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IN VITRO REASSOCIATION OF PHYCOBILIPROTEINS AND MEMBRANES TO FORM FUNCTIONAL MEMBRANE-BOUND PHYCOBILISOMES

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A membrane-bound phycobilisome complex has been isolated from the cyanobacterium *Fremyella diplosiphon* grown in green light, thus containing phycoerythrin in addition to phycocyanin and allophycocyanin. The complex was dissociated by lowering the salt concentration. In the mixture obtained, no energy transfer from phycoerythrin to chlorophyll (Chl) *a* was observed. Reassociation of the phycobiliproteins and membrane mixture was carried out by a gradual increase of the salt concentration. The complex obtained after reassociation was characterized by polypeptide composition, absorbance and fluorescence emission spectra and electron microscopy. These analyses revealed similar composition and structure for the original and reconstituted membrane-bound phycobilisomes. Fluorescence emission spectra and measurements of Photosystem II activity demonstrated energy transfer from phycoerythrin to Chl *a* (Photosystem II) in the reconstituted complex. Reassociation of mixtures with varying phycoerythrin/Chl ratio showed that the phycobiliprotein concentration was critical in the reassociation process. Measurements of the amount of phycobilisomes reassociated with the photosynthetic membrane did not show saturation of binding when increasing the phycobiliprotein concentration. The ratio phycoerythrin/Chl *a* in the native complex was 7:1 (mg/mg). When the phycobiliprotein concentration was increased during the reassociation process, a ratio of 13–15 mg phycoerythrin/mg Chl *a* could be obtained. Under these conditions, only part of the phycobilisomes attached to the thylakoids was able to transfer energy to Photosystem II.

Introduction

The energy-collecting system of cyanobacteria consists largely of two types of pigments: Chl *a* and phycobiliproteins [1,2]. The chlorophyll is organized in chlorophyll-protein complexes which form both reaction centers of PS II and I [3], as well as chlorophyll-protein antennae complexes which are intrinsic components of the photosynthetic membranes. The phycobiliproteins are water-soluble polypeptides to which bile pigments are covalently bound [1]. These are able to associate primarily via hydrophobic interactions to form large complexes (molecular mass $3.5–7.5 \cdot 10^6$) [4], the phycobilisomes, which in vivo are attached to the photosynthetic membranes and transfer the absorbed light energy to Chl *a* [1,2]. While extensive information is available on the spectral properties and interconnections between the various

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Abbreviations: Chl, chlorophyll; PS, photosystem; DCIP, 2,6-dichlorophenolindophenol; LDS, lithium dodecyl sulfate.

polypeptides participating in the formation of the phycobilisomes [1,2,4–8], little is known on the properties and specificity of interaction between phycobilisomes and membranes. Katoh and Gantt [9] have reported the isolation of membrane-bound phycobilisomes from *Anabaena variabilis*. In the present work we have attempted to isolate such a complex from *Fremyella diplosiphon* and determine whether it would be possible to dissociate and reassociate it in vitro in order to obtain an experimental system from which more detailed information on the parameters influencing the specific binding and energy transfer between the phycobilisomes and the membrane could be obtained.

Materials and Methods

F. diplosiphon cells were grown at 26 ± 1°C in the mineral medium described by Hughes et al. [10] and as modified by Allen [11]. Semicontinuous cultures were obtained by use of a New Brunswick Microferm fermentor connected to a 20 l carboy containing growth medium. The connection could be exchanged under sterile conditions using the method described before [12]. The cultures (4–6 l) were diluted periodically and kept at a relatively constant cell density, corresponding to the end of the logarithmic phase of growth. The operation of this system could be maintained for several months without interruption, permitting a daily supply of cells. The fermentor was covered with cellophane filter transmitting light between 425 and 600 nm (green). Illumination was by six fluorescent lamps of 40 W, and the stirring rate was 200 rpm. The cultures were aerated by 5% CO_2 in air at a rate of 0.2–0.5 l/min. Cells were harvested by centrifugation and washed in buffer as described below before further use.

Preparation of membrane-bound phycobilisomes and purified membranes

Membrane-bound phycobilisomes were prepared by a method similar to that described by Katoh and Gannt [9]. Cells were washed three times with 0.5 M potassium phosphate buffer solution containing 0.3 M sodium citrate, pH 7 (phosphate-citrate buffer), and the pellet obtained from an initial culture of 3 l was resuspended in 60 ml of the same buffer with the addition of lysozyme (Sigma, 0.4 mg/ml). The suspension was stirred at 25°C for 1 h. The cells were then washed in phosphate-citrate buffer, resuspended in the same buffer with the addition of 15 mg/ml bovine serum albumin, and the suspension passed through an ice-cold French press operated at 8000 lb/inch^2 twice. The resulting homogenate, which contained very few unbroken cells, was loaded on a continuous sucrose gradient (0–50%, w/v) prepared in phosphate-citrate buffer, and centrifuged at 10°C in a Beckman SW27 rotor at 25000 rpm for 2 h. The band containing the membrane-bound phycobilisomes was collected, pelleted by centrifugation, and stored at −80°C for up to 3 weeks. Membrane-bound phycobilisome fractions were dissociated in Tris-HCl (50 mM, pH 8) to yield thylakoids and soluble phycobilisome components. The thylakoids were pelleted and washed twice in Tris-HCl (50 mM, pH 8) by centrifugation at 35000 rpm at 5°C in a Beckman 40 rotor for 15 min (80000 × g).

