The Chromophore of Phytochrome

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Summary. The chromophore of phytochrome, the plant photomorphogenic pigment, was cleaved from the associated protein. Chromatographic and spectral properties indicated that it was a bilirubin closely similar to but distinct from the chromophore of C-phycocyanin and allophycocyanin.

The photoreversible changes in absorbance of phytochrome are properties of a specific chromophore attached to a protein. Comparison between action spectra for photomorphogenic responses of higher plants and absorption spectra of the algal chromoproteins C-phycocyanin and allophycocyanin indicated that the several chromophores were probably similar (1-3). The algal chromophores are known to be bile-type pigments (4-6). Purification of phytochrome from plant tissue (7) afforded an opportunity for study of the phytochrome chromophore which is reported here.

Materials and Methods

Phytochrome (P) from dark-grown oat seedlings was purified by repeated dicalcium phosphate and gel-filtration chromatography as previously described (7,8). Six kg lots of seedlings gave an average of 30 ml of a purified phytochrome solution having a Δ (ΔOD) of about 0.8/cm between the 2 forms, Pₚ and Pᵣ at 660 and 725 mμ respectively. This amounts to about 1 x 10⁻⁷ moles of chromophore/ml if α at 660 mμ for Pᵣ is taken as 1 x 10⁻⁴, which is the value indicated by the action spectra for photoconversion in vitro if the quantum efficiency is near one (3). Phycocyanin and allophycocyanin from Plectonema boryanum were purified by chromatography on dicalcium phosphate essentially by the procedure of Haxo, OhEocha and Norris (9) or by fractional ammonium sulphate precipitation.

The method of denaturing the proteins was a factor affecting cleavage of the chromophores as will be discussed. The procedure adopted was denaturation by making the protein solutions to 5% trichloroacetic acid. The precipitated protein was washed with water, followed by repeated washing with CH₃OH at the centrifuge. The CH₃OH washing removed a yellow impurity from phytochrome. Cleavages of the chromophores were effected by refluxing the denatured proteins in CH₃OH for 3 to 4 hours. The solution was filtered, water was added, and the pigments were partitioned into CHCl₃. The CHCl₃ solution was reduced to a volume of 0.05 to 0.5 ml by heating at 60° in a stream of N₂. This final small volume was blue if the cleavage was successful.

Cleavage of the phytochrome chromophore from 5 ml of the purified phytochrome solution gave an average of 0.20 OD/cm in 1 ml of CH₃OH-5% HCl at the absorbancy maximum (690 mμ). This corresponds to about 5 to 10% yield if α for the chromophore is 10 or 5 x 10⁻⁸ (3) or about 1 x 10⁻⁸ moles. The yield was not changed by prolonging boiling of the CH₃OH beyond 4 hours, by repeated treatment, or by refluxing under N₂. Assuming the chromophore has a molecular weight near 600, the yield is about 6 μg of pigment from 5 ml of purified phytochrome solution.

Because of the small amounts of cleavage product obtained from phytochrome, examinations were restricted to measurements of absorbancies (table I) on a Cary 14 recording spectrophotometer, and thin-layer chromatography on silica gel (Adsorbosil 1, Applied Science Laboratories, State College, Pennsylvania). Chromatograms were developed with the several solvent systems shown in the tables II and III. They were examined under visible and ultraviolet (310 to 420 mμ) radiations before and after spraying. Spray reagents used were: (1) a saturated solution of zinc acetate in ethanol to form the zinc salts of the bile pigments which have distinctive fluorescent properties and (2) 0.01% I₂ in ethanol containing 0.2 ml NH₄OH/10 ml which serves as an oxidant.

Bile pigments were prepared or obtained for chromatographic comparisons. Urobilins (d- and i-) were obtained from Dr. P. Barrett, National Institutes of Health, Bethesda, Maryland. Glucobilin (mesobiliverdin) and mesobiliviolin were prepared by ferric chloride oxidation of d-urobilin and i-urobilin, respectively (10). Biliverdin was

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prepared by ferric chloride oxidation of bilirubin (11). Methylation of the pigments was achieved by refluxing with 10 % BF₃ in CH₃OH for 2 minutes. Partially esterified materials were obtained by adding 5 ml of methanol and 0.01 ml of 12 N HCl to dry samples of pigments and evaporating the solutions at 60° in a stream of N₂.

Results and Discussion

Phytochrome denatured with trichloroacetic acid was previously observed to remain blue colored, although the photoreversibility was destroyed (12). When it was denatured with CH₃OH or by boiling in water the denatured protein was only very lightly colored and no chromophore was obtained upon boiling with CH₃OH. Attempts to cleave the chromophore from trichloroacetic acid-denatured protein by hydrolysis with alkali or 12 N HCl (13,14), which gives products with algal biliproteins, were ineffective with phytochrome. A poor yield of the phytochrome chromophore was obtained by refluxing the trichloroacetic acid-denatured phytochrome protein in 1 % ascorbic acid in CH₃OH was satisfactory, but not more so than CH₃OH alone. Fujita and Hattori (15) refluxed CH₃OH-washed cells of Tolypothrix tennis and Anabaena cylindrica in 1 % ascorbic acid in CH₃OH and obtained a blue pigment. They recognized the product as a bilirubine-type of bile pigment, but because of the poor yield and mild conditions of cleavage suggested that it was not the chromophore of phycoerythrin but that it was a precursor of the pigment. The yield of chromophore obtained by us from allophycocyanin and phycoerythrin, never exceeded 20 % based on absorbancies and the yield from phytochrome did not exceed 10 %. The prior purification of these materials indicates that the cleaved product is a constituent of the chromoprotein and is the chromophore. The low yields rather seem to involve some feature of the denaturation process that is not now understood. The yield was not improved, but often greatly reduced, by many variations of procedure and use of other cleaving agents.

