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Light-harvesting chlorophyll c-like pigment in *Prochloron*

(endosymbiosis/phylogeny/Prochlorophyta)

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ABSTRACT **A chlorophyll c-like pigment, similar to magnesium-3,8-divinyl pheoporphyrin a_5 monomethyl ester, has been isolated from *Prochloron* sp. obtained from five species of didemnid ascidians from the Great Barrier Reef, Australia, and from Palau, Micronesia. The pigment represents 4–15% of the total chlorophyll content and is shown to function in a light-harvesting pigment protein complex of *Prochloron*. The observation that all of the major chlorophylls (a+b+c) function in a light-harvesting role in *Prochloron* and possibly in other prochlorophytes is discussed in terms of the phylogeny of the prochlorophytes.**

The discovery of *Prochloron* sp., a symbiotic prokaryotic alga with chlorophyll (Chl) b and Chl a (1, 2), stimulated debate as to the origin of chloroplasts. Discussion has continued to the present time, fueled by the recent discoveries of two other prochlorophytes—the filamentous freshwater alga *Prochlorothrix hollandica* (3) and the small, unicellular, free-living planktonic alga *Prochlorococcus marinus* (4). Some view the prochlorophytes as allied to the ancestors of green chloroplasts (4, 5), while others consider them as members of the cyanobacteria, which have lost phycobiliproteins and gained Chl b, probably independently, in each group (6–10). The presence of a Chl c-like pigment, tentatively identified as magnesium 3,8-divinylphaeoporphyrin a_5 monomethyl ester (MgDVP), has been claimed for *Prochlorococcus* (4, 11), although only the peak wavelengths of the natural and demetallated pigment were provided (11). It also has been claimed that MgDVP occurs in *Prochlorothrix hollandica* (11) in low concentrations. Here we present evidence, based on the spectra of pigments resolved by thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC), that a Chl c-like pigment tentatively identified as MgDVP occurs in *Prochloron* in concentrations that are 4–15% of the total Chl. Moreover, the pigment is shown to be located in a light-harvesting Chl pigment–protein complex (LHC) and to act as a light-harvesting pigment.

MATERIALS AND METHODS

Prochloron spp. were isolated from five didemnid ascidians (Table 1) at One Tree Reef, Capricorn Section, Great Barrier Reef, as described (15).

Analysis of the pigments of *Prochloron* was done on site by reverse-phase HPLC using a Waters 600E quaternary pump system with a Waters 990 photodiode array detector and a Shimadzu RF-551 spectrofluorimeter. The reverse-phase system was used with a C_{18} column (3.9 × 150 mm Waters Novapak 36975; 4-μm particle size, spherical end-capped material with a low carbon load), and gradient elution with acetonitrile/methanol/water (method 1) was applied as follows: initial conditions, 95% solvent A/5% solvent B; linear gradient to 1% A/99% B in 15 min; and then held at 1% A/99% B for 15 min [solvent A = 50% water/25% acetonitrile/25% methanol (vol/vol); solvent B = 50% acetonitrile/50% methanol (vol/vol); flow rate, 2 ml·min^{-1}].

Further analyses used (*i*) TLC with polyethylene (Super Rylan 7007, sample no. PRX 1013, Koppers, Warrington, PA) and with acetone as the mobile phase or (*ii*) the same polyethylene used in an HPLC system (16) (hand-packed 4.6 × 250 mm steel column) with a step solvent system (method 2) as follows: system preequilibrated with 65% acetone/35% water; step gradient to 100% acetone at 0 min and continued for 15 min; flow rate, 2 ml·min^{-1}.

Eluant from the Chl peaks separated by HPLC was collected, frozen, and freeze-dried under vacuum. Pigments were redissolved in 90% acetone/10% water, 100% acetone, or 100% ether, and their spectra were analyzed in a Beckman DU-8 spectrophotometer. Chl ratios were determined on site in 90% acetone/10% water by using an Hitachi U-2000 spectrophotometer and the trichromatic equation of Jeffrey and Humphrey (13). Demetallated Chl (i.e., removal of Mg from the porphyrin ring) was produced by adding 50 μl of 0.1 M HCl to 2–4 ml of acetone or ether extracts. The LHC of *Prochloron* was isolated as described (15). Low-temperature (77 K) fluorescence spectra were measured with a Perkin Elmer 44B Fluorimeter.

