical difference spectrum of material extracted from band A-2 revealed the absence of the Photosystem I reaction center, *P*-700. The percent of chlorophyll associated with band A-2

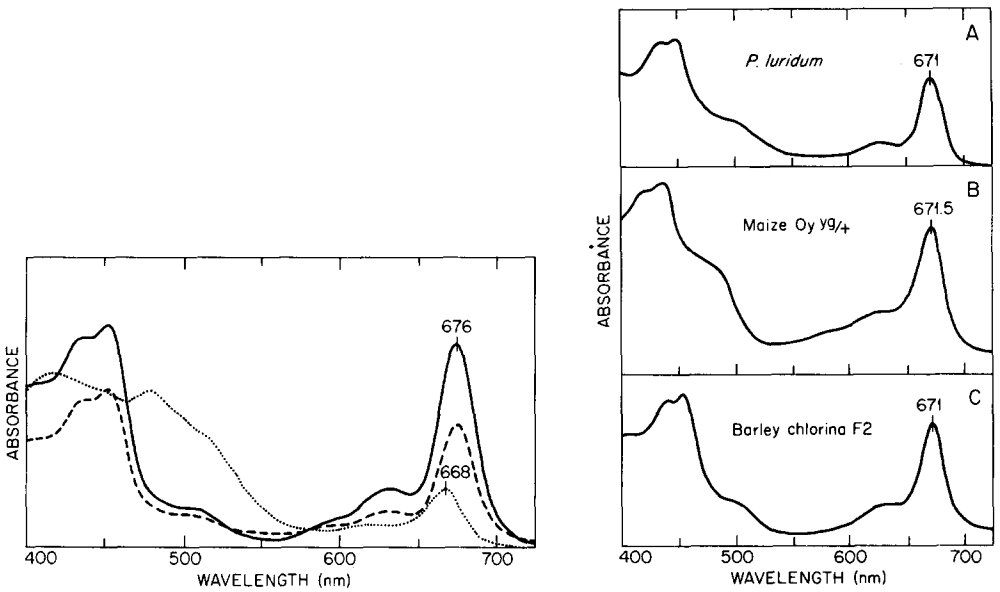

Fig. 2. Absorption spectrum of chlorophyll-containing bands of *P. luridum* cut from a gel similar to that shown in Fig. 1B. ———, band A; - - - - - -, band A-1; · · · · · ·, F zone. Full scale absorbance of 1.0.

Fig. 3. Absorption spectra of the 58 ± 5 kilodaltons chlorophyll-containing band. (A) Band A-2 from *P. luridum*; full scale absorbance of 1.0. (B) Complex IV from $Oy^{yg/+}$; full scale absorbance of 0.5. (C) From barley chlorina-F2; full scale absorbance of 1.0.

depended on the length of time of electrophoresis. After 30 min band A-2 constitutes 10—13% of the total chlorophyll, and thereafter the percent decreases until the band eventually disappears. No attempt has yet been made to optimize the stability of band A-2. However, when electrophoresis was through an 8% acrylamide gel rather than 5%, the stability of complex A-2 improved. A few preparations of blue-green algal membranes did not exhibit this complex on electrophoresis, while in a few other preparations two chlorophyll-protein bands were seen in this region of the gel. The reason for the occasional variability is unknown. When the protein in the A-2 zone was extracted from the gel matrix, dissociated and electrophoresed, at least eight polypeptides were revealed, ranging in molecular weight from 15 to 72 kilodaltons.

The characteristics of band A-2 suggested that this chlorophyll-protein might be analogous to complex IV observed by Hayden and Hopkins [3], and proposed by them to be associated with Photosystem II. On electrophoresis of wild-type organisms, complex IV is obscured by the presence of large quantities of a chlorophyll *a/b*-protein complex. These authors circumvented this problem by using the maize mutant, $Oy^{yg/+}$, which when grown under high light intensity has very little chlorophyll *b* present and hence little of the chlorophyll *a/b*-protein. To investigate the similarity of band A-2 and complex IV, the maize mutant was examined using the identical experimental procedures used for blue-green algal membranes. The spectrum of complex IV from $Oy^{yg/+}$ as resolved by the Markwell et al. system [7] is presented in Fig. 3B. Complex IV exhibited the same electrophoretic mobility as band A-2.

