Chromophores of Allophycocyanin and R-Phycocyanin

By D. J. CHAPMAN, W. J. COLE AND H. W. SIEGELMAN

Department of Biology, Brookhaven National Laboratory, Upton, N.Y. 11973, U.S.A.

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The biliprotein allophycocyanin was purified from *Phormidium luridum*, *Anabaena variabilis* and *Plectonema boryanum*. R-phycocyanin was purified from *Rhodysmenia palmata*. The chromophores were cleaved from the denatured protein by methanol hydrolysis. They were purified and crystallized as the dimethyl esters. Chromatographic and absorption-spectral (visible-ultraviolet and infrared) comparisons with reference material have established phycocyanobilin as the chromophore of allophycocyanin. Phycocyanobilin and phycoerythrobilin were shown to be the chromophores of R-phycocyanin.

C-phycocyanin, R-phycocyanin and allophycocyanin are the blue photosynthetically active biliproteins of the blue-green and red algae. C-phycocyanin is the principal biliprotein of the blue-green algae (Cyanophyceae) and certain red algae (Rhodophyceae). Allophycocyanin appears to be a minor component of both groups of algae. R-phycocyanin is occasionally the major phycocyanin of some Rhodophyceae (Ó hEocha, 1965; Chapman, Cole & Siegelman, 1967b). The chromophore of C-phycocyanin is the bilitriene phycocyanobilin (Chapman, Cole & Siegelman, 1967a; Cole, Chapman & Siegelman, 1967). Ó hEocha (1963) noted that the absorption spectra of allophycocyanin and C-phycocyanin denatured with hydrochloric acid and 50% ethanol were similar, and suggested that the chromophores might be identical. Siegelman, Turner & Hendricks (1966) obtained spectral and chromatographic evidence that phycocyanobilin was the chromophore of allophycocyanin. Lemberg (1930) isolated both phycoerythrobilin and 'phycocyanobilin' from R-phycocyanin. Ó hEocha (1958), however, obtained a blue pigment different from phycocyanobilin on cleavage of R-phycocyanin, but did obtain evidence for phycoerythrobilin.

Using recently developed techniques for the cleavage, preparation and crystallization of phycocyanobilin (Siegelman *et al.* 1966; Chapman *et al.* 1967a) we have prepared phycocyanobilin from allophycocyanin and phycoerythrobilin and phycocyanobilin from R-phycocyanin.

MATERIALS AND METHODS

Allophycocyanin was prepared from *Phormidium luridum*, *Anabaena variabilis* and *Plectonema boryanum*. The algae were grown in Kratz & Myers (1955) D medium in a mass-culture apparatus (Lyman & Siegelman, 1967). R-phycocyanin was isolated from *Rhodysmenia palmata* ('Dulse')

obtained from Fletcher W. Harvey, Grand Manan Island, New Brunswick, Canada. Silica gel (Adsorbosil 5) and magnesium silicate (Adsorbosil M-1) and BF₃-methanol solutions were obtained from Applied Science Laboratories, State College, Pa., U.S.A. Reference phycocyanobilin dimethyl ester was prepared from C-phycocyanin isolated from *Phormidium luridum* (Chapman *et al.* 1967a). The phycocyanins were released from the algae by successive freezing and thawing at pH 7, then at pH 6, in 0.1 M-potassium phosphate buffer. The biliproteins were released from 10 lb. of dried *Rhodysmenia* by successively grinding the moistened blades through 10-, 20-, 50- and 100-mesh sieves. The resulting slurry was then successively frozen and thawed to further the release of the biliproteins. The phycocyanins from *Phormidium*, *Anabaena* and *Plectonema* were fractionated by (NH₄)₂SO₄-gradient elution chromatography (Chapman *et al.* 1967a). The allophycocyanin-rich fractions were combined and precipitated by the addition of solid (NH₄)₂SO₄ to a further 15% saturation. Hereafter percentages of (NH₄)₂SO₄ refers to percentage of saturation in aqueous solution. A modified elution gradient was used to fractionate the allophycocyanin, R-phycocyanin and R-phycoerythrin in *Rhodysmenia*. The Celite column was eluted with a linear gradient of 3 l. of 40%-saturated (NH₄)₂SO₄ and 3.2 l. of 25%-saturated (NH₄)₂SO₄ and then with 2 l. of 25%-saturated (NH₄)₂SO₄. This gradient eluted allophycocyanin and an R-phycoerythrin fraction. R-phycocyanin started to appear in the eluate at the end of the gradient. A second linear gradient of 5 l. of 25%-saturated (NH₄)₂SO₄ and 5.5 l. of 5%-saturated (NH₄)₂SO₄ was then applied to the column. This gradient eluted the R-phycocyanin-rich fractions (*E*₆₁₅/*E*₅₆₅ ratio > 0.75). These were combined and the R-phycocyanin was precipitated by the addition of further (NH₄)₂SO₄ to increase the concentration by 20% saturation.

