Short Communication

Formation, Chromophore Composition, and Labeling Specificity of Cyanidium caldarium Phycocyanin

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Phycocyanin is an accessory pigment found in the photosynthetic apparatus of blue green, cryptomonad, and red algae (1). The phycocyanin molecule consists of a protein to which are bound approximately 20 to 30 residues of the straight-chained tetrapyrrolic (bile pigment) chromophore, phycocyanobilin (10). Conventional methods used to determine the chromophore composition of proteins with polyarylactic prosthetic groups, e.g., hemoglobin, myoglobin (5), which rely on quantitative splitting and recovery of the pigment constituents, fail to liberate phycocyanobilin from phycocyanin apoprotein (Troxler, unpublished observations). Phycocyanobilin is released from trichloroacetic acid-denatured phycocyanin, however, during reflux in absolute methanol albeit in limited yield as first described by Siegelman et al. (13, 14).

Cells of the alga, Cyanidium caldarium, synthesized phycocyanobilin-labeled phycocyanin-14C (16, 17) when illuminated in liquid suspension containing the porphyrin-bile pigment precursor, \( \Delta \)-aminolevulinic acid-4-14C (ALA). The percent phycocyanobilin (by weight) in phycocyanin can be determined from the ratio of the specific radioactivity (dpm/\( \mu \)g) of the chromophore to that of the entire bile pigment-protein complex. The specific radioactivity ratios method is valid, however, only if labeling in the molecule is restricted to the prosthetic group. The present communication describes the quantitative relationship between the chromophore and apoprotein in C. caldarium phycocyanin, and the specificity of ALA labeling in the phycocyanin molecule.

Materials and Methods

C. caldarium, mutant III-D-2, were grown in darkness for 7 days in nutrient medium supplemented with glucose at 40 to 45\(^\circ\) (15). Dark-grown cells (ca. 10 g fr wt) lacking phycocyanin and chlorophyll-\( \alpha \) were collected by centrifugation and resuspended in a glucose-free medium (250 ml) to which was added carrier diluted ALA-4-14C (specific radioactivity 0.15 to 0.30 mc/mumole, New England Nuclear) or ALA-3-5-\( \alpha \)H prepared by enolization exchange (9) (specific radioactivity 1.5-3.0 mc/mumole), or both 14C-labeled and tritiated ALA. Suspensions were agitated on a shaker under a bank of fluorescent lights (GE F40 CW) which provided about 1.4 \( \times \) 10\(^4\) ergs/cm\( ^2 \)sec\(^{-1}\) (read directly with a Kettering YSI thermistor radiometer) at the surface of the shaker platform.

After incubation in suspension with radiolabeled ALA for 48 hr, the III-D-2 cells containing phycocyanin and chlorophyll-\( \alpha \) were washed with water, disrupted by sonic vibration at 10 to 20\(^\circ\) in 2 volumes of 0.1 M potassium phosphate buffer, pH 6.5, and centrifuged at 35,000g for 30 min. Phycocyanin-14C, or -3H, or phycocyanin labeled with both isotopes, present in the supernatant was purified by ammonium sulfate fractionation and column chromatography on brushite as described previously (17). Pigment in column eluates was crystallized from 25% ammonium sulfate at 10\(^\circ\).

Crystalline C. caldarium phycocyanin (\( E_{436}^{0.1\%} = 7.74 \) at 618 nm in 0.05 M phosphate buffer, pH 6.5, \( E_{620/280}^{0.1\%} = 4.5 \), Troxler, unpublished data) labeled with the respective isotopes was dialyzed against distilled water for 24 hr at 4\(^\circ\) and denatured by the addition of an equal volume of 0.44 M trichloroacetic acid (TCA). The precipitated pigment was washed with water (3X), methanol (3X), and refluxed for 12 hr in absolute methanol. Radiolabeled phycocyanobilin released from protein during the methanol reflux was converted to the dimethylster derivative by heating the bile pigment free acid in methanol containing 7% BF\(_3\) to 60\(^\circ\) for 3 min (4, 13, 16). Phycocyanobilin dimethylster was separated from impurities by thin-layer chromatography on adsorbosil-5 (Applied Science Labs, State College, Pennsylvania) plates developed with carbon tetrachloride, methyl acetate, 2:1. Phycocyanobilin

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dimethyl ester \[ \epsilon = 38,000 \] at 685 nm in methanol-5\% HCl \((4,17)\) was eluted from adsorbosil-5 with absolute ethanol. Triplicate samples of phycocyanin and of the purified chromophore split from it were counted in 15 ml of Bray's (2) solution in a Tricarb liquid scintillation spectrometer.

