Properties and *N*-Terminal Sequence of Allophycocyanin from the Unicellular Rhodophyte *Cyanidium caldarium*

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Allophycocyanin from the unicellular rhodophyte *Cyanidium caldarium* was purified by $(\text{NH}_4)_2\text{SO}_4$ fractionation and ion-exchange chromatography on brushite (calcium phosphate) columns and on DEAE-Sephadex A-25 columns. The specific absorption coefficient ($A_{1\text{cm}}^{0.1\%}$) at 650 nm of purified allophycocyanin was 6.35 in 0.05 M-potassium phosphate buffer, pH 7.0. Absorption maxima of allophycocyanin occurred at 650, 618 (shoulder), 350 and 275 nm. Circular-dichroic spectra displayed positive-ellipticity bands at 658 and 630 nm and a major negative-ellipticity band at 340 nm. Computer analysis of the circular-dichroic spectrum of allophycocyanin from 207 to 243 nm indicated 42% α -helix and 58% β -form. The estimated molecular weight of purified allophycocyanin on calibrated Sephadex G-200 columns at pH 7.0 was 196 000. Electrophoretic examination of allophycocyanin on sodium dodecyl sulphate/polyacrylamide gels revealed a single band with apparent mol. wt. 16 000. The presence of two polypeptide subunits, with nearly the same molecular weight, was revealed on polyacrylamide gels by using a modified electrophoresis buffer. Spectral analysis of the allophycocyanin subunits resolved by ion-exchange chromatography on Bio-Rex 70 columns indicated that a single phycocyanobilin chromophore was present on each polypeptide chain. Treatment of allophycocyanin with 8 M-urea (pH 3.0) and subsequent removal of urea by dialysis against water yielded a derivative phycobiliprotein with spectroscopic characteristics similar to those of phycocyanin. The original allophycocyanin spectrum was regenerated after incubation in phosphate buffer, pH 7.0. Automated sequence analysis of the *N*-terminus of allophycocyanin showed that (a) the sequences of the two subunits were different from one another and were different from the subunits of phycocyanin from the same alga, (b) the subunits occurred in a molar ratio of 1:1 and (c) the sequence homology at the *N*-terminus among α - and β -subunits of allophycocyanin from blue-green and red algae approached 90%.

Allophycocyanin, phycocyanin and phycoerythrin are bile pigment-protein complexes which function as photosynthetic accessory pigments in red (Rhodophyta), blue-green (Cyanophyta) and cryptomonad (Cryptophyta) algae (O'hEocha, 1965). Phycobiliproteins are located on the outer surfaces of thylakoid membranes in large aggregated complexes known as phycobilisomes in red and blue-green algae (Gantt & Conti, 1966). Action spectra for oxygen evolution show that light absorbed by phycobilisomes is efficiently utilized to drive algal photosynthesis (Myers, 1971).

Phycocyanin and phycoerythrin are the most thoroughly studied phycobiliproteins, owing to their abundance in algal cells (40–50% of the soluble protein) and the ease with which they are extracted and purified (Bogorad, 1975; Bennett & Siegelman, 1977). Considerably less is known about allophycocyanin, both because it is less abundant (5–20% of the soluble protein) and because it is more difficult to purify to homogeneity. In fact, earlier work on allophycocyanin was concerned with demonstrating that it occurred naturally (Haxo *et al.*, 1955; Hattori & Fujita, 1959*a,b*), and was not a denatured form of

phycocyanin, as had been suggested by Lemberg & Bader (1933). Despite the spectral differences between allophycocyanin (visible absorption maximum, 650 nm) and phycocyanin (visible absorption maximum, approx. 620 nm), the linear tetrapyrrole phycocyanobilin is the only chromophore associated with each of these phycobiliproteins (Chapman *et al.*, 1967). Thus the spectral difference between these pigments has been ascribed to intrinsic differences in the nature of chromophore-protein interaction.

Renewed interest in allophycocyanin occurred when its spectral similarity to phytochrome, the bile pigment-protein complex involved in photo-control of physiological responses in higher plants, was recognized (Briggs & Rice, 1972; Bennett & Siegelman, 1977). For example, refined action spectra for photosynthesis in three species of blue-green algae have shown that wavelengths of light absorbed predominantly by allophycocyanin promoted oxygen evolution more efficiently than wavelengths of light absorbed primarily by phycocyanin (Lemasson *et al.*, 1973). These results are compatible with (a) fluorescence studies on isolated phycobilisomes

which indicate that allophycocyanin mediates transfer of absorbed light energy between other phycobili-proteins and chlorophyll *a* (Gantt & Lipschultz, 1973; Gray & Gantt, 1975; Gantt *et al.*, 1976) and (b) a structural model for phycobilisomes which predicts that allophycocyanin occurs as a central core surrounded by phycocyanin, which is itself surrounded by phycoerythrin (Gantt *et al.*, 1976).

