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Photosynthetic unit size, carotenoids, and chlorophyll–protein composition of *Prochloron* sp., a prokaryotic green alga

(photosynthesis/P700/light-harvesting/symbiont)

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ABSTRACT **Six samples of the prokaryotic, unicellular algae *Prochloron* sp., which occur in association with didemnid ascidians, were collected from various localities in the tropical Pacific Ocean, and their pigments and chlorophyll-protein complexes were identified and characterized. No phycobilin pigments were detected in any of the species. Chlorophylls *a* and *b* were present in ratios of *a*/*b* = 4.4–6.9. The major carotenoids were β-carotene (70%) and zeaxanthin (20%). Minor carotenoids of one isolate were identified as echinenone, cryptoxanthin, isocryptoxanthin, mutachrome, and trihydroxy-β-carotene; no ε-ring carotenoids were found in any sample. Except for the absence of glycosidic carotenoids, the overall pigment composition is typical of cyanobacteria. A chlorophyll *a*/*b*–protein complex was present in *Prochloron*; it was electrophoretically and spectrally indistinguishable from the light-harvesting chlorophyll *a*/*b*–protein of higher plants and green algae. It accounted for 26% (compared to ~50% in green plants) of the total chlorophyll; 17% was associated with a P700-chlorophyll *a*–protein. The photosynthetic unit size of 240 ± 10 chlorophylls per P700 in *Prochloron* was about half that of eukaryotic green plants. A model is proposed for the *in vivo* organization of chlorophyll in *Prochloron*.**

Prochloron is a unicellular, prokaryotic marine alga that, to date, has been found to be associated only with didemnid ascidians and, recently, has been placed in a new division, the Prochlorophyta (1). These algal cells have been shown (*i*) to lack membrane-bound organelles and a defined nucleus, (*ii*) to lack phycobilisomes normally present in cyanophyta, (*iii*) to possess chlorophyll *b*, an accessory pigment hitherto found only in eukaryotes, and (*iv*) to show some stacking of photosynthetic lamellae (1–6).

Since the pigment composition is unusual for a prokaryotic alga, it seemed possible to us that the organization of chlorophyll *in vivo* and the carotenoid composition of the photosynthetic lamellae may also be at variance with other prokaryotes. Recognizing from previous studies (7, 8) that chlorophyll *b* in green plants is located solely in the major chlorophyll-binding protein, termed the light-harvesting chlorophyll *a*/*b*–protein or Complex II (8), we were interested to determine if a homologous component was also present in *Prochloron*. We also looked for the photosystem I reaction-center complex, the P700–chlorophyll *a*-protein or Complex I (8, 9), a ubiquitous component of all examined chlorophyll *a*-containing organisms (7, 8). From such investigations it proved possible to compare the organization of these chlorophyll–proteins in *Prochloron* with their organization in the green plant and in the cyanobacterial photosynthetic unit (PSU) described previously (8). The studies reported here provide some insight into the position and role of this organism in the evolution of the prokaryotic and eukaryotic algal groups and of the higher plant chloroplast.

MATERIALS AND METHODS

The algae were collected from the field as described (2–4). Six samples of *Prochloron* cells were collected from five locales: (*i*) endozoic in *Lissoclinum patella:* Rodda Reef, N. Queensland, Australia, (*iii*) endozoic in *Diplosoma virens:* Magnetic Island, Queensland, Australia, (*iii*) endozoic in *D. virens:* Coconut Island, Kaneohe Bay, Oahu, HI, (*iv*) sporadically epizoic on *Didemnum* sp. (grey colonies): Isla San José, Baja California, Mexico, (*v*) sporadically epizoic on *Didemnum* sp. (white colonies): Isla San José, Baja California, Mexico, (*vi*) endozoic in *Tridemnum cyclops:* Eniwetok, Marshall Islands, United States Trust Territories of the Pacific. The algal cells from all of these sites appeared similar, though those from *Didemnum* were smaller (6–8 μm) and those from *Lissoclinum* were larger (12–16 μm) than those from *Diplosoma* and *Tridemnum* (8–12 μm). The algal cells were centrifuged at 2000 × *g* and lyophilized. In addition, a sample of viable *Prochloron* cells was transported in seawater, by airplane, from Hawaii to California and used to measure their liquid nitrogen (77K) absorption spectrum on a single beam spectrophotometer (10). A fourth-derivative spectrum was calculated with a number of digitizing intervals (dX) equal to 10.

