A CHLOROPHYLL *c*-CONTAINING PIGMENT–PROTEIN COMPLEX FROM THE MARINE DINOFLAGELLATE, *GLENODINIUM* SP.

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1. Introduction

Photosynthetic pigments of green plants are associated with distinct proteins in the thylakoid membranes [1,2]. Investigations of the organization of pigments in other plant phyla have received much less attention. This applies particularly to the chl *a*–chl *c*–carotenoid systems of brown algae, diatoms, and dinoflagellates (however, see [3–5]). These groups of algae are responsible for the majority of the primary productivity in marine environments, and therefore are of considerable interest.

Dinoflagellates differ from brown algae and diatoms in that they contain only one chl *c* type [6]; and peridinin rather than fucoxanthin is the major light-harvesting carotenoid [7]. Initial investigation of the organization of dinoflagellate chlorophylls and carotenoids has resulted in the characterization of two major chl *a*-containing proteins. Peridinin was obtained as a water-soluble protein complex with chl *a*, and this complex, PCP, was shown to function as a major light-harvesting component in several dinoflagellates [8]. Second, a P700-chl *a*–protein was isolated from both *Glenodinium* sp. and *Gonyaulax polyedra*.

This study furthers the investigation of the photosynthetic apparatus in *Glenodinium* sp. It concerns itself with the development of procedures for solubilization and fractionation of dinoflagellate thylakoid membrane components. A partial characterization of 4 pigment protein complexes is presented. One complex of particular interest appears to be a chl *a*–chl *c*–protein-containing fraction which contains most of the chl *c* in the organism.

Abbreviations: SDS, sodium dodecyl sulfate; chl, chlorophyll; LS, sodium-*N*-lauroyl sarcosine; TLC, thin-layer chromatography

2. Materials and methods

2.1. *Growth conditions*

Standard conditions for the mass-culturing of *Glenodinium* sp. (L. Provasoli, M. Bernard Strain; UCSB code no. 5M29) have been detailed [8]. From illuminated 180 liter vats of enriched seawater medium (22°C, 500 μW . cm^{-2}) [9], late exponential cultures were harvested by continuous flow centrifugation (Sharples, Model T-1). The cells were resuspended in 50 mM Tris/HCl–1 mM EDTA (pH 8.0), and readjusted to pH 8.0 with HCl if necessary. The cells were frozen rapidly in liquid N_2 and then stored at −14°C.

2.2. *Sample preparation*

Thawed cell suspensions were sonicated at 0°C (Sonifier model W185; 70 W for 2 min on/off intervals) until 80–90% cell breakage was achieved, as estimated by microscopic examination. A total of 8–10 min sonication time was usually necessary. Whole cells and larger cellular debris were separated by centrifugation at 270 × *g*, 10 min, 4°C. The supernatant was clarified further by centrifugation (27 000 × *g*, 70 min, 4°C). The pellet containing thylakoid fragments was washed by recentrifugation (27 000 × *g*, 40 min, 4°C) in 50 mM Tris/HCl (pH 8.0) containing 1 mM EDTA. The final pellet was resuspended in buffer, quick-frozen in a liquid nitrogen bath and stored at −14°C. Membrane preparations were stored for ≤2 weeks and showed no spectral changes during this time.

Chloroplast lamellae were solubilized in 1.5% SDS

at a ratio of SDS/chl *a* of 33:1. The mixture was homogenized in a Ten Broeck tissue grinder (Corning) and centrifuged (40 000 × *g*, 15 min, 10°C). SDS extract (7 μl) was loaded onto each polyacrylamide tube gel (0.5 cm × 8.0 cm) [10]. The gels contained 5% acrylamide, 0.25% *N,N'*-methylene bis acrylamide, 0.125% ammonium persulfate, 0.1% (v/v) *N,N,N',N'*-tetramethylethylene diamine, 6.2 mM Tris, 48 mM glycine, and 0.1% Deriphat 160. Electrophoresis was carried out at 65 V for 20–30 min with an electrode buffer of 12.4 mM Tris, 96 mM glycine, and 0.2% Deriphat 160. Immediately following electrophoresis, gel scans were made at 670 nm on a Beckman Acta III Spectrophotometer equipped with a gel scanner attachment, photocopied, and the peaks cut out and weighed for determination of relative chl *a* distribution. Chl-containing bands were eluted by excising gel slices and forcing them through discs of Nitex netting placed at the bottom of a syringe. The resulting material was loaded onto a Bio-Gel P-150 (BioRad) column (1.2 cm × 2.6 cm) and the colored material eluted with electrophoresis buffer.