Table I. Wavelength Maxima of the Phytochrome, Allophycocyanin, and Phycocyanin Chromophores and Authentic Bile Pigments

<table>
<thead>
<tr>
<th>Compound</th>
<th>Wavelength maxima (nm)</th>
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<tbody>
<tr>
<td>Phytochrome chromophore</td>
<td>380, 690</td>
</tr>
<tr>
<td>Phycocyanin chromophore</td>
<td>375, 685</td>
</tr>
<tr>
<td>Allophycocyanin chromophore</td>
<td>375, 685</td>
</tr>
<tr>
<td>Biliverdin</td>
<td>372, 680</td>
</tr>
<tr>
<td>Glaucobilin</td>
<td>358, 682</td>
</tr>
<tr>
<td>Mesobiliviolin</td>
<td>327*, 505*</td>
</tr>
</tbody>
</table>

* Gray et al. (24).

Availability of authentic bile pigments and the chromophores of phytochrome, allophycocyanin, phycoerythrin permitted spectral and chromatographic intercomparisons. The absorbance maxima (table I) of the algal biliprotein and phytochrome chromophores in 5 % HCl-CH₃OH were similar to, but not identical with, the maxima of the bilirubines, biliverdin and glaucobilin but differed markedly from the maxima of the bilidiene, mesobiliviolin.

The Rₚ values of the dimethyl esters of allophycocyanin and phycocyanin chromophores were identical in several solvents on thin-layer chromatograms (table II) and were distinctly different from Rₚ values of biliverdin, glaucobilin, and mesobiliviolin dimethyl esters. Phytochrome dimethyl ester Rₚ values differed from all of these. Three spots were present on the chromatograms of the partially esterified methyl esters of each material (table III). The Rₚ values of the phytochrome esters were only slightly different in the particular solvents from those of phycoerythrin and allophycocyanin which were equal. The lutidine-H₂O-NH₄OH solvent is known to separate porphyrin pigments according to the number of carboxyl groups (16–18). The 3 spots were in accord with presence of 2 carboxyl groups as the free acids, mono-, and di-methyl esters.

Fluorescence under ultraviolet radiation and changes in color and fluorescence after spraying the chromatograms gave further information. The phytochrome, phycocyanin and allophycocyanin chro-

<table>
<thead>
<tr>
<th>Compound</th>
<th>Rₚ values in various solvents</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>C₆H₅Cl₃</td>
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<tr>
<td>Phytochrome chromophore</td>
<td>0.61</td>
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<tr>
<td>Allophycocyanin chromophore</td>
<td>0.52</td>
</tr>
<tr>
<td>Phycocyanin chromophore</td>
<td>0.52</td>
</tr>
<tr>
<td>Biliverdin</td>
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<tr>
<td>Glaucobilin</td>
<td>0.48</td>
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<tr>
<td>Mesobiliviolin</td>
<td>0.24</td>
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</tbody>
</table>
mophores, biliverdin, and glaucobilin, all were blue and did not fluoresce under uv-light. Mesobilivio-
lvin was red colored and was reddish-purple fluores-
cent under uv-light. After spraying with zinc ace-
atet solution, mesobilivin remained red fluores-
cent and the other compounds did not fluoresce. The chromatograms sprayed with zinc acetate were
oversprayed with the alkaline I₂ solution. The mesobilivin changed to an orange color and was
greenish fluorescent, and the other pigments became
bright-red fluorescent. The fluorescence of the un-
treated mesobilivin was probably due to heavy
metal contamination of the thin layer bed.

The allophycocyanin and phycocyanin chromo-
ophores are identical on the basis of our findings. This is in agreement with the earlier conclusions of
O'hEocha (14, 19) who considered that the chromo-
phorogenic forms were very closely related, if not identical. He further considered the phycocyanin
chromophore, phycocyanobilin, prepared by acid
cleavage of the purified biliprotein was intermediate in properties between a verdin and a violin (14).
The phytochrome chromophore is similar in struc-
ture to the phycocyanin chromophore and is a dia-
cid but must differ in some detail. Complete physical
and chemical examination of the readily obtainable

![Absorption spectra of allophycocyanin (APC) and C-phycocyanin (CPC) from Plectonema boryanum and the red-absorbing form of phytochrome from Avena (Pᵣ).](image)

The chromophores of phytochrome, phycocyanin, and allophycocyanin are apparently very similar, the proteins might not be related and surely differ in the number of chromophores per molecule. The numbers are probably 1 for phytochrome (7) 12 for allophycocyanin (20) and about 22 for C-
phycocyanin (20). This is in agreement with the earlier conclu-
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<table>
<thead>
<tr>
<th>Chromophore</th>
<th>Rᵣ value in:</th>
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<tbody>
<tr>
<td></td>
<td>H₂O</td>
</tr>
<tr>
<td>Phycocyanin</td>
<td>0.03, 0.23, 0.98</td>
</tr>
<tr>
<td>Allophycocyanin</td>
<td>0.03, 0.23, 0.98</td>
</tr>
<tr>
<td>Phytcochrome</td>
<td>0.03, 0.25, 0.98</td>
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</table>

<table>
<thead>
<tr>
<th>Chromophore</th>
<th>NH₄OH vapor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phycocyanin</td>
<td>0.03, 0.23, 0.98</td>
</tr>
<tr>
<td>Allophycocyanin</td>
<td>0.03, 0.23, 0.98</td>
</tr>
</tbody>
</table>

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Literature Cited

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