Pigments were extracted by sonicating lyophilized cells successively in 100%, 90%, and 80% acetone and transferring the extracts to ether as described (3). The chlorophyll *a* and *b* contents were determined spectrophotometrically (11). Acetone extracts of *Scenedesmus obliquus* were used to provide authentic chlorophyll *b* for cochromatography on thin layers (12).

Carotenoids were separated by column chromatography on neutral Al_2O_3 (activity grade III) developed with ether/pe-

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Abbreviations: PSU, photosynthetic unit; $NaDodSO_4$, sodium dodecyl sulfate.

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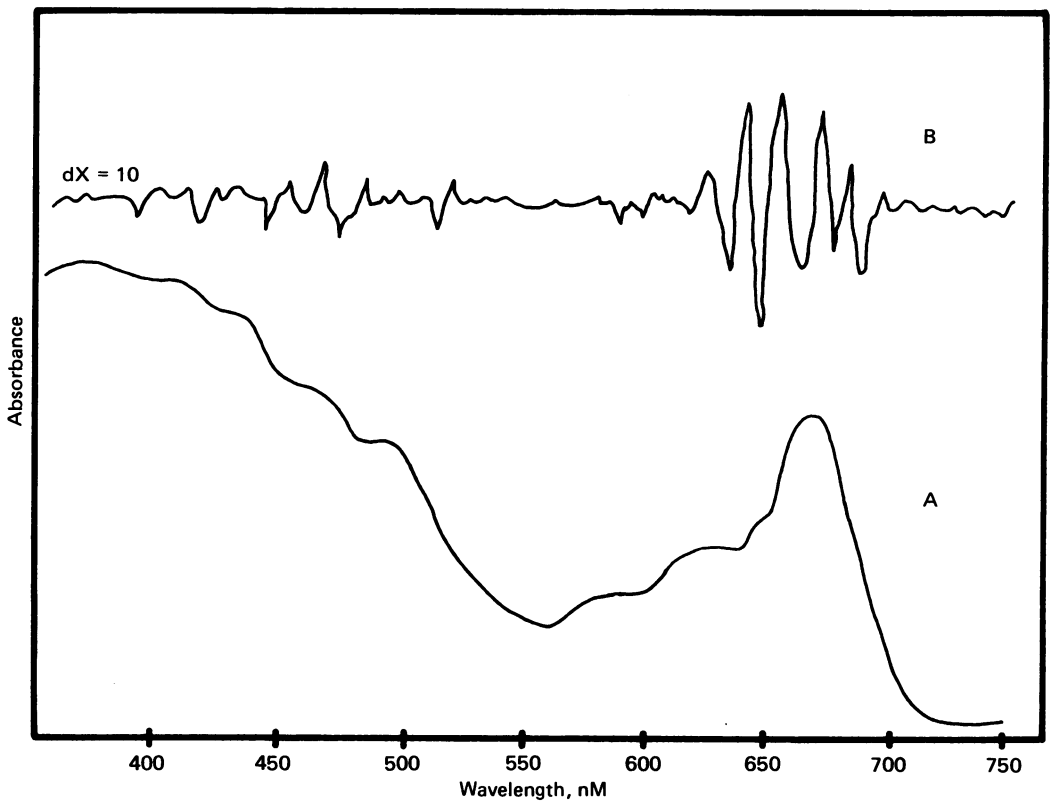

FIG. 1. Absorption spectrum of intact, viable *Prochloron* cells (from host didemnid, *Diplosoma virens;* Coconut Island, Kaneohe Bay, Oahu, HI). Curve A: Absorption spectrum of whole cells recorded at 77 K (12). Curve B: Fourth-derivative of curve A with a dX (number of digitizing intervals) equal to 10 (12).

