A New Form of Chlorophyll c Involved in Light-Harvesting

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ABSTRACT

A new form of chlorophyll c has been isolated from the pyrimesiophyte Pavlova gyran Butcher. This pigment is spectrally similar to chlorophyll c2, but all the absorption maxima (454, 583, and 630 nm in diethyl ether) are shifted 4 to 6 nanometers to longer wavelengths. The new pigment can be separated from other chlorophyll c-type pigments by reversed-phase high performance liquid chromatography and thin layer chromatography. Both chlorophylls c1 and c2 are found with the new chlorophyll c pigment in P. gyran, and it has also been detected in the chrysophyte Synura petersenii Korsh. The light-harvesting function of the new chlorophyll c pigment is indicated by its presence along with chlorophyll c1 and c2 in a light-harvesting pigment-protein complex isolated from P. gyran in which chlorophyll c pigments efficiently transfer absorbed light energy to chlorophyll a.

The Chl c pigments are widely distributed among marine and freshwater algae. These Chl forms differ from both Chl a and Chl b in that ring IV is unsaturated and there is a lack of a hydrophobic esterified phytol side chain. This combination of characteristics is also found in the protochlorophyllides, and Chl c forms have been called chlorophyllides c (27). Originally, two forms of Chl c were recognized, both of which have acrylic acid groups at C1 (15). Chl c1 has an ethyl group present at position C4 in ring II of the Chl macrocycle, whereas Chl c2 has a vinyl group in this position (4, 25). This difference is not sufficient to enable separation in most conventional chromatographic systems, such as cellulose TLC or RP2-HPLC. A polyethylene-based TLC separation system was used by Jeffrey (15) to determine that Chl c1 and c2 occur together in chrysophytes, diatoms, brown algae, and a few dinoflagellates, while most dinoflagellates and cryptomonads possess only Chl c2 (17). Where studied, both of these pigments have been shown to be involved in light-harvesting. Chromophores in which light-harvesting pigment-protein complexes containing some form of Chl c have been isolated include brown algae (2), diatoms (6, 10, 22), cryptomonads (14), dinoflagellates (3), pyrinesiophytes (7, 13), and a chrysophyte (28).

Jeffrey and Wright (20) and Vesk and Jeffrey (26) have recently isolated and spectrally characterized a third Chl c species, which they called Chl c5. This pigment migrated with Chl c2 in the polyethylene TLC system, but could be separated from both Chl c1 and c2 by reversed-phase high performance TLC. Chl c5 has also been separated from Chl c1 and c2 by RP-HPLC (9). Chl c5 is also implicated in light-harvesting in the pyrimesiophyte Emiliania huxleyi, since the Chl c present in this alga has been shown to contribute to light harvesting (12). Stuber and Jeffrey (24) recently found several diatoms with Chl c5, and it is likely that it is to be found in more organisms.

Another pigment which is structurally very similar to Chl c1 and Chl c2 is magnesium 2,4-divinylpheoporphyrin a5, monomethyl ester, which was initially isolated from a photosynthetic bacterium (21). This pigment is also found in several small green flagellates (Micromonadophyceae) (23), although the actual identity of the Chl c-like pigment in these organisms has been questioned (29). In these green algae, Mg 2,4-divinylpheoporphyrin a5 monomethyl ester is present in a light-harvesting complex which also contains Chl a and Chl b (8, 30). In Mg 2,4-divinyl-pheoporphyrin a5 monomethyl ester, the acrylic acid group at C7 is replaced by a propionic acid group.

I recently (5) described a RP-HPLC system for the separation of pigments from the pyrimesiophyte Pavlova gyran. Using that procedure, it appeared that Chl c1 and Chl c2 were being separated. However, in trying to extend that procedure to other organisms, it soon became apparent that Chl c1 and Chl c2 were not being separated. Rather, a new form of Chl c with spectral characteristics similar to Chl c5 is present in P. gyran. Here I describe the characteristics and function of this new Chl c pigment and compare it to previously described forms of Chl c.

