Mass-Spectral Identification and Purification of Phycoerythrobilin and Phycocyanobilin

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The bile-pigment chromophores of C-phycoerythrin (phycoerythrobilin) and C-phycocyanin (phycocyanobilin) were cleaved from their respective proteins with boiling methanol or butan-1-ol. They were purified as dicarboxylic acids by preparative reverse-phase liquid chromatography. Each pigment existed in two principal forms, which were characterized by using 20 pmol samples by proton-transfer chemical-ionization mass spectroscopy. These two principal forms were isomeric species, and all had protonated parent molecular ions with m/e 587, corresponding to a molecular weight of 586.

Phycocyanobilin and phycoerythrobilin are the blue and red bile-pigment chromophores respectively of the photosynthetically active biliproteins of the cyanobacteria and the red and cryptomonad algae (O'Carra & Ó hEocha, 1976). Characterization of these pigments has been complicated by the apparent multiplicity of products obtained on cleavage (Cole *et al.*, 1968). Purification of the algal bile pigments by chromatography on silica (Cole *et al.*, 1968; Beuhler *et al.*, 1976) requires prior esterification, which can introduce further complexity. The diacid forms of the pigments can be purified by reverse-phase liquid chromatography. The lesser volatility of the bile-pigment diacids requires the utilization of newly

developed volatility-enhancement techniques for mass-spectral analysis (Beuhler *et al.*, 1976). These techniques permit examination of fragile molecular systems under conditions that provide an opportunity to resolve the problems of molecular weights determined by mass spectroscopy. Crespi *et al.* (1967), and subsequently Schram & Kroes (1971), observed the parent molecular ion of phycocyanobilin diacid with m/e 588. Cole *et al.* (1968) proposed that the phycocyanobilin diacid had a mol.wt. of 586, based on the determination of an m/e value of 614 for phycocyanobilin dimethyl ester. Chapman *et al.* (1967) reported an m/e value of 614 for phycoerythrobilin dimethyl ester, corresponding to a mol.wt. of 586 for the diacid. The schematic structures of (a) phycocyanobilin and (b) phycoerythrobilin are shown in Fig. 1.

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Fig. 1. Schematic structures of (a) phycocyanobilin and (b) phycoerythrobilin

The present paper reports mass-spectral analyses of phycocyanobilin and phycoerythrobilin cleaved from their respective proteins. Reverse-phase liquid chromatography was used to separate the pigments. Newly developed techniques were used to evaporate the pigments to obtain proton-transfer chemical-ionization mass spectra. Rapid evaporation of 20 pmol samples of the pigments from a matrix of oxalic acid yielded protonated parent molecular ions with m/e values of 587, corresponding to a mol.wt. of 586 for the free diacids. Furthermore, the mass spectra established the identity of the major components isolated from the protein as isomeric species.

Experimental

Protein purification

The cyanobacteria *Phormidium luridum*, *Phormidium persicinum* and *Tolypothrix tenuis* were grown in mass culture (Siegelman & Guillard, 1971). The bili-proteins were extracted and purified as described previously (Cole *et al.*, 1968) and freeze-dried. The C-phycoerythrin and C-phyocyanin of *T. tenuis* were separated by fractional precipitation with $(\text{NH}_4)_2\text{SO}_4$, dialysed against water and freeze-dried.

Cleavage

The bile pigments were cleaved from 1 g-portions of freeze-dried biliprotein by alcoholysis. The protein was put in a folded filter paper (Whatman 114V: 18.5 cm diameter) and placed in the inner tube of a Soxhlet extractor. The pigments were cleaved by refluxing alcohol, and the alcohol was removed and replaced with fresh alcohol every 30 min. Methanol cleavage continued for a total of 240 min, and butan-1-ol cleavage for 90 min. The individual alcohol extracts were concentrated by rotary evaporation at 35°C; the evaporation of butan-1-ol was enhanced by the addition of water to form an azeotropic mixture. The dried cleavage products of C-phyocyanin were carefully extracted two or three times with 0.5 ml portions of ethanol. The ethanol extracts were not further examined. The extracted blue residue was dissolved in methanol, dried by rotary evaporation at 35°C and stored at -15°C. The dried cleavage products of C-phycoerythrin were extracted with 3×1.0 ml of aq. 30% (v/v) methanol, and the extracts were not further examined. The extracted red residue was dissolved in methanol and dried and stored as described above. Yield data were not determined, but chromatographic analyses showed that the products obtained during any 30 min period were identical and insensitive to cleavage yields.

