Light-Harvesting System of the Red Alga *Gracilaria tikvahiae*¹

II. PHYCOBILISOME CHARACTERISTICS OF PIGMENT MUTANTS

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**ABSTRACT**

Phycobilisomes were isolated from wild type *Gracilaria tikvahiae* and a number of its genetically characterized Mendelian and non-Mendelian pigment mutants in which the principal lesions result in an increase or decrease in the accumulation of phycoerythrin. Both the size and phycoerythrin content of the phycobilisomes are proportional to the phycoerythrin content of the crude algal extracts. In most of the strains examined, the structure and function of the phycocyanin-allophycocyanin phycobilisome cores are the same as in wild type. The phycobilisome architecture is derived from wild type by the addition or removal of phycoerythrin. The same pattern is observed for the phycobilisome of *mos* which contains a large excess of phycocyanin that is not bound to the phycobilisome. The single exception is a yellow, non-Mendelian mutant, *NM7-1*, which makes functional phycobilisomes composed of phycoerythrin and allophycocyanin with almost no phycocyanin. Characterization of the 'linker' polypeptides of the phycobilisome indicates that a 29 kilodalton protein is required for the stable incorporation of phycocyanin into the phycobilisome. Evidence is provided for the requirement of nuclear and cytoplasmic genes in phycobilisome synthesis and assembly. The symmetry properties of the phycobilisome are considered and a structural model for the reaction center II-phycobilisome organization is presented.

Phycobilisomes are photosynthetic light-harvesting assemblages which are composed of pigment-protein complexes and are found associated with the photosynthetic membranes of the cyanobacteria and red algae (5). In many species, the phycobilisomes are quite abundant and fill much of the interthylakoidal space (5, 6). All phycobilisomes contain the pigment-proteins APC and PC, and some cyanobacteria and most red algae contain PE. APC is closely associated with the photosynthetic membrane and is attached to rods composed of only PC, or PC and PE (PC:PE rods) in a ratio of six rods per phycobilisome.

This structural model is based on the dissociation kinetics of the isolated phycobilisome which demonstrate that PE is the most rapidly dissociated and therefore the most peripheral component of the phycobilisome (5), on the observation of six rods attached to negatively stained, isolated phycobilisomes from cyanobacteria and red algae (2, 10, 19, 25), and by the isolation and characterization of PC:PE rods from a unicellular red alga (10).

Excitation of either PC, PE, or APC in isolated intact phycobilisomes results in a major emission band with a maximum at 670 nm. The absorption and emission properties of the isolated biliproteins, the arrangement of the biliproteins in the phycobilisome, and the fluorescence emission properties of the isolated phycobilisomes all indicate that excitation energy is transferred from PE to PC to APC and emitted as fluorescence at 670 nm. *In vivo*, the phycobilisomes transfer excitations directly to Chl (5). This functional interpretation of the organization of the phycobilisome is supported by time-resolved fluorescence rise kinetics in isolated phycobilisomes and *in vivo* (16, 20).

The models of phycobilisome structure and function are derived from work on cyanobacteria and unicellular red algae. We sought to characterize the structural and functional organization of the phycobilisome of the macrophytic red alga *Gracilaria tikvahiae*. Because a number of pigment mutants of this species have been isolated and genetically characterized (23, 24), the genetic regulation of phycobilisome structure, assembly, and function can be examined. Some of the *Gracilaria* pigment mutants (13) have a lower PE/PC ratio than wild type, while others have a higher PE/PC ratio than wild type. Therefore, we examined whether the phycobiliprotein composition of the phycobilisomes isolated from the different *Gracilaria* strains corresponds to the biliprotein composition of the whole alga in order to test the model for phycobilisome structure. Further, we examined whether the presence of particular 'linker' polypeptides in the *Gracilaria* phycobilisome was correlated with any phycobilisome structural parameters such as PC and PE content or phycobilisome size. Based on these observations, the structure of the *Gracilaria* phycobilisome is discussed and the genetic control of synthesis and assembly considered.

**MATERIALS AND METHODS**

*Alga Material and Culturing.* The *Gracilaria tikvahiae* stocks were cultured in a modified f/2 medium (11) and *Anacystis nidulans* (UTEX 625) was cultured in BG-11 (1, 12). Field-collected *G. tikvahiae* was obtained at Waquoit Bay, near Falmouth, MA. All analyses of *Gracilaria* were carried out with cultured material unless indicated otherwise.