Allophycocyanin-like pigments apparently play a significant role in developmental processes in blue-green algae. Lazaroff & Schiff (1962) showed that the action spectrum for photo-induction of the cell cycle in the filamentous cyanophyte *Nostoc muscorum* resembled the absorption spectrum of allophycocyanin. Diakoff & Scheibe (1973) demonstrated that the action spectrum for phycocyanin formation in another filamentous cyanophyte *Tolypothrix tenuis* had peaks of effectiveness at 660 and 360 nm characteristic of allophycocyanin. The photo-reversible pigment thought to be involved in regulation of phycocyanin synthesis in *T. tenuis* has been partially purified and is nearly spectrally identical with allophycocyanin (Scheibe, 1972). At least three other photo-reversible bile-pigment-protein complexes have been described (Bjorn & Bjorn, 1976) and may be synonymous with 'adaptochromes' which regulate phycobiliprotein synthesis in red and blue-green algae (Bogorad, 1975).

Our previous work described the physical-chemical properties and the sequence at the *N*-terminus of allophycocyanin from the filamentous cyanophyte *Phormidium luridum* (Brown *et al.*, 1975). The present paper is concerned with a characterization of allophycocyanin from the unicellular rhodophyte *Cyanidium caldarium*. This provides the basis for comparing some parameters of allophycocyanin from prokaryotic and eukaryotic organisms.

Experimental

Organism

Cyanidium caldarium is a unicellular eukaryote which can be grown heterotrophically in the dark in medium containing glucose (Allen, 1959). Under these conditions this organism is unable to make photosynthetic pigments. When dark-grown cells are placed in the light in glucose-free medium, they synthesize chlorophyll *a*, phycocyanin and allophycocyanin (Troxler & Bogorad, 1966). Pigment synthesis ceases on maturation of a single cup-shaped chloroplast in each algal cell (Mercer *et al.*, 1962; Bogorad *et al.*, 1963). Confusion surrounds the taxonomy of *C. caldarium*, and the rationale for considering it a member of the Rhodophyta has been discussed (Bogorad, 1975; Troxler *et al.*, 1975).

Culture conditions

The *C. caldarium* strain used in the present work has been described by Allen (1959), and the culture

conditions used for growth of this organism in the laboratory have been reported (Troxler & Lester, 1967).

Preparation of phycobiliproteins

Pigmented algal cells were washed twice with 0.1 M-potassium phosphate buffer, pH 7, and disrupted in the same buffer (1 vol. of cells:5 vol. of buffer) by sonic vibration (100 W) with a Branson sonic oscillator (model W 185). The disrupted cells were centrifuged for 30 min at 30000g and the phycobiliproteins in the resulting supernatant were precipitated by the addition of solid $(\text{NH}_4)_2\text{SO}_4$ to 50% saturation. The precipitate was dissolved in water, dialysed exhaustively against water at 4°C and centrifuged at 100000g for 1 h. The resulting supernatant was chromatographed on a column (2.5 cm × 20 cm) of brushite ($\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$), which was developed with potassium phosphate buffers (pH 7.0) of increasing ionic strength as described previously (Troxler & Lester, 1967). Brushite was prepared from 5 litres of 0.5 M- K_2HPO_4 and 5 litres of 0.5 M- $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, which were pumped (100–150 ml/h) from separate containers into a 12-litre plastic bucket with a peristaltic pump (Harvard Apparatus Co., model no. 600-000). The resulting brushite (calcium phosphate) precipitate was washed with 5 × 10 litres of water and stored at room temperature (25°C) until used (Levin, 1962; Siegelman & Firer, 1964). Phycocyanin was eluted from the column in 0.01–0.02 M-potassium phosphate buffer and allophycocyanin in 0.05–0.10 M-phosphate buffer. Eluted phycobiliproteins were precipitated in 80%-satd. $(\text{NH}_4)_2\text{SO}_4$ and dialysed against water at 4°C.

