The Native Forms of the Phycobilin Chromophores of Algal Biliproteins

A CLARIFICATION

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Pigments released from phycoerythrins and phycocyanins by treatment with hot methanol are currently regarded as equivalent to the native chromophores phycoerythrobilin and phycocyanobilin. However, evidence presented here confirms the original views of O'Carra & O'hEocha [(1966) Phytochemistry 5, 993–997] that these methanol-released pigments are artefacts differing in their chromophoric conjugated systems from the native protein-bound prosthetic groups. By contrast, the native spectral properties are retained in pigments released by careful acid treatment of the biliproteins and these acid-released phycobilins, rather than the methanol-released pigments, are therefore regarded as the protein-free forms of the native chromophores. The conclusion reached by Chapman, Cole & Siegelman [(1968) J. Am. Chem. Soc. 89, 3643–3645], that all the algal biliproteins contain only phycoerythrobilin and phycocyanobilin, is shown to be incorrect. The identification of a urobilinoid chromophore, phycourobilin, accompanying phycoerythrobilin in B- and R-phycoerythrins is confirmed and supported by more extensive evidence. The cryptomonad phycocyanins are shown to contain a phycobilin chromophore accompanying phycocyanobilin. This further phycobilin has the spectral properties of the class of bilins known as violins and the provisional name ‘cryptoviolin’ is proposed pending elucidation of its structure.

The algal biliproteins are a group of brilliantly coloured fluorescent proteins that function as photosynthetic pigments in red, blue–green and cryptomonad algae (O'hEocha, 1965, 1966). They are subdivided into two major categories on the basis of visual appearance: the phycoerythrins are red with a golden fluorescence; the phycocyanins are blue with a red fluorescence (O'hEocha, 1965, 1966). Several subtypes of these chromoproteins are distinguished on the basis of differences in absorption spectra. R-, B- and C-phycoerythrins and R-, C- and allophycocyanins are found in various members of the Rhodophyta (red algae) and Cyanophyta (blue–green algae). Cryptomonad algae contain a complex group of further variants [see O'hEocha et al. (1964) for a review of these].

The brilliant coloration of the phycoerythrins and the phycocyanins is imparted by open-chain tetrapyrrole chromophores that are covalently attached to the apoproteins (O'hEocha, 1965, 1966). These chromophores are termed phycobilins. They have labile structures and are difficult to release from the apoproteins without alteration. This has greatly hindered the complete structural characterization of the pigments. We believe that the structures currently accepted for the major phycobilins, phycoerythrobilin and phycocyanobilin, apply to artefact derivatives of the native chromophores. Since the confusion of native and artefact forms has been extensive (Beuher et al., 1976; Chapman et al., 1967, 1968; Cole et al., 1967; Crespi & Katz, 1969; Crespi et al., 1968; Glazer & Fang, 1973; Glazer & Hixson, 1975; Troxler et al., 1978), we decided that it was appropriate to clarify this issue before proceeding to a consideration of the structures of the native chromophores [see the following paper (Killilea et al., 1980)].

A further issue needing clarification is the question as to whether phycoerythrobilin and phycocyanobilin are the only phycobilins occurring in
algal biliproteins, as claimed by Chapman et al. (1968). O’Héocha & O’Carra (1961) and O’Carra et al. (1964) suggested the occurrence of a third type of phycobilin with urobilinoid spectral properties in R-phycocerythrin, and evidence presented here strongly supports this. In addition, spectral evidence suggest that, as well as containing phycocyanobilin, cryptomonad phycocyanins have a further type of phycobilin with violinoid spectral properties (O’Héocha et al., 1964). We propose that these further phycobilins be termed respectively phycourubilin and cryptoviolin.

**Experimental**

**Biliprotein preparations**

R-phycocerythrin from the red alga *Rhodymenia palmata*, C-phycocerythrin from the red alga *Phormidium persicinum*, C-phycocyanin from the blue–green alga *Nostoc punctiforme* and R-phycocyanin from the red alga *Ceramium rubrum* were isolated and purified as by O’Carra (1965). Cryptomonad phycocyanin was isolated from *Hemiselmis virescens* as by O’Héocha et al. (1964).