The centrifuged allophycocyanin precipitate was redissolved in water. Residual (NH₄)₂SO₄ was removed by passage through Sephadex G-50 equilibrated with 1 mM-potassium phosphate buffer, pH 6. Equilibrated allophycocyanin was applied to a column of hydroxyapatite (Siegelman, Wiczorek & Turner, 1965) equilibrated with 1 mM-potassium phosphate buffer, pH 6, containing KCl (0.2 M). Residual C-phycocyanin was eluted with 10 mM-potassium phosphate buffer, pH 6. Allophycocyanin was

then eluted with 0.5M-potassium phosphate buffer, pH 6. The purified allophycocyanin was denatured with 1% (w/v) trichloroacetic acid.

The centrifuged R-phycocyanin precipitate was redissolved in 10mM-potassium phosphate buffer, pH 6, containing KCl (0.1M). The solution was applied to a brushite column (Siegelman *et al.* 1965) and equilibrated with the same solution. The R-phycocyanin was eluted with 20mM-potassium phosphate buffer, pH 6, containing KCl (0.1M). Residual R-phycoerythrin was removed by elution with 0.2M-Na₃PO₄ solution. The pure R-phycocyanin was denatured with 1% (w/v) trichloroacetic acid.

The chromophores were cleaved from the denatured proteins by methanol hydrolysis (Siegelman *et al.* 1966). Preparative chromatography of the free acids was performed on silica gel-0.3M-oxalic acid (Ellfolk & Sievers, 1966) with benzene (or toluene)-propan-2-ol (11:9, v/v) or on magnesium silicate with butan-2-one-acetic acid (19:1, v/v). The free acids were methylated with 7% (w/v) BF₃ in methanol, chromatographed on silica gel with carbon tetrachloride-methyl acetate (2:1, v/v) and crystallized from chloroform-methanol (Chapman *et al.* 1967a). Analytical thin-layer chromatography was performed on silica gel with two additional two-solvent systems: benzene-ethanol (9:1, v/v) and ethylene dichloride-ethyl acetate (7:3, v/v). For phycoerythrobilin dimethyl ester a fourth system, methylene dichloride-pentan-3-one (8:3, v/v), was used.

The visible-ultraviolet-absorption spectra of the fully protonated methyl esters were measured in 5% (w/v) HCl in methanol, and those of the zinc complexes in ethanol saturated with zinc acetate, with a Cary model 14 spectrophotometer. Infrared-absorption spectra were recorded in KBr disks with a Perkin-Elmer Infracord instrument.

RESULTS

The chromophore of allophycocyanin was found to be phycocyanobilin. The dimethyl ester crystallized as the characteristic fibrous crystals. Identity with the reference phycocyanobilin dimethyl ester was established by the visible-ultraviolet-absorption spectrum in 5% hydrochloric acid in methanol ($\lambda_{\max}$, 685, 374m μ) and mixed chromatography with the three solvent systems. Phycocyanobilin dimethyl ester from allophycocyanin (from *Phormidium*) and the reference phycocyanobilin dimethyl ester also had closely similar infrared-

absorption spectra. The material from *Anabaena* and *Plectonema* was insufficient for recording the infrared-absorption spectrum.