Reassociation experiments

Pellets of the membrane-bound phycobilisome fraction were resuspended in Tris-HCl (50 mM, pH 8) buffer for half an hour. The thylakoids were separated from the phycobilisomes by centrifugation in a Beckman 40 rotor at 35000 rpm for 15 min as above.

In all experiments phycoerythrin was used as a marker for phycobilisome quantitation and Chl for quantitation of membranes.

Suspensions that contained thylakoids (8–170 μg Chl/ml) and disrupted phycobilisomes (0.3–0.75 mg phycoerythrin/ml) were dialysed in 1 cm diameter Visiking tubing (The Scientific Instrument Centre Ltd., London) against 1 l of phosphate-citrate buffer for 3 h at 10°C. The content of the dialysis bag was then centrifuged in a Beckman 40 rotor at 35000 rpm and 10°C for 7 min. The pellet was used for spectral and polypeptide analysis and for photosynthetic activity measurements.

Photosynthetic activities

Measurements of photosynthetic activities were carried out spectrophotometrically using diphenylcarbazide as an electron donor and DCIP as an electron acceptor for PS II [13] or reduced DCIP

and methyl viologen as donor and acceptor for PS I activity [14].

Analysis of polypeptide pattern

The polypeptide pattern of native complexes, purified membranes and dissociated phycobiliprotein fractions was analyzed by electrophoresis on polyacrylamide gels in the presence of LDS using the method described by Delepelaire and Chua [15]. For resolution of all polypeptides present, the solution of membranes in 0.1% LDS was heated at 90°C for 1 min. Slab gels containing a polyacrylamide gradient (7.5–15%) were used and staining was done with Coomassie brilliant blue G.

Fluorescence emission spectra

Room temperature spectra were recorded using a Perkin-Elmer spectrofluorimeter model MPF4. In order to excite all phycobiliproteins, excitation was at 380 nm [16]. Emission spectra were recorded in 'energy' mode.

Fluorescence emission spectra at 77 K were normalized to the highest peak and were recorded as described by Gershoni and Ohad [17] but using a glass dewar of 1 cm exterior diameter, fitted in the cuvette holder of the Perkin Elmer spectrofluorimeter.

Chlorophyll was extracted in absolute methanol from pelleted complexes or membrane fractions and quantitated from absorption measurements according to the method of Bennett and Bogorad [18].

Electron microscopy

Thin sections. Membrane-bound phycobilisome preparations were fixed for 2.5 h in 2% glutaraldehyde (Polyscience) in 0.05 M cacodylate buffer. After fixation the preparations were washed three times in the same buffer and post-fixed overnight in 1% osmium tetroxide in the same buffer. The fixed material was washed twice in bidistilled water, mixed with 1% agar to prevent dispersion and stained 'en bloc' with 1% uranyl acetate for 1 h at room temperature. Preparations were then dehydrated in a graded series of acetone and embedded in the low-viscosity embedding medium of Spurr [19].

Negative staining. In order to stabilize the interactions between phycobiliproteins and membranes, glutaraldehyde was added to the preparations to a final concentration of 0.4%. Best results were obtained when samples were applied to the carbon-coated collodion-covered grids within 1 min after addition of the glutaraldehyde. After application of the sample, grids were washed with a few drops of 0.1 M NaCl and negatively stained with 1% potassium phosphotungstate, pH 7.2.

Preparations were viewed with a Phillips EM 400 electron microscope operating at 80 kV and micrographs were recorded on Kodak emulsion 4489.

Results

Dissociation and reassociation of phycobilisome-membrane complexes

It has been previously reported that phycobilisome-membrane complexes isolated in the presence of high phosphate-citrate buffer dissociate when the buffer concentration is reduced below 0.1 M [9]. Dissociation of the phycobilisomes from the membrane should result in a reduction of the fluorescence emission of Chl *a* (680 nm) caused by phycobiliprotein excitation. Further dissociation of the phycobilisomes into their constituent phycobiliproteins should result in a decrease in the fluorescence emission at 665 and 675–680 nm and a corresponding increase in the individual phycobiliprotein fluorescence (660, 645 and 580 nm for allophycocyanin, phycocyanin and phycoerythrin, respectively [20,21]). The fluorescence emission of a phycobilisome-membrane complex following incubation in various buffer concentrations for various amounts of time is shown in Fig. 1. It is evident that one can select dissociation conditions under which (a) intact free phycobilisomes or (b) dissociated phycobiliproteins prevail. In all reassociation experiments carried out in this work dissociation of the native complex was carried out as in b for 1 h. Reassociation of the dissociated mixture was obtained by a gradual rise in the buffer concentration by dialysis of the mixture gainst 0.5 M phosphate and 0.3 M citrate buffer, pH 7.4, as described in Materials and Methods. The degree of reassociation was almost complete (greater than or equal to 80%) as ascertained by measurements of the amount of phycoerythrin and phycocyanin found in the reas-