Chromatography

Analytical liquid-chromatography separations were performed on a column (0.4 cm $\times$ 15 cm) packed with

either a bonded octyl phase on 5 μm particle-size silica (RP-8; E. Merck, Darmstadt, Germany) at a flow rate of 1 ml/min at 1500 lb/in² (1.03×10^7 Pa) or a column (0.4 cm $\times$ 15 cm) packed with a bonded octadecyl phase on 5 μm particle-size silica (RP-18; E. Merck) at a flow rate of 1.5 ml/min at 1900 lb/in² (1.3×10^7 Pa). Preparative reverse-phase liquid chromatography used a column (1.0 cm $\times$ 25 cm) packed with the bonded octyl phase described above with a flow rate of 2 ml/min or a column (2.5 cm $\times$ 45 cm) of bonded octadecyl phase on 25–40 μm particle-size silica (RP-18; E. Merck) at a flow rate of 2 ml/min. The chromatographic solvent for the octyl-phase columns was acetone/water/acetic acid (150:50:1, by vol.). The chromatographic solvent for the octadecyl columns was acetone/water (3:1, v/v) containing 2 mM- H_3PO_4 and 0.5 mM- NaH_2PO_4 for analytical chromatography, and acetone/water (3:1, v/v) containing 0.5 mM- H_3PO_4 and 0.013 mM- NaH_2PO_4 for preparative chromatography. Phycocyanobilin and phycoerythrobilin were detected spectrophotometrically at 365 and 535 nm respectively. The separated pigments were extracted from the chromatographic solvent with chloroform after the addition of water. The chloroform layer was washed with water until neutral and dried by rotary evaporation at 35°C or with a He jet. All fractions were stored dry at -15°C.

Mass spectrometry

Mass spectra were obtained by rapid evaporation of approx. 20 pmol samples of bile pigment from a matrix of approx. 1–3 nmol of oxalic acid. Heating rates estimated to be in excess of 1000°C/s were used to flash the sample into the gas phase in a chemical-ionization mass-spectrometer ion source. A plot of the relative intensity of the protonated parent-molecule ion as a function of time, shown in Fig. 2, with results normalized to the maximum intensity at about 20 ms after the onset of sample heating, shows almost complete exhaustion of the pigment sample in less than 50 ms. Spectra were scanned with an Extranuclear Laboratories (Pittsburg, PA, U.S.A.) Quadrupole Mass Analyzer, in 5-mass-unit steps at very low resolution and maximum sensitivity. Under these circumstances the time available for recording a spectrum severely limited the mass range in each scan. With minimum dwell times on a peak in the spectrum of 0.2 ms, spectra were taken with repetitive scans of less than 50 steps per scan by using varying resolution of a quadrupole mass analyser to determine the active-mass regions of mass spectra. The mass range from $m/e = 250$ to $m/e = 700$ was covered in three overlapping runs. Higher-resolution runs (capable of unit resolution at mass 600) were then made for the purpose of mass identification in the mass region corresponding to the

Fig. 2. Plot of the time-dependence of the relative intensity of the protonated parent molecule ion obtained in the NH_4^+ chemi-ionization mass spectra of a sample of phycocyanobilin II evaporated from an oxalic acid matrix

The sample size was 20 pmol of phycocyanobilin II in a matrix of 3 nmol of oxalic acid.

protonated parent and wherever peaks with relative intensities in excess of 10% of that of the protonated parent ion were observed. The operation of the mass analyser was controlled with a PDP 8E (Digital Equipment Corp., Maynard, MA, U.S.A.) mini-computer, which also served as a data-acquisition and -processing system. The chemical-ionization source was operated with ammonia or, occasionally, methanol as the solvent carrier gas.

The detector system used in these experiments was a major factor in the successful determination of low-resolution mass spectra with 20 pmol samples of pigment. Ions exiting from the mass analyser were post-accelerated on to a collector with 30–40 keV. Secondary electrons produced from the energetic ion-impact process were focused on to an ORTEC solid-state detector. Ion post-acceleration enhances the sensitivity of detection of heavy molecular ions and also provides the option of noise rejection by discrimination against one- or two-electron pulses, with most of the phycocyanobilin or phycoerythrobilin ion signal giving pulses well over six or seven electrons. Details of the techniques of heavy-ion detection have been presented elsewhere (Beuhler & Friedman, 1977).

