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Functional Organization of Chlorophyll *a* and Carotenoids in the Alga, *Nannochloropsis salina*[1]

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ABSTRACT

Chlorophyll-protein complexes were isolated from a yellow-green alga, *Nannochloropsis salina* after mild detergent treatment and gel electrophoresis. Three different complexes were obtained which correspond to the three major kinds of chlorophyll-proteins isolated from spinach chloroplasts by the same procedure and previously identified as reaction center complexes for photosystems I and II and a light-harvesting complex. The analogy between the algal complexes and those from spinach was drawn from their absorption and fluorescence spectra and relative pigment content. The identities and amounts of the major carotenoids associated with each isolated complex were determined by HPLC. Although the reaction center complexes accounted for only 14% of the total chlorophyll, they were highly enriched in β-carotene, whereas the light-harvesting complex contained a high proportion of xanthophylls (mainly violaxanthin and vaucheriaxanthin-ester). Fluorescence excitation spectra of the algal membranes showed that one or both of the major xanthophylls may act as antenna pigment for photosynthesis.

In higher plant chloroplasts, photosynthetic pigments are noncovalently bound to at least three different intrinsic membrane protein complexes (26). We know that the two reaction centers, where primary conversion of radiant to chemical energy occurs, together with Chl *a* and β-carotene are closely associated with two different protein complexes (PSI and PSII). In addition, about 50% of the Chl *a* and most of the Chl *b* and xanthophylls are bound to a third class of pigment-protein complexes called LHCII.[2] The LHCII complexes serve as antenna by absorbing and transferring light energy to the Chl *a* that is closer to the reaction centers.

A better understanding of the role that Chl *b* and certain xanthophylls play in light-harvesting and of their organization into pigment-protein complexes may be obtained by comparing LHCII complexes from higher plants with those from algae which do not have Chl *b*. The alga *Nannochloropsis salina* is particularly well suited for this comparison because it lacks both Chl *b* and Chl *c* but, like higher plants, contains a high proportion of violaxanthin (25).

N. salina is a very small unicellular marine alga. When the Eustigmatophyceae and Xanthophyceae were divided, based in part on morphological evidence (15), *N. salina* (formerly called GSB Sticho) was placed with the former. Violaxanthin and vaucheriaxanthin-ester are the major carotenoids in this species (19, 27).

It may be assumed that if energy transfer between other pigments and Chl *a* can be demonstrated by fluorescence excitation spectra for Chl *a* emission, those pigments can function as antenna for photosynthesis. By that criterion, the light-harvesting ability of several xanthophylls including fucoxanthin, peridinin, and siphonaxanthin has been documented (1, 16). Although the occurrence of fucoxanthin is widespread in diatoms and brown algae, the distribution of the others is limited to a few algal groups. Recently LHCII complexes from higher plants have been isolated with their xanthophylls still firmly attached, and excitation spectra of these complexes clearly show a contribution by these pigments (mainly lutein and violaxanthin) to Chl *a* emission (6, 21); but the presence of Chl *b* with absorption overlapping that of the carotenoids complicates an interpretation of the excitation spectra. Therefore, study of *Nannochloropsis*, which has violaxanthin but not Chl *b*, may provide evidence for the role of these pigments as antenna for photosynthesis.

Here we describe three different Chl-protein complexes from *N. salina*, present an analysis of the pigments in each complex, and show that energy transfer can occur from the xanthophylls to Chl *a* in the algal membranes. This is the first description of native Chl-proteins, similar to those in higher plants, in a eucaryotic alga of a group which has only Chl *a* (except for Chrystal and Larkum [10]).

MATERIALS AND METHODS

Preparation of Cell Membranes. *Nannochloropsis salina* Hibberd, strain GSB Sticho was obtained from the SERI Microalgae Culture Collection, Golden, CO. The cells were grown in 3.5 L of enriched seawater (14) for 7 to 8 d, stirring and bubbling with air at 22°C under a 16:8 h light-dark cycle at about 100 $\mu E \cdot m^{-2} s^{-1}$. They were harvested by centrifugation, resuspended in 0.1 M sorbitol, 2 mM Tris-maleate buffer (pH 8), 0.5 mM ε-amino caproic acid, and 1 mM phenylmethylsulfonyl fluoride, and were broken by passing through a French pressure cell twice. The broken cell membranes were centrifuged and washed two times with 5 mM EDTA, 2 mM Tris-maleate buffer (pH 7).