**Results and Discussion**

The specific radioactivities \( \text{dpm/\(\mu\)g} \) of phycocyanin and of the corresponding phycocyanobilin obtained during methanol reflux are shown in table I. Each set of values represents pigment from a different batch of algal cells. Although the dimethyl ester derivative \([\text{molecular weight} 614 (4)]\) of phycocyanobilin was used in performing the spectro-photometic and radiochemical determinations, specific activity values have been corrected to that of the bile pigment free acid \([\text{molecular weight} 584 (4)]\) because the latter molecular species is the naturally occurring form. The phycocyanin in each experiment yielded a phycocyanobilin which contained approximately 27 times as much radioactivity per \(\mu\)g as did the starting bile pigment-protein complex. The ratio of the specific radioactivity of phycocyanin to that of phycocyanobilin represents the percent (by weight) of chromophore in chromoprotein. This value was calculated to be 3.60 \pm 0.06\% in pigment samples obtained from algal cells exposed previously to either \(^{14}\text{C}\)- or \(^{3}\text{H}\)-labeled ALA (table I).

The reproducibility of these data was tested further by preparing crystalline phycocyanin from III-D-2 cells incubated with both ALA-4-\(^{14}\text{C}\) and ALA-3-5-\(^{3}\text{H}\) during the period of rapid pigment synthesis in light \((15)\). In 2 separate experiments TCA-denatured phycocyanin-\(^{14}\text{C}\)-\(^{3}\text{H}\) liberated a “double labeled” phycocyanobilin in which the specific radioactivity of each isotope was again about 27 times greater than that in the starting phycocyanin (table II). The suitability of either labeled precursor for experimentation and the reliability of the method were suggested by the nearly identical isotope ratios \(^{3}\text{H}/^{14}\text{C}\) found in phycocyanin and in phycocyanobilin split from it (table II).

One critical condition upon which the validity of the foregoing rests is that precursor ALA must be incorporated exclusively into the chromophore and not into the apoprotein moiety of phycocyanin. Experience with labeling of at least 1 other chromoprotein with a polypyrrolic prosthetic group, \(i.e\), hamster myoglobin \((5)\), has suggested that ALA is incorporated only into the pyrrolic moiety of the molecule. In the case of phycocyanin, however, the pyrrolic and proteinaceous moieties cannot be separated quantitatively by the usual methods so that the labeling specificity was demonstrated directly by amino analysis.

A 3 mg sample of phycocyanin-\(^{3}\text{H}\) \((100 \text{ dpm/\(\mu\)g}; \text{total radioactivity} = 3 \times 10^6 \text{ dpm})\) was hydrolyzed in a sealed glass vial (not evacuated) in \(6 \times \text{HCl}\) for 24 hr at \(10^\circ\). After evaporation of HCl from the hydrolysate, acid stable amino acids were placed on the column of a Technicon Amino Acid Analyzer equipped with a stream splitting device. Elution was carried out as described by Hamilton \((6)\), the portion of the eluate not analyzed being collected in 1.6 ml fractions. Radiochemical assay was performed on 1.0 ml of each fraction in 15 ml of Bray's solution (2) as described above. Most of the radioactivity applied to the column was obtained in early “acidic” fractions which were located between cysteic and aspartic acids. Significant quantities of radioactivity were not detected in fractions which contained ninhydrin-positive compounds between aspartic acid and arginine \((6)\). It is conceivable that the acidic, radiolabeled, ninhydrin-negative substances are carboxymethyl- or carboxylethyl-pyrrolo-dicarboxylic acid breakdown products of phycocyanobilin similar to those obtained from acid oxidation of biliverdin \((11)\). In any event, significant quan-