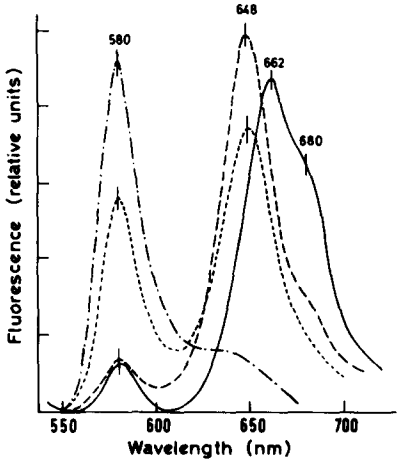

Fig. 1. Fluorescence emission spectra at 25°C of a membrane-bound phycobilisome preparation following gradual dissociation by lowering salt concentration. A preparation in phosphate citrate buffer, containing 2 μg Chl/ml (———), was diluted with distilled water (a) 4-fold (— — —) and (b) 10-fold (- - - - - -), and fluorescence emission spectra recorded after 10 min. The spectrum of the 10-fold diluted sample was recorded again after 2 h (·—·—·). Excitation was at 380 nm.

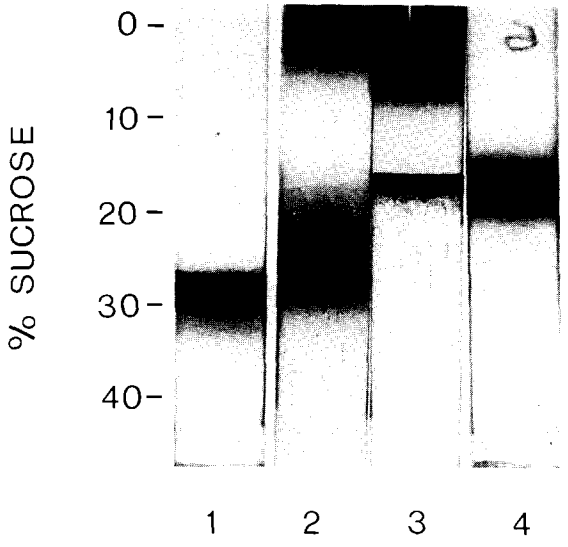

Fig. 2. Separation of various preparations, containing phycobilisomes and membranes, by sucrose density gradient centrifugation. (1) Membrane-bound phycobilisomes prepared in high phosphate-citrate buffer. (2) Membrane-bound phycobilisomes reassociated by dialysis against phosphate-citrate buffer for 3 h. (3) Membrane-bound phycobilisomes after dissociation in 10-fold diluted buffer for 1 h. (4) Purified membranes. The amount of material loaded on the gradients corresponds to about 186 μg Chl. The sucrose gradients (0–50%) were prepared in 0.5 M phosphate and 0.3 M citrate buffer.

sociated complex relative to that initially present in the association mixture prior to dialysis.

Sedimentation of the reassociated complexes on a sucrose gradient prepared in a high concentration of phosphate-citrate buffer showed that the buoyant density of the reassociated complex is essentially identical to that of the native complex and significantly different from that of phycobilisome-free membranes or dissociated phycobilisomes (Fig. 2).

The phycobiliprotein composition of the reassociated complex was also similar to that of the native complex as estimated from absorption spectra (Fig. 3) and analysis of polypeptide pettern by LDS-polyacrylamide gel electrophoresis (Fig. 4).

The polypeptide pattern of the soluble fraction obtained after dissociation of a native complex and sedimentation of the membranes showed predominance of phycoerythrin, phycocyanin and allophycocyanin and subunit polypeptides which could be identified from their relative mobility and retention of their specific color throughout electrophoresis [18]. These polypeptides appear as heavily Coomassie brilliant blue-stained bands in the molecular mass range 10–18 kDa (Fig. 4). In addition, polypeptides considered as colorless constituents of the phycobilisomes [22,23] in the molecular mass range 27–94 kDa were also present.

Membranes from which the phycobilisomes

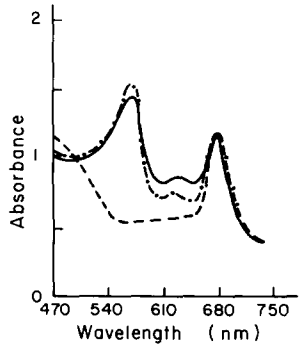

Fig. 3. Absorption spectra of native membrane-bound phycobilisome complex (———), reassociated (·—·—·) and purified membranes (- - - - - -).