Rapid heating sublimation was conducted in a high vacuum under conditions that permitted collection of the products of pulsed heating of samples of C-phycocyanin and alcohol-cleaved phycocyanobilins. The products were collected on a stainless-steel foil situated a few mm above the sample probe filament. The probe-heating technique was similar to that used in the mass-spectrometer ion source. Samples of about 100 μg of C-phycocyanin yielded about 10 nmol of the chromophore on sublimation. The products collected on the foil in approx. 2π geometry were dissolved in methanol and subjected to liquid-chromatography

analyses. Similar results were obtained with samples of phycocyanobilin, which indicated an effective sample size of about 2 pmol of gaseous chromophore in the mass-spectrometer ion source when solid samples were heated rapidly.

Results and Discussion

Cleavage

The principal objective of this study is the identification of the algal bile pigments obtained by methanol and butan-1-ol cleavages of C-phycoerythrin and C-phycocyanin. The algal bile pigments were purified without prior esterification by reverse-phase liquid chromatography. Methanol cleavage of C-phycocyanin yielded phycocyanobilin I and phycocyanobilin II after a 30-min hydrolysis, and, similarly, C-phycoerythrin yielded two phycoerythrobilins (Fig. 3). Careful washing of the dried extracts after cleavage removed unidentified trace components. Cleavage of C-phycocyanin with butan-1-ol also yielded two phycocyanobilins. The two phycocyanobilins from the methanol and butan-1-ol cleavages were chromatographically identical. The algal bile pigments were unstable in solution and were kept dry at -15°C except during experimental use.

Mass spectroscopy

The object of the mass-spectrometric studies of the bile-pigment chromophores was to identify molecular weights and, if possible, to derive structural information from the patterns of fragment ions. With relatively fragile, complex and non-volatile molecular systems, mass-spectrometric determination of molecular weights can be achieved only if both volatilization and ionization of the molecular system take place with limited molecular decomposition. The replacement of electron-impact ionization by chemical-ionization techniques minimizes ionization-decomposition reactions. However, structural changes during volatilization remain as a serious limitation in the mass spectroscopy of non-volatile complex molecules.

A problem in establishing the molecular weights of phycocyanobilin and phycoerythrobilin diacids became apparent when parent molecule-ion m/e values of 614 were found for the respective dimethyl esters (Chapman *et al.*, 1967; Cole *et al.*, 1968). Previous studies cited above showed that the only volatile component obtained from batch purification of the phycocyanobilin diacid on silica gel had a mol.wt. of 588. The rather remote possibility of a dehydrogenation reaction taking place during the course of the esterification reaction was seriously considered. An alternative explanation for the experimental observations was that hydrogenation reactions were involved

Fig. 3. Elution diagram of phycocyanobilins (PCB, —) and phycoerythrobilins (PEB, ----) cleaved from *C-phycocyanin* and *C-phycoerythrin* respectively

The separation was on a column (0.4cm $\times$ 15cm) of bonded-octyl-phase silica with acetone/water/acetic acid (150:50:1, by vol.). Detection was at 365 nm for phycocyanobilins and 535 nm for phycoerythrobilins.

in the formation of the volatile component responsible for previously determined mass spectra. Such reactions have been observed in the study of the mass spectra of complex quinones (Muraca *et al.*, 1967; McLafferty, 1973).

Chemical-ionization mass spectra of the two forms of the diacids of phycocyanobilin and phycoerythrobilin, with the NH_4^+ ion as the proton-transfer ionizing reagent, all show an intense protonated parent molecule ion at m/e 587. Thus the mass-spectra molecular weight of these compounds, obtained from very rapid heating of approx. 20 pmol matrix-isolated samples, is 586. Slower evaporation of larger samples (of the order of more than 100 pmol

on the sample probe) gave evidence of species having molecular weights of 586 and 588. The question of whether a dehydrogenation reaction takes place on rapid heating in an oxalic acid matrix or hydrogenation when an amorphous or microcrystalline deposit of the solid is heated more slowly is not conclusively settled. Since neither oxalic acid nor its decomposition products are considered as particularly good dehydrogenating agents and results with the dimethyl ester derivatives provide corroborating evidence, we conclude that the molecular weight of phycocyanobilin and phycoerythrobilin is 586.