MATERIALS AND METHODS

Culture Methods

Pavlova gyran Butcher (Culture Collection of Marine Phytoplankton, Bigelow Laboratory for Ocean Sciences, West Boothbay Harbor, ME (CCMP), clone MPPA; Phaeodac- tyium tricornutum Bohlin (University of Texas at Austin Culture Collection of Algae [UTEX] 6466); Mantoniella squamata (Manton & Park) Desikachary (UTEX 990), and Prym- nesium parvum Carter (CCMP clone PRYM) were grown in modified ES enriched artificial seawater medium (ESA/SW) (7) and Synura petersenii Korsch. UTEX 845 was grown in modified Woods Hole freshwater medium (8). Cultures of all organisms were maintained in one to 2 L Erlenmeyer flasks. Marine organisms were continually bubbled with air, while S. petersenii was not. Temperature was constant at 20°C, and illumination was provided by cool-white fluorescent lights at about 50 μE/m2/s with a 14 h:10 h light:dark cycle.
Pigment Extraction

Algae were harvested from dense, late-log phase cultures by centrifugation and pigments were extracted using three methods: (a) extraction of the cell pellet with at least nine volumes of ice-cold 100% acetone; (b) extraction of the cell pellet with at least nine volumes of ice-cold 100% methanol; and (c) freezing the pellet with liquid nitrogen, followed by thawing the pellet in at least nine volumes of 100% acetone. During extraction, all procedures were performed on ice. For HPLC, pigment extracts were centrifuged, and 20 to 100 μL of supernatant was injected into the HPLC system. For TLC, the pigments were transferred to fresh anhydrous diethyl ether before application.

HPLC

The HPLC system used was that described previously (5). This is a RP system consisting of an Alltech Econosphere 5 μm C18 cartridge column (25 cm × 4.6 mm). The solvent system used was B) 50% methanol, 50% acetonitrile (v/v); A) 50% water, 50% B (v/v). Pigments were eluted from the column using a linear gradient of 0 to 80% B in 10 min, followed by a linear gradient from 80 to 98% B in 10 min, with continued elution with 98% B until all pigments were removed from the column. The initial flow rate of 1.5 ml/min was changed to 2.5 ml/min at 30 min. Fractions were collected as they eluted from the column.

TLC

Cellulose TLC to separate Chl c pigments from all other pigments was performed by the method of Jeffrey (18), except that commercial plates (EM Science No. 5577) were used. Polyethylene (Polysciences No. 2719) TLC to separate Chl c1 from c2 was performed using the method of Jeffrey (16), except that the adsorbent layer was 250 μm and the solvent was 100% acetone.

Spectral Characterization

Pigments separated by HPLC were transferred from the elution solvent to diethyl ether, and those purified by TLC were eluted directly into the appropriate solvent. Absorption spectra were recorded using a Beckman DU-7 spectrophotometer. Magnesium-free derivatives were prepared in acetone by the method of Jeffrey and Shibata (19).

Isolation of P. gyrans Light-Harvesting Complex

A light-harvesting pigment-protein complex was isolated from P. gyrans by the method of Fawley et al. (7). Pigments were extracted in methanol as described above.

RESULTS

Two fractions containing Chl c pigments were separated by RP-HPLC of pigment extracts of Pavlova gyrans (Fig. 1A). As previously reported (5), the first fraction had spectral characteristics similar to Chl c1, and the second, Chl c2. However, RP-HPLC of pigment extracts of Phaeodactylum tricornutum revealed only a single Chl c fraction (Fig. 1B). Since P. tricornutum is known to possess both Chl c1 and Chl c2 (25), it was apparent that the fraction from P. gyrans which had been identified as Chl c2 was in fact a previously undescribed pigment. One potential concern was that this new pigment might be a chromatographic artifact. To eliminate this possibility, fraction 1 and fraction 2 were isolated by RP-HPLC and subjected to a second run with the RP-HPLC system. Rechromatography of fraction 1 did not result in the production of fraction two, and no Chl c1 or c2 resulted when fraction 2 was rechromatographed (data not shown).

To better characterize this pigment, it was compared to all other known forms of Chl c. Chl c1 and Chl c2 were isolated by polyethylene TLC from P. tricornutum (16), Chl c3 was isolated by the pynnesiophytes Prymnesium parvum (9) by RP-HPLC, and Mg-2,4-divinylpheoporphyrin a5 monomethyl ester from the micromonadophyte Mantoniella squamata (23) by RP-HPLC and cellulose TLC.