Micromonas pusilla (culture CS-86 from the Culture Collection, CSIRO Fisheries, Hobart, Tasmania) was grown in GP medium (17) under natural but low light (maximum, 200 μE·m^{-2}·s^{-1}).

RESULTS AND DISCUSSION

Analysis by HPLC in the field of the 90% acetone extracts of each of five isolates revealed the presence of a chlorophyll c-like pigment (Table 1) that was eluted at 14 min; Chl a, Chl b, and carotenoids were also present. A typical elution profile and an on-line spectrum of the Chl c-like pigment are shown in Fig. 1. Comparisons (not shown) of the extracts from *Prochloron* with a brown alga (possessing Chl c_1 + c_2) and a dinoflagellate (Chl c_2) indicated that the HPLC system separated the Chl c-like pigment from Chl c_1 and Chl c_2, which themselves were partially separable from each other.

The use of HPLC in the field was crucial for the discovery of the Chl c-like pigment(s) in *Prochloron*. At present *Prochloron* cannot be cultured (18), and didemnid ascidians, the major source of material, release acids when handled. Thus, unless fresh material is extracted and processed carefully and immediately, Chl breakdown products (e.g., chlorophyllides and pheoporphyrins) can be generated, which would obscure the presence of Chl c-like pigments. However, further laboratory work was necessary, using polyethylene chromatography (14, 16, 19), to verify the presence of Chl

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Abbreviations: Chl, chlorophyll; BChl, bacteriochlorophyll; LHC, light-harvesting Chl pigment–protein complex; MgDVP, magnesium 3,8-divinylphaeoporphyrin a_5 monomethyl ester.

Table 1. The ratios of Chl a/b- and Chl a/c-like pigment found by reverse-phase HPLC analysis (method 1) for *Prochloron* sp. from five didemnid ascidians collected at One Tree Reef, Capricorn Section, Great Barrier Reef

Species of ascidian host for *Prochloron* sp.	Chl a/b	Chl a/c-like
Didemnum molle (Herdmann, 1886)	3.1	15.8
Diplosoma virens (Hartmeyer, 1908)	2.9	5.0
Lissoclinum patella (Gottschaldt, 1898)	3.1	6.2
Lissoclinum bistratum (Sluiter, 1905)	7.6	9.6
Tridemnum paracyclops (Kott, 1980)	3.55	6.1

Quantitation based on absorbance of individual peaks and extinction coefficients in methanol (12). Note that 90% acetone/10% water extracts of the symbionts gave similar values for the Chl a/b ratio and, surprisingly, also for the Chl a/c-like ratio (13), despite the difference in absorption maximum of Chl c and MgDVP in the red region of the spectrum (14).

c-like pigments. Thus, carefully prepared frozen or freeze-dried samples of *Prochloron* from *L. patella* were further analyzed in Sydney (the frozen samples came from a site at 15-m depth near Lizard Island, Great Barrier Reef; the freeze-dried samples were a gift to G.C.C. from R. Lewin and were collected in Palau, Micronesia). TLC analysis (14, 19) revealed the presence of two Chl c-like compounds that had a significantly greater R_f value than those of either Chl c_1 or Chl c_2 (Fig. 2) and migrated in the relative position of MgDVP (14, 19). There is also evidence from the fluorescence spectra of two related pigments (see below). The presence of a Chl c-like pigment was also confirmed by reverse-phase HPLC with a polyethylene column, but only one broad peak was resolved (Fig. 3). The spectral peaks of the compound(s) eluted at 7.9 min (Fig. 3 *Lower*, solid curve) (438 nm, 576 nm, and 626 nm—in 90% acetone/10% water) agree well with those of MgDVP (14, 19) in the same solvent. With extracts of brown algae or dinoflagellates, Chl c_1 or Chl c_2 or both were eluted later (not shown). The spectral peaks of the demetallated Chl c-like pigment (Table 2) (419 nm, 568 nm, and 588 nm) match well with those of demetallated MgDVP (11, 20). In addition, cells of *M. pusilla* were extracted in 90%

