Characterization of R-Phycocyanin

CHROMOPHORE CONTENT OF R-PHYOCYANIN AND C-PHYCOERYTHRIN*

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R-Phycocyanin was purified from two independent isolates of the unicellular red alga Porphyridium cruentum. At pH 7.0, the protein sediments as a single component with $s_{20,w}$ of 5.98 S (at 2 mg/ml, $c/2 = 0.02$). Over a protein concentration range of 0.2 to 0.5 mg/ml ($c/2 = 0.16$), sedimentation equilibrium gave a molecular weight of 103,000 ± 6,000, with no evidence of heterogeneity. In common with C-phycocyanins, R-phycocyanin consists of $\alpha$ and $\beta$ subunits of molecular weights of 18,200 and 20,500, determined by electrophoresis in sodium dodecyl sulfate-polyacrylamide gels. Isoelectric focusing in polyacrylamide gels resolves two bands, blue (at pI of 5.2), and purple (at pI of 5.3), believed to correspond to the $\alpha$ and $\beta$ subunits, respectively. The native protein gave a single precipitin band when tested against the homologous antiserum by the Ouchterlony double diffusion technique. No cross-reaction was observed with antiserum to the allophycocyanin from the same organism.

The absorption spectrum of native trimeric R-phycocyanin at pH 7.0 exhibited $e_m (555 \text{ nm})$ of 1.51 x $10^{5} \text{ M}^{-1} \text{ cm}^{-1}$, $e_m (618 \text{ nm})$ 2.55 x $10^{5} \text{ M}^{-1} \text{ cm}^{-1}$, and $A_{560}^{\%}$ of 70.0. The circular dichroism spectrum of the native protein was characterized by the following molecular ellipticity maxima in deg cm$^2$ per dmol x $10^{-7}$: $[\theta]_{555} = -2.36$, $[\theta]_{618} = -3.27$, $[\theta]_{560} = 4.67$, and $[\epsilon]_{650} = -6.27$. All of these values were based on an $\alpha\beta$ molecular weight of 36,300, calculated from the amino acid composition.

To permit quantitative estimation of the chromophore composition of R-phycocyanin, the absorption properties of Aphanocapsa sp. C-phycoerythrin were determined. At pH 7.0, native C-phycoerythrin exhibited $e_m (562 \text{ nm})$ of 4.88 x $10^{5} \text{ M}^{-1} \text{ cm}^{-1}$, and $A_{560}^{\%}$ of 125, based on an $\alpha\beta$ molecular weight of 38,400 calculated from the amino acid composition.

The molar extinction coefficients for polypeptide-bound phycoerythrobilin were calculated from the spectrum of denatured C-phycoerythrin in 8 M urea at pH 1.9, on the assumption that each $\alpha\beta$ unit contains six such chromophores. The analogous data for phycocyanobilin was available from an earlier study (Glazer, A. N., and Fang, S. (1973) J. Biol. Chem. 248, 659-662).

The absorption curve of denatured R-phycocyanin was fitted with high precision by a theoretical curve calculated for a mixture of two phycoerythrobilin and one phycocyanobilin chromophore. The amino acid analyses of R-phycocyanin and of its separated $\alpha$ and $\beta$ subunits demonstrated a 1:1 stoichiometry for the subunits in the native protein. The absorption spectra of the isolated subunits were consistent with the conclusion that the $\alpha$ subunit carries a single phycocyanobilin chromophore, while one phycoerythrobilin and one phycocyanobilin chromophore are bound to the $\beta$ subunit.

Because of the uncertainty as to the precise extinction coefficients for free phycoerythrobilin in aqueous acid, and the possible perturbation of the chromophore spectrum by the covalent links to the protein, the data do not rigorously rule out the possibility that C-phycoerythrin may contain 12 phycoerythrobilin chromophores per $\alpha\beta$ unit, and the $\beta$ subunit of R-phycoycyanin may contain two of these chromophores. However, this possibility is considered unlikely because of the high degree of similarity between R- and C-phycocyanins. The $\beta$ subunit of the latter carries two phycocyanobilin chromophores.

The major path for resonance energy transfer from the biliproteins to chlorophyll a follows the sequence: phycoerythrin to phycocyanin to allophycocyanin to chlorophyll a. Our earlier results (see citation above) taken in conjunction with the present study demonstrate that the chromophore content per $\alpha\beta$ unit, of similar molecular weight, follows the same order—six for C-phycoerythrin, three for C-phycocyanin, and two for allophycocyanin.

The chromophore density and relative orientation are of key importance to ensuring that interactions of both like and of dissimilar proteins within the phycobilisome will all lead to maximum efficiency of

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The phycobiliproteins are the photosynthetic accessory pigments of the prokaryotic blue-green and eukaryotic red algae. In both groups of organisms, these proteins are assembled into structures (phycobilisomes) regularly arranged on the photosynthetic lamellae (1-3). Depending on the organism, as many as four phycobiliproteins, each with distinctive spectroscopic properties, may be present in the phycobilisome (4-6). The ability to absorb light in the visible region of the spectrum is conferred upon these proteins by the presence of covalently linked linear tetrapyrrole derivatives, the phycobilins (7). The classification of the phycobiliproteins is complex and is summarized in Table I, to facilitate further discussion.