2.3. *Characterization*

Gels were stained for protein with 0.2% Coomassie Blue R in methanol/H_2O/acetic acid (5:5:1, by vol.) following overnight fixation in 10% acetic acid, and destained in methanol/H_2O/acetic acid (5:5:1, by vol). Proteins used in electrophoretic estimation of the molecular sizes of the algal pigment protein complexes were: myosin (200 000); β-galactosidase (130 000); phosphorylase B (92 000); bovine serum albumin (68 000); ovalbumin (43 000); carbonic anhydrase (31 000); trypsin inhibitor (21 000); and lysozyme (14 300). The marker proteins were denatured by boiling for 3 min in 6.2 mM Tris/48 mM glycine/1% SDS/1% 2-mercaptoethanol/10% (v/v) glycerol, then run simultaneously with the unknowns on the Deriphat electrophoresis system.

Eluted band II was re-electrophoresed using a Laemmli [12] buffer system. The sample was boiled for 2 min in 1% 2-mercaptoethanol, 10% (v/v) glycerol, and 1% SDS or applied directly to the gel. Marker proteins were used as above.

Absorption spectra of solutions and gel slices were measured by an Aminco DW-2 spectrophotometer. [Chl] and pigment ratios were determined using the procedures in [8]. Some samples were extracted with diethyl ether and concentrated under N_2 for chromatographic analysis of carotenoids. TLC was carried out on Silica Gel 60 plates (Merck) developed in isooctane/acetone/diethyl ether (3:1:1, by vol.).

Tris, LS, SDS, ammonium persulfate, and glycine were purchased from Sigma, St Louis, MO; acrylamide (electrophoresis purity), Triton X-100, and *N,N'*-methylene-bis acrylamide from BioRad, Richmond, CA; and *N,N,N',N'*-tetramethylethylenediamine from Eastman Kodak, Rochester, NY Deriphat 160 was a generous gift from the Henkel, Kanakee, IL.

3. Results

Before the electrophoretic fractionation system in section 2 had been adopted as the standard separation procedure, other electrophoretic systems had been examined. Pelleted membranes of *Glenodinium* sp. suspended in 50 mM Tris/HCl 1 mM EDTA (pH 8.0) were treated with different detergents and detergent concentrations to evaluate their ability to solubilize the chloroplast membranes. The supernatants of the different detergent-solubilized thylakoids were loaded onto a variety of acrylamide gel systems, so that fractionation band patterns and band stability could be judged.

Triton X-100 used either as the solubilizing detergent or in electrophoresis buffers proved inferior to SDS. Triton X-100 incompletely solubilized the membranes at a ratio of 75:1 g/g chl *a*, and what was solubilized migrated as free pigment (cf. [1]). Thus, SDS was used as the solubilizing detergent. However, as indicated in [5,11] all pigment complexes, with the exception of P700–chl *a*–pigment protein, on gel electrophoresis in 5% polyacrylamide gels containing 0.1% SDS quickly dissociated to yield free pigment. The ratio of SDS/chl *c* with the extract medium was varied from 10/1 to 25/1. But, if the electrophoretic system of [10] is used, in which SDS is replaced in the electrophoretic buffer and gel by a zwitterionic detergent, Deriphat 160-C (monosodium *N*-lauryl-β-iminodiproprianate), then chl–protein complexes from *Glenodinium* sp. remained stable through 30 min electrophoresis. During this time, little or no free pigment was formed. An optimum SDS/chl *a* ratio of 33:1 was arrived at. We tried substituting LS for some of the SDS but without improving the separating system. Thus we arrived at the separation procedure in section 2 which we found to give a reproducible pattern.

The gel banding pattern for the system is shown in

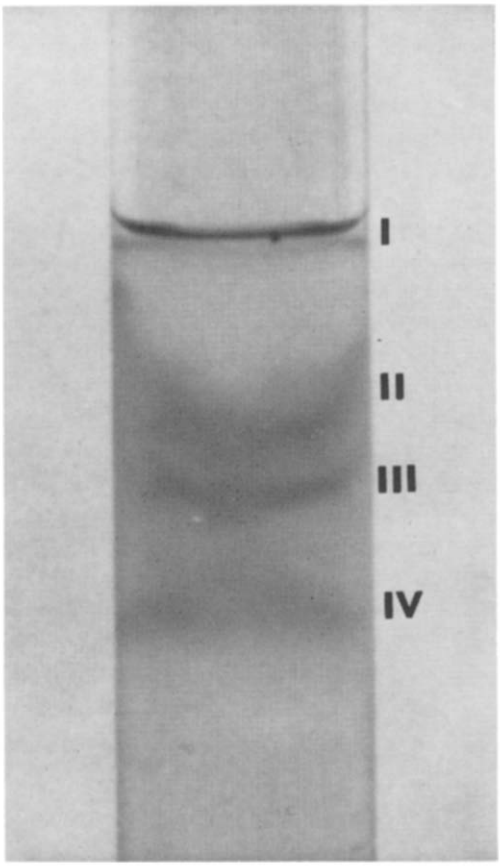

Fig.1. Electrophoretic pattern of *Glenodinium* sp. SDS-solubilized thylakoid membranes.