Figure 2 shows the absorption spectra, in diethyl ether, of the new Chl c pigment from P. gyrans, Chl c1, and c2 from P. tricornutum, Chl c3 from P. parvum, and Mg-2,4-divinylpheoporphyrin a5 monomethyl ester from M. squamata.
porphyrin \(a_5\) monomethyl ester from \(M.\ squamata\). Maxima and band ratios of these spectra are presented in Table I. Values are in close agreement with published results (15, 16, 20, 24). The new Chl c form is similar to Chl c2, in that the ratio of band II to band I is about 1; however, the absorption maxima of the two pigments are different by 4 to 6 nm. The most notable difference between the new pigment and other Chl c forms is the Soret maximum, which at 454 nm in diethyl ether is the longest wavelength Soret maximum of any of these pigments.

Differences among the Chl c pigments are also evident in retention characteristics using different chromatographic separation systems (Table II). Three systems were used: cellulose TLC, polyethylene TLC, and RP-HPLC. With the three systems, Chl c1 and c2 migrate identically except in polyethylene TLC, where Chl c1 migrates further than Chl c2. Chl c3 can be separated from Chl c1 and c2 by RP-HPLC, but all three pigments have the same RF values in cellulose TLC, and Chl c3 and c2 have the same RF values in polyethylene TLC. Mg-

**Table I. Spectral Characteristics of Chl c Pigments in Diethyl Ether**

All spectra at room temperature.

<table>
<thead>
<tr>
<th>Alga</th>
<th>Pigment*</th>
<th>Absorption Maxima*</th>
<th>Band Ratios</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pavlova gyrans</td>
<td>Chl c</td>
<td>454, 583, 630</td>
<td>13.0 1.07</td>
</tr>
<tr>
<td>Pavlova gyrans</td>
<td>Chl c1</td>
<td>444, 577, 626</td>
<td>8.7 0.66</td>
</tr>
<tr>
<td>Pavlova gyrans</td>
<td>Chl c2</td>
<td>447, 580, 627</td>
<td>13.2 1.16</td>
</tr>
<tr>
<td>Phaeodactylum tricornutum</td>
<td>Chl c1</td>
<td>444, 577, 626</td>
<td>8.7 0.64</td>
</tr>
<tr>
<td>Phaeodactylum tricornutum</td>
<td>Chl c2</td>
<td>448, 579, 627</td>
<td>12.1 0.99</td>
</tr>
<tr>
<td>Prymnesium parvum</td>
<td>Chl c3</td>
<td>452, 585, 625</td>
<td>34.0 3.67</td>
</tr>
<tr>
<td>Mantoniella squamata</td>
<td>MgDV</td>
<td>437, 574, 624</td>
<td>10.0 0.52</td>
</tr>
</tbody>
</table>

*Chl c* = new Chl c pigment. MgDV = magnesium-2,4-divinylphaeoporphyrin \(a_5\) monomethyl ester. *Maxima are rounded to nearest integer.

**Table II. Retention of Chl c Pigments in Different Chromatography Systems**

<table>
<thead>
<tr>
<th>Pigment*</th>
<th>Cellulose TLC (RF)</th>
<th>Polyethylene TLC (RF)</th>
<th>RP-HPLC (Rf, min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chl c</td>
<td>0.07</td>
<td>0.80</td>
<td>13.9</td>
</tr>
<tr>
<td>Chl c1</td>
<td>0.17</td>
<td>0.66</td>
<td>8.5</td>
</tr>
<tr>
<td>Chl c2</td>
<td>0.17</td>
<td>0.47</td>
<td>8.5</td>
</tr>
<tr>
<td>Chl c3</td>
<td>0.16</td>
<td>0.50</td>
<td>7.2</td>
</tr>
<tr>
<td>MgDV</td>
<td>0.15</td>
<td>ND*</td>
<td>8.5</td>
</tr>
</tbody>
</table>

*Chl c* = new Chl c pigment from \(P.\ gyrans\), MgDV = magnesium-2,4-divinylphaeoporphyrin \(a_5\) monomethyl ester from \(M.\ squamata\). Chl c1 and c2 were isolated from \(P.\ tricornutum\) and Chl c3 from \(P.\ parvum\). ND* = not determined.