From a variety of studies on whole cells (see Ref. 17 and citations therein), as well as on phycobilisomes (5, 8), the major pathway for the transfer of trapped light energy from the phycobiliproteins to chlorophyll a follows the sequence:

\[
\text{phycocyanin} \rightarrow \text{phycoerythrin} \rightarrow \text{allophycocyanin} \rightarrow \text{chlorophyll a}
\]

The structural features of these molecules which are of the most central interest are those which relate to their function as donors and acceptors in the transfer of energy.

Inspection of Table I shows that R-phycoerythrin is the sole biliprotein possessing both phycocyanobilin and phycoerythrobilin prosthetic groups (18). The native protein displays absorption maxima characteristic of both C-phycoerythrin and C-phycoerythrin. The fluorescence emission maximum of R-phycoerythrin is at 636 nm, close to that of C-phycoerythrin, showing efficient energy transfer from the phycocyanobilin to the phycoerythrobilin chromophores. In common with other phycobiliproteins carefully examined to date, R-phycoerythrin is an oligomeric protein made up of two nonidentical subunits, \(\alpha\) and \(\beta\). Upon separation by electrophoresis in polyacrylamide gels containing sodium dodecyl sulfate, the bands corresponding to the \(\alpha\) and \(\beta\) subunits are blue and red-purple, respectively (6, 16). On the basis of this qualitative observation, O’Carra (16) concluded that the \(\alpha\) subunit contained solely phycocyanobilin, and the \(\beta\) subunit solely phycoerythrobilin.

Because of its unique prosthetic group composition, R-phycoerythrin provides an opportunity to examine in detail the architecture of a macromolecule which evolved for the effective transfer of energy between a limited number of both chemically distinct and of identical chromophores. Further, R-phycoerythrin may be of considerable interest as a surviving intermediate species in an evolutionary pathway leading from phycocyanin to phycoerythrin.

Inter alia, the studies reported here have led to an unambiguous determination of the subunit stoichiometry in R-phycoerythrin, and of the chromophore content of the protein, as well as that of each subunit. Comparative studies of the absorption spectra of R-phycoerythrin and of C-phycoerythrin, following denaturation in acid urea, have permitted quantitation of the number of phycoerythrobilin chromophores in the latter protein.

**EXPERIMENTAL PROCEDURE**

**Materials**

*Algae—An axenic culture of *Porphyridium cruentum* was obtained from Mr. R. Derman of the Department of Botany at the University of California, Berkeley, through the courtesy of Ms. Riyo Kunisawa. A separate isolate, *Porphyridium cruentum* B, was obtained from the Marine Biology Research Division, Scripps Institution of Oceanography, La Jolla, California. Except where specifically noted in the text, the studies described below utilized material from cells originating from the culture obtained from Berkeley.*

*The cells were grown axenically at 23-25° in Fernbach flasks equipped with magnetic stirrers in the medium described by Jones et al. (19). Cultures were continuously illuminated with a bank of fluorescent tubes (General Electric Warm White DeLuxe) at a light intensity of 50-60 foot-candles.*

**Table I**

Classification of phycobiliproteins of blue-green and red algae

The data were obtained on dilute aqueous solutions of the native biliproteins at near neutral pH.

<table>
<thead>
<tr>
<th>Biliprotein</th>
<th>Distribution</th>
<th>Representative organism</th>
<th>Major absorption maxima in the visible region</th>
<th>Fluorescence emission maxima</th>
<th>Refs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Allophycocyanin</td>
<td>Blue-green and red algae</td>
<td><em>Porphyridium cruentum</em></td>
<td>565(PCB) &gt; 555(PEB) &gt; 548(S)(PUB?)</td>
<td>600</td>
<td>8</td>
</tr>
<tr>
<td>C-Phycocyanin</td>
<td>Blue-green and red algae</td>
<td><em>Anacystis nidulans</em></td>
<td>620(PCB) &gt; 578(PEB) &gt; 548(S)(PUB?)</td>
<td>648</td>
<td>9</td>
</tr>
<tr>
<td>C-Phycoerythrin</td>
<td>Blue-green algae</td>
<td><em>Schizothrix calcicola</em></td>
<td>565(PCB) &gt; 578(PEB) &gt; 548(S)(PUB?)</td>
<td>577</td>
<td>10</td>
</tr>
<tr>
<td>R-Phycocyanin</td>
<td>Red algae</td>
<td><em>Porphyridium cruentum</em></td>
<td>617(PCB) &gt; 555(PEB) &gt; 488(PUB?)</td>
<td>636</td>
<td>8</td>
</tr>
<tr>
<td>R-Phycoerythrin</td>
<td>Red algae (Blue green?)</td>
<td><em>Ceramium rubrum</em></td>
<td>567(PEB) &gt; 538(PEB) &gt; 488(PUB?)</td>
<td>578</td>
<td>11, 12</td>
</tr>
<tr>
<td>B-Phycoerythrin</td>
<td>Red algae (Blue-green?)</td>
<td><em>Porphyridium cruentum</em></td>
<td>54(S) &gt; 565(PEB) &gt; 488(S)(PUB?)</td>
<td>575</td>
<td>8</td>
</tr>
</tbody>
</table>

*Organism which served as the source of the protein for which the spectroscopic properties are given.