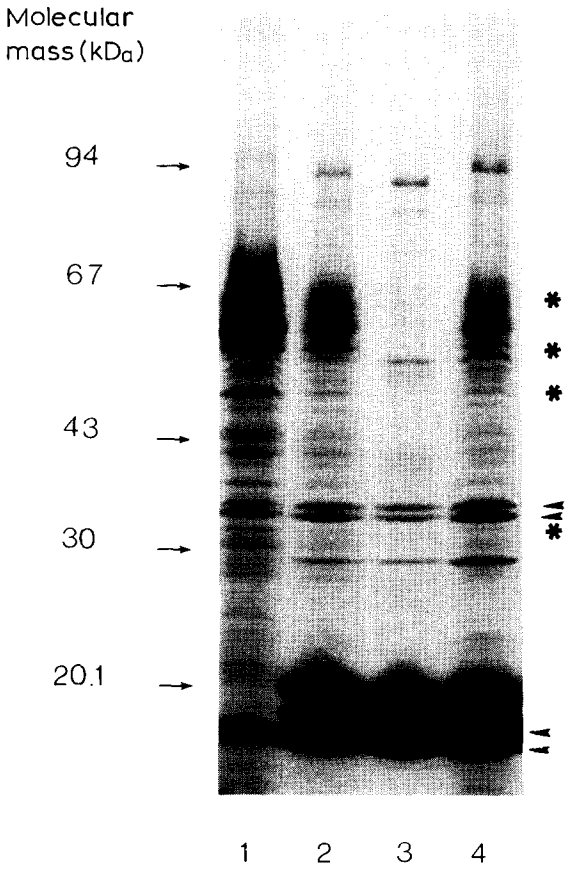

Fig. 4. Polypeptide pattern on LDS-polyacrylamide gel electrophoresis of isolated membranes (1), reassociated complex (2), dissociated phycobilisomes (3), and native complex (4). Asterisks indicate (top to bottom) polypeptides in the molecular mass range corresponding to P-700 apoprotein, CF_1 α- and β-subunits, PS II Chl *a*-binding polypeptides and herbicide-binding site [13,15,36–38]; arrowheads indicate polypeptides of 34.5, 33.5, 18.5 and 17.5 kDa present in both phycobiliprotein fractions and isolated thylakoids.

were dissociated exhibited a very different polypeptide pattern in which polypeptides of molecular masses similar to those identified in other membrane systems [13,15] as the P-700 apoprotein (molecular mass 69 kDa), CF_1 α- and β-subunits (molecular mass 56.5 and 54 kDa), PS II Chl *a* binding polypeptides (molecular mass 47–50 kDa) and the herbicide-binding site (molecular mass 32.5 kDa) could be detected (Fig. 4). In addition, four polypeptides having molecular masses similar to those found in the phycobiliprotein fraction (34.5, 33.5, 18.5 and 17.8 kDa) were also present. Whether these were identical polypeptides partitioning between the membrane and soluble fractions of membrane specific polypeptides was not established. The polypeptide pair of molecular mass 17.8–18.5 kDa in the membrane fraction did not appear to be pigmented prior to staining of the gel. The polypeptide pattern of the undissociated native complex appeared as the sum of both above patterns (Fig. 4) and was identical to that of the reassociated complex with the exception that the relative concentration of a polypeptide of molecular mass 53 kDa seemed to be significantly reduced in the reassociated complex (Fig. 4).

Preparations of the native and reconstituted phycobilisome-membrane complexes were monitored by electron microscope examination of thin sections (Fig. 5a) and on negatively stained glutaraldehyde-fixed material. Profiles of membrane-associated phycobilisomes were seen in both the native and reassociated complex (Fig. 5a–d). Occasionally, tubular-like structures of varying lengths consisting of stacked phycobiliprotein subunits were also observed (Fig. 5d). Phycobilisomes or subunit profiles were absent in purified membrane preparations (Fig. 5e).

Energy transfer to Chl a in native and reassociated complexes

Energy transfer between phycobilisomes and Chl *a* of the membranes in native isolated complexes was reported before [2]. The data presented so far demonstrate that all components present in the membrane-bound phycobilisomes reassociated with the membrane to form phycobilisome-like structures. The question arises as to whether these reassociated complexes are functional and thus able to transfer energy to Chl *a* of PS II similar to the complex in vivo.