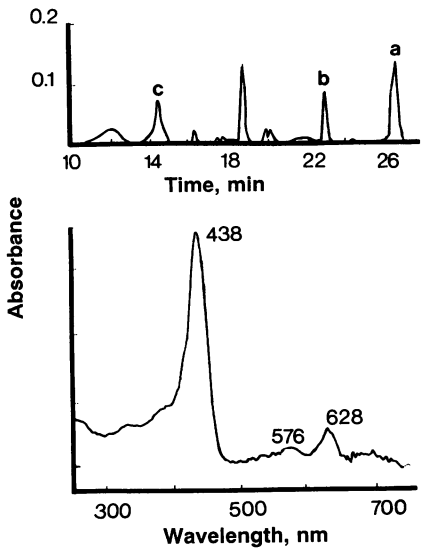

FIG. 1. (*Upper*) An elution profile (λ_{det} = 440 nm) of the 90% acetone extract of *Prochloron* sp. from *Diplosoma virens* separated by reverse-phase HPLC (method 1; see Table 1) on a Novapak C_{18} column (Table 1). Bands: a, Chl a; b, Chl b; c, Chl c-like pigment; others, carotenoids. (*Lower*) On-line spectrum of Chl c-like pigment eluted as band c at 14.4 min. [This band was also detected by fluorescence emission in the red region of the spectrum (excitation, 440 nm) with a maximum at 635 nm (not shown).]

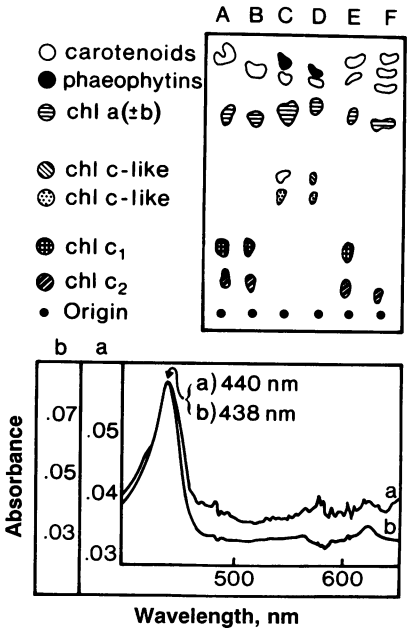

FIG. 2. (*Upper*) Diagram of a developed thin-layer chromatogram (14) of the following 90% acetone extracts. Lanes: A, *Endarachne binghamiae* (Phaeophyta); B, *Colpomenia sinuosa* (Phaeophyta); C, *Prochloron* sp. (*L. patella* from Palau); D, *Prochloron* sp. (*L. patella* from Lizard Island); E, *Pavlova lutheri* (Prymnesiophyta); F, *Amphidinium carterae* (Pyrrophyta). The developing solvent was acetone. In lane C only the lower Chl c-like pigment was clearly visible, since the upper Chl c-like pigment was overlain by an orange pigment, probably a carotenoid. (*Lower*) Spectra of two Chl c-like pigments in 90% acetone from lane D in *Upper*. Spectra: a, spectrum of lower Chl c-like (stippled) pigment; b, spectrum of upper Chl c-like (hatched) pigment. (λ_{max} for the major peak of a and b spectra are shown.)

acetone/10% water and analyzed by HPLC with a C_{18} column (method 1). A Chl c-like pigment was present, which was coeluted with the major Chl c-like pigment from *Prochloron*. It also had similar spectral properties to the Chl c-like pigment of *Prochloron* (Table 2). The Chl c-like pigment in *Micromonas* spp. has been tentatively identified as MgDVP (12, 14, 19) on the basis of its spectral properties and elution characteristics on TLC and HPLC.