*The abbreviations, given in parentheses, for the tetrapyrrole prosthetic groups responsible for the absorption maxima are: PCB, phycocyanobilin; PEB, phycoerythrobilin; PUB, phycoerythrin.*

*Data from Ref. 13.*
intensity of ~3000 lux at the surface of the culture vessel. The cultures were gassed above the level of the medium with 1% CO\textsubscript{2}/99% N\textsubscript{2} (v/v). The cells were grown to a density of 2.5 to 3.0 g per liter (7 to 10 days), harvested by centrifugation, and the pellets stored at -20°.

The unicellular blue-green alga of _Aphanocapsa_ sp. (strain 6701), originally obtained from the Berkeley collection (20), was cultured as described by Stanier et al. (20). The cells were grown to a density of approximately 1.5 g per liter, harvested by centrifugation, and the pellets stored at -20°.

**Chemicals**—Prespersion Whatman microgranular DEAE-cellulose DE52 was obtained from Reeve Angel, Clifton, New Jersey; Bio-Rex 70 (minus 400 mesh) from Bio-Rad, Richmond, California; and Sephadex G-200 from Pharmacia Fine Chemicals Inc., Piscataway, New Jersey. Ampholine carrier ampholytes (40% w/v, Batch 41) were obtained from LKB Produkter AB, Sweden. Ultrapure urea was purchased from Schwarz/Mann, Orangeburg, New York, and electrophoresis grade acrylamide from Eastman Organic Chemicals, Rochester, New York. Hydroxyapatite was prepared as described by Siegelman et al. (21) and stored at 4°. All other chemicals were of reagent grade.

**Methods**

**Preparation of R-Phycocyanin**—All buffers mentioned in this paper contained 10\textsuperscript{-3} M β-mercaptoethanol and 10\textsuperscript{-2} M sodium azide unless otherwise specified.

Frozen _Porphyridium cruentum_ cells were thawed and suspended in 0.01 M K-phosphate buffer at pH 7.0 (50 g wet weight of cells per 150 ml of buffer) at 4°. The thick suspension was gently homogenized by hand in an all-glass tissue grinder, and then passed through an Amino French Pressure Cell at 15,000 p.s.i. at 4°. The broken cell suspension was centrifuged at 25,000 × g for 20 min. The supernatant was decanted and the pellets washed twice with cold buffer. Solid (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4} was added slowly with stirring to the pooled supernatants, at 4°, to 65% of saturation. After 18 hours at 4°, the precipitate was collected by centrifugation and the supernatant discarded. The precipitate was dissolved in a minimum volume of 0.001 M K-phosphate/0.1 M NaCl, pH 7.0, and dialyzed to equilibrium against the same buffer at 4°. The viscous dialyzed solution was added to a thick slurry of hydroxyapatite (50 g wet weight of cells required 250 ml of gel as is shown in Fig. 1). The mixture was stirred slowly for 5 min and centrifuged at 4,300 × g for 10 min at 4°. The supernatant was discarded, and the hydroxyapatite pellet was washed once with the pH 7.0 buffer. The phycobiliproteins were then eluted from the hydroxyapatite with 0.2 M K-phosphate, 0.2 M Na-acetate, pH 5.5, and precipitated by the addition of solid (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4} to 65% of saturation. The precipitate was washed with cold buffer at pH 7.0. The precipitated phycobiliproteins were dissolved in 0.01 M K-phosphate buffer, pH 7.0, buffer (adsorption of the biliproteins from 50 g wet weight of cells required 250 ml of gel). The phycobiliprotein solution was then dialyzed exhaustively against 0.05 M Na-acetate buffer at pH 5.5 at -1°C for 18 hours at 4°. The precipitate was collected by centrifugation, and the supernatant discarded. The dialyzed, precipitated phycobiliprotein solution containing both R-phycoerythrin and R-phycocyanin was loaded, as 0.1 ml volumes, on DEAE-cellulose DE52 (5.3 x 8.2 cm), previously equilibrated with 0.02 M Na-acetate buffer at pH 5.5, with a linear gradient of 0.05 M Na-acetate buffer at pH 5.5 to 0.25 M Na-acetate buffer at pH 5.5, in the mixing chamber and 1200 ml of 0.05 M Na-acetate buffer, pH 5.5, in the reservoir, at a constant flow rate of 100 ml per hour. After completion of the gradient, the column was eluted with 600 ml of 0.55 M Na-acetate, pH 5.50 (a representative elution profile obtained on a smaller column is shown in Fig. 1). R-Phycocyanin was eluted in the early portion of the gradient. Fractions characterized by a ratio of A\textsubscript{585}/A\textsubscript{620} > 0.42 were pooled, and the protein concentration determined by the method of Yphantis (27). The eluted proteins were applied to a column of Sephadex G-200 at neutral pH.