troleum ether mixtures (0–100%, vol/vol) and by thin-layer chromatography on silica gel G and MgO/Kieselguhr, 1:1. Carotenoids were identified by comparison of their absorption and mass spectral characteristics with those of authentic samples and by cochromatography with standards. Confirmation of the presence of echinenone in *Prochloron* was obtained by its reduction (KBH_4 in ethanol) to isocryptoxanthin followed by comparisons of the reduced product with standard isocryptoxanthin. Published $E^{1\%}_{1\,cm}$ values for quantitative estimates of carotenoids were employed (12). The standard carotenoids—zeaxanthin, β-carotene, cryptoxanthin, and echinenone—were obtained from Hoffmann–La Roche; isozeaxanthin and isocryptoxanthin were obtained by KBH_4 reduction of authentic canthaxanthin and echinenone (Roche), respectively.

Mass spectra were determined with an AEI-MS 12 mass spectrometer using a direct probe at an ionizing voltage of 70 eV with an ion source temperature of 180°.

Sodium dodecyl sulfate ($NaDodSO_4$) solubilized lamellae were prepared (7, 13) from cells of *Prochloron* (samples 1 and 3 of Table 2). Cells were broken by one pass through a French pressure cell at 12,000 pounds per square inch (83 MPa). The lamellae were pelleted from the homogenate and washed once in 50 mM Tris·HCl, (pH 8.0). The washed lamellae were solubilized in $NaDodSO_4$ ($NaDodSO_4$/chlorophyll = 10:1, wt/wt) as described (13). The $NaDodSO_4$ extracts were separated by $NaDodSO_4$ gel electrophoresis using described procedures (7, 13). Cells of *Scenedesmus obliquus* were treated similarly and the *Scenedesmus* extract was co-electrophoresed with extracts from *Prochloron*. The PSU size in *Prochloron* was calculated from the ratio of total chlorophyll to P700, each determined separately (9) in Triton X-100 extracts of the photosynthetic lamellae (9).

RESULTS

Chlorophyll and light-harvesting pigments

The absence of phycobilin pigments in *Prochloron* sp. was indicated by the absence of absorption bands typical of phycoerythrin, phycocyanin, and allophycocyanin between 550 and 650 nm (Fig. 1). Furthermore, aqueous extraction of sonicated cells did not yield any detectable biliproteins. These observations, together with some previous studies on the fluorescence properties (3–5) of these algae, establish that bilipigments, which are characteristic of cyanobacteria, are not present in *Prochloron* sp. One of the chlorophylls extracted from these algae (samples 1–5 of Table 2) cochromatographed with authentic chlorophyll *b* and had the same absorption maxima as those published (14) for chlorophyll *b*. Absorption and fourth-derivative spectra of whole cells (Fig. 1) showed that chlorophyll *a* (maxima at 660, 677, and 688 nm) was present as well as chlorophyll *b* (maximum at 647 nm). The chlorophyll *a*/*b* ratios ranged from 4.4 to 6.9. The total chlorophyll *a* content of *Prochloron* sp. (sample 1) was 6.74 mg/g dry weight.

The occurrence of chlorophyll *b* in *Prochloron* cells was further substantiated by detection on polyacrylamide gel electrophoretograms (7, 8, 13) of a chlorophyll–protein complex that had a spectrum (red maxima at 652 and 671 nm) and electrophoretic mobility (Fig. 2) identical to those reported for the chlorophyll *a*/*b*–protein (Complex II) of green plants (7, 8, 13) (see Fig. 2). The amount of this complex present in cells of this alga (*Prochloron* sample 1) is precisely what would be expected from cells with a chlorophyll *a*/*b* ratio of 6.6 (cf. ref. 8). The $NaDodSO_4$-altered form of the P700–chlorophyll *a*–protein (Complex I) was also detected on $NaDodSO_4$ gels (Fig. 2). It accounted for 17% of the total chlorophyll, a value inter-