**Bilin preparations**

Methanol-released ‘purple pigment’ from R-phycocerythrin and ‘blue pigment’ from C-phycocyanin were prepared as by O’Carra & O’Héocha (1966). Preparations of phycocerythobilin and phycocyanobin from R-phycocerythrin and C-phycocyanin, respectively, were obtained by incubating the biliproteins for successive periods of 15 min at 18°C in 11.5–12M-HCl (O’Carra et al., 1964; O’Héocha, 1963). Phycocyanobilin was similarly released from R-phycocyanin when most of the phycocerythobilin had been first released from the protein by two successive incubations with HCl (O’Carra, 1962; Murphy, 1968). T.l.c. on silica-gel G was used for analysis of the free diacid forms of the phycobilin preparations and for purification of individual bilin constituents (Murphy, 1968). The solvent systems used were: carbon tetrachloride/acetic acid (2:1, v/v); carbon tetrachloride/acetic acid (4:8:1, by vol.); heptane/acetone/acetic acid (16:16:3, by vol.). Bilins were esterified (Murray, 1966) in a solution (1 ml) of BF₃ in methanol (14%, v/v; Sigma, Poole, Dorset, U.K.) for 25 min at 4°C. Ice-cold water (4 ml) was then added and the esterified pigment was extracted with three 2 ml portions of chloroform.

**Tryptic digestion of biliproteins**

Previous studies have shown that, under some conditions used to release phycocerythobilin from phycocerythrins, random artefact covalent linkage of the bilin to the polypeptide chains of the apoprotein can take place and the chromophore is also converted into a urobilinoid artefact derivative (O’Carra et al., 1964; Rüdiger & O’Carra, 1969). Since the artefact attachment takes place by condensation of the vinyl-side chain group of ring D of phycocerythobilin with thiol groups on the polypeptide chains (O’Carra et al., 1964; Rüdiger & O’Carra, 1969), blocking of the thiol groups by reduction and aminoethylation (Raftery & Cole, 1966) before tryptic digestion was carried out to eliminate this complication. To further protect the chromophores, all procedures used were carried out in the dark and under nitrogen. Tryptic digests were carried out as follows. Reduced and aminoethylated biliprotein (50 mg) was suspended in 10 ml of 0.2M-sodium phosphate buffer, pH 7.0. A solution (0.1 ml) of diphenylcarbamoyl chloride-treated trypsin (2.5 mg; Sigma) in 1mM-HCl was added. The mixture was incubated with stirring for 6h at 25°C under nitrogen, and any insoluble material was removed by centrifugation. After the addition of a further portion (0.1 ml) of trypsin solution to the supernatant, digestion was continued for 16h. Digests were stored at -20°C under nitrogen.

**Starch-gel electrophoresis of tryptic digests of R-phycocerythrin**

Electrophoresis of tryptic digests of R-phycocerythrin was carried out on horizontal starch gels by using ‘Starch Hydrolysed’ (Connought Medical Research Laboratories, Toronto, Ont., Canada) and adopting the procedure of Smithies (1955). The buffer systems used were: glycine/HCl, pH 2.2; sodium acetate, pH 4.0, 4.5, 5.0 and 5.5; Tris/malate, pH 7.4 and 8.3. For the glycine/HCl and sodium acetate buffers, the gel buffer concentration was 0.025M and the bridge buffer concentration was 0.05M. For the Tris/malate buffers, the gel buffer concentration was 0.005M and the bridge buffer concentration was 0.1M. Electrophoresis was carried out at 4°C in the dark for 16h at 7.5 V/cm. After electrophoresis the starch gels were soaked in ethanol/pyridine (1:1, v/v) for 10 min. The bathing fluid was replaced for each of two subsequent washings. The gels were then placed in a saturated solution of zinc acetate in ethanol for 10 min to convert the bilin chromophores into their zinc complexes. Although the peptide bands that contained bilin chromophores could be detected visually after electrophoresis, much greater sensitivity was achieved by viewing the fluorescent zinc complexes under u.v. light, when phycocerythobilin and phycourobilin exhibit orange and green fluorescence respectively. At each pH value used for electrophoresis of the tryptic digest, only one peptide containing phycocerythobilin and one containing phycourobilin could be detected. Similar results were obtained when the digests were subjected to electrophoresis on polyacrylamide disc gels by the method of Dietz & Lubrano (1967).
Results and Discussion

To simplify discussion, the phycobilins are considered individually.