**Electrophoresis**—Electrophoresis at pH 8.1 on 7.5% polyacrylamide gels was performed as previously described (22, 26).

**Acrylamide Gel Electrophoresis**—Electrophoresis at pH 8.1 on 7.5% polyacrylamide gels (8 cm long) was performed by the procedure of Andrews et al. (20). The column was then washed with 0.10 M K-phosphate buffer, pH 7.0. The supernatant was discarded, and the hydroxyapatite column was then washed with 0.10 M Na-phosphate buffer, pH 7.0, and dialyzed to equilibrium against the same buffer at 4°. The weight of R-phycocyanin at protein concentrations of 0.25, 0.37 and 0.50 mg/ml was measured. The column was then washed with 0.10 M K-phosphate buffer, pH 7.0, and dialyzed to equilibrium against the same buffer at 4°. The weight of R-phycocyanin at protein concentrations of 0.25, 0.37 and 0.50 mg/ml was measured. The column was then washed with 0.10 M K-phosphate buffer, pH 7.0, and dialyzed to equilibrium against the same buffer at 4°. The weight of R-phycocyanin at protein concentrations of 0.25, 0.37 and 0.50 mg/ml was measured. The column was then washed with 0.10 M K-phosphate buffer, pH 7.0, and dialyzed to equilibrium against the same buffer at 4°.

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Their mobility on calibrated Na dodecyl-SO₃-polyacrylamide molecular weights estimated for the α and β subunits from trace of material of higher molecular weight (Fig. 2, Gel 4). The dodecyl-SO₃-polyacrylamide gel electrophoresis as well as a band to the β subunit. Such behavior was observed for a hybrid C-phycocyanin described in an earlier report (22). Two bands R-phycocyanin dissociates on isoelectric focusing to its cy and β subunits. The most probable explanation of this finding is that were tric focusing (Fig. 2, Gel 3), two colored red-fluorescent bands single purple, red-fluorescent component (Fig. 2, Gel 1). On isoelectric electrophoresis, the R-phycocyanin preparation migrated as a faster moving colorless component (Fig. 2, Gel 2). Oxidation was allowed to proceed for 5 hours at 0°C. At the end of that time, the performic acid was removed by lyophilization, 50 μl of aqueous phenol solution (5% w/v) were added to the sample, and the lypsyliization was repeated. The oxidized protein was then hydrolyzed with 6 N HCl in vacuo for 24 hours.

RESULTS

Characterization of R-Phycocyanin—On polyacrylamide gel electrophoresis, the R-phycocyanin preparation migrated as a single purple, red-fluorescent component (Fig. 2, Gel 1). Staining revealed a small amount (~4% by densitometry) of a faster moving colorless component (Fig. 2, Gel 2). On isoelectric focusing (Fig. 2, Gel 3), two colored red-fluorescent bands were obtained—a blue band at pH 5.2 and a purple band at pH 5.3. The most probable explanation of this finding is that R-phycocyanin dissociates on isoelectric focusing to its α and β subunits, the blue band corresponding to the α and the purple band to the β subunit. Such behavior was observed for a hybrid C-phycocyanin described in an earlier report (22). Two bands corresponding to the α and β subunits were obtained on Na dodecyl-SO₃-polyacrylamide gel electrophoresis well as a trace of material of higher molecular weight (Fig. 2, Gel 4). The molecular weights estimated for the α and β subunits were obtained on Na dodecyl-SO₃-polyacrylamide gels were 18,200 and 20,500, respectively, in reasonable agreement with previously reported values of 16,400 and 18,400 (6). Examination of all gels, prior to staining, under near-ultraviolet illumination indicated absence of traces of phycocerythrin.

Amino Acid Analysis—These were performed by conventional procedures (30). Amino acid compositions were calculated to give the best fit to integral numbers of residues, and to the monomer molecular weights as determined by Na dodecyl-SO₃-polyacrylamide gel electrophoresis. For the determination of tryptophan, hydrolysis was performed with 3 N p-toluenesulfonic acid/0.2% 3(2-aminoethyl)indole as described by Liu and Chang (31). For the determination of half-cystine content, lyophilized protein (~0.7 mg) was dissolved in 0.25 ml of ice-cold performic acid (32). Oxidation was allowed to proceed for 5 hours at 0°C. At the end of that time, the performic acid was removed by lyophilization, 50 μl of aqueous phenol solution (5% w/v) were added to the sample, and the lypsyliization was repeated. The oxidized protein was then hydrolyzed with 6 N HCl in vacuo for 24 hours.