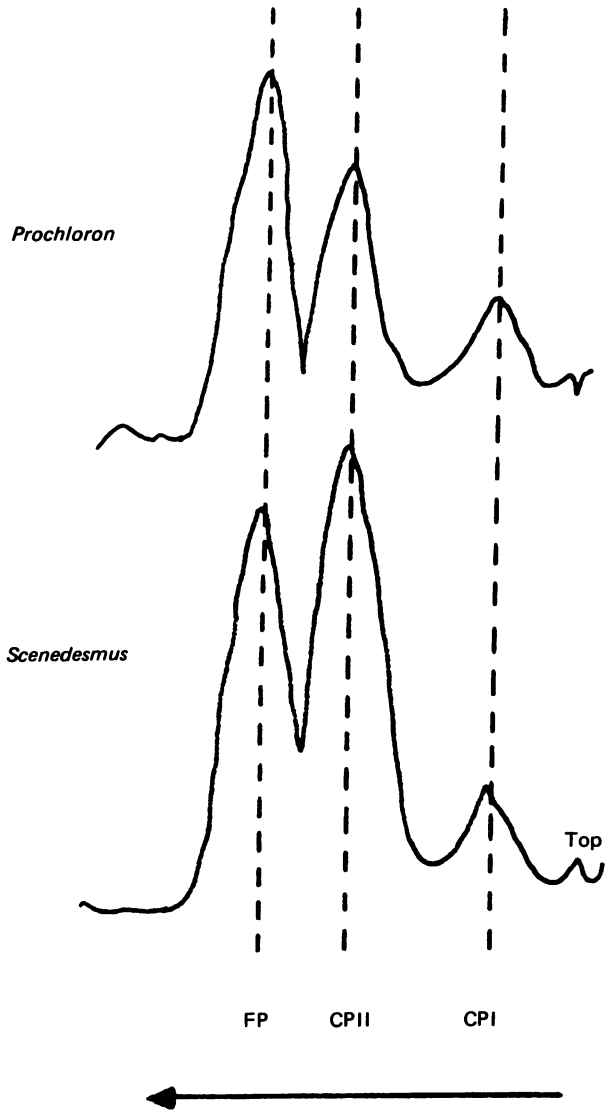

FIG. 2. Electrophoretograms of $NaDodSO_4$-solubilized lamellae of *Prochloron* sp. (sample 1) and *Scenedesmus obliquus*, showing the common presence and electrophoretic mobilities of Complex I (CPI) (the $NaDodSO_4$-altered form of the P700–chlorophyll *a*–protein), Complex II (CPII) (the light-harvesting chlorophyll *a*/*b*–protein), and free pigment (FP).

mediate between the values found in cyanobacteria (~30%) and eukaryotic chlorophytes (~10%) (8). The ratio of total chlorophyll to P700, a measure of the PSU size, was 240 ± 10 chlorophylls per P700 in *Prochloron*. This value is also intermediate between those found in cyanobacteria (180/1) and green plants (400/1) (8).

Carotenoids

β-Carotene, zeaxanthin, β-cryptoxanthin, echinenone, mutatochrome, and isocryptoxanthin were identified in *Prochloron* sp. (sample 1). Total carotenoids amounted to 1.77 mg/g dry weight. The quantitative carotenoid composition of *Prochloron* sp. (sample 1) is given in Table 1. The major carotenoids of the five other *Prochloron* isolates examined were essentially the same (Table 2).

Table 1. Carotenoid composition of *Prochloron* sp.*

Carotenoid	% of total
β-Carotene (β,β-carotene)	65†
Mutatochrome (5,8-epoxy-5,8-dihydro-β,β-carotene)	3
Isocryptoxanthin (β,β-caroten-4-ol)	1
Cryptoxanthin (β,β-caroten-3-ol)	7
Zeaxanthin (β,β-caroten-3,3′-diol)	20
Echinenone (β,β-caroten-4-one)	3
Trihydroxy-carotene (β,β-carotene-3,4′,4-triol?)‡	1
Total	100

*Associated with the didemnid ascidian *L. patella* on Rodda Reef, Heron Island, Australia (sample 1 in Table 2).
† β-Carotene amounted to 3% (by weight) of the total hydrocarbons in *Prochloron* sp.
‡ Assignment of hydroxyl group positions only tentative.