The chemical-ionization mass spectra further show that the two major components obtained by chromatographic separation of the cleavage products of the pigments from the respective proteins are isomeric species. Low-resolution mass spectra of the components isolated from *C-phycocyanin* are given in Fig. 4. Limitations of data-acquisition rate and sample size precluded the determination of a significant segment of the mass spectrum at higher resolution. Mass determination of the protonated parent molecule ion was made at much higher resolution by scanning over a limited range of several masses. The low-resolution mass spectra serve to 'fingerprint' adequately the respective molecules under conditions of highest possible sensitivity with a minimum distortion of

Fig. 4. Low-resolution mass spectra of phycocyanobilin I (a) and II (b) covering the mass range 265–625

Spectra were scanned in steps covering 5 mass units/step with dwell times of 0.2 ms/step.

relative ion intensities arising from mass discrimination in the quadrupole mass analyser. In addition to the identity of the masses of the protonated parent molecule, Fig. 4 shows minor differences in the relative yields of the fragment ions. In the phycocyanobilin-II mass spectrum in Fig. 4 there is a band of peaks in the mass range 480–490, which is not seen in the spectrum of phycocyanobilin I. The only significant yields of ion fragments in the phycocyanobilin-I spectrum are found at much lower mass, between 265 and 355, with most abundant fragment-ion yields between 280 and 310. The general absence of these particular ions in the phycocyanobilin-II mass spectrum of Fig. 4 should be noted. Ions formed in the mass range 280–310 are readily identified as products of bond-rupture processes at the central methine linkage in the tetrapyrrole system (see Fig. 1). Fragmentation reactions with the loss of less than 100 mass units from the protonated parent molecule ions cannot be ascribed to a bond-rupture process in which either ring A or D is eliminated. The band of unresolved ions in the m/e range 480–490 is probably formed in decomposition reactions involving the propionic acid side chains on rings B and C, if decomposition reactions are assumed to follow generally accepted mechanisms favouring processes that involve minimum expenditure of energy. Decarboxylation of one of the propionic acids with elimination of acetic acid from the other would account for the loss of 104 mass units from the parent ion, giving an ion fragment of m/e 483. Alternatively, the elimination of oxalic acid from the diacid with subsequent loss of one of the methyl side chains would also give an ion in the same mass band. Failure to observe ions in the m/e range 480–490 in the mass spectrum of phycocyanobilin I can be explained by the assumption that the isomers are *cis-trans* molecular systems with the *cis* system capable of decomposition reactions in which the elimination of the carboxylic acid functional groups was accompanied, in a concerted reaction, by the formation of a new ring by bonding the alkyl side-chain residues to each other as follows:

This ring would reinforce the attachment of the B and C rings in the tetrapyrrole system, minimizing the probability of decomposition reactions giving ions in the m/e range 280–300. Thus the major differences in the observed spectra can be accounted for by assumption of *cis-trans* isomerism with restricted

rotation about the central methine-bridge double bond. Both phycocyanobilins I and II may be characterized as in the *Z* form if the nomenclature applied to bilirubin and to dihydropyrromethane ring systems is used (Bonnett *et al.*, 1976).

Isomerism based on restricted rotation about the central methine carbon-carbon single bond of conjugated tetrapyrrole conformers is not expected unless the energy required for rotation about this carbon-carbon bond can be increased by hydrogen-bonding at several possible sites in the *cis* and *trans* tetrapyrrole system. Schematic possible structures showing hydrogen-bonding in the *cis* and *trans* isomers of phycocyanobilin are shown in Fig. 5. Attempts to interconvert isomeric species by treating solutions of phycocyanobilin I and II separately with BF_3 in diethyl ether at 0°C and 25°C respectively showed no evidence of interconversion of isomers. However, 14% (v/v) BF_3 in methanol (65°C) caused an interconversion of phycocyanobilins II into I and methyl esterification. These experiments indicate that phycocyanobilin I is the more stable isomeric form. Direct evaporation of phycocyanobilin from C-phycocyanin was achieved under high vacuum by using rapid heating. The phycocyanobilin isomers sublimed from C-phycocyanin had ratios of phycocyanobilins I and II similar to those obtained by alcohol cleavage.

Fig. 5. Schematic structures of phycocyanobilin in (a) *cis* and (b) *trans* configuration showing possible hydrogen-bonding that would stabilize the respective structures

Similar mass-spectral data were not obtained with the phycoerythrin free acids, because the fragmentation reactions of these molecules were dominated by cleavage of the terminal ring D at the methylene linkage. Phycoerythrobilin presented a much more difficult mass-spectrometric problem because successful spectroscopy was possible only with very freshly prepared samples.

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