Fig. 1. The chromatographic fractionation of Porphyridium cruentum phycobiliproteins on a column (3.9 x 50 cm) of DEAE-cellulose DE52, developed at a flow rate of 80 ml/hour with a linear gradient of 0.05 M to 0.25 M Na-acetate at pH 5.5, followed by elution with 0.35 M acetate at pH 5.5. The phycobiliprotein mixture applied to this column was obtained from 100 g of cells (wet weight). For experimental details, see text. The R-phycocyanin-containing fraction was pooled as indicated by the solid bar.

Fig. 2. Electrophoresis and isoelectric focusing of purified R-phycocyanin. Gels 1 and 2, electrophoresis at pH 8.1 in 7.5% polyacrylamide gel; Gel 1, photographed before staining (arrow indicates the position of the tracking dye, bromphenol blue); Gel 2, the same gel photographed after staining with Amido black. Gel 3, isoelectric focusing of R-phycocyanin in 7.0% acrylamide gel containing Ampholines covering the pH range 3 to 10. Gel 4, electrophoresis of R-phycocyanin in Na dodecyl-SO₃-polyacrylamide gel; pattern photographed after staining with Coomassie brilliant blue.

Native R-phycocyanin, over the protein concentration range 0.12 to 1.2 mg/ml, in 0.02 M Na-acetate buffer, pH 7.0, gave an A₅₄₀nm/A₄₈₅nm ratio of 0.60. This compares favorably with the ratio of 0.63 obtained by Gantt and Lipschultz (6) for Porphyridium cruentum R-phycocyanin, and the value of 0.67 reported by Haxo et al. (33) for their purest preparation of R-phycocyanin from Porphyra perforata, and again indicates the absence of phycocerythrin contamination.

The fluorescence emission maximum of R-phycocyanin in 0.02 M Na-acetate buffer at pH 7.0 was a 634 nm upon excitation at 275, 320, or 380 nm, in reasonable agreement with the value of 636 nm reported by Gantt and Lipschultz (6) for their preparation in 0.1 M K-phosphate buffer at pH 6.8.

At pH 7.0, at a protein concentration of 2 mg/ml, R-phycocyanin sediments as a single component with a sedimentation coefficient of 5.98 S. Determination of the molecular weight of the native protein in 0.1 M K-phosphate buffer at pH 7.0 by sedimentation equilibrium yielded a value of 103,000 ± 6,000 with no evidence of heterogeneity. Based on a molecular weight for the monomer (αβ) of 36,300 (see below), the protein is trimeric under these conditions.

R-Phycocyanin gave a single purple, red-fluorescent, precipitin band when tested against the homologous antiserum by the Ouchterlony double-diffusion technique (Fig. 3A). The protein gave no reaction with antiserum towards Porphyridium cruentum allophycocyanin (Fig. 3B), indicating absence of allophycocyanin contamination.

Amino Acid Analysis of R-Phycocyanin—The amino acid analysis of R-phycocyanin, shown in Table II, was calculated on the assumption that the protein contains a single residue of histidine. The minimum molecular weight for the protein calculated from these data is 36,300, assuming the presence of three tetapyrrole chromophores (molecular weight 586 each) per αβ unit. This is in excellent agreement with the monomer molecular weights of 38,500 reported here, and of 34,800 reported by Gantt and Lipschultz (6), as sums of the molecular weights of the α and β subunits, as determined by Na dodecyl-SO₃-polyacrylamide gel electrophoresis.
The amino acid composition of Porphyridium cruentum R-phycocyanin and of its $\alpha$ and $\beta$ subunits is presented in Table III. The preparation and other properties of this protein were described earlier (23).

A molecular weight of 38,400 may be calculated for the $\alpha$B monomer based on the amino acid composition and the assumption that each monomer contains six phycocerythrobilin prosthetic groups (see discussion below). This value is in reasonable agreement with the sum of the subunit molecular weights, 20,000 ± 700 for $\alpha$ and 22,000 ± 600 for $\beta$, determined by electrophoresis in calibrated Na dodecyl-SO₄-polyacrylamide gels (23).

The absorption spectra of native C-phycoerythrin and of the protein in acid urea are shown in Fig. 4. $\lambda_{\text{max}}$ (562 nm) for Aphanocapsa sp. C-phycoerythrin was determined to be 127. A molecular weight of 38,400 may be calculated for the $\alphaB$ monomer based on the amino acid composition and the assumption that each monomer contains six phycocerythrobilin prosthetic groups (see discussion below). This value is in reasonable agreement with the sum of the subunit molecular weights, 20,000 ± 700 for $\alpha$ and 22,000 ± 600 for $\beta$, determined by electrophoresis in calibrated Na dodecyl-SO₄-polyacrylamide gels (23).