**Phycobilisome Isolation.** Three methods of phycobilisome isolation were employed.

*Method 1.* In all of the initial experiments, phycobilisomes...
were prepared using a method similar to that of Gantt and coworkers (7). This procedure was carried out at 20°C, using 0.75 M NaK P (pH 7.2) for extraction, and an alga to buffer ratio of 1 g to 10 ml. Although this method results in good phycobilisome yields for unicellular algae and many macrophytes including wild type *Gracilaria* and *ora*, an R-PE overproducer, it is not satisfactory for PE-deficient mutants of *Gracilaria* which have low solubility in 0.75 M NaK P, buffer.

**Method II.** All steps were carried out at 5 to 10°C. Algae were suspended in 0.85 to 1.2 M NaK P buffer containing 4% (w/v) sucrose at a ratio of 1 g alga to 1 to 5 ml of extraction buffer and homogenized in a French press at 20,000 p.s.i. The homogenate was chromatographed on Nondenat P-40 or Triton X-100 incubated for 10 min, and centrifuged for 2 min at 44,000 g. The blue or red supernatant was removed from beneath a green Chl-containing layer and the centrifugation was repeated twice more. The crude phycobilisome-containing extract was concentrated 2- to 8-fold using Sephadex G-25. One ml was loaded onto a 31-ml linear sucrose gradient of 10 to 35% or 10 to 25% sucrose-containing 0.75 M NaK P, buffer, and centrifuged for 24 and 30 h, respectively, in a SW 25.1 rotor at 53,000 g and 10°C. The gradients were fractionated and the absorbance measured at 546, 620, or 660 nm. Careful gradient preparation, centrifugation, and fractionation permitted reproducible sedimentation patterns to be obtained. Further details are given in Kursar (11).

**Method III.** R-PE and PC bound to phycobilisomes and that which was free were separated in less than 1 h using a method similar to that described by Rigbi et al. (18). A crude phycobilisome extract was prepared as described in method I using 0.85 M NaK P, (pH 7.2) containing 20% sucrose. The Chl-free crude phycobilisome extract was made 15% (w/v) in polyethylene glycol 4000 (PEG), incubated for 20 min at 20°C, and centrifuged for 2 min at 44,000 g. The upper phase contained the free phycobiliproteins, with phycobilisomes concentrated at the interface, and the lower phase was colorless or light green (11).

**Electron Microscopy.** Electron microscopy was performed in a Siemens 101 operated at 80 kV. The primary magnification of the negatively stained preparations was 40,000. Copper grids (200 mesh) were prepared with a Formvar film and carbon coated. The grids were glow discharged in a Balzers apparatus just before use. Phycobilisome preparations were prefixed in glutaraldehyde, applied to the grid, and stained with uranyl acetate (2).

**Ultracentrifuge Measurements.** Ultracentrifugal analyses were carried out in a model E analytical ultracentrifuge operated at 20,000 to 28,000 rpm and 25°C. UV optics were employed and the sample A at 265 nm was 0.4 to 0.6. The phycobilisomes were dialyzed against 0.75 M NaK P, buffer (pH 7.2) and the $S_{0.0}$ values were corrected to $S_{0.0}$ coefficients using a correction factor of 2.00 (8).

**Spectroscopic Measurements.** All spectroscopic measurements were made at room temperature. Absorption measurements were made in an Amino DW-2 dual beam spectrophotometer, and fluorescence measurements were made with an Amino SPF-500 spectrofluorometer.

**Phycobiliprotein Analyses.** Phycobilisome preparations were dialyzed against 50 mM NaP, (pH 5.5) for 12 to 48 h. The absorbances at 498, 614, and 651 nm were determined. PC and APC concentrations were calculated as described (13). Inasmuch as no extraneous material absorbing at 498 nm was present in the fractions containing phycobilisomes, the R-PE concentration (µg/ml) was calculated using equation 1 which is derived solely from the properties of isolated biliproteins.

\[
R-PE = 155.8 \times A_{498} - 6.67 \times A_{614} - 1.75 \times A_{651}
\]

(1)

**Electrophoresis.** Samples were dissociated in 5 mM Tris-glycine (pH 8.3) containing 1% SDS and 1% 2-mercaptoethanol by heating for 2 min at 80°C. Electrophoresis was carried out in 12.5% polyacrylamide gels overlaid with a 3% stacking gel as described by Laemmli (14) except that the temperature was 12°C and the gels were run at constant voltage (150 v). The gels were stained overnight in 50% methanol, 10% TCA, and 0.1% Coomassie Brilliant Blue R and destained in 10% acetic acid.