Neither those carotenoids specific to the Chlorophyta (α-carotene, lutein, loroxanthin, violaxanthin, and neoxanthin) nor those specific to the Cyanobacteria (β,β-carotene-2,3,3′-triol, β,β-carotene-2,3,2′,3′-tetraol, and myxoxanthophyll) were detected in *Prochloron* sp. (15, 16). One minor carotenoid, with the properties of a β,β-carotene triol (λ max at 475, 449, and 425 nm in light petroleum; mass spectrum with a molecular ion at *m/e* 584), was detected and could be readily separated from β,β-carotene-2,3,3′-triol (from *Anacystis nidulans*) (16) by thin-layer chromatography. The ready loss of water from the parent ion, shown in the mass spectrum of the *Prochloron* compound, indicated the presence of at least one allylic hydroxyl group. Indication of this water loss was much stronger in the mass spectrum of the *Prochloron* compound than in that of β,β-carotene-3,4,3′-triol, although the two samples could not be separated by thin-layer chromatography. It is concluded that the *Prochloron* compound could be β,β-carotene-3,4′,4-triol, though no authentic sample was available for comparison.

DISCUSSION

The photosynthetic pigment complement of *Prochloron* sp. combines features of both cyanobacteria and chlorophytes. The combined presence in *Prochloron* of β-carotene and zeaxanthin (70 and 20%, respectively) as major carotenoids and of echinenone, cryptoxanthin, and isocryptoxanthin as minor constituents is typical of cyanobacteria (15). However, the glycosidic carotenoids (e.g., myxoxanthophyll) and β,β-carotene-2,3,3′-triol and 2,3,2′,3-tetraol commonly found in cyanobacteria (16) are absent. Furthermore, *Prochloron* lacks ε-ring carotenoids (α-carotene, lutein, and loroxanthin) and violaxanthin and neoxanthin, which are common to the chlorophytes (17). The carotenoid composition of *Prochloron* sp. (sample 1) (Table 1) is, however, both qualitatively and quantitatively similar to that of *Phormidium persicinum*, a red, filamentous cyanobacteria that has no glycosidic carotenoids (15).

The measured chlorophyll *a*/*b* ratios of five samples ranged from 4.36 to 6.92 (mean = 6.1), indicating that proportionally less chlorophyll *b* is synthesized by *Prochloron* than by eukaryotic green algae and higher plants, which typically have ratios in the range of 2–3 (7). A ratio of 6 is similar to that found in *Euglena* (7). The presence in *Prochloron* of chlorophyll *b* and its localization *in vivo* in the light-harvesting chlorophyll *a*/*b*–protein are uniquely characteristic of green algae and higher

Table 2. Major carotenoids (expressed as % total) and chlorophyll *a*/*b* ratios of six samples of *Prochloron* sp.

Location and host ascidian	Rodda Reef, *L. patella*	Magnetic Island, *D. virens*	Coconut Island, *D. virens*	Isla San José, *Didemnum* sp. white var.	Isla San José *Didemnum* sp. grey var.	Eniwetok, *Tridemnum* cyclops
Carotenoids						
β-Carotene	65	63	71	70	68	+++
Zeaxanthin	20	25	20	19	20	++
Cryptoxanthin	7	5	2	ND	ND	
Minor carotenoids	8	6	6	11	12	+
Total	100	99	99	100	100	
Chlorophyll *a*/*b*	6.6	6.4	6.3	4.36	6.9	ND

ND, Not determined.

plants (7, 8). However, in *Prochloron* 26% of the total chlorophyll is associated with the chlorophyll *a*/*b*-protein, a value considerably lower than that (approximately 50%) generally found in green algae and higher plants (7, 8).

Typically, the PSU size of cyanobacteria is about 180 chlorophylls per P700, whereas the ratio in chlorophytes is usually around 400 (9). The smaller PSU size in cyanobacteria is partly attributable to the fact that a large proportion of the light-harvesting pigment is represented by bilipigments, which do not contribute to the PSU size as measured by the chlorophyll/P700 ratio. In fact, a recent (18) measurement in a cyanobacterium of the PSU size, in terms of the ratio of total light-harvesting pigments to reaction center, indicated a size still smaller than that in chloroplasts but surprisingly similar to that found in *Prochloron*. Based on the smaller proportion of chlorophyll contributed by the chlorophyll *a*/*b*-protein in *Prochloron*, it can be calculated (8) that the PSU size in this organism should be only 60% of that typically found in green algae and higher plants. This is precisely the size measured (240 ± 10 chlorophylls per P700).