Information on energy transfer between phycobilisomes and Chl *a* can be obtained from evaluation of low-temperature (77 K) fluorescence emission spectra in which the emission of individual phycobiliproteins (phycoerythrin, 580 nm, phycocyanin, 645 nm; allophycocyanin, 660 nm) as well as that of the phycobilisomes (686 nm), PS II and PS I Chl *a* (696 and 730 nm, respectively) is well resolved. The fluorescence emission spectra at 77 K of native, dissociated and reassociated complexes are shown in Fig. 6. The spectra of both the

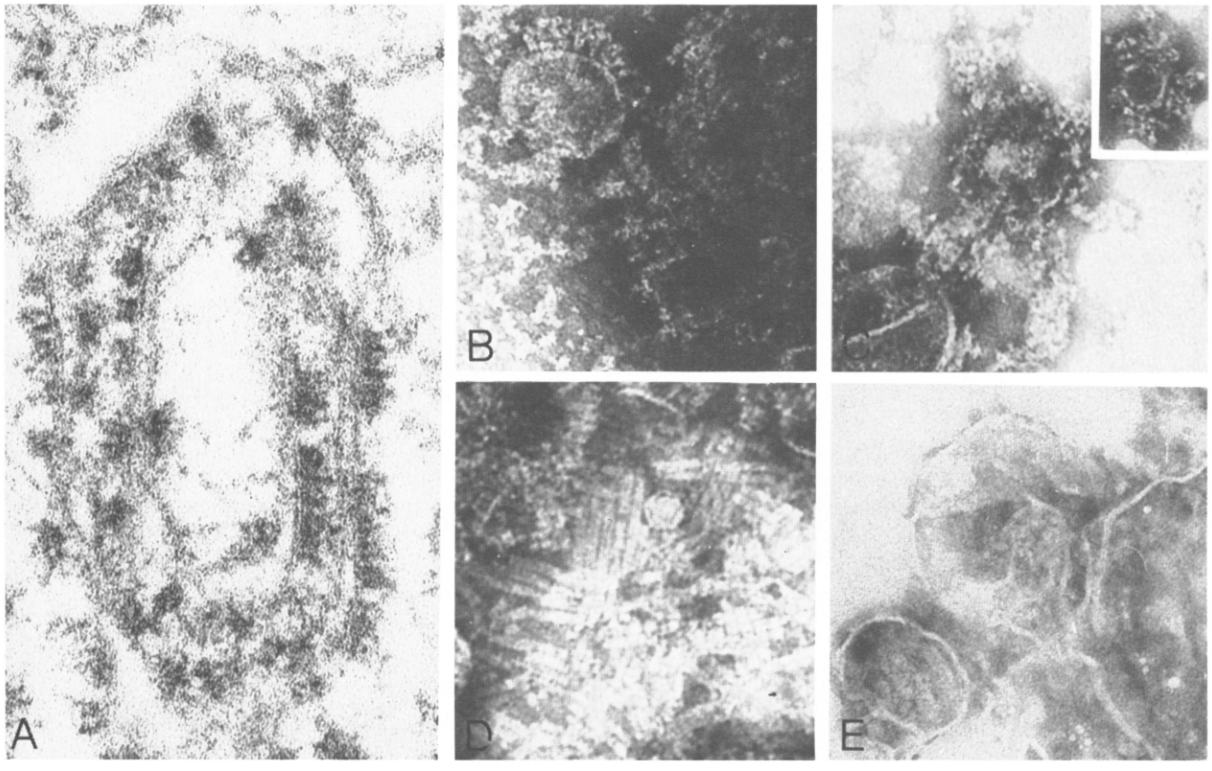

Fig. 5. Electron micrographs. (a) Thin section of membrane-bound phycobilisomes showing the appearance of phycobilisomes attached to the membranes. When favorably oriented to the plane of section, phycobilisomes are seen as discrete structures composed of a number of finger-like projections. (b) Negatively stained membrane-bound phycobilisomes. Individual phycobiliproteins are seen as 130 Å diameter disks with a strongly stained center. These are assembled on the membrane as stacks comprised of between 5 and 10 individual disks. Views are also obtained when the stacks are seen lying across the membrane surface. (c) Negatively stained reassociated membrane-bound phycobilisome preparations. Assemblies of stakes of phycobiliprotein disks are seen in association with membrane fragments. (d) Negatively stained reassociated membrane-bound phycobilisome preparations. Assemblies of unusually long stacks in association with membrane fragments. Individual phycobiliprotein disks are clearly resolved in side view. (e) Negatively stained membranes after dissociation of phycobiliproteins. The smooth appearance of the membrane is in striking contrast to a and b. Magnification, ×131600.

native and reassociated complex are similar and show major fluorescence emission bands at 686 nm (allophycocyanin I), 696 nm (PS II Chl *a*) and 730 nm (PS I Chl *a*). The dissociated mixture of phycobiliproteins and membranes shows fluorescence emission maxima for phycoerythrin (580 nm), phycocyanin and allophycocyanin (645 nm and 660 nm) and a barely detectable emission of Chl *a* when all conditions including relative concentration of phycobiliproteins, membranes, Chl *a* and instrumental settings are similar.