**RESULTS**

The isolated phycobilisomes of wild type *Gracilaria* have absorption peaks in the visible at 498, 545, and 565 nm assigned to PE and 614 nm assigned to PC and a shoulder at 650 assigned to APC (Fig. 1a). Excitation of the PE in intact phycobilisomes at 498 nm results in a fluorescence emission spectrum with a peak at 670 to 674 nm (Fig. 1a); this is characteristic of intact, functional phycobilisomes and indicates that the transfer of excitation energy from PE to PC and then to APC occurs efficiently. Excitation of dissociated phycobilisomes at 498 nm results in an emission spectrum with a peak at 575 nm characteristic of PE. Analogous results are obtained upon excitation of APC or PC in either intact or dissociated phycobilisomes (Fig. 1b).

When a crude phycobilisome extract of *Gracilaria* is separated by sedimentation velocity on a linear sucrose density gradient, most of the biliproteins migrate as phycobilisomes (Fig. 2).

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**FIG. 1.** Absorption and fluorescence spectra of the isolated phycobilisomes of wild type *Gracilaria*. Phycobilisomes were isolated by method I (see "Materials and Methods"), and fraction 17 (see Fig. 2) of the isolation gradient was analyzed. In the upper box (a) is the absorption spectrum ($A_{565} = 0.65$) of the isolated phycobilisomes (-----) and the fluorescence emission ($A_{565} = 0.05$) of the isolated phycobilisomes (-----) in 0.75 M NaK P. The sample was excited at 498 nm (10 nm bandpass) and the emission (1.0 nm bandpass) was corrected on a quantum basis. The corrected fluorescence emission spectra of the dissociated phycobilisomes are in the lower box (b). Phycobilisomes were dissociated by dialysis against 50 mM NaP, (pH 7.0) and $A$ at 565 nm was 0.05. The sample was excited at either 498 nm (10 nm bandpass) (-----), 615 nm (15 nm bandpass) (-----), or 640 nm (10 nm bandpass) (-----) resulting in emission peaks at 574 nm, 640 to 660 nm, and 660 nm, respectively, using a 1.0 nm emission bandpass.

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Correlated with intensities emission at excited by phycobilisomes from geneous reflects the great which absorbs than 5. About 620 nm into 0.75 nm is absorbed practically by phycobilisomes.

Fractions 10 or higher contain functionally intact phycobilisomes inasmuch as the ratio of 670 to 575 nm fluorescence is greater than 5. About 90% of the material recovered from the gradient which absorbs at 498 nm (primarily PE) is found in the phycobilisome band. The high UV absorbance at the top of the gradient may be due largely to residual Triton.

The ratio of $A$ at 498 to 612 nm, which approximates the PE/PC ratio, is 1.3 in fraction 10 and increases with increasing fraction number to a value of 2.6 in fraction 20 (Fig. 2). Larger phycobilisomes have a higher PE content. The phycobilisomes of *Gracilaria* freshly collected in the field are even more heterogeneous and contain two major size classes of phycobilisomes. That the larger phycobilisomes also have a higher PE/PC ratio is demonstrated by a comparison of the gradients scanned at 565 nm, which is absorbed primarily by PC, and at 620 nm, which is absorbed primarily by PE (Fig. 3). The absorption spectra of phycobilisomes from the two peaks in the 620 nm scan in Figure 3 have quite different biliprotein compositions (11). Therefore, the great width of the phycobilisome-containing band accurately reflects the size heterogeneity of phycobilisomes in wild type *Gracilaria*.

For wild type and two mutants, phycobilisome size also is correlated with PE content. Negatively stained preparations of wild type phycobilisomes taken from fraction 17 of a gradient (equivalent to that shown in Fig. 2) have average sizes of 40.0 ($\sigma = 6.5$) by 31.0 nm ($\sigma = 6.0$). The phycobilisomes of *ora*, a PE overproducer, are 45.0 ($\sigma = 6.6$) by 32.0 nm ($\sigma = 6.0$). The phycobilisomes of vrt$^2$, a PE-deficient mutant, scored as rods 26.0 nm ($\sigma = 1.5$) by 11.0 nm which may represent dissociated phycobilisomes, or as ellipses 24.0 nm ($\sigma = 3.5$) by 19.5 nm ($\sigma = 3.5$) (Fig. 4). The measured sedimentation coefficients of the total population of phycobilisomes of wild type and *ora* are 92 s and 116 s, respectively. The absorption spectra of the isolated phycobilisomes of *ora* and vrt$^2$ phycobilisomes are quite different (Fig. 5); *ora* phycobilisomes are composed primarily of PE, whereas vrt$^2$ phycobilisomes are composed primarily of PC and APC. Since the gross changes in phycobilisome size and com-
Excitation at 498 or 580 nm of isolated phycobilisomes from all of the strains which we have analyzed yields fluorescence emission spectra equivalent to that of wild type phycobilisomes. Phycobilisome sizes were characterized more precisely by running crude phycobilisome extracts on standard linear sucrose gradients. Using this method with the wild type and 10 mutants, a simple relationship is found between the algal pigment content, the biliprotein composition of the phycobilisomes, and the size of the isolated phycobilisomes. In most strains, the PE content of the isolated phycobilisome corresponds to the PE content of the Chl-free aqueous extract. For example, wild type, ora, and vrt2 have 57%, 75%, and less than 5%, respectively, of the total biliprotein as PE in their aqueous extracts and the isolated phycobilisomes are 57%, 77%, and 6.8% PE, respectively (Table I, columns b, and c). In most strains, a higher fraction of PE in the isolated phycobilisome corresponds to a proportionate increase in the PE/PC ratio. Wild type, ora, and vrt2 have PE/PC ratios of 2.2, 5.9, and 0.14, respectively (Table I, column e). A larger PE/PC ratio corresponds to a longer PE:PC rod and hence to a larger phycobilisome. The relative migration distances of wild type, ora, and vrt2 are 13.5, 18.6, and 7.8, respectively (Table I, column d; Fig. 6). The altered ultrastructural and sedimentation characteristics (Table I; Figs. 4, 5, and 6) of ora and vrt2 phycobilisomes can be adequately accounted for by changes in the phycobilisome masses rather than phycobilisome densities. Similar results regarding these three points are presented for NMG-2, uai, obr, mos2, Pur, and NMY-1 and for three alleles of the vrt gene, vrt, vrt2, and vrt3 (Table I; Fig. 6).