2,4-divinyl phaeoporphyrin \(a_5\) monomethyl ester has the same retention time (Rf) as Chl c1 and c2 in RP-HPLC and the same Rf as Chl c1, c2, and c3 in cellulose TLC. Polyethylene TLC of Mg-2,4-divinylphaeoporphyrin \(a_5\) monomethyl ester was not performed, but Jeffrey and Wright (20) indicate that this pigment migrates faster than Chl c1 in polyethylene TLC. The new Chl c pigment can be separated from other Chl c pigments by all three methods. It has a longer Rf in RP-HPLC than any of the other Chl c pigments, has a lower Rf value in cellulose TLC than any of the other pigments, and a higher Rf than Chl c1 in polyethylene TLC.

The absorption spectrum of the new Chl c pigment in acetone and also of the Mg-free derivative (pheophytin) in acetone are presented in Figure 3. Table III lists the absorption maxima of all the Chl c pigments and Mg-free derivatives in acetone for comparison. The spectra of the new Chl c pigment once again differ from those of other Chl c pigments in that the Soret maximum is at longer wavelength. The Mg-free derivative of the new pigment (Fig. 3) exhibits features generally attributed to porphyrins, namely four maxima, a low absorption for band I, and a shift of about 20 nm with the transition from Chl to pheophytin (11).
To determine the full Chl c pigment complement of *P. gyrans*, pigment extracts were subjected to cellulose TLC, which separated the new Chl c pigment from other Chl c pigments. The spot containing the other Chl c pigments was rechromatographed on polyethylene TLC. Two Chl c fractions were produced, with absorption characteristics very similar to Chl c1 and c2 from *P. tricornutum* (Table I). The new Chl c pigment, therefore, is present with both Chl c1 and c2 in *P. gyrans*.

To determine if the new Chl c pigment functions in light-harvesting as do other forms of Chl c, a light-harvesting complex known to have transfer of energy from Chl c pigments to Chl a (7) was isolated from *P. gyrans*. The isolated complex was pelleted by ultracentrifugation, and pigments extracted with methanol. The RP-HPLC chromatogram of this extract is presented in Figure 4. Based on retention times (5), the pigments present included Chl c1 and/or c2, the new Chl c pigment, fucoxanthin, diadinoxanthin, diatoxanthin, and Chl a. Polyethylene TLC of pigments extracted from this light-harvesting complex revealed the presence of both Chl c1 and c2. This light-harvesting complex from *P. gyrans* therefore contains four Chl forms and three carotenoids.

One other organism has thus far been shown to possess this new Chl c pigment. The Chrysophyte *Sympodium petersenii* was previously reported to possess only Chl c1 (1); however, the RP-HPLC chromatogram of pigment extracts from *S. petersenii* indicated a fraction with retention time identical to the new Chl c pigment (data not shown). The identity of this fraction as the new Chl c pigment was verified by absorption spectroscopy (Fig. 5). The new Chl c pigment was also detected in pigment extracts from UTEX 992, the only other clone of *P. gyrans* which has been examined (data not shown).

**DISCUSSION**

The new Chl c pigment can be distinguished from previously described forms on the basis of its apparent lower polarity in RP-HPLC and its longer wavelength Soret maximum in either diethyl ether or acetone. The absorption band ratios of this new pigment are similar to those of Chl c2. Overall the best discriminator of the five Chl c pigments studied here is the Soret maximum in diethyl ether. Mg-2,4-divinylphaeoporphyrin *a*5 monomethyl ester has the shortest wavelength Soret maximum at 437 nm, followed by Chl c1 at 444 nm, Chl c2 at 448 nm, Chl c3 at 452 nm, and the new Chl c pigment at 454 nm.

To separate these pigments, a combination of methods must be used. Chl c1 and the new Chl c pigment can be separated from other Chl c pigments by RP-HPLC, but the three remaining pigments cannot be separated by this system. Cellulose TLC will separate the new Chl c pigment from all others, but the RΓ is so low that care must be taken to avoid confusion with chlorophylls and other derivatives which can appear in mishandled extractions. It would appear that the best method presently available to separate these pigments with confidence is the combination of cellulose TLC, polyethylene TLC, and RP-TLC used by Jeffery and Wright (20) and Stauber and Jeffery (24). However, RP-HPLC provides an excellent first method to determine if either Chl c1 or the new Chl c pigment is present. The TLC separation system(s) needed to determine the presence of other Chl c forms can then be chosen.