Isolation of Chl-Protein Complexes. The procedure of Picaud *et al.* (20) for isolating Chl-protein complexes from spinach was followed. Washed membranes were resuspended in 2 mM Tris-maleate buffer (pH 7), and digitonin was added to give a final Chl concentration of 0.8 to 1 mg/ml and a digitonin to Chl ratio of 60 (w/w). This mixture was stirred at 25°C for 1 h. After centrifugation at 100,000*g* for 1 h at 4°C to remove undissociated membranes, an aliquot of the supernatant (about 300 μg Chl) was loaded onto a 5% polyacrylamide gel containing 0.05% sodium deoxycholate (DOC) in a 14 mm diameter tube, and electrophoresis was carried out at 2 mamp per tube overnight at 4°C. The running buffer contained 50 mM Tris-glycine (pH 8) and 0.05% DOC.

Each green band was excised from the gels and ground in a mortar with distilled H_2O. Gel pieces were removed by centri-

[1] DPB-CIW No. 942.

[2] Abbreviations: LHCII, light harvesting complex; DOC, deoxycholate; FP, free pigment.

fugation and repeatedly extracted with water until they were colorless. The Chl-protein complexes in the combined supernatants were concentrated with Centriflo membrane cones (Amicon CF-25). The amount of Chl *a* in each gel band was estimated from the absorbance at the red maximum and the volume of each extract before concentration.

Pigment Analysis. Pigments were extracted from membranes or isolated complexes into acetone:water (4:1, v/v) and analyzed with a 1080 Series Liquid Chromatograph from Hewlett-Packard equipped with a Variable Wavelength Detector. Two different HPLC columns (Alltech Assoc.) were used: a semipreparative, RSIL, C8, 10 μ column and an analytical econosphere C18, 5 μ column. The former column was used for obtaining sufficient amounts of each pigment to enable its identification by absorption spectroscopy and use as a standard for calibrating the latter column. Routinely a gradient solvent system of methanol:water (4:1, v/v) to methanol:acetone (4:1, v/v) was used, and the pigments were detected by their absorption at 445 nm (18). The flow rate, gradient time, and initial solvent mixture were varied depending on the pigment composition of the sample and column used.

Spectroscopy. Absorption spectra were measured at 77 K with a Cary 17 spectrophotometer equipped with scattering transmission and Dewar attachments (7). Fluorescence spectra were measured at 77 K with a Perkin-Elmer MPF-3L fluorometer (8). All spectra were collected and stored in a Hewlett-Packard minicomputer. P700 was measured with a Perkin-Elmer Dual Beam Spectrophotometer from the light-induced decrease in absorption at 700 nm (4).

RESULTS

Three different Chl-protein complexes were recovered by gel electrophoresis of dissociated algal membranes. Each green band ran in the gel at the same relative rate as corresponding bands from spinach chloroplasts which have been previously identified as PSI (slowest migrating), PSII, and LHCII (5, 20). *Nannochloropsis salina* had a much smaller proportion of the PSI and PSII bands than spinach or barley and considerably more free Chl than normal higher plants (Table I).

The complexes were identified by the following characteristics: absorption and fluorescence emission spectra measured at 77 K (Fig. 1) and pigment content (Table II). The spectra of PSI are typical, with both more absorption and fluorescence emission at longer wavelengths. However, the long wavelength emission maximum is near 725 nm compared to 735 nm in spinach. Like spinach, the fluorescence yield of the algal PSI is very low.

The absorption spectrum of the PSII complex is also similar to that of spinach with a maximum at 670 nm and shoulder near 680 nm. The emission maximum near 686 nm is similar to that from spinach PSII, but the shoulder near 695 nm is not as prominent.

The absorption spectrum of the algal LHC complex is very different from that of spinach LHCP which has a peak at 676 nm and distinct shoulder at 670 in addition to a maximum at 650 nm from Chl *b*. The emission maximum of the algal LHC near 684 nm is at slightly longer wavelengths than that of spinach LHCP.

Table I. *Percentage of Total Chl Found in Each Chl-protein Band and in the FP Zone after Gel Electrophoresis*

Spinach data from Ref. 5 and barley from Ref. 12.