The material at the top of the gradients (Figs. 3 and 6) is residual Chl, free biliproteins, and light scattering debris, whereas the main phycobilisome-containing bands migrated away from this region. Most of the material which remains at the top of the gradients is absent from PEG-isolated phycobilisomes (11). If

position described above were found in the _Gracilaria_ mutants, the phycobilisomes from these and a number of other mutants were analyzed in more detail.

**Table 1. Pigment Composition and Migration Distance of Phycobilisomes**

<table>
<thead>
<tr>
<th>Species and Strains</th>
<th>PE Content of Crude Extract (a)</th>
<th>PE Content of Phycobilisomes (b)</th>
<th>Peak Fraction Number (d)</th>
<th>PE/PC Mole Ratio (e)</th>
<th>PC/APC Mole Ratio (f)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Gracilaria tikvahiae</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NMG-2</td>
<td>&lt;5</td>
<td>11*</td>
<td>0.24</td>
<td>1.7</td>
<td></td>
</tr>
<tr>
<td>uai</td>
<td>&lt;5</td>
<td>6.5*</td>
<td>0.13</td>
<td>1.6</td>
<td></td>
</tr>
<tr>
<td>vrt2</td>
<td>&lt;5</td>
<td>6.8*</td>
<td>7.8</td>
<td>0.14</td>
<td>1.5</td>
</tr>
<tr>
<td>vrt</td>
<td>46</td>
<td>37</td>
<td>11.0</td>
<td>0.98</td>
<td>2.1</td>
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<tr>
<td>vrt3</td>
<td>51</td>
<td>43</td>
<td>11.8</td>
<td>1.3</td>
<td>1.8</td>
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<tr>
<td>obr</td>
<td>53</td>
<td>58</td>
<td>13.1</td>
<td>2.2</td>
<td>2.3</td>
</tr>
<tr>
<td>wild type</td>
<td>57</td>
<td>57</td>
<td>13.5</td>
<td>2.2</td>
<td>2.0</td>
</tr>
<tr>
<td>mos2</td>
<td>13</td>
<td>62</td>
<td>14.5</td>
<td>2.1</td>
<td>2.4</td>
</tr>
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<td>Pur</td>
<td>62</td>
<td>64</td>
<td>15.7</td>
<td>3.0</td>
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<tr>
<td>ora</td>
<td>75</td>
<td>77</td>
<td>18.6</td>
<td>5.9</td>
<td>1.9</td>
</tr>
<tr>
<td>NMY-1</td>
<td>62</td>
<td>74</td>
<td>7.8</td>
<td>0.045</td>
<td></td>
</tr>
</tbody>
</table>

*If the isolation gradient contains 0.75 mM NaK Pi, 50 to 80% of the vrt2, uai, or NMG-2 phycobilisomes form a pellet and the remaining phycobilisomes have the PE content indicated in the above table. If 0.50 mM NaK Pi is substituted for 0.75 mM NaK Pi, the vrt2 phycobilisomes are fully soluble and the isolated vrt2 phycobilisomes contain less than 2% of PE. The higher PE content of vrt2 phycobilisomes reported in the above table is probably due to the preferential precipitation, in the high phosphate buffer, of those vrt2 phycobilisomes which are the most deficient in PE (11).
Fig. 6. Profiles of the phycobilisome isolation gradients of (a) vr
(b) wild type, (c) or, (d) vr, (e) NMY-1, and (f) vr. Phycobilisomes
were isolated by method II except that a 10 to 20% gradient and a
centrifugation time of 30 h were used for (d), (e), and (f). The gradients
were all scanned at 620 nm and or was also scanned at 546 nm (--.--).