The presence of Chl c alteration products in pigment extracts have been noted (15, 16); however, none of the alteration products have the characteristics of the pigment described here. To minimize the possibility that the new Chl c pigment might be an artifact that had not previously been characterized, three pigment extraction procedures were used. The new Chl c pigment was present in all of these extracts, as determined by RP-HPLC. One of these procedures, extraction with acetone on ice, did produce some alteration products from both *P. tricornutum* and *P. parvum*, but not from *P. gyrans*. In addition, rechromatography by RP-HPLC of both Chl c fractions from *P. gyrans* did not result in any additional Chl
The pigment from Chl complex harvesting the peaks, indicating that the new pigment was not an artifact of the separation system.

That the new Chl c pigment is able to function in light-harvesting is strongly suggested by its presence in a light-harvesting complex which is known to have transfer of energy from Chl c pigments to Chl a, as evidenced by fluorescence excitation spectra and a lack of Chl c fluorescence emission (7). These fluorescence data indicate that light energy absorbed by all the Chl c pigments is transferred to Chl a; however, it is not possible to separate the individual forms of Chl c to be certain the new form is actually transferring energy. The new Chl c pigment, or one of the other forms of Chl c could be inactive. This seems very unlikely, since no fluorescence attributable to Chl c forms is detected in the fluorescence emission spectrum (7), indicating that all the Chl c is coupled to Chl a. Another possibility is that the new Chl c pigment is not fluorescent in this complex, but Chl pigments always fluoresce or transfer energy in light-harvesting complexes. The most reasonable interpretation of these results is that the new Chl c pigment is a functional light-harvesting pigment.

The presence of multiple forms of Chl c in the light-harvesting complex from P. gyrans is in keeping with the presence of both Chl c1 and c2 in a light-harvesting complex from the diatom P. tricornutum (22) which is homologous to that of P. gyrans (7). However, the light-harvesting complex from P. gyrans is the first pigment-protein complex known to contain four forms of Chl.

The discovery of a new form of Chl c brings to five the number of described Chl c-like pigments in eukaryotic organisms. Four of these pigments are found in the chlorophyte algae: Chl c1, Chl c2, Chl c3, and the new Chl c pigment. One is found in the Micromonadophyceae (Chlorophyta): Mg-2,4-divinylpheoporphyrin a5 monomethyl ester. It is apparent that structural investigations of both the new form of Chl c and Chl c3 must be undertaken so that a more meaningful system of nomenclature can be devised than that presently in use, and to determine if all of these pigments should be grouped as Chl c pigments.

Not only does the chemical structure of the new Chl c pigment need to be determined, but also its distribution in the various groups of algae. Thus far, the new Chl c pigment has been found in two clones of the prymnesiophyte Pavlova gyrans and one isolate of the chrysophyte Synura petersenii. S. petersenii is interesting because it had previously been determined that this organism contains only Chl c1 (1), one of a group of freshwater chrysophytes which are the only organisms known to possess Chl c1 but not Chl c2. This

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**Figure 4.** Separation of pigments from a light-harvesting complex of P. gyrans by RP-HPLC. Isolated light-harvesting complex was extracted with at least nine volumes of ice-cold methanol and 30 μL injected into the HPLC system described in the text. Detection was at A440nm, 0.16 full scale. Peak identities as given in Figure 1.

**Figure 5.** Visible absorption spectrum in diethyl ether of the new Chl c pigment isolated by RP-HPLC from pigment extracts of S. petersenii.
investigation clearly shows that at least this single isolate also possesses the new Chl c.

All four Chl c forms described in the Chromophyte algae have now been detected in the Prymnesiophyceae, although the combinations vary. Some prymnesiophytes have Chl c1 and c2 (17), others have Chl c2 and Chl c3 (20), one has Chl c1, c3, and c2 (9), and now *P. gyrans* has Chl c1, c2, and the new Chl c pigment. The organization of all these forms of Chl c in the photosynthetic apparatuses of these organisms is presently unknown except for the light-harvesting function. This should be a fertile area for more research.

**LITERATURE CITED**