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Table III

Amino acid composition of Aphanocapsa sp. (strain 6701)

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Integral ratios</th>
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</tr>
</thead>
<tbody>
<tr>
<td>Lysine</td>
<td>13</td>
<td>Alanine</td>
<td>54</td>
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<tr>
<td>Histidine</td>
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<tr>
<td>Serine\textsuperscript{d}</td>
<td>29</td>
<td>Leucine</td>
<td>35</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>24</td>
<td>Tyrosine</td>
<td>13</td>
</tr>
<tr>
<td>Proline</td>
<td>12</td>
<td>Phenylalanine</td>
<td>6</td>
</tr>
<tr>
<td>Glycine</td>
<td>21</td>
<td>Tryptophan\textsuperscript{d}</td>
<td>1</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Values calculated from 24-, 48-, and 72-hour hydrolysates. The integral ratios were calculated on the assumption that the monomer contains 2 histidine residues. All values were rounded off to the nearest integer.

\textsuperscript{\textit{b}}Determined as S-carboxymethylcysteine by the procedure of Crestfield et al. (36).

\textsuperscript{\textit{c}}Determined by the method of Liu and Chang (31), see text.

\textsuperscript{\textit{d}}Determined by the method of Luo and Chang (31), see text.

FIG. 4. Absorption spectra in the visible region of Aphanocapsa sp. C-phycoerythrin. (A) in 0.05 M ammonium acetate buffer at pH 6.8, at a protein concentration of 0.6 mg/ml; (B) in 8 M urea-10 mM β-mercaptoethanol at pH 1.9, at a protein concentration of 0.12 mg/ml.

FIG. 5. Absorption spectrum of Porphyridium cruentum R-phycoerythrin (1.2 mg/ml) in 0.02 M ammonium acetate buffer at pH 7.0. The inset shows the absorption and CD spectra of R-phycoerythrin after 30 min in 0.015 M ammonium acetate buffer at pH 7.0, containing 1% (v/v) Na dodecyl-SO\textsubscript{4} at 24°.

Serum albumin as standard. Comments on this approach to the determination of extinction coefficients are offered in Footnote 2.

Chromophore Content of R-Phycoerythrin—The absorption spectrum of R-phycoerythrin in acid urea is shown in Fig. 6. The absorbance at wavelengths higher than 620 nm is expected to arise entirely from the phycocyanobilin chromophores since acid urea-denatured C-phycoerythrin does not absorb in this region (see Fig. 4B). The value of $\epsilon_{662}^{\text{nm}}$ for R-phycoerythrin was 72.4 x 10$^3$, very close to the value of 71 x 10$^3$ expected for the molar contribution of two phycocyanobilin chromophores.

The molar extinction coefficients of polypeptide-bound phycocyanobilin (data from Ref. 34), and phycoerythrobilin (data from Fig. 4B), in acid urea, are given in Table IV. A theoretical absorption curve was computed for R-phycoerythrin from these values on the assumption that the molecule contains one phycoerythrobilin and two phycocyanobilin chromophores. The results of this computation, presented in Fig. 6, demonstrate convincingly that the assumed chromophore composition of

\begin{table}
\begin{tabular}{|c|c|c|}
\hline
Amino acid & Integral ratios & Amino acid & Integral ratios \\
\hline
Lysine & 13 & Alanine & 54 \\
Histidine & 2 & Half-cystine\textsuperscript{a} & 3 \\
Arginine & 21 & Valine & 21 \\
Aspartic acid & 37 & Methionine & 7 \\
Threonine\textsuperscript{c} & 15 & Isoleucine & 16 \\
Serine\textsuperscript{d} & 29 & Leucine & 35 \\
Glutamic acid & 24 & Tyrosine & 13 \\
Proline & 12 & Phenylalanine & 6 \\
Glycine & 21 & Tryptophan\textsuperscript{d} & 1 \\
\hline
\end{tabular}
\end{table}
R-phycoerythrin is indeed correct, and further that the \( \epsilon_m \) of phycoerythrobilin is indeed \(~43,300\). The spectroscopic data on R-phycoeranin rule out a value of 86,000 per phycoerythrobilin chromophore. The \( \epsilon_m \) of R-phycoerythrin in acid urea is 58,000 per \( \alpha \beta \) unit. Of this absorbance, 15,600 is contributed by the two phycoerythrobilin chromophores (see Table IV). The residual molar absorbance values for a mixture of one phycoerythrobilin and two phycoerythrobilin chromophores, calculated from the data in Table IV.