### Table I. Phycocyanobilin Composition of Phycocyanin-\(^{14}\text{C}\) Prepared From C. caldarium, Mutant III-D-2

| Precursor | Specific radioactivity | \% Chromophore by wt
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<tbody>
<tr>
<td></td>
<td>Phycocyanin (\text{dpm/(\mu)g})</td>
<td>Phycocyanobilin (\text{dpm/(\mu)g})</td>
</tr>
<tr>
<td>1. ALA-4-(^{14}\text{C})</td>
<td></td>
<td></td>
</tr>
<tr>
<td>a)</td>
<td>22</td>
<td>620</td>
</tr>
<tr>
<td>b)</td>
<td>109</td>
<td>2980</td>
</tr>
<tr>
<td>2. ALA-3-5-(^{3}\text{H})</td>
<td></td>
<td></td>
</tr>
<tr>
<td>a)</td>
<td>180</td>
<td>5100</td>
</tr>
<tr>
<td>b)</td>
<td>170</td>
<td>4660</td>
</tr>
</tbody>
</table>

### Table II. Chromophore Composition of Phycocyanin-\(^{14}\text{C}-^{3}\text{H}\) Prepared From C. caldarium, Mutant III-D-2

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Specific radioactivity and isotope ratios</th>
<th>% Chromophore by wt</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Phycocyanin (^{14}\text{C})</td>
<td>(^{3}\text{H})</td>
</tr>
<tr>
<td>I.</td>
<td>140</td>
<td>160</td>
</tr>
<tr>
<td>II.</td>
<td>95</td>
<td>52</td>
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tities of tritium were not found in fractions of phycocyanin-²H hydrolysate containing amino acids.

The foregoing results have shown that phycocyanobilin constitutes about 3.6%, by weight, of C. caldarium phycocyanin. Previous estimates of phycocyanobilin in phycocyanin performed on pigment from other algal species have ranged from 2.0 to 5.1%. These values were obtained, however, by methods which destroy phycocyanobilin (e.g., hydrolysis of phycocyanin in concentrated HCl) or which rely on measurements of fluorescence lifetime (τ), fluorescence yield (ϕ), and on establishing the molecular weight of the chromoprotein by sedimentation analysis in the ultracentrifuge (3, 8). Since the phycocyanin molecule consists of subunits which associate and dissociate reversibly with changes in pH and ionic strength of the buffer solution (12), and since phycocyanin is not homogeneous in the ultracentrifuge between pH 4.5 and 9.0 (1, 12), the “true” molecular weight of phycocyanin would appear to be an enigma. Moreover, values of about 30,000 and 46,000 have been reported for the molecular weight of the phycocyanin monomer (7, 12). Therefore, the specific radioactivity ratios method for determining the chromophore composition of this chromoprotein has advantages over other methods due to methodologic restrictions imposed by strong bonding between phycocyanobilin and pigment apoprotein and by the complex sedimentation behavior of the entire bile pigment-protein complex.

An attempt is now being made to determine the molecular weight of the phycocyanin subunit by sedimentation analysis under conditions which favor dissociation of the pigment into small units, e.g., at pH values greater than 8.0. The minimum molecular weight of Pleconema calothricoides phycocyanin based on amino acid analyses was reported to be 15,200 (7). If the phycocyanobilin composition of phycocyanin from this alga were similar to that of pigment in C. caldarium, that is, 3.6%, it would be anticipated that 1 “protein equivalent” based on the calculated minimum molecular weight: 0.036 × 15,200 = 547; molecular weight of phycocyanobilin = 584 (4). On the other hand, phycocyanin subunits with molecular weights of 30,000 and 46,000 would be expected to exist in association with 2 and 3 phycocyanobilin residues, respectively.

Added Note. After submission of this manuscript, Crespi et al. (Biochemistry 7, 2232, 1968) reported the chromophore composition of phycocyanin from Phormidium luridum and Synechococcus lividus to be 4%. Although this value was based on spectrophotometric analysis of phycocyanin after treatment with proteolytic enzymes at alkaline pH, and therefore, was somewhat less direct, Crespi et al. concluded that a subunit weighing 45,000 would contain 3 chromophoric residues which is in agreement with the results of the present investigation.

Literature Cited