An additional way to estimate energy transfer as well as photosynthetic activity of the membrane in the native and reassociated complex is to measure the rate of electron flow of both PS II and PS I at limiting light intensity. An increase in the electron-flow rate due to excitation of the absorption band of phycoerythrin (560–570 nm) as compared to the rate elicited by excitation of Chl *a* (greater than or equal to 665 nm) should indicate energy transfer from phycoerythrin to Chl *a*. Results of such an experiment are shown in Tables I and II. Excitation at 560 nm results in about 40% of the activity of PS II elicited by excitation of Chl *a* at 665 nm or greater in both the native and reassociated complexes, but only 12% of this activ-

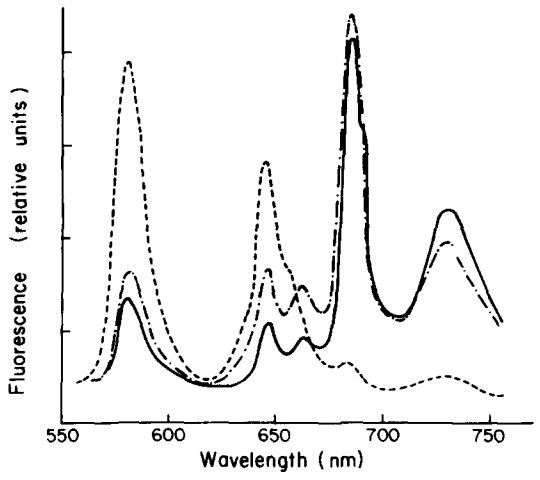

Fig. 6. Fluorescence emission spectra at 77 K of a native complex (———), reassociated complex (·–·–·) and isolated membranes (— — —). The Chl concentration was about 3–4 μg/ml and excitation was at 380 nm.

ity in membranes lacking phycobilisomes (Table I). Similar measurements of PS I activity showed a less pronounced effect on electron flow while exciting the phycoerythrin as compared with PS II activity (Table II). These results can be interpreted as evidence for specific energy transfer from phycobilisomes to PS II which is preserved also in the reassociated complex.

Binding specificity

Further indications for specificity of phycobilisome reassociation with the membranes could be obtained if one could demonstrate that the amount of functional binding sites on a chlorophyll basis is similar to that found in the native complex. To test this possibility, reassociation experiments were carried out under experimental conditions in which the ratio of phycoerythrin (used as a marker for the phycobiliprotein mixture) to Chl *a* (used as a marker for the amount of membranes) was varied. Results of such experiments are shown in Fig. 7. When a constant amount of phycobiliproteins was added (1.75 mg phycoerythrin, 0.583 mg phycoerythrin/ml) and the amount of membranes was gradually increased in the association mixture, it was found that all the phycoerythrin present was bound when the Chl *a* content was 0.14 mg (0.047 mg/ml) or at a phycoerythrin/Chl ratio of 12 (mg) in the dialysed mixture. The ratio phycoerythrin bound/Chl *a* increased continually as the ratio phycoerythrin/Chl *a* in the association mixture increased, showing no tendency for saturation (Fig. 7). The highest ratio (mg) of phycoerythrin bound/Chl *a* achieved in these experiments was 13 as compared with only 7 mg phycoerythrin bound/Chl *a* in the native complex.

When the Chl *a* was kept constant (0.1 mg Chl *a*; 0.033 mg Chl *a*/ml) and the amount of phycobiliproteins was increased in the association mixture a linear relationship was found between the amount of phycoerythrin added and that bound to the membrane. No tendency for saturation was observed up to a ratio of 15 mg phycoerythrin bound/mg Chl *a*.

These results demonstrate that at least part of the reassociated phycobilisomes might be non-

TABLE I

ENERGY TRANSFER FROM PHYCOERYTHRIN TO PS II IN NATIVE AND REASSOCIATED PHYCOBILISOME-MEMBRANE COMPLEXES

For experimental details see Materials and Methods.

Experimental system	Activity (μmol DCIP reduced/mg Chl per h)		Activity ratio 560 nm/665 nm	Phycoerythrin bound/Chl (mg/mg)
	Excitation 560 nm	Excitation ≥ 665 nm		
Native complex	44.8	113.1	0.40	7
Membranes	11.7	100.6	0.12	≤ 0.1
Membranes + dissociated phycobilisomes	13.5	102.5	0.13	≤ 0.1
Reassociated complex	40.0	114.1	0.35	6.3

TABLE II

ENERGY TRANSFER FROM PHYCOERYTHRIN TO PS I IN NATIVE AND REASSOCIATED PHYCOBILISOME-MEMBRANE COMPLEXES

For experimental details see Materials and Methods.