Phycobilisomes prepared using PEG are analyzed on a sucrose
gradient, 40 to 70% of the biliprotein sediments to the bottom
and the remaining soluble phycobilisomes migrate as though
aggregated (11). The integrity of the phycobilisomes is main-
tained during the isolation procedure. After isolation on a sucrose
gradient or after rapid separation of free biliproteins from phy-
cobilisomes using PEG, in most cases 80 to 90% of the PE or
PC recovered is found in the phycobilisomes (data not shown).
Two exceptions are mos2 in which 90% of the PC is not phycobil-
osome bound and NMY-1 in which essentially all of the PC and
40% of the PE are not phycobilisome bound.

The percent of PE in each of the major phycobilisome-contain-
ing sucrose gradient fractions of vr, vr, wild type, mos2,
Pur2 gradients and or is presented in Table II. The PE content
of the phycobilisomes in fraction 12 of vr, vr, wild type, mos2,
and Pur2 is between 43.9 and 57.2% (Table II). Similarly, the PE
content in fraction 17 of wild type, mos2, Pur2, and or is between
66.5% and 74.1%. The differences between strains in the PE
content of equivalent fractions are probably due to diffusion and
convection from the region of highest particle concentration. In
the absence of such effects, the PE content of a particular sucrose
gradient fraction should be nearly equivalent in all of the strains
presented in Table II. If so, the gross architecture of the phycob-
ilisome must be the same in all strains (except NMY-1) and the
phycobilisome sedimentation properties depend primarily on
their content of PE.

A population of phycobilisomes which are heterogeneous in
size should also be heterogeneous in biliprotein composition. In
wild type, the phycobilisomes are 49.9% PE in fraction 12, 54.3% in
fraction 13, which increases to 66.5% PE in fraction 17 (Table
II). Analogous results were obtained for vr, vr, Pur2, and mos2.
These data confirm that, in a particular strain, an increase in
phycobilisome size is associated in an increase in the PE content
of the phycobilisomes and that in most strains the phycobilis-
omes are heterogeneous in size. The phycobilisomes of NMY-1
are unusual in this regard; all of the NMY-1 phycobilisome
fractions in Figure 6e have nearly the same biliprotein compo-
nition (Table II). Or phycobilisomes also are relatively homo-
genous as shown by inspection of Table II and by comparison of
gradient scans at 620 and 546 nm (Fig. 6c; cf. Fig. 3).

Most of the strains containing significant amounts of PE make
phycobilisomes which are quite heterogeneous in size. To inves-
tigate size heterogeneity of strains with small phycobilisomes,
samples were separated on a shallow sucrose gradient (10 to
20%). The phycobilisomes of NMY-1 and vr form a broad band
and are heterogeneous (Fig. 6, e and f), whereas vr2 phycobilis-
omes form a narrow, symmetrical band suggesting that they are
homogeneous in size (Fig. 6d).

The PC/APC should be independent of fraction number. For
the four strains above and obr, the ratios of PC to APC of the
individual gradient fractions are 2.0 ± 0.5 (data not shown); the
average PC/APC of the main phycobilisome band in wild type
and of the mutants examined is also 2.0 ± 0.5 (Table I, column
f). The uncertainty is due to the concentration dependence of
the PC and APC extinction coefficients. Additional evidence on

Table II. PE Content of the Principal Phycobilisome-Containing Sucrose Gradient Fractions of Wild Type
and Selected Mutants of Gracilaria