Phycerythrobilin

This red phycobilin imparts the characteristic red colour to the phycoerythrins. In 1958, O'hEocha reported that careful treatment of phycoerythrins with cold concentrated HCl releases a chloroform-soluble pigment from the proteins. The free bilin was considered to be closely related to the native protein-bound chromophore, and this was confirmed later by O'Carra et al. (1964). The acid treatment, however, resulted in many side reactions producing artefacts, so that yields of the acid-released pigment were low.

O'Carra & O'hEocha (1966) showed that treatment of phycoerythrins with hot methanol slowly released a more stable derivative of the chromophore. This methanol-released pigment was shown to be identical with a bilin extracted from algae with hot methanol and previously considered by Fujita & Hattoni (1962, 1963) to be a biosynthetic precursor of phycoerythrobilin. The results of O'Carra & O'hEocha (1966) showed that the extracted pigment was a derivative rather than a precursor of phycoerythrobilin. O'Carra & O'hEocha (1966) considered the acid-released pigment to be much closer in properties to the native protein-bound pigment than the methanol-released 'purple pigment', which was regarded as a spectrally altered artefact. However, in much subsequent work by other authors this distinction has been disregarded. Since the methanol-releasing procedure offers a more convenient method of obtaining protein-free pigment, much of the recent structural work (Frackowiak & Skowron, 1978; Gossauer & Weller, 1978; Fu et al., 1979) has concentrated on the methanol-released derivative (cf. e.g. Chapman et al., 1967; Crespi et al., 1968).

The spectral properties, summarized in Table 1, clearly demonstrate a distinction between the acid-

released and methanol-released pigment preparations. The two preparations retain their spectral distinctiveness through a variety of derivatization procedures, e.g. formation of the hydrochloride and zinc complex (Table 1), and also when converted into the dimethyl esters. The pigments as dimethyl esters travel with similar Rf values when subjected to t.l.c. on silica-gel G in some solvent systems, and this may have helped to create the impression that they were identical. In other solvent systems, however [benzene/light petroleum (b.p. 100–120°C)/methanol (9:5:1, by vol.); carbon tetrachloride/acetic acid (1:1, v/v) carbon tetrachloride/ethyl acetate (1:1, v/v)], the two pigment preparations are readily separated.

In Table 1 and Fig. 1 the spectral properties of the released pigments are compared with those of denatured C-phycoerythrin. Protein-denaturing conditions were used to ensure unfolding of the polypeptide chains of the phycoerythrin, thus exposing the covalent bound chromophore to the same solvent conditions as for the protein-free-pigment

![Absorption spectra in 0.1 M HCl of (---) denatured C-phycoerythrin, (-----) acid-released phycoerythrobilin and (⋯⋯) methanol-released 'purple pigment' derivative of phycoerythrobilin](image_url)

Table 1. Spectral absorption maxima of denatured phycoerythrins, phycoerythrin-derived bilins and i-urobilin

The major maxima are italicized and minor maxima or absorption shoulders are in parentheses. The absorption maximum at 278 nm is attributable to aromatic amino acids.