	N. salina	Spinach	Barley	
			Wild Type	Chl *b*-less
PSI	7	34	34	40
PSII	7	15	14	12
LHCII	58	50	50	32
FP	28	1	2	16

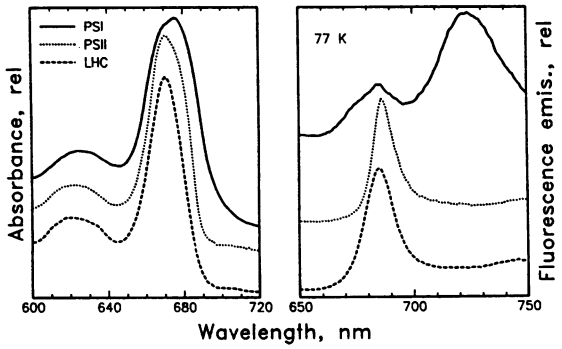

FIG. 1. Absorption and fluorescence emission spectra of three Chl-protein complexes from *N. salina*. Excitation at 438 nm, slitwidth = 10 nm, emission slitwidth = 3 nm.

Table II. *Number of Carotenoid Molecules for Each 100 Chl a Molecules Found in Cell Membranes, Each of the Isolated Chl-protein Complexes, and in the FP Zone following Gel Electrophoresis*

The retention times (t_R) are from the HPLC analyses (Fig. 2).

	t_R	Membrane	PSI	PSII	LHCII	FP
	min		*mol/100 mol Chl a*			
Neoxanthin	4.5	3	tr[a]	tr[a]	2	4
Violaxanthin	6.8	29	6	4	26	36
Vaucheriaxanthin[b]	8–10	21	4	1	25	12
β-Carotene	21	3	29	15	4	0

[a] Trace. [b] Sum of 3 HPLC bands.

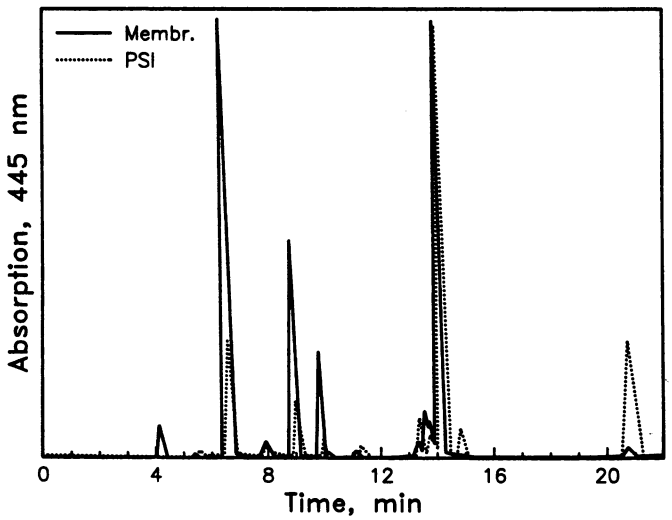

FIG. 2. Chromatograms of the pigments in *N. salina* membranes and PSI complexes, normalized to the peak at 14.2 min (Chl *a*), were performed with a C18 analytical column. The solvent gradient was from methanol:H_2O:acetone (8:1:1) to methanol:acetone (4:1) in 10 min.

Figure 2 shows chromatograms of the pigments extracted from *N. salina* membranes and isolated PSI complexes. They have been normalized to the height of the Chl *a* band at 14.2 min. The areas under these bands before normalization were used to calculate corresponding values in Table II. The chromatograms shown in Figure 2 were performed with an analytical column, but a similar chromatographic separation of the pigments from the algal membranes using a preparative column gave a sufficient amount of each pigment to enable measurement of its absorption

spectrum directly. Although the absorption maxima we observed are not identical to those given by Davies (11) because of solvent differences, they are close enough to justify the identification of neoxanthin at 4.5 min and violaxanthin at 6.8 min from their relative polarity and similar absorption maxima near 416, 440, and 470 nm. As expected, acidification caused the maxima of the monoepoxide, neoxanthin to shift downward by about 20 nm and those of the diepoxide, violaxanthin by 40 nm.