fig.1. Four pigmented bands are resolved. All bands stain for protein (free pigment can be observed running below band IV particularly when higher detergent concentrations are used). A green band I, with a molecular weight equivalence of 160 000 contained ~20% of the chl *a* loaded on the gel. The absorption spectra of band I is shown in fig.2. The eluted material was extracted into organic solvent and the pigments examined: both the chl *a*/chl *c* ratios and TLC indicated that chl *a* was the predominant chl form present.

Green band II contained ~30% of the chl *a* and most of the chl *c* in the gel and had a molecular weight equivalence of 50 000. The chl *c*/chl *a* molar ratio of 4.8 ± 0.6 (n = 5) is highly enriched as compared to the ratios in the initial homogenate (c/a = 1.4) or whole cells (c/a = 1.0). TLC confirmed the presence of chl *a* and chl *c* and showed the presence of yellow xanthophylls, (probably dinoxanthin and diadino-

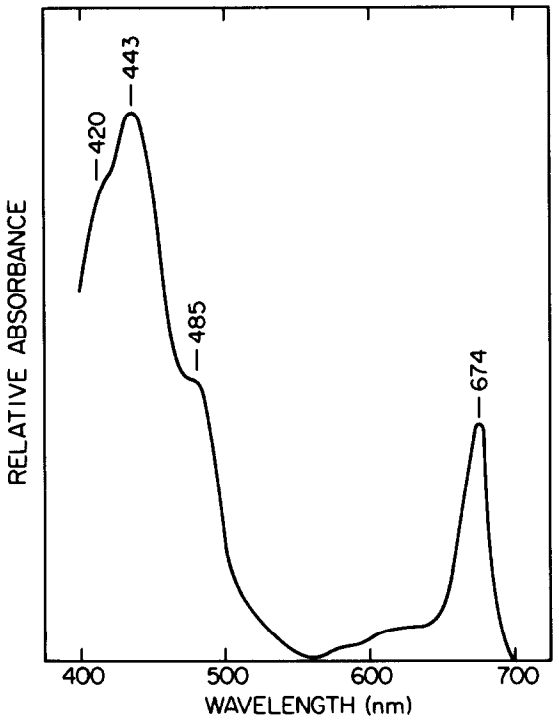

Fig.2. Room temperature absorption spectrum of band I recorded in the gel slice.

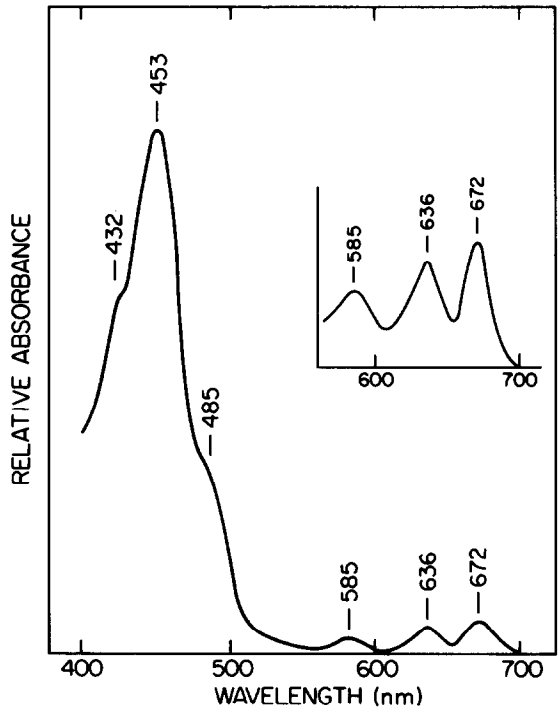

Fig.3. Room temperature absorption spectrum of band II of *Glenodinium* sp. recorded in gel slice.

xanthin, although peridinin was also sometimes observed). The absorption spectrum of band II gel slices has maxima or shoulders at 432, 453, 485, 585, 636 and 672 nm (fig.3), which were conserved upon elution. Maxima at 453, 585 and 636 nm are attributed to chl *c* and those at 432 and 672 nm to chl *a*. The red absorption maxima blue shifts upon heating (5 min at 100°C), suggesting detachment of the chlorophylls from their protein component. Preliminary results from Laemmli gels indicate a major polypeptide of 20 000 mol. wt in the dissociated sample.