MgDVP has a fully unsaturated porphyrin macrocycle and does not carry a long-chain esterifying alcohol at C-17 and differs from Chl c_2 only in the presence at C-17 of a propionic acid instead of an acrylic acid (14). Evidence suggests that MgDVP is an intermediate in the pathway of Chl a [and bacteriochlorophyll (BChl)] biosynthesis (21). It was first documented in the photosynthetic bacterium *Rhodobacter sphaeroides* grown in the presence of 8-hydroxyquinoline, but it probably occurs also in some mutants of *R. sphaeroides* as a result of a lesion in the normal synthesis of BChl (21). MgDVP was also identified as a pigment of unknown function in several marine flagellates (22) including the prasinophyte *M. pusilla*, where it has since been shown to be present in an LHC as a light-harvesting pigment (23, 24). However, it should be pointed out that the precise structure of the Chl c-like pigment in *M. pusilla* and the related alga *Mantoniella squamata* is debated (12, 20) [both algae are placed in the special group, the micromonadophytes (25)]. MgDVP is also probably present in the prochlorophyte *Prochlorococcus marinus* (11), although full details have yet to be documented.

On the basis of the present evidence, we conclude that a chl c-like pigment, similar to MgDVP, occurs in *Prochloron* sp. together with a closely related pigment; however, both pigments need to be further examined by nuclear magnetic

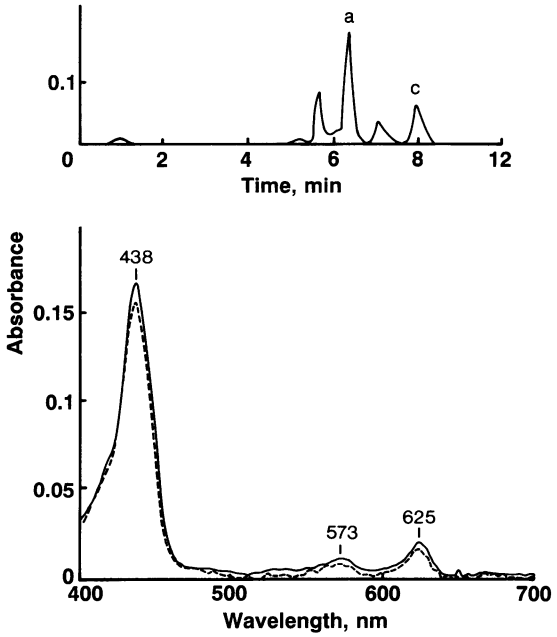

FIG. 3. (*Upper*) Profile of pigments extracted in 90% acetone from *Prochloron* sp. (*L. patella* from Palau) and separated by reverse-phase HPLC on a polyethylene column (17) (method 2) monitored at 440 nm (the eluant was also monitored by a fluorescence detector, which confirmed the fluorescence of Chl c-like at 635 nm in the band eluted at 7.9 min). Bands: a, Chl a + b; c, Chl c-like; others, carotenoids. Extracts from a brown alga *E. binghamiae* and a dinoflagellate alga *A. carterae* showed that Chl c_1 was eluted at 9.2 min and Chl c_2 was eluted at 11.8 min. (profiles not shown). (*Lower*) On-line spectrum of the Chl c-like band (c) eluted at 7.9 min. (solid curve) and of the pigment extracted and chromatographed in the same position from the LHC (dashed curve).

resonance spectroscopy. It is possible that only one of the pigments is native and that the other is a product of the preparative procedures. We reject the possibility that both pigments are the result of contamination by another alga or photosynthetic bacterium. The preparations used were monitored microscopically and showed less than 0.3% contamination on a cell count basis (<0.03% by mass) by other photosynthetic organisms. Chl c-containing algae generally have Chl a/c ratios of much more than 3 (26). Thus, any contaminant would have to be present at 50% or more, and unless some other novel organism was present, significant amounts of Chl c [c_1, c_2, or c_3 (14)] would have been observed (or for a photosynthetic bacterium, BChl).

An important question remained to be answered. Is the Chl c-like pigment(s) present and active in the LHC (15), and not merely present because of the accumulation of a biosynthetic intermediate in the synthesis of Chl a (21)? If the Chl c-like pigment were functional in photosynthesis, then it would transfer absorbed energy to Chl a molecules, which under appropriate conditions would emit this energy maximally at 683 nm at 77 K.