<table>
<thead>
<tr>
<th>Fraction</th>
<th>NMY-1</th>
<th>vr</th>
<th>vr</th>
<th>Wild</th>
<th>mos2</th>
<th>Pur2</th>
<th>or</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>PE (%)</td>
<td>PE (%)</td>
<td>PE (%)</td>
<td>PE (%)</td>
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<td>PE (%)</td>
<td>PE (%)</td>
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<tr>
<td>Crude extract</td>
<td>62</td>
<td>46</td>
<td>51</td>
<td>57</td>
<td>13.5</td>
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<td>75</td>
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<td>Fraction 9</td>
<td>20.9</td>
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<tr>
<td>Fraction 10</td>
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<td>32.0</td>
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<td></td>
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<tr>
<td>Fraction 11</td>
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<td>38.0</td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>Fraction 12</td>
<td>43.9</td>
<td>44.2</td>
<td>49.9</td>
<td>53.1</td>
<td>57.2</td>
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<tr>
<td>Fraction 13</td>
<td>48.4</td>
<td>48.5</td>
<td>54.3</td>
<td>58.0</td>
<td>60.2</td>
<td></td>
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</tr>
<tr>
<td>Fraction 14</td>
<td>71.7</td>
<td>51.7</td>
<td>58.9</td>
<td>60.6</td>
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<tr>
<td>Fraction 15</td>
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<td>61.3</td>
<td>64.7</td>
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<td>67.5</td>
<td>68.4</td>
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<tr>
<td>Fraction 17</td>
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<td>69.7</td>
<td>70.7</td>
<td>74.1</td>
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<tr>
<td>Fraction 18</td>
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<tr>
<td>Fraction 19</td>
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<tr>
<td>Fraction 20</td>
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the amount of phycobilisomes 
were independent of emission or absorption.

Measurements, fluorescence (Table I, 366 KURSAR 17 (-)) The whole algal pigment contents of the 'bright green' mutants vrt2, uai, and NMG-2 are quite similar (13); the pigment compositions of the isolated phycobilisomes are also quite similar (Table I, columns c, e, and f). In particular, their phycobilisome PC/APC ratios are low, 1.5 to 1.7, indicating that they probably have an incomplete complement of PC. The phycobilisomes of all three mutants also contain PE; excitation of the dissociated phycobilisomes at 498 nm generates a fluorescence emission spectrum characteristic of PE (data not shown [see 13]). The phycobilisomes of vrt2, uai, and NMG-2 have essentially identical sedimentation patterns (data not shown). At this level of analysis, these three mutants have the same phenotype; therefore, both Mendelian and non-Mendelian mutations can result in similar and marked changes in PE synthesis and incorporation into the phycobilisome. 

The phycobilisomes of NMY-1 are exceptional in several regards. Their composition is 71% PE and 27% APC, with negligible amounts of PC. NMY-1 phycobilisomes migrate as two bands on sucrose gradients having sedimentation properties similar to phycobilisomes which contain only 40% PE (for example, vrt in Figure 6f). Even though NMY-1 phycobilisomes contain essentially no PC, they have a fluorescence emission peak at 670 to 674 nm upon excitation at 498 nm (Fig. 8). Apparently PE is attached directly to APC and transfers excitation energy to APC efficiently. The phycobilisomes of NMY-1 are also unstable on a time scale of days, during which wild type phycobilisomes remain essentially intact. In preliminary experiments on NMY-1 cultured in Halifax, shipped to Chicago and maintained under slow growing conditions, the PC/APC ratio of the isolated phycobilisomes was about 0.5. These features suggest that NMY-1 may be a leaky mutation whose phenotype depends on growing conditions.

Phycobilisomes of Gracilaria were shown by SDS-PAGE to contain a number of larger polypeptides, termed linker polypeptides, in addition to the α and β subunits of the biliproteins (12–22 kD). The smallest phycobilisomes, those of vrt2 and uai, which are extremely deficient in PE, contain primarily 29 and 89 kD linker polypeptides (Fig. 9, lanes 4, 5, 9, and 10), while...
vt and wild type and ora phycobilisomes with a PE/PC ratio of 1 to 6, contain a 34 kD protein in addition to the 29 and 89 kD proteins (Fig. 9, lanes 1, 2, 3, 6, 7, and 8). The phycobilisomes of ora also contain a fourth linker polypeptide which appears as a diffuse band at 31 kD (Fig. 9, lanes 1 and 6). The phycobilisomes of NMY-I contain only the 89 kD linker polypeptide (Fig. 9, lane 11). Other less intensely stained bands may be contaminants, degradation products of the main bands, or represent additional phycobilisome-specific linker polypeptides.

**DISCUSSION**

The present investigation demonstrates that the phycobilisomes of several strains of the macrophytic red alga *G. tikvahiae* can be isolated in forms representative of their *in vivo* state, that the general architecture of the phycobilisomes among the strains is conserved, and that phycobilisome size is directly related to R-PE content. Further, this study demonstrates that cooperative interactions between nuclear and cytoplasmic genomes must control phycobilisome pigment composition and size, and consequently the functional organization of the light-harvesting assemblages and the reaction centers of photosynthesis in red algae.