Allophycocyanin was further purified by chromatography on DEAE-Sephadex A-25 columns. Allophycocyanin was dialysed against 0.05 M-sodium acetate buffer containing 1 mM-mercaptoethanol, pH 5.5, and applied to a column (1.5 cm × 38 cm) of DEAE-Sephadex A-25 (Pharmacia Fine Chemicals, Piscataway, NJ, U.S.A.) equilibrated with the same buffer. The columns were initially washed with 150 ml of 0.05 M-acetate buffer, pH 5.5, followed by 100 ml of 0.15 M-acetate buffer, pH 5.5, which eluted trace amounts of phycocyanin. Allophycocyanin was then eluted with 0.2 M-acetate buffer, pH 5.5. All acetate buffers contained 1 mM-2-mercaptoethanol. Allophycocyanin in which the A_{650}/A_{620} and A_{650}/A_{280} ratios were greater than 1.4 and 4.0 respectively was considered pure. Fractions not meeting these criteria were rechromatographed on a DEAE-Sephadex A-50 column under the conditions described above.

The specific absorption coefficient ($A_{1\text{cm}}^{0.1\%}$) of *C. caldarium* allophycocyanin was determined by weighing samples (which were dried on a hot-plate) of a solution of known A_{650} in tared aluminium pans on a Cahn micro-balance. The specific absorption

coefficient of allophycocyanin at 650 nm in 0.05 M-potassium phosphate buffer (pH 7.0) was 6.35. The specific absorption coefficient of phycocyanin under the same conditions was 7.74.

Gel-filtration chromatography

Sephadex G-200 was equilibrated for 5 days in 0.05 M-potassium phosphate buffer (pH 7.0) containing 0.02% NaN_3 and poured into a 1.25 cm $\times$ 50 cm column. The column was calibrated with cytochrome *c* (mol.wt. 11 700), myoglobin (mol.wt. 17 200), ovalbumin (mol.wt. 43 000), horse haemoglobin (mol.wt. 65 000), catalase (mol.wt. 250 000) and Blue Dextran (Pharmacia) as described by Andrews (1965). The flow rate was 30 ml/h and was maintained with a Pharmacia peristaltic pump (model no. P3) attached to the bottom of the column. Samples were applied to the top of the column and eluent flowed down through the column, through the pump and into a Buchler fraction collector (Fractometre 200).

Spectroscopic measurements

Absorption spectra were determined on a Beckman DB-GT recording spectrophotometer. C.d. (circular-dichroic) spectra were determined on a Cary 61 spectropolarimeter calibrated with a (+)-camphor-10-sulphonic acid standard (1 mg/ml; Eastman Kodak Co., Rochester, NY, U.S.A.) in water. All c.d. measurements were performed at 27°C. Mean residue ellipticity $[\theta]$ in degrees $\cdot \text{cm}^2 \cdot \text{dmol}^{-1}$ was determined by using mean residue weights of 110 for both allophycocyanin and phycocyanin. Mean residue weights were calculated from the amino acid compositions of these phycobiliproteins. The c.d. of these phycobiliproteins from 207 to 243 nm was analysed by the method of Chen *et al.* (1972), modified as described by Brown *et al.* (1975), by using an IBM 370/145 computer.

Gel electrophoresis

Polyacrylamide-gel electrophoresis was performed on 10% polyacrylamide gels (0.5 cm $\times$ 9 cm) by the method of Weber & Osborn (1969), at a ratio of *NN'*-methylenebisacrylamide to acrylamide of 1:18.5 (w/w). Freeze-dried phycobiliproteins (1 mg) were placed in 1.0 ml of 0.01 M-sodium phosphate buffer, pH 7.0, containing 1% sodium dodecyl sulphate and 1% 2-mercaptoethanol. The resulting solutions were heated at 100°C for 3 min, cooled to room temperature and applied to the gels.

The electrophoresis buffer in the upper and lower compartments of the electrophoresis apparatus (Buchler Polyanalyst) contained 3.9 g of $\text{NaH}_2\text{PO}_4 \cdot 7\text{H}_2\text{O}$, 19.3 g of $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ and 1 g of sodium dodecyl sulphate per litre. In some experiments the

concentration of the electrophoresis buffer (modified electrophoresis buffer) was decreased to one-quarter (i.e. 0.97 g of $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 4.82 g of $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ and 0.25 g of sodium dodecyl sulphate per litre). The gels were run either for 4–6 h at 8 mA/tube or overnight at 2 mA/tube. All reagents were purchased from Bio-Rad Laboratories, Richmond, CA, U.S.A. Gels were stained with 0.2% Coomassie Brilliant Blue in 10% (v/v) methanol/7% (v/v) acetic acid for 40 min at 37°C. Diffusion destaining was carried out overnight in the same solvent.