Experimental system	Activity (μmol O_2 reduced/mg Chl per h)		Activity ratio 560 nm/665 nm	Phycoerythrin bound/Chl (mg/mg)
	Excitation 560 nm	Excitation $\geq$ 665 nm		
Native complex	113.8	175	0.65	7
Membranes	99.5	215	0.46	$\leq$ 0.1
Membranes + dissociated phycobilisomes	89.1	175	0.51	$\leq$ 0.1
Reassociated complex	127.4	215.2	0.59	6.3

specifically bound. To test this possibility the energy transfer from phycoerythrin to Chl *a* was measured as above – in reassociated complexes having increasing ratios of phycoerythrin/Chl *a*. The results of such an experiment are shown in Fig. 8. It is evident that electron-flow activity

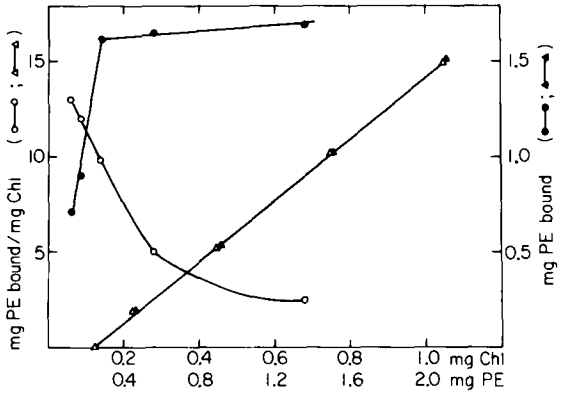

Fig. 7. Reassociation of phycobiliproteins and thylakoid membranes as a function of their relative concentrations. Mixtures of phycobiliproteins and theylakoids, in which either the membranes or phycobiliproteins concentration was kept constant while that of the other component varied, were dialysed as described in Material and Methods. The resulting phycobilisome-membrane complexes were isolated and the ratio phycoerythrin/Chl *a* in these complexes was estimated. (▲——▲, △——△) Membrane concentration constant (33 μg Chl/ml), phycobiliprotein concentration variable (130 to 660 μg phycoerythrin/ml); (●——●, ○——○) phycobiliprotein concentration constant (583 μg phycoerythrin/ml) and membrane concentration variable (66 to 330 μg Chl *a*/ml). The numbers on the abscissa represent the total amount of phycoerythrin (PE) and Chl present in the reassociation mixtures.

elicited by phycoerythrin excitation increases as more phycoerythrin becomes associated with the membranes and a plateau is reached at a ratio (mg) phycoerythrin bound/Chl *a* of approx. 7.5 as in the native complex. This indicates that at this ratio of phycoerythrin/Chl *a*, all PS II specific binding sites are occupied. The additionally bound phycoerythrin (mg phycoerythrin/mg Chl *a* approx. 15) might thus be bound nonspecifically.

Fluorescence emission spectra at 77 K of intact free and bound phycobilisomes always show an emission peak at 580 nm ascribed to a fraction of the energy absorbed by phycoerythrin which is not transferred to allophycocyanin I (emission at 686 nm). Energy transfer from allophycocyanin I to Chl *a* should result in a lowering of its emission peak, without affecting the phycoerythrin energy

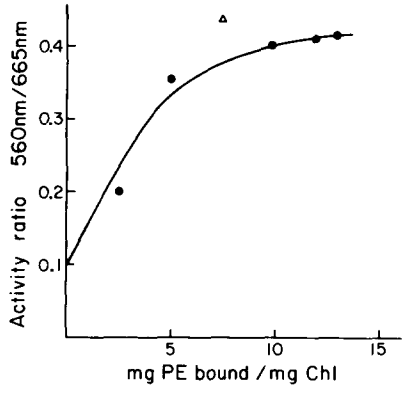

Fig. 8. Energy transfer from phycobilisomes to PS II in reconstituted complexes containing phycobilisomes bound to the membrane; (△) value of the native complex. PE, phycoerythrin.

loss at 580 nm. Examination of the ratio 686 nm/580 nm emission at 77 K in reassociated complexes having increased amounts of phycoerythrin bound to the membranes showed indeed an increase in this ratio from 1.5 to 2.2 to 3.0 for complexes having 5.5, 9.1 and 16.1 mg phycoerythrin/mg Chl *a*, respectively. These results support the view that energy absorbed by phycobilisomes bound in excess to the available PS II sites is released as fluorescence of the allophycocyanin I complex of the intact phycobilisomes.