### Table IV

<table>
<thead>
<tr>
<th>( \lambda ) (nm)</th>
<th>Phycocythrobilin</th>
<th>Phycoerythrobilin</th>
<th>Phycocyanobilin</th>
<th>Phycoerythrobilin</th>
<th>Phycoerythrobilin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( \epsilon_m \times 10^4 ) M^-1 cm^3</td>
<td>( \epsilon_m \times 10^4 ) M^-1 cm^3</td>
<td>( \epsilon_m \times 10^4 ) M^-1 cm^3</td>
<td>( \epsilon_m \times 10^4 ) M^-1 cm^3</td>
<td>( \epsilon_m \times 10^4 ) M^-1 cm^3</td>
</tr>
<tr>
<td>330</td>
<td>4.3</td>
<td>27.7</td>
<td>480</td>
<td>6.3</td>
<td>1.4</td>
</tr>
<tr>
<td>340</td>
<td>4.3</td>
<td>2.35</td>
<td>490</td>
<td>9.7</td>
<td>1.4</td>
</tr>
<tr>
<td>350</td>
<td>5.0</td>
<td>35.1</td>
<td>500</td>
<td>14.3</td>
<td>1.5</td>
</tr>
<tr>
<td>360</td>
<td>5.7</td>
<td>34.6</td>
<td>510</td>
<td>20.5</td>
<td>1.9</td>
</tr>
<tr>
<td>370</td>
<td>5.8</td>
<td>30.0</td>
<td>520</td>
<td>27.7</td>
<td>2.6</td>
</tr>
<tr>
<td>380</td>
<td>5.5</td>
<td>25.0</td>
<td>530</td>
<td>34.3</td>
<td>3.4</td>
</tr>
<tr>
<td>390</td>
<td>4.5</td>
<td>18.2</td>
<td>540</td>
<td>39.5</td>
<td>4.5</td>
</tr>
<tr>
<td>400</td>
<td>3.2</td>
<td>11.0</td>
<td>550</td>
<td>42.8</td>
<td>6.0</td>
</tr>
<tr>
<td>410</td>
<td>1.7</td>
<td>7.0</td>
<td>560</td>
<td>42.8</td>
<td>7.8</td>
</tr>
<tr>
<td>420</td>
<td>0.8</td>
<td>5.0</td>
<td>570</td>
<td>38.3</td>
<td>10.2</td>
</tr>
<tr>
<td>430</td>
<td>0.8</td>
<td>3.7</td>
<td>580</td>
<td>28.2</td>
<td>12.9</td>
</tr>
<tr>
<td>440</td>
<td>0.6</td>
<td>2.6</td>
<td>590</td>
<td>16.0</td>
<td>16.2</td>
</tr>
<tr>
<td>450</td>
<td>1.3</td>
<td>1.9</td>
<td>600</td>
<td>6.8</td>
<td>19.7</td>
</tr>
<tr>
<td>460</td>
<td>2.3</td>
<td>1.6</td>
<td>610</td>
<td>8.8</td>
<td>23.3</td>
</tr>
<tr>
<td>470</td>
<td>3.8</td>
<td>1.4</td>
<td>620</td>
<td>0.5</td>
<td>26.3</td>
</tr>
</tbody>
</table>

The spectrum is characterized by two pronounced positive peaks at 627 nm (\( [\theta]_{238} = 4.67 \times 10^4 \) deg cm^2 per dmol) and two smaller negative peaks at 343 nm (\( [\theta]_{238} = -3.27 \times 10^4 \) deg cm^2 per dmol) and 311 nm (\( [\theta]_{238} = -2.36 \times 10^4 \) deg cm^2 per dmol). Based on our earlier studies of the CD spectra of C-phycoerythrin aggregates (28), the peaks at 627 and 343 nm are attributed to the phycoerythrobilin chromophores, while those at 552 and 311 nm originate largely from the phycoerythrobilin chromophores (see Fig. 4A, for example, where the major absorbance bands of native C-phycoerythrin are located at 560 and 307 nm). Comparison of the absorption and CD spectra of R-phycoerythrin (Figs. 5 and 7), obtained on the same protein solution, reveals that the spectra correspond quite closely in shape and relative magnitude in the range of 480 to 560 nm. Thus, the CD spectrum indicates that the phycoerythrobilin chromophores of R-phycoerythrin are not involved in strong exciton-type interaction either with each other or the phycoerythrobilin chromophores. In contrast, the CD band corresponding to the phycoerythrobilin chromophores is displaced significantly to longer wavelengths (\( [\theta]_{238} \) at 627 nm) relative to the absorption band (\( [\theta]_{238} \) at 618 nm). This situation is similar to that observed for C-phycoerythrin where association of the monomer to higher aggregates was demonstrated to result in a red shift and enhancement of the CD band relative to the absorption band, and could be interpreted in terms of strong interaction of the phycoerythrobilin chromophores of one \( \alpha \beta \) monomer with those of another in the aggregate (28). The quantitative data are also consistent with this interpretation. In the C-phycoerythrin hexamer, \( \lambda_{238} \) at 621 nm, and the maximum molecular ellipticity, at 636 nm, is \( 3.03 \times 10^4 \) deg cm^2 per dmol, calculated per phycoerythrobilin chromophore (see Ref. 28, Fig. 1, and Table I)—there are three chromophores per \( \alpha \beta \) monomer in C-phycoerythrin (34). In R-phycoerythrin, \( [\theta]_{238} \) is \( 3.14 \times 10^4 \) deg cm^2 per dmol per phycoerythrobilin. The near identity of these values is a strong indication of considerable similarity in chromophore orientations and interactions of the phycoerythrobilin chromophores within aggregates of R- and C-phycoerythrin.