Band III and IV, orange components, contained ~21% and 29%, respectively, of the chl *a* distributed on the gel and have an apparent molecular weights of ~42 000 and 32 000. Their absorption spectra are shown in fig.3,4. Neither component contains appreciable amounts of chl *c*; however, peridinin was detected in the excised slices of both bands. Upon elution from the gel, all bands, except band II, showed some spectral shifts indicating disruption of chl organization.

4. Discussion

Major obstacles to the study of the chl organization in the dinoflagellate thylakoid membrane have been the problems inherent in using detergent solubilization techniques: the generation of free pigment and the incomplete solubilization of the membrane in cells. Using SDS solubilization coupled with the electrophoretic system of [10] four discrete, reproducible pigment–proteins were fractionated from the thylakoid membrane of the dinoflagellate *Glenodinium* sp. Excised band I, upon re-electrophoresis on SDS gels [5] gave rise to a single high molecular weight band in a position similar to that of conventional CPI, P700–chl *a*–protein complex of *Glenodinium* sp. [5]. However, preliminary absorption spectrum indicated a 675 nm chl *a* peak, a secondary 660 nm peak (perhaps indicative of dissociating chl *a*), and the continued presence of the 485 nm shoulder.

Band II contained the chl *c* and 30% of total chl *a*. This material is the first chl *c*-containing component isolated from a dinoflagellate [5]. Previous arguments [5] made it likely that chl *a* and *c* occurred in vivo in a pigment–protein complex highly enriched in chl *c*. Based on calculations, a chl *c*/chl *a* ratio of 5–8/1 was suggested. Such a ratio occurs in band II (4.8 ± 0.6). The reproducible pigment ratio tends to indicate that the pigments in band II occur in a single entity, rather than in two different complexes. Initial studies of the excitation spectra of fluorescence indicate some energy transfer between the two pigments, supporting the idea of a single complex. The 20 000 mol. wt polypeptide observed upon dissociation of band II may be the chl-binding apoprotein.

The absorption maxima of band II at 585, 636 and 672 nm, compare closely to those seen in [4] in a chl *a*/chl *c*-containing fraction from the brown alga, *Acrocarpia paniculata*; however, the chl *c*/chl *a* ratio in the chl *a*–chl *c*-complex from *Glenodinium* sp. at 4.8/1 is much greater than that found in the complex from *A. paniculata* (0.33/1). This is relected in the relative intensities of the chl *c* and chl *a* associated peaks in these algae. It should be noted that *Glenodinium* sp. contains only the c_2 form of chl *c*, while *A. paniculata* contains both c_1 and c_2.

A fraction of peridinin in *Glenodinium* sp. appears closely associated with the membrane, and can only be partially released by subsequent repeated aqueous extraction [11]. It is tempting to suggest that bands III and IV may represent membrane-bound peridinin.

Based on the previously described distribution of chl in dinoflagellates, a working model for the organization of these components into a functional PSU has been proposed [5]. The existence of a second major light-harvesting component in dinoflagellates, an integral chl *a*–chl *c*–protein complex, as predicted by this model appears to be confirmed.

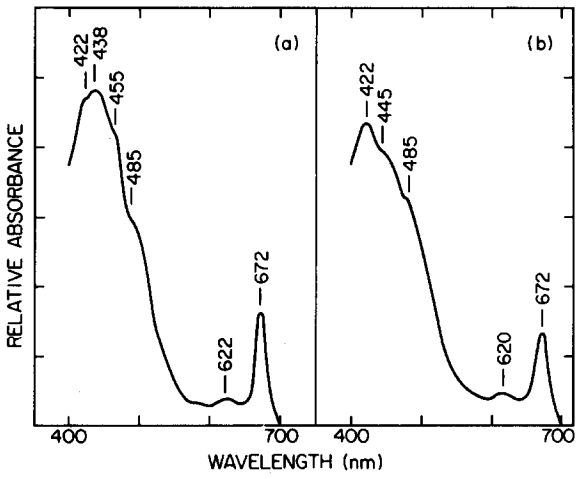

Fig.4. Room temperature absorption spectra of bands III and IV recorded in gel slice.

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