R-phycocyanin was found to contain phycocyanobilin and phycoerythrobilin. Chromatography of the free acids from the R-phycocyanin hydrolysate revealed two principal zones. A blue-green band (phycocyanobilin) chromatographed just in front of a red-violet band (phycoerythrobilin). On silica gel the order of the two zones was reversed. Other very minor zones were considered to be artifacts. The two principal zones were eluted from the silica gel in methanol, transferred to chloroform and washed with water. The resultant solutions were deep blue (phycocyanobilin) and deep red (phycoerythrobilin). The two bile pigments were methylated in boron trifluoride-methanol solutions and chromatographed on silica gel. Phycocyanobilin dimethyl ester was crystallized as fibrous hairs from chloroform-methanol. The phycoerythrobilin was insufficient for crystallization. Spectral and chromatographic analyses were therefore done on solutions freshly eluted from the plate.

Identity of the phycocyanobilin dimethyl ester with reference phycocyanobilin dimethyl ester was established by visible-ultraviolet-absorption spectra (Table 1), closely similar infrared-absorption spectra and chromatography. Mixed chromatography on silica gel with the four solvent systems showed no separation of the reference and sample phycocyanobilin dimethyl ester.

The phycoerythrobilin dimethyl ester was compared spectrally and chromatographically with reference phycoerythrobilin dimethyl ester prepared from C-phycoerythrin (from *Phormidium persicinum*) by the methanol cleavage procedure described for phycocyanobilin (Chapman *et al.* 1967a). Similar absorption spectra (Table 1) and the failure of the reference and sample diester to separate on mixed chromatography in the four solvent systems established the identity of the phycoerythrobilin dimethyl ester prepared from R-phycocyanin. The material was insufficient for recording the infrared-absorption spectra.

Table 1. *Absorption-spectral maxima of phycocyanobilin and phycoerythrobilin from R-phycocyanin*

Compound	$\lambda_{\max}$, (m μ)				
	5% HCl in methanol		Satd. zinc acetate in ethanol		
	Band 1	Band 2	Band 1	Band 2	Band 3
Phycocyanobilin dimethyl ester (R-phycocyanin)	689	374	663		375
Phycocyanobilin dimethyl ester	688	374	664		375
Phycoerythrobilin dimethyl ester (R-phycocyanin)	592	327	604	559	336
Phycoerythrobilin dimethyl ester	591	327	603	556	334

DISCUSSION

The chromophore of allophycocyanin was cleaved, purified and crystallized as the dimethyl ester. It is phycocyanobilin, the chromophore of C-phycocyanin. C-phycocyanin and allophycocyanin have different absorption spectra in the native state, although the denatured forms appear similar (Ó hEocha, 1963). Since both phycocyanins possess phycocyanobilin, and presumably only phycocyanobilin, as the chromophore, it is likely that the protein environment in the region of the chromophoric group can markedly alter the spectral absorption. Absorption spectral differences, at least in the region 580–660m μ , in denatured or dissociated phycocyanins cannot therefore be attributed to different chromophores. The only valid test for different chromophores is cleavage from the protein and characterization of the individual bile pigments.

Hattori & Fujita (1959) proposed that R-phycocyanin was not a specific biliprotein, but was C-phycocyanin contaminated with phycoerythrin. Ó Carra & Ó hEocha (1965) considered that R-phycocyanin was a distinct biliprotein. Phycoerythrobilin and phycocyanobilin were cleaved from R-phycocyanin and separated as the free acids before esterification. A small amount of phycocyanobilin dimethyl ester is formed from phycoerythrobilin by the esterification procedure. Comparison of phycocyanobilin dimethyl ester and phycoerythrobilin dimethyl ester with reference dimethyl esters of phycocyanobilin and phycoerythrobilin by chromatographic and spectral analyses established that phycocyanobilin and phycoerythrobilin are the chromophores of R-phycocyanin.

There was no evidence for additional chromophores in either allophycocyanin or R-phycocyanin. Chromatography of the esterified cleavage products from allophycocyanin yielded phycocyanobilin and the violinoid artifact (Chapman *et al.* 1967a) as the principal components. Chromatography of the cleavage products from R-phycocyanin yielded phycocyanobilin and phycoerythrobilin as the principal fractions.

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