<table>
<thead>
<tr>
<th>Bilin or denatured phycoerythin</th>
<th>Chromophore as hydrochloride</th>
<th>Chromophore as zinc complex</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>In 0.1 M HCl</td>
<td>In acidified choloroform</td>
</tr>
<tr>
<td>Denatured C-phycoerythrin</td>
<td>287, 307, 556</td>
<td>Insoluble</td>
</tr>
<tr>
<td>Phycoerythrobilin (acid-released)</td>
<td>307, 556</td>
<td>312, 576</td>
</tr>
<tr>
<td>'Purple pigment' derivative of phycoerythrobilin</td>
<td>326, 590, 329, 602</td>
<td></td>
</tr>
<tr>
<td>Denatured R-phycoerythrin</td>
<td>287, 307, 498, 556</td>
<td>Insoluble</td>
</tr>
<tr>
<td>i-Urobin</td>
<td>495</td>
<td>499</td>
</tr>
</tbody>
</table>

Fig. 1. Absorption spectra in 0.1 M HCl of (---) denatured C-phycoerythrin, (-----) acid-released phycoerythrobilin and (⋯⋯) methanol-released 'purple pigment' derivative of phycoerythrobilin
preparations. In the native proteins the chromophores are buried in the interior by the folding of the polypeptide chains and lie in environment(s) different from the external medium (O’hEocha & O’Carra, 1961; Lavorel & Moniot, 1962; O’Carra & O’hEocha, 1965; Murphy & O’Carra, 1970). That the denaturing conditions cause no covalent alteration of the bound chromophore is shown by the fact that C-phycoerythrin readily renatures when the denaturing conditions, low pH or 8 M urea, are removed. The spectral properties of the native protein then return in a rapid spontaneous process.

It can be seen that the protein-bound chromophore is almost identical in spectral properties with the acid-released phycoerythrobilin preparations and differs considerably from the methanol-released pigment. Muckle & Rüdiger (1977) also emphasized that the methanol-released pigment exhibits spectral properties, including absorption coefficients, different from those of protein-bound chromophore.

**Phycocyanobilin**

The situation regarding phycocyanobilin and the blue pigment released from C-phycoerycyanin by hot methanol is analogous to that outlined above for phycoerythrobilin and its methanol-released ‘purple pigment’ derivative. The ‘blue pigment’ released from blue-green algae by methanol treatment was originally considered by Fujita & Hattori (1962, 1963) to be a biosynthetic precursor of phycocyanobilin, but was shown by O’Carra & O’hEocha (1966) to be an artefact derived from protein-bound phycocyanobilin. As can be seen from the spectral properties recorded in Table 2, this ‘blue pigment’ differs spectrally from protein-bound phycocyanobilin. When C-phycoerycyanin was treated by the method of O’hEocha (1963) with 12 M HCl, a phycobilin preparation that absorbed maximally at 630–645 nm in acidified chloroform was obtained. By t.l.c. analysis this preparation was shown to contain a mixture of pigments. The predominant component was a blue–green bilin with an absorption maximum at 630 nm, and this is referred to as ‘phycoerythrin–630’. It could be further resolved on chromatograms into two closely spaced components that were identical by other criteria, including absorption spectra, acid/base properties, isomerizations in acids and alkali, chemical reactions and degradation products (Rüdiger & O’Carra, 1969).

The two forms of phycocyanin–630 may reflect spatial isomerism, which does not involve any rearrangement of the double-bond system. The other bilin components were a violinoid pigment, which may be a hydrated derivative of phycocyanobilin–630 (Murphy, 1968), and a bilin, which was chromatographically and spectrally identical with the methanol-released blue pigment. The proportion of the components extracted into chloroform varied with the concentration of HCl used to treat the C-phycoerycyanin. Time-course studies with C-phycoerycyanin and with chromatographically purified phycocyanobilin–630 in HCl at various concentrations between 10 M and more than 13 M, however, showed that the blue–green phycocyanobilin–630 was the bilin initially released. In 10–12 M HCl it was converted into the blue pigment together with some violin, but, as the concentration of HCl was increased to over 13 M, the conversion into violin became the major transformation. These observations were confirmed with acid-released phycocyanobilin–630 from R-phycoerycyanin.

Spectral comparison of denatured C-phycoerycyanin with the chromatographically purified phycocyanobilin–630 shows that this pigment closely resembles the native protein-bound chromophore (Fig. 2, Table 2).