A rough quantitative estimate of the contribution of the native protein structure to the absorption and CD spectra of R-phycoerythrin may be made from the data presented in the inset in Fig. 5. Treatment of the protein at neutral pH with 1% Na dodecyl-SO₄ produces a 3- to 4-fold decrease in the molar absorbance and an 8- to 9-fold decrease in molecular ellipticity.

### Properties of \( \alpha \) and \( \beta \) Subunits of R-Phycocyanin—The amino acid analyses of the \( \alpha \) and \( \beta \) subunits of R-phycoerythrin are shown in Table II. Based on these analyses, and Na dodecyl-SO₄-polyacrylamide gel electrophoresis, the subunits appeared to be free of cross-contamination.
unrelated immunologically (Fig. 3, A and B).

P. cruentum R-phycocyanin and allophycocyanin appear to be cross-reacted strongly with the antiserum to the C-phycocyanin of Cyanidium caldarium. The immunological relatedness of the two proteins (Table II) are very similar; the sole significant differences are in the relative contents of serine and glycine.

**DISCUSSION**

The single remarkable feature of R-phycocyanin is its chromophore composition. In many other respects, it is strikingly similar to C-phycocyanins of blue-green algal origin. For each class of phycocyanins, the monomer consists of two dissimilar subunits, \( \alpha \) and \( \beta \). In each case, the \( \alpha \) subunit carries a single phycocyanobilin chromophore and contains histidine and tryptophan (22, 34, 44). Assuming the assignment of chromophore content (six) for C-phycoerythrin to be indeed correct, the \( \beta \) subunit of R-phycocyanin carries one phycocyanobilin and one phycoerythrobilin group, in strict analogy with the \( \beta \) subunit of C-phycocyanin which carries two phycocyanobilin chromophores (34). The immunological relatedness of the \( \alpha \) and \( \beta \) phycocyanin lends further support to the concept of a common evolutionary origin for these proteins.

Due to the lack of precise molar extinction coefficients for phycoerythrobilin in aqueous acid solution, and of the possible perturbation of the chromophore spectrum by the covalent links to the protein, the data presented here do not rigidly exclude the possibility that C-phycoerythrin contains 12 phycoerythrobilin chromophores per monomer unit. If this were true, then the phycoerythrobilin content of the \( \beta \) subunit of R-phycocyanin would be two rather than one. The obvious homology between R- and C-phycocyanins would argue strongly against this possibility. However, definite resolution of this question will have to await isolation and chemical characterization of the chromopeptides from either R-phycocyanin or C-phycoerythrin.

It is obvious that, as an absolute minimum, C-phycoerythrin carries two phycoerythrobilin chromophores on the \( \alpha \) subunit and four on the \( \beta \) subunit.

As stated in the introductory remarks, in blue-green and red algae, the main path of energy transfer to chlorophyll \( a \) follows the sequence

phycoerythrin \( \rightarrow \) phycoerycin \( \rightarrow \) allophycocyanin \( \rightarrow \) chlorophyll \( a \)

There are indications, both from cross-linking experiments on intact cells (45), and from studies of phycobilisomes (4), that the above order parallels the physical arrangement of the phycobiliproteins on the thylakoid, i.e. allophycocyanin is closest to the surface of the photosynthetic lamellae and phycoerythrin is the most distant.

The striking correlation which has emerged from our studies is that the number of chromophores per protein monomer is directly related to the position of the phycobiliprotein in the energy transfer chain. Per phycobiliprotein monomer, the chromophore content increases from two for allophycocyanin (34), to three for phyococyanin (34), to six (or more) for phycoerythrin.

It is obvious that the efficiency of a biliprotein as a trap for light energy is proportional to its chromophore content. Another and possibly more important consequence of the increase in the number of chromophores per molecule is the generation of more paths for both intra- and intermolecular energy...
migration, and, hence, a higher efficiency of energy transfer (9). A corollary of this may be that a number of spatially different arrangements of molecules, e.g. those resulting from phycocerythrin-phycocerythrin contacts, and those resulting from phycocerythrin-phycocyanin contacts, are still compatible with a very high efficiency of energy transfer.

The study of the higher assembly forms of the phycobiliproteins, at present in its early stages (46), should provide a basis for a more critical examination of the above suggestions.

Acknowledgments—We are indebted to Douglas M. Brown for performing the ultracentrifugal studies and to Dorothy McNall for the amino acid analyses. We are grateful to Dr. Michael Faith for his assistance in obtaining antisera.

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