That the phycobilisomes are isolated in a native state is evidenced by the presence of 80 to 90% of the R-phycocerythrin and PC in the phycobilisomes. This high recovery is similar to that reported for *Anacystis* and *Neoagaridia* (12). Furthermore, the isolated phycobilisomes show fully coupled energy transfer from PE to APC (Fig. 1). The phycobilisome architecture of wild type *Gracilaria* and a number of mutants is remarkably constant. For most strains, the phycobilisome PC/APC ratios are about two even though the phycobilisome PE/PC ratio is highly variable. This includes the Mendelian mutant mos2 which contains a great excess of PC over APC. Nevertheless, the PC/APC ratio of the mos2 phycobilisomes is only 2.5 and, relative to other strains, there is no dramatic increase in PC incorporation into the phycobilisomes.

Our observations suggest specific functions for at least two linker polypeptides in the phycobilisomes of *Gracilaria*. The non-Mendelian mutant NMY-I makes PC, yet the phycobilisomes of this strain probably contain less than one PC for every six APCs. Yamanaka and Glazer (26) provide evidence that 29 and 89 kD polypeptides are required to assemble the PC/APC core of the cyanobacterial phycobilisome. Mutant NMY-I lacks a 29 kD polypeptide, which, if essential for the attachment of PC to APC, would explain the absence of PC in NMY-I phycobilisomes. Therefore, our studies are consistent with the proposed functional role for the 29 kD linker polypeptide in phycobilisomes and demonstrate its common occurrence in a macrophytic red alga. Further, our data suggest that non-nuclear genes may code for polypeptides essential for the structural integrity of the red algal phycobilisome. Except in NMY-I, a 34 kD polypeptide is associated with the presence of PC; this polypeptide may be the γ subunit of PE or it may be required to attach PE subunits either to PC or to the PE:PC rods of the red algal phycobilisome.

*Gracilaria* probably contains PE:PC rods. In sucrose gradient fractions 5 and 6 of ora, particles composed primarily of PE and PC are consistently observed. On a weight basis, the apparent APC content is only 1 to 4% and the PE/PC ratio is 3.0 to 3.6. Upon excitation at 498 nm, these particles fluoresce at 576 and 641 nm; the 641 nm peak is more intense by a factor of 1.2 to 1.7 (spectra not shown). These particles which are also present in wild type have many of the properties of the PE:PC rods which have been described in a unicellular red alga (10).

While there are six biliprotein rods per phycobilisome in other species, it is not clear whether this is true in *Gracilaria*. The mass of *Anacystis* phycobilisomes is 4.9 ± 0.9 × 10⁶ (12). If we assume that *Gracilaria* and *Anacystis* phycobilisomes having the same mass also have the same frictional coefficient, it is possible to use the *Anacystis* phycobilisomes to calibrate one point in a sucrose gradient. The phycobilisomes of *Anacystis* migrate with a peak fraction number of 13.2 on a standard sucrose gradient (Table I). Wild type and vt phycobilisomes at this fraction number are 55.2 and 50.2% PE, respectively (interpolated from Table II). If we assume that the *Gracilaria* phycobilisomes are 15% uncolored proteins (17, 22) and have a PC/APC ratio of 2.0, the number of PC hexamers per *Gracilaria* phycobilisome is calculated to be 5.8 in wild type and 6.6 in vt. Therefore, the phycobilisomes of *Gracilaria* and most of the mutants are probably composed of three APC hexamers, six PC hexamers, and a variable amount of PE.

The phycobilisome structure proposed by several groups lacks rotational symmetry (2, 10, 17) (Fig. 10, model 1). The presence of linker polypeptides in the phycobilisome may generate the phycobilisome asymmetry. The γ subunit of B-PE is asymmetrical (3) and if the linker polypeptides are also asymmetric. The 3-fold rotational symmetry of the subunits (3) and the well-known role of molecular symmetry in biological self-assembly processes (9) argue that the phycobilisome structure may be rotationally symmetric as proposed here (Fig. 10, model 2).

In this structure, the overlapping electron dense material would not necessarily allow resolution of PE:PC rods; such images are frequently observed in electron micrographs (2, 8, 10, 25). The trimer core with six attached rods may be observed only when the rods stick to the grid surface.

A 3-fold symmetry axis could confer several advantages to phycobilisome structure which are entirely unrelated to a self-assembly process. Three-fold rotational symmetry is found in two other light-harvesting pigment-proteins, a bacteriochlorophyll-protein and a bacteriorhodopsin (3). Fisher et al. (3) point out that 3-fold rotational symmetry is the lowest symmetry for which there are no preferred oscillator angles. Therefore, a randomly oriented chromophore array should be most efficient for harvesting unpolarized light.