As for phycoerythrobilin, the structure and properties currently accepted (Brown & Troxler, 1977; Troxler et al., 1978; Frackowiak & Skowron, 1978; Gossauer & Hinze, 1978) for phycocyanobilin (Cole et al., 1967; Crespi et al., 1968) relate to the methanol-released ‘blue pigment’. This is now seen to be identical with a spectrally altered artefact derived from the native phycocyanobilin chromophore. Because of the differences between the

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**Table 2. Spectral absorption maxima of denatured C-phycoerycyanin and its released bilins**

The major maxima are italicized. To form the zinc complex of the chromophore in C-phycoerycyanin, the biliprotein was dissolved in 8 M urea containing 1% (w/v) zinc acetate. The zinc complex of protein-free bilins was formed in chloroform, which contained a drop of ethanolic zinc acetate and a trace of pyridine to neutralize protons. The absorption maximum at 278 nm is attributable to aromatic amino acid residues.

<table>
<thead>
<tr>
<th>Chromophore as hydrochloride</th>
<th>In aq. methanolic 0.1 M-HCl</th>
<th>In acidified chloroform</th>
<th>Chromophore as zinc complex in 8 M-urea or chloroform</th>
</tr>
</thead>
<tbody>
<tr>
<td>Denatured C-phycoerycyanin</td>
<td>278, ~350, 655–660</td>
<td>Insoluble</td>
<td>278, 375, 630</td>
</tr>
<tr>
<td>Phycocyanobilin (acid-released phycocyanobilin–630)</td>
<td>~360, 655</td>
<td>357, 630</td>
<td>375, 630</td>
</tr>
<tr>
<td>‘Blue pigment’ derivative of phycocyanobilin</td>
<td>374, 684</td>
<td>680</td>
<td>378, 665</td>
</tr>
</tbody>
</table>

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spectral properties of the methanol-released pigment and those of native phycocyanobilin bound to the protein, Muckle & Rüdiger (1977) state that the use of the absorption coefficient of the methanol-released pigment to calculate the chromophore content of phycocyanin can give erroneous results. The phycobilin–630 released by brief acid treatment consists predominantly of a pigment very close in properties to the native chromophore.

**Phycocyanobilin**

The grounds on which Chapman *et al.* (1968) have disputed the suggestions that R-phycoerythrin contains a urobiniloid chromophore (phycourobilin) accompanying phycoerythrobilin may be summarized as follows. (1) The spectral properties of phycoerythrobilin are distorted by the interaction of the chromophore with the native protein environment and thus the peak at 495–598 nm might be attributable to such non-covalent modulation of the properties of phycoerythrobilin in the native protein. (2) Free phycoerythrobilin readily undergoes covalent conversion into urobiniloid artefacts both by isomerization and by oxidation reactions, and the appearance of urobiniloid material in denatured R-phycoerythrin might be attributable to such artefact material formed while the chromophore is still attached. (3) Refluxing R-phycoerythrin in methanol releases only the ‘purple pigment’ equated with phycoerythrobilin by Chapman *et al.* (1967, 1968), and no urobin is released.

In order to check the first two objections, the spectral data shown in Table 1 and Fig. 3 were obtained with fully denatured R-phycoerythrin in which the effects of the native protein environment are eliminated by unfolding of the polypeptide chains. The possibility that the procedures used for obtaining these spectra might cause covalent modification of phycoerythrobilin was investigated by subjecting purified acid-released phycoerythrobilin and denatured C-phycoerythrin to exactly the same procedures. Their spectral properties are compared in Table 1 and Fig. 1. No significant amount of urobiniloid material was formed in either case.

As Table 1 and Fig. 3 show, a distinctive peak, characteristic of urobin, persists in the spectrum of denatured R-phycoerythrin. Quantitative subtraction of the absorption spectrum of phycoerythrobilin, by using an adjusted spectrum of denatured C-phycoerythrin, leaves a spectrum almost identical with that of i-urobin. Addition of Zn$^{2+}$ causes the characteristic shift of the urobiniloid absorption peak (Table 1) associated with the formation of the zinc complex. The fluorescence spectrum of the zinc-complexed denatured R-phycoerythrin shows two fluorescence bands, one ($\lambda_{\text{max}}$ 520 nm) characteristic of urobin—zinc and the other ($\lambda_{\text{max}}$ 600 nm) attributable to phycoerythrobilin—zinc.