The chlorophyll *b*-less barley mutant chlorina f-2 [18] also presents an opportunity of examining the chlorophyll-protein content of higher plant chloroplast membranes without the interference of the chlorophyll *a/b* proteins. Electrophoresis of solubilized thylakoids of the barley mutant using the procedure of Markwell et al. [7] revealed three green zones: (a) one analogous to A-1 or CPI, (b) one to the F zone, and (c) one of the same electrophoretic mobility as band A-2 of blue-green algae and complex IV of the maize mutant. The spectrum of barley zone (c) is presented in Fig. 3c. The measured molecular weight equivalencies of blue-green algal band A-2, of complex IV of the maize mutant and zone (c) of the barley mutant lies between 58 000 ± 5000.

The possible association of band A-2 with Photosystem II was examined more directly. The occurrence of the A-2 band in heterocyst cells, apparently lacking Photosystem II activity [20], and in Photosystems I and II preparations was examined. Fig. 4A shows a densitometer tracing of the pigmented zones of the solubilized vegetative cells of *A. cylindrica* while Fig. 4B shows a tracing of solubilized heterocyst cells. The A-2 band, present in the vegetative cells, is missing from the heterocyst cells.

The results of an examination of Photosystem I- and II-enriched fractions are shown in Fig. 5A—C. Although greater than 80% of the total chlorophyll was contained in the Photosystem I fractions, no band comparable to A-2 was observed on the gels (Fig. 5B and C). Band A-2 was observed only in the Photosystem II fraction (Fig. 5A). Fig. 5D demonstrates that Triton-solubilized photosynthetic membranes fractionated on the Markwell et al. system [7] yields a pattern of chlorophyll-proteins identical to that observed when SDS-solubilized membranes are examined (Fig. 1B).

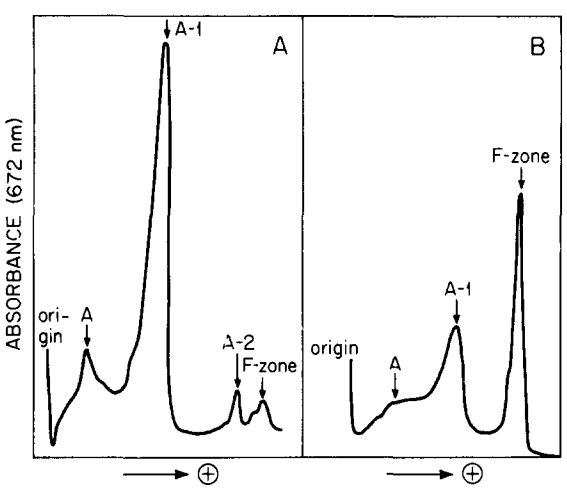

Fig. 4. Densitometer tracings following electrophoretic fractionation of SDS extracts of membranes of (A) vegetative cells of *A. cylindrica;* (B) heterocyst cells of *A. cylindrica.* The gels were electrophoresed for 30 min at which time the FP zone had migrated 5 cm from the origin.

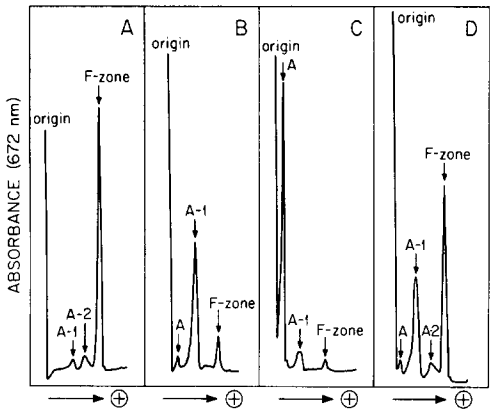

Fig. 5. Densitometer scans following electrophoretic fractionation of subchloroplast particles obtained by the solubilization of *P. luridum* membranes in Triton X-100 followed by sucrose density centrifugation. (A) light fragment, L, enriched in Photosystem II. (B) Heavy fragment, H, enriched in Photosystem I. (C) H', enriched in Photosystem I. (D) Triton-solubilized membranes. L, H and H' are the same abbreviations used by Ogawa et al. [15]. The gels were electrophoresed for 30 min at which time the FP zone had migrated 5 cm from the origin.

Discussion

Blue-green algae are photosynthetically similar to higher plants but have the experimental advantage that a large proportion of the photosynthetic pigments, phycobiliproteins, can be removed from the organism without disrupting the structure of the membrane. Hence the resulting membrane preparation should contain fewer pigmented components and make the study of the organization of chlorophyll in these membranes somewhat simpler than in higher plants. A recognized problem in such studies is that a considerable proportion of the total chlorophyll occurs as free pigment when the membranes are solubilized with detergents. It is essential for a total understanding of the organization of chlorophyll in vivo to determine whether or not this chlorophyll is conjugated with protein before dissolution of the membranes. A recently developed electrophoretic fractionation technique [7] aided in a solution to this problem.