Discussion

It has been demonstrated before that purified phycobiliproteins can be reassociated in vitro to form molecular aggregates containing phycoerythrin, phycocyanin or allophycocyanin [24]. The reassociation of phycobiliproteins and 'colorless proteins' seems to be based on specific hydrophobic interactions between the phycobiliproteins and results in the formation of complexes in which the pattern of energy transfer is preserved as in the original phycobilisomes [7,8,25]. Recently, complete reassembly in vitro of *Nostoc* sp. phycobilisomes including all phycobiliproteins phycoerythrin, phycocyanin and allophycocyanin as well as the so-called colorless proteins was also reported [26]. In the present work it is demonstrated that complete reassociation of all *F. diplosiphon* phycobilisome components and the membrane to form phycobilisome-membrane complexes in vitro is possible. Electron microscopy has confirmed the existence of a discrete structural complex between the photosynthetic membranes of *F. diplosiphon* and phycobiliproteins reassembled into phycobilisomes on the membrane. The association in native isolated membranes has been previously visualized by Katoh and Gantt [9]. Our observations essentially confirm their findings, but we further show that the reconstituted complex is very similar in appearance to the native complex consisting of stacks of phycobiliprotein disks extending outwards from the membrane surface. The number of disks per stack is variable and is most likely due to not completely stable conditions prevailing during the preparations for electron microscopy. In the vicinity of the complexes individual phycobiliproteins could always be seen, characterized by a 130 Å diameter ring with a strongly staining center.

Electron microscopic observation of reassociated complexes does not provide information on their functional association, nor does the analysis of their polypeptide composition. The final proof for effective reassociation as shown in this work resides in results of energy-transfer measurements. Based on studies of energy transfer [27,28] and examination of freeze-fracture faces of membranes to which phycobilisomes were attached [29,30] it was implied that phycobilisomes bind specifically to PS II sites. It was also suggested that a colorless polypeptide of about 90–95 KDa might be involved in this process [22,31–34]. The specificity of binding of phycobilisomes to PS II sites of the membranes is also demonstrated in this work. However, nonspecific binding can also occur when the relative concentration of the phycobiliproteins exceeds that of available specific sites. Possibly, the amount of the 90–95 kDa polypeptide present in the membrane and assumed to participate in the process of specific binding of phycobilisomes to the thylakoids [22,31,33,34] is limiting the amount of bound phycobilisomes which might transfer energy to PS II. Thus, one could propose that the above polypeptide(s) might be part of the PS II complex. The nonspecific binding might be due to hydrophobic interactions facilitated by the high salt present in the associated mixture. Such interactions could occur between free phycobilisomes and PS II-associated phycobilisomes or phycobilisome-free membrane areas. However, such binding does not result in energy transfer. This conclusion is based on the fact that energy transfer reaches a 'plateau' at a ratio of 7.5 mg phycoerythrin bound/mg Chl *a* and is in agreement with the concept that energy transfer requires the participation of allophycocyanin I and the 90–95 KDa polypeptides [33,34].

Based on the data reported here one can calculate tentatively the number of phycobilisomes bound per PS II unit and the number of sites per unit area of the membrane. The number of Chl *a* molecules per electron-transfer chain in *F. diplosiphon* appears to be about 400. This will be equivalent to 2.5 nmol electron-transfer chains or PS II units per mg Chl *a*. Since effective binding of

phycobilisomes transferring energy to PS II reached a plateau when about 7.5 mg phycoerythrin were bound per mg Chl *a* and if one takes the molecular mass of a phycoerythrin unit $(\alpha\beta)_6$ to be about 229000 [18], one can calculate 32 $(\alpha\beta)_6$ phycoerythrin units bound to 2.5 PS II sites or about 13 $(\alpha\beta)_6$ phycoerythrin units per site, as compared with 18 units reported for various algae and based on analysis of isolated phycobilisomes [4,2,35]. Assuming a membrane thickness of 100 Å one can calculate a surface of 10^{14} μm^2 of membrane per cm^3. The buoyant density of *F. diplosiphon* thylakoids appears to be about 1.20 g/cm^3, the ratio Chl/protein is about 0.14 (w/w) and the ratio protein/lipid 1.1 (unpublished data). One can calculate that 1 cm^3 of membrane contains 88 mg Chl, or 220 mmol sites per 10^{14} μm^2 membranes. This will be equivalent to about 1300 sites/μm^2 membranes. The number of sites reported for other algae varies between 20 and 1400 [2]. Using a molecular mass value of 229000 for the $(\alpha\beta)_6$ unit of phycoerythrin [18] and 18 such units per phycobilisome [4,35] one can calculate a 72% occupancy of the PS II sites when 7.5 mg phycoerythrin are bound per mg Chl *a*. In a similar way a 100% occupancy can be calculated if only 13 $(\alpha\beta)_6$ phycoerythrin units are present per phycobilisome. These calculations are in good agreement with the observed plateau reached in energy transfer at the above ratio of phycoerythrin bound per Chl *a*.

The fact that part of the phycobilisomes bind nonspecifically is rather unexpected. One should note that binding of phycobilisomes to the membranes does not occur if intact phycobilisomes are mixed with membranes stripped of phycobilisomes. Reassociation was found to occur only when dissociated phycobiliproteins were mixed with stripped membranes prior to the gradual increase of salt concentration. The experimental system described here permits the testing of requirements for various phycobilisome and membrane polypeptides for the correct or specific binding as well as the possibility of reassociation of heterologous systems using phycobilisomes and membranes of different organisms. Such experiments are now in progress.

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