The pigments collected at 8.2, 9.1, and 10 min had similar absorption maxima near 420, 444, 472 nm, and each showed the 20 nm downward shift after acidification characteristic of a monoepoxide. Vaucherioxanthin-ester has these spectral properties and is a major xanthophyll in these algae (19). Antheraxanthin also has similar polarity and spectral properties and has been reported to be present as a minor component (2).

The component at 14.2 min had a spectrum characteristic of Chl *a*; the small peaks just before it are probably oxidized products of Chl *a*. Chromatograms of the pigments from the Chl-proteins frequently showed a component just after Chl *a* at 14.9 min which may be a Chl *a* isomer (24). The pigment at 20.8 min had the spectral properties and polarity typical of β-carotene (11).

Nannochloropsis cells (not shown) and washed membranes (Table II) had the same pigment content. P700 was detected only in the PSI complex where it was present in a ratio of about one per 160 Chl *a* molecules and enriched about 10 times compared to the cell membranes. Likewise, PSI and also PSII complexes were highly enriched (10 and 5 times, respectively) in β-carotene compared to the original membranes. On the other hand, the major xanthophylls were associated primarily with the LHC complex. Violaxanthin comprised about 55% of the total xanthophylls in the membranes. Our own experiments with spinach chloroplasts have corroborated those of Siefermann-Harms (23) which showed that the xanthophylls (especially violaxanthin) are less tightly bound to the Chl-proteins than β-carotene or Chl *b* and more easily detached during detergent treatment. Therefore, it is not surprising that a high proportion of violaxanthin ran into the free pigment zone. Vaucheriaxanthin-like pigments were also found mainly with the LHC complex.

Figure 3 shows absorption and fluorescence excitation spectra between 400 and 500 nm for Chl *a* emission of the washed membranes and isolated LHC complexes. Similar spectra from intact cells (not shown) were the same as those of the membranes. Chl *a* has an absorption maximum near 440 nm and shoulder around 420 nm. The carotenoids have several overlapping absorption bands throughout the blue spectral region, but are most clearly seen here between 450 and 500 nm. The broad excitation bands near 465 and 490 nm in the membrane spectra show that light energy absorbed by certain of these carotenoids can be transferred to Chl *a*.

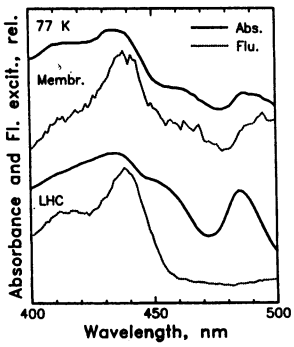

FIG. 3. Absorption and fluorescence excitation spectra of *N. salina* membranes and LHCII complexes. Emission at 685 nm, slitwidth = 6 nm, excitation slitwidth = 4 nm.

After treatment of the membranes with digitonin, the heights of the carotenoid excitation bands were decreased somewhat (not shown). Following exposure to DOC and electrophoresis, the xanthophylls still associated with the LHC complexes were no longer able to transfer energy to Chl *a*. By comparison, when spinach chloroplasts were treated similarly, the xanthophylls (primarily lutein and violaxanthin) retained considerable capacity for energy transfer to Chl *a* (6).

DISCUSSION

The results reported here provide further evidence to support the hypothesis that photosynthetic pigments are bound to certain specific membrane protein complexes in all plants. Although we have not yet analyzed the proteins themselves, we could isolate three Chl-protein complexes from *Nannochloropsis* with spectral characteristics and pigment content analogous to those isolated from higher plants. There are, however, some interesting differences which may help us to understand pigment organization in both algae and higher plants.

One difference is between the relative amounts of Chl *a* in PSI and light-harvesting complexes (Table I). In higher plants about 35% of the Chl *a* is closely associated with PSI whereas in *Nannochloropsis salina* this value falls to less than 10%. The high Chl/P700 ratio in the alga (about 1500) compared to about 400 for spinach chloroplasts shows that there are nearly 4 times fewer PSI reaction centers per Chl in the alga. Therefore, it is not surprising that we found relatively less PSI in the gels. When isolated by the procedure used here, spinach PSI includes a core complex containing the reaction center, P700, and an antenna complex, LHCI (26). LHCI is a Chl *a-b*, low mol wt complex, and when bound to the core complex causes the fluorescence emission with a maximum near 735 nm at 77 K. The Chl *a*-core complex alone has an emission maximum near 725 nm. The PSI complex isolated from *N. salina* has an emission maximum near 725 nm and seems to be analogous to the core complex of higher plants, although its unit size is much greater (160 Chl/P700 compared to 60 to 80 for a mutant barley core complex [12]).