The LHC was obtained from *Prochloron* by sodium dodecyl sulfate (SDS) treatment and sucrose density gradient centrifugation (15) and was extracted with 90% acetone/10% water. HPLC analysis revealed the presence of a Chl c-like pigment (Fig. 3) in amounts, relative to Chl a, greater than in the whole-cell extract.

Low temperature (77 K) fluorimetry showed that the Chl c-like pigment, as well as Chl b, acted in a light-harvesting capacity in the LHC. The isolated LHC emitted fluorescence at 683 nm (Fig. 4*A*), whether it was excited at 440 nm (Chl a and Chl c-like) or 470 nm (Chl b). SDS was added to the LHC to a concentration of 2%, and the mixture was heated to 70°C for 5 min. This shifted the major fluorescence peak to 679 nm because of partial dissociation of the Chl a from the protein complex. Peaks at either 636 nm (Chl c-like) or 655 nm (Chl b) now appeared when excited at 440 nm and 470 nm, respectively (Fig. 4 *D* and *E*). Addition of acetone (to a final concentration of 95%) to the LHC shifted the emission maximum to 672 nm because of complete dissociation of the Chl a from the protein. An emission band, from the dissociated Chl c-like pigment at 628 nm (Fig. 4*B*), was now observed when excited at 440 nm. However, when excited at 470 nm, a major band of fluorescence appeared at 658 nm, presumably arising from Chl b (Fig. 4*C*). These results are interpreted to indicate that Chl b and the Chl c-like pigment pass excitation energy to Chl a in the native LHC but fluoresce individually when the complex is denatured. The excitation spectrum for the fluorescence emission peaks of the Chl c-like pigment at 628 nm or 636 nm (after treatment, respectively, with acetone or SDS plus heat) had a split peak with a maximum at 438 nm and a shoulder at 442 nm (not shown), suggesting the presence of a second Chl c-like pigment or a breakdown product.

This evidence shows conclusively that some of the Chl c-like pigment is present in the LHC and is a functional component of the LHC (compare refs. 24 and 27). A photosystem I chlorophyll–protein fraction isolated by the same

Table 2. Absorption and fluorescence maxima (nm) and band ratios of the chlorophyll c-like pigment from *Prochloron* sp., MgDVP from *M. pusilla*, and their demetallated derivatives, isolated by method 1

		Absorption, nm			Band Ratio		Fluorescence	
	Solvent	III	II	I	III:I	II:I	F_{ex}	F_{em}
Chl c-like pigment								
(*Prochloron*)	Acetone	438	576	625	7.9	0.54	438	635
							440	*634*
	Diethyl ether	437	574	625	9.7	0.57	—	—
MgDVP	Acetone	438	576	625	8.1	0.52	439	634
							440	*633*
	Diethyl ether	438	574	624	9.8	0.62	—	—
Demetallated Chl								
c-like (*Prochloron*)	Acetone	422		568	12.5		419	654 (714)
							418	*659 (712)*
	Diethyl ether	423		569	11.8		—	—
Demetallated DVP	Acetone	422		568	12.4		419	655 (715)
							419	*660 (712)*
	Diethyl ether	423		569	12.1		—	—

Values in italics were measured at 77 K; other values were measured at 20°C. F_{ex}, peak of fluorescence excitation spectrum; F_{em}, peak of fluorescence emission spectrum. Numbers in parentheses are subsidiary peaks.