To further eliminate any influence of the protein environment, denatured R-phycoerythrin was digested with trypsin. The digestions were carried out under carefully controlled conditions (neutral pH, exclusion of oxygen) designed to minimize any isomerization or oxidative conversion of phycoerythrobilin units into artefact urobiniloid material. Again, the procedures used were fully checked by

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*Fig. 2. Absorption spectra in 0.1 m HCl of (----) denatured cryptomonad phycocyanin, (---) denatured C-phycocyanin and (---) acid-released phycocyanobilin (phycobilin-630)*

*Fig. 3. Absorption spectra in 0.1 m HCl of (----) denatured R-phycoerythrin, (---) acid-released phycoerythrobilin and (---) i-urobin*
control experiments in which R-phycoerythrin was replaced with C-phycoerythrin, and negligible conversion of phycocrythobilin, the only chromophore of C-phycoerythrin, into a urobinil was observed. The tryptic digests of R-phycoerythrin retained the same spectral properties as the denatured biliprotein, and when the peptide mixture was fractionated on the basis of a number of independent properties (e.g., size or charge characteristics), a clear-cut separation of the phycocrythobilin-containing and phycourobilin-containing peptides was observed (Figs. 4 and 5). The results show that the two chromophores reside in quite distinct sections of the polypeptide sequence, further eliminating the possibility that the urobinil might be an artefact form of phycocrythobilin.

Refluxing the phycourobilin-containing peptide fraction, isolated by gel-filtration, with methanolic KOH for 5 min released the chromophore and converted it rapidly into a green product that was identified as the bilin mesobiliverdin. This result confirms that phycourobilin is an open-chain linear tetrapyrrrole.

Treatment of the phycourobilin-containing peptide with hot methanol did not release the pigment. Structural studies show that the attachment of phycourobilin to the apoprotein is much stronger than that of phycocrythobilin (P. O'Carra & S. D. Killilea, unpublished work). Thus the failure of Chapman et al. (1968) to release urobinil material by hot methanol is readily explained.

Although the experiments described here provide strong evidence that phycourobilin is a discrete and natural chromophore of R-phycoerythrin, this concept now also receives acceptance by some other groups (Frackowiak & Skowron, 1978; Glazer & Hixson, 1977).

Cryptocyanin

All the cryptomonad phycocyanins in the native state exhibited an absorption peak at about 588 nm in addition to varied spectral characteristics at longer wavelengths, usually manifested by a peak at either 615, 630 or 645 nm (O'hEocha et al., 1964).
When these phycocyanins are unfolded by denaturation, the longer-wavelength absorption in all cases collapses to a spectral peak characteristic of phycocyanobilin. The shorter-wavelength peak also shifts, but remains clearly distinct from the phycocyanobilin spectrum. Fig. 2 shows the spectrum of a typical denatured cryptomonad phycocyanin in dilute acid; all cryptomonad phycocyanins give almost identical spectra under such denaturing conditions. The short-wavelength absorption peak is at 610 nm under these conditions, but if the spectrum is analysed by subtracting out the phycocyanobilin absorption spectrum with an adjusted spectrum of acid-denatured C-phycocyanin, the true absorption maximum of the peak is found to be about 590 nm. This maximum and the shape of the analysed peak coincides exactly with the absorption characteristics of the class of bilins known as violins; the absorption maximum of mesobiliviolin in dilute acid is at 590–600 nm.

The bilin nature of the extra chromophore in cryptomonad phycocyanin was indicated by the observation that both it and the phycocyanobilin chromophore were converted into mesobiliverdin when samples of the phycocyanin were refluxed in methanolic 1M-KOH for 30 min.

The extra chromophore seems clearly to have a violinoid conjugated system and it is tempting to suggest the term phycoviolin, which would be in line with the terminology of the other phycobilins. A similar term, phycobiliviolin, has been used by Rüdiger (1971) for the 'purple pigment' derivative of phycoerythrobilin, so, to avoid confusion, it is suggested that the violinoid chromophore of cryptomonad phycocyanin be termed cryptoviolin.

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