Brown et al. [19] reported that 30% of the total chlorophyll of blue-green algal membranes is associated with the only chlorophyll-protein observed (i.e. CPI) after electrophoresis of SDS-solubilized membranes using the procedure of Thornber and Highkin [18]. The procedure [7] used in this report results in 85—90% of the total chlorophyll being complexed to three chlorophyll-protein complexes (A, A1, A2; Fig. 1B) after electrophoresis. Each complex contains carotenoids in addition to chlorophyll and protein.

The presence of *P*-700, the molecular weight equivalency, and the absorption spectrum (Fig. 2) of band A-1 indicate that this band is analogous to the A-1 band observed previously in higher plants [7] and to CPI present in higher plants and algae [2]. A chlorophyll-protein complex of higher molecular weight, band A, has an absorption spectrum very similar to that of band A-1 (Fig. 2). A chemical difference spectrum reveals the presence of *P*-700. The occurrence of more than one major chlorophyll-protein band containing *P*-700 is comparable to the three chlorophyll-protein bands resolved electrophoreti-

cally from SDS-treated membranes of the blue-green alga, *Spirulina platensis* [21]. The pigment-protein complexes of *S. platensis* had molecular weight equivalencies greater than 100 000 and the spectra of all three bands were similar to each other and to the spectra of bands A and A-1 in this report. It has been suggested [21] that these CPI-type bands represent a heterogeneity of Photosystem I reaction centers. Further studies are needed and are in progress to understand the function and interrelationship, if any, of these multiple, high molecular weight pigment-protein complexes present in SDS extracts of a blue-green alga.

Band A-2, electrophoretically resolved from the two algae examined (Figs. 1B and 4A), has a red absorbance maximum at 671 nm (Fig. 3A) and a molecular weight equivalency of 58 ± 5 kilodaltons. A chemical difference spectrum revealed the absence of *P*-700. A chlorophyll-protein complex with identical electrophoretic mobility and spectral properties to band A-2 of blue-green algae was also detected in mutants of higher plants (Fig. 3B and C). This band was not observed in solubilized membranes of higher plants separated by the Markwell et al. [7] system (Fig. 1A) probably because one of the chlorophyll *a/b*-containing bands obscured its presence.

The characteristics of band A-2 are similar to those reported by others for electrophoretically resolved pigment-complexes of higher plants thought to be associated with Photosystem II [3,5,6,8,9]. These complexes have red absorption maxima between 671 and 673 nm, contain carotenoids but very little, if any, chlorophyll *b*, and account for a small portion (less than 10%) of the total chlorophyll; the reported molecular weight equivalencies span a range of 30—60 kilodaltons. If these complexes are equivalent to each other and to band A-2, discrepancies in the molecular weight of the holocomplexes are probably the result of different conditions of solubilization and electrophoresis. Hence we have noted that when thylakoids of the maize mutant $Oy^{yg/+}$ are electrophoresed using the procedure of Markwell et al. [7] the molecular weight of complex IV is the same as band A-2, but not the same as originally reported [3]. The close correspondence of the properties of band A-2 with those of a 'Photosystem II band' in higher plants suggests a possible function for the algal complex.

Additional evidence that the blue-green algal A-2 band might be associated with Photosystem II activity was accumulated: heterocyst cells of N-fixing blue-green algae, which lack Photosystem II activity [20] do not have an A-2 band whereas the band is present in the photosynthetically normal vegetative cells (Fig. 4); furthermore band A-2 is present in Photosystem II-enriched fractions but not in Photosystem I-enriched preparations. The polypeptide composition of band A-2 was complex and while there were polypeptides present of a size anticipated for the Photosystem II complex, it could not be ascertained that these were related to the chlorophyll-containing component. It is impossible to assign unequivocally a function to band A-2 without performing an extensive examination of the partial electron transfer reactions, flash-induced rapid absorbance changes and fluorescence properties of the material in this band.

It is likely that bands A and A-1 contain the chlorophylls associated with Photosystem I and band A-2 those with Photosystem II. Bands A and A-1

account for 80—85% of the chlorophyll with less than 15% of the chlorophyll in band A-2. This distribution of chlorophyll between the two photosystems in blue-green algae agrees extremely well with that determined by other methods [22].

Note added in proof

Further research has revealed that two major polypeptides of 49 and 51 kilodaltons are present in the material extracted from the A-2 zone. These may be involved in binding chlorophyll.

Acknowledgments

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