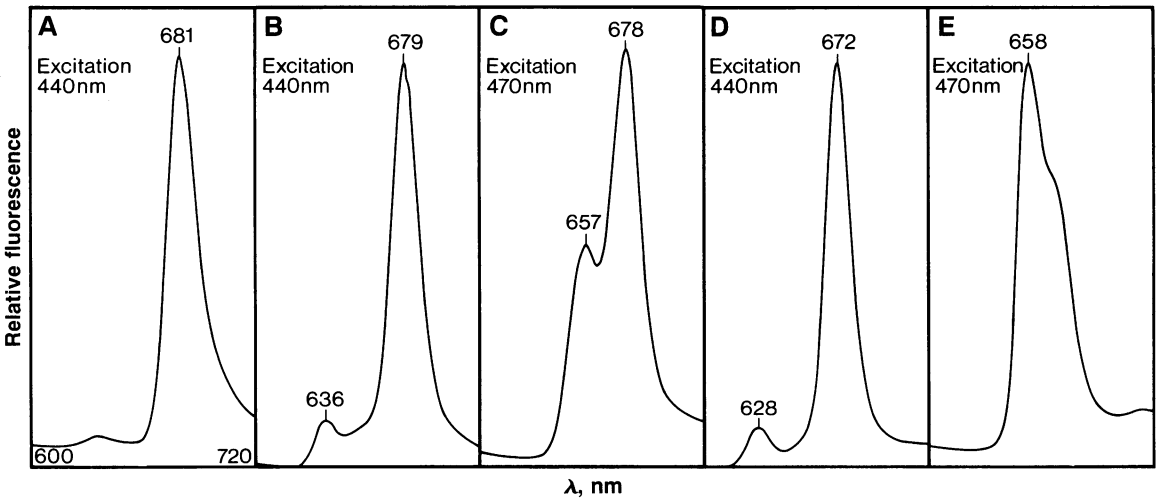

FIG. 4. Low-temperature (77 K) fluorescence emission spectra of the LHC isolated from *Prochloron* sp. (from *L. patella* found at 10-m depth on One Tree Reef). (*A*) Isolated LHC excited by light at 440 nm. (*B*) LHC extract in 95% acetone/10% water excited at 440 nm. (*C*) LHC extract in 95% acetone/5% water excited at 470 nm. (*D*) LHC in 2% SDS and heated to 70°C for 5 min, excited at 440 nm. (*E*) LHC in 2% SDS and heated to 70°C for 5 min, excited at 470 nm.

procedure (15) as the LHC had no detectable levels of the Chl c-like pigment, although Chl a and b were present, as shown previously (15). This evidence suggests that the Chl c-like pigment is not intimately connected to photosystem I. The nature and function of the major LHC in *Prochloron* sp. and the presence of other LHCs have yet to be fully elucidated. It may be similar to LHC II of chlorophytes and higher plants and therefore associated with photosystem II (15), but neither its molecular location nor its primary structure has been reported.

The occurrence of Chls a, b, and c in a light-harvesting role in *Prochloron* raises many intriguing questions. The presence of an identical or similar Chl c-like pigment to that in *Prochloron* seems likely in *Prochlorococcus marinus*, although it has yet to be shown in the latter organism whether the pigment acts in a light-harvesting capacity and is found in an LHC. Is *Prochlorothrix hollandica*, the other prochlorophyte, similar? Preliminary pigment analysis of that organism indicates that MgDVP may be present in low concentrations (11). If this is substantiated, then all three prochlorophytes are likely to contain the Chl c-like pigment, although it would still be necessary to show that it acts in a light-harvesting capacity. At the moment it is possible to say that *Prochloron* sp. and possibly each of the three prochlorophytes are more closely allied to the micromonadophyte chloroplast line (25, 28) than to any other chloroplast line on the evidence that they all share a Chl c-like pigment. However, the distinct differences in the LHCs between *Prochloron* (15) and *Prochlorothrix* (29) on the one hand (the type of LHC in *Prochlorococcus* is not known) and the micromonadophytes on the other hand (20, 30) (where the LHC is of the chromophytic type) stand against a close affinity between these two groups. The recent finding of divinyl Chl a and b in *Prochlorococcus* (4, 11) and the evidence that both *Prochloron* and *Prochlorococcus* share the presence of a Chl c-like pigment raise the possibility that divinyl chlorophylls a and b also exist in *Prochloron*.

The occurrence of a Chl c-like pigment and Chl b [or divinyl-Chl b (11)] in all of the prochlorophytes would weaken the argument that these organisms are merely cyanobacteria, where Chl b originated independently in each of the three groups (7–10). We believe that the phylogenetic tree analysis on which that argument was based is flawed (31–33). Another explanation is that the prochlorophytes are an anciently diverged group (34) widely separated from the Cyanobacteria, as originally suggested (2). Thus, all three known prochlorophytes may be only very distantly related to the green chloroplast and may have diverged considerably from each other. Finally, the present evidence lends support to the recent suggestion that Chl c-like pigments were present in the earliest photosynthetic prokaryotes (28).

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