The proposed model for the organization of chlorophyll into photosynthetic units in *Prochloron* sp., based on a PSU size of 240 and a chlorophyll *a*/*b*-protein content of 26% (equivalent to 60 of the 240 chlorophylls in the PSU), is given in Fig. 3. This model is consistent with one proposed previously (8) for the green plant and cyanobacterial unit. In the photosynthetic apparatus of this prokaryotic oxygen-evolving organism, the phycobilin-proteins typical of cyanobacteria are almost certainly replaced as light-harvesting components by the chlorophyll *a*/*b*-protein (see above). Thus, this chlorophyll-protein would be expected to feed the bulk of its gathered light energy to the photosystem II reaction center (Fig. 3) (cf. ref. 4). That an efficient organization of light-harvesting and reaction-center components exists in *Prochloron* is indicated by the fact that these cells have a photosynthetic capacity equivalent to that of a green alga such as *Chlorella* (4).

The prokaryotic alga *Prochloron* sp. thus embodies photosynthetic features of both green plants and cyanobacteria. This new genus possesses many characteristics that could place it as a transition organism between the cyanobacteria and the chlorophytes; it may even be a descendant of the postulated progenitor of the green plant chloroplast.

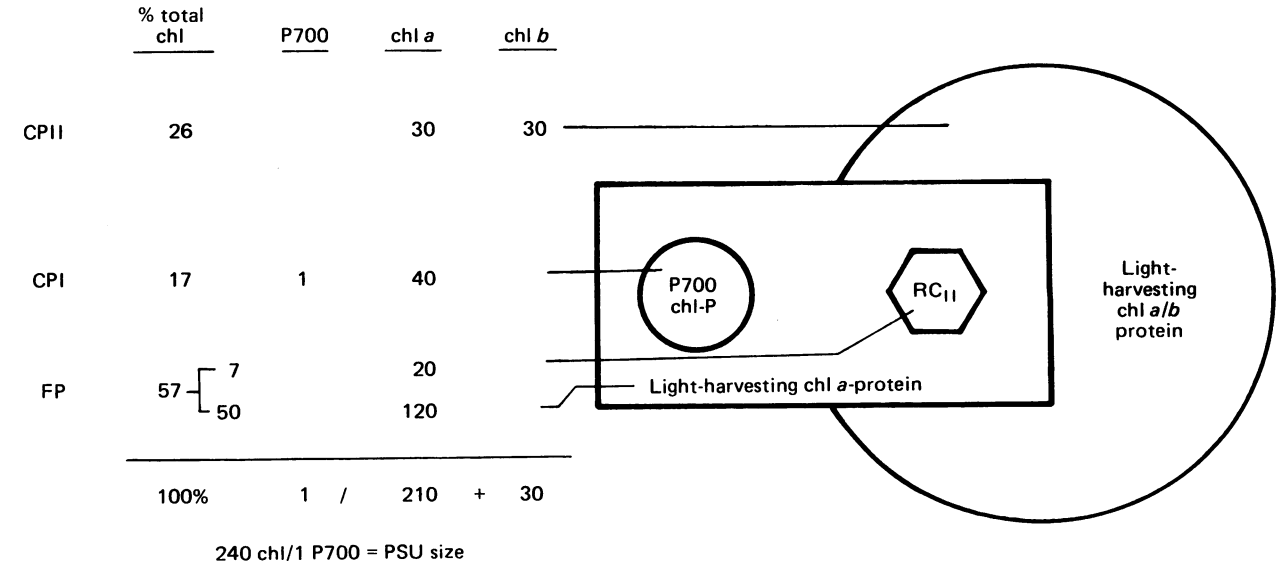

FIG. 3. A proposed model for the *in vivo* organization of chlorophyll (chl) in *Prochloron* sp., based on a PSU size of 240 chlorophylls to 1 P700 and the presence of 26% of the total chlorophyll of the PSU in the light-harvesting chlorophyll *a*/*b*-protein [after Thornber *et al.* (8)]. Chl-P refers to chlorophyll-protein.

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