The position in the gel after electrophoresis, absorption spectrum, and pigment content of the algal PSII complex are all properties similar to PSII from higher plants; but, like PSI, its relative proportion seems to be lower (7% compared to 15% for spinach, Table I).

In general, aquatic plants (like shade plants) have a higher proportion of their pigments serving as antenna than have most land plants (13). *N. salina* is no exception with most of its Chl *a* and xanthophylls associated with a LHCII complex. The relatively higher proportion of free pigment following electrophoresis of the alga compared to spinach under the same solubilization conditions suggests that its pigments are more loosely associated with protein, or the proteins are less stable than are those of spinach (Table I). It is more likely that most of the free pigments are detached from the LHC rather than the reaction center complexes because of the high proportion of xanthophylls observed in the FP zone. Others have noted that even in higher plants, the xanthophylls are much more loosely bound to the Chl-proteins than are Chl *b* and β-carotene (21, 22). Until recently, no antenna Chl *a*-protein complexes equivalent to LHCII were observed in Chl *b*-less mutants (9), probably because these pigment-proteins without Chl *b* are extremely labile and more susceptible to both protease and detergent action (12). The procedure used to isolate a Chl *a* antenna complex from the Chlorina-f2 barley mutant was the same as that applied here to *N. salina*, and the gel pattern for the fastest running green bands (LHCII and FP) were similar (Table I). The polypeptides of the mutant antenna complexes are similar to those of wildtype barley (12). Therefore, it will be of interest to compare the *N. salina*

polypeptides with those of Chl *b*-containing plants.

Lutein is the major xanthophyll in higher plants, but violaxanthin is also present in relatively large amounts (3, 17, 22). A light-induced de-epoxidation cycle between violaxanthin, antheraxanthin, and zeaxanthin has been described (25). Although *N. salina* has no lutein, violaxanthin is a major component, and there are significant amounts of several vaucheriaxanthin-like pigments (Fig. 2; Table II). Our experimental procedure did not allow a distinction between vaucheriaxanthin and antheraxanthin. Both types are monoepoxides and have similar absorption spectra and polarity. However, Norgard *et al.* (19) identified the major xanthophylls in GSB Sticho (now *N. salina*) as violaxanthin and vaucheriaxanthin-ester. Because of a possible de-epoxidation cycle *in vivo*, the small peak at 8.2 min could be antheraxanthin; the peaks at 9.1 and 10.1 min may be forms of vaucheriaxanthin. A violaxanthin cycle has been observed in *Pleurochloris commutata*, a close relative of *N. salina* (25). The Xanthophyceae, on the other hand, have a diadinoxanthin-diatoxanthin cycle, but no violaxanthin (25), and therefore are less like higher plants in this respect.

Our results show (Fig. 3) that pigments absorbing between 450 and 500 nm are able to transfer absorbed light energy to Chl *a* in the cell membranes. Although the isolated LHC complexes are enriched in xanthophylls and have comparatively more absorption in this spectral region, energy transfer ability has been lost. As discussed above, this loss is most probably because of a relatively loose attachment between pigments and protein. By use of different detergents and procedures, it may be possible to keep the pigments more closely bound. We can conclude that either violaxanthin and/or vaucheriaxanthin are able to act as antenna pigment for photosynthesis, but further work will be necessary to distinguish between them. Recently an LHCII complex was isolated from *Polyedriella helvetica*, an Eustigmatophyte with a pigment composition very similar to *N. salina* (10). These authors also concluded that violaxanthin is an antenna pigment, but did not rule out a similar function for the other xanthophylls. β-Carotene is probably not a significant antenna pigment because of its very low amount in the LHC complexes, but it may play a role in transferring absorbed energy directly to the reaction centers. The photoprotective role of β-carotene has been considered elsewhere (16).

Comparison of the protein part of the isolated pigment complexes with those of other plants should give information about the phylogenetic position of the Eustigmatophyceae and is planned for future experiments.

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