A second consideration favoring 3-fold symmetry is that strong chromophore-chromophore interactions can effectively create dimeric or higher order structures which may quench excitations (15), thereby reducing the efficiency of energy transfer. All evenly symmetric pigment-protein structures would generate pairs of oscillators which have parallel transition moments. A parallel orientation of transition moments will increase the probability of strong interactions and the consequent quenching of excitations. Odd rotational symmetry results in the weakest angular dependence for interactions between the equivalent oscillators on each subunit. The angle between symmetrically related oscillator pairs for odd symmetric structures is 60° for 3-fold, 72° for 5-fold, and 51.4° for 7-fold rotational symmetry. The exact relationship between the mutual transition moments and the strength of any interactions which might generate quenching sites depends upon the quantum mechanical model used to describe such interactions. For example, for the Förster model of weak dipole-dipole coupling (4), the interaction energy of chromophores related either by 3-fold or 5-fold rotational symmetry is 10 times less than for the case of parallel orientation. Therefore, the self-assembly of a chromophore array which lacks quenching sites may be significantly more probable for an odd symmetric structure than for an even symmetric assembly.

Based on the data presented here and in a companion report, a model for the organization of the light-harvesting systems and reaction centers in the cyanobacterial and red algal photosynthetic apparatus is proposed. Two characteristics of the photosynthetic lamellae, the Chi to reaction center I and Chi to reaction center II ratios, appear to be highly conserved in a number of cyanobacterial and red algal species (12, 13). A third property, the mass of biliprotein packed into the interthylakoidal space is

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**PHYCOBILISOMES OF GRACILARIA PIGMENT MUTANTS**

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FIG. 10. Left, Alternative models of phycobilisome structure. APC is indicated by circles and the PE:PC rods are indicated by rectangles. Model 1 has been proposed by Köller et al. (10) and others (2, 17). Model 2 conserves the 3-fold rotational axis of symmetry of the subunits. Right, Model of phycobilisome-RC II association in the cyanobacteria and red algae. Phycobilisomes are indicated by circles and RC IIIs by squares. (a), ora; (b), wild type; and (c), vrt2.

quite similar in *Neoagarididella*, *Gracilaria*, and *Anacystis* (Table I, column I, in Reference 13). One consequence of having fewer, larger phycobilisomes is that the ratio of reaction center IIIs must increase. This idea is supported by the demonstration that the reaction center II to phycobilisome ratio is more than 2-fold greater in *Neoagarididella* (4.1) than in *Anacystis* (1.7) which have phycobilisome masses of about 10–15 × 106 and 5 × 106, respectively (12). The *Gracilaria* strains allow one to study the relationship between phycobilisome size and packing within a single species. For example, the total biliprotein per Chl of wild type, *ora* and *vrt2* phycobilisomes probably differs from wild type by 2- to 3-fold, the ratio of biliprotein to Chl in these strains only changes by 10 to 20%. The abundance, on a Chl basis, of APC and PC in *ora* is about 60% of the value in wild type (13). Therefore, it appears that *ora*, which has larger phycobilisomes than the wild type, probably has a decreased number of phycobilisomes on a Chl or reaction center II basis. If the mass of biliprotein per area of lamellae does not change, then an increase in biliprotein per area of lamellae size must result in a decrease in the number of phycobilisomes per area of lamellae or per Chl. The mutant *vrt2*, which makes very small phycobilisomes, has APC to Chl and PC to Chl ratios which are about 180% of the wild type value (13). This mutant should have more phycobilisomes per Chl than does wild type. If the structure of the Chl-containing membrane is the same in the mutant *pur2* and wild type, a more general model than the above will be required in order to include the purple mutants of *Gracilaria*. The phycobilisomes of *pur2* are similar to wild type in size and composition (Tables I and II), yet relative to Chl, *pur2* contains 6-fold more biliprotein than wild type (Tables I, column I, in Reference 13). Even though phycobilisome packing appears to constrain the structure of the photosynthetic apparatus in *Gracilaria*, *pur2* may contain many more phycobilisomes per area of membrane than are found in wild type. The biochemistry and anatomy of the purple mutants of *Gracilaria* remain to be studied.

A change in phycobilisome size when the amount of biliprotein per area of lamellae is held constant could result in a coordinate change in the ratio of reaction center IIIs to phycobilisomes. For example, in Figure 10b, the reaction center II to phycobilisome ratio is 1 to 2 in wild type, 3 to 4 in *ora*, and only 1 in *vrt2*. A second case, not shown in Figure 10b, is that of an increase or decrease in the amount of biliprotein per area of lamellae, such as may be occurring in *pur2*. The model assumes a uniform thylakoid system, unlike that found in green plants (21), and is applicable to *Anacystis* or *Neoagarididella* and those cyanobacterial or red algal species which contain or lack PE. Therefore, changes in phycobilisome size, whether controlled directly by genetic lesions or environmental factors, have large and functionally significant influences on the organization of the photosynthetic unit of the cyanobacteria and red algae.

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