Subunit preparation

Purified allophycocyanin (10 mg) was dissolved in 12% (v/v) formic acid containing 0.01 M-mercaptoethanol and applied to a 1 cm $\times$ 10 cm column of Bio-Rex (– 400 mesh; Bio-Rad Laboratories) which had been equilibrated in 12% formic acid. The column was developed stepwise by a modification of the procedure of Glazer & Fang (1973) by using aqueous urea solutions of increasing concentration: 2 M-urea (10 ml), 4 M-urea (30 ml), 6 M-urea (50 ml), 7.5 M-urea (55 ml) and 8 M-urea (50 ml). The developing solutions were adjusted to pH 3.0 with conc. HCl and made 0.01 M with respect to mercaptoethanol. One allophycocyanin subunit was eluted in 7.5 M-urea and the other in 8 M-urea. The fractions containing the subunits were separately pooled, dialysed exhaustively against water and freeze-dried.

Amino acid analyses

Allophycocyanin was hydrolysed *in vacuo* in 6 M-HCl for 24, 48 and 72 h at 110°C. Hydrolysates were analysed with a Jeolco 6AH automated analyser using a two-column system. The two separated allophycocyanin subunits were hydrolysed for 22 h at 110°C in 6 M-HCl. As a general protocol, 0.2 mg of each phycobiliprotein sample was hydrolysed in 1 ml of acid and half of the hydrolysate was subjected to amino acid analysis.

Automated sequence analysis

Sequencing was carried out with automatic stepwise degradation (Beckman model 890C sequencer) by the procedure of Edman & Begg (1967). A 7 mg sample of allophycocyanin was subjected to automated sequencing (protein-sequencing program 071472) by using a Quadrol buffer system (Beckman Sequencing Manual, 1969). Norleucine phenylthiohydantoin served as an internal standard in all samples. All fractions were converted into the amino acid phenylthiohydantoin derivatives and extracted into ethyl acetate. A portion (10%) of the ethyl acetate extract was analysed on a Beckman model 65 gas chromatograph by a modification (Foster *et al.*,

1973) of the method of Pisano & Bronzert (1969). Amino acid phenylthiohydantoin were hydrolysed in 6M-HCl containing 0.25% SnCl₂ for 4 h at 150°C (Mendez & Lai, 1975) and identified with a Jeolco 6AH amino acid analyser with a two-column system.

Approx. 4mg of the allophycocyanin β -type subunit was sequenced as described above. The phenylthiohydantoin were subjected to analysis by g.l.c., hydrolysed in HI (Smithies *et al.*, 1971) and analysed in a Beckman (model 119) analyser on a one-column system.

Results

Allophycocyanin purification

Allophycocyanin comprised approx. 18% and phycocyanin approx. 45% of the total soluble protein extracted from *C. caldarium* cells and precipitated by 50%-satd. (NH₄)₂SO₄. Chromatography of allophycocyanin on a second brushite column did not yield pigment with satisfactory A_{650}/A_{620} and A_{650}/A_{280} ratios. Although spectrally pure allophycocyanin was obtained when samples eluted from brushite columns were chromatographed on DEAE-Sephadex A-25 columns, nearly 20% of the pigment remained irreversibly bound. Allophycocyanin losses were less when DEAE-Sephadex A-50 columns were used.

Table 1. *Molecular weight of C. caldarium phycobiliproteins*

The molecular weight of *C. caldarium* phycobiliproteins was determined by gel-filtration chromatography on calibrated Sephadex G-200 columns at pH 7.0 in 0.05M-phosphate buffer. Deduced aggregates were assigned by assuming that allophycocyanin monomers contain two polypeptide subunits, each with mol.wt. 16000 (see Fig. 3); phycocyanin from this alga is composed of α - and β -subunits with mol.wts. of 17000 and 19500 respectively (Troxler *et al.*, 1975). It is also assumed that the phycobiliprotein monomers comprise one α - and one β -subunit.

Phycobiliprotein	Aggregate mol.wt.	Deduced aggregate
Allophycocyanin	196000*	Hexamer
	660000†	Uncertain
Phycocyanin	228000	Hexamer

* This allophycocyanin sample was chromatographed twice on a Sephadex G-200 column at concentrations of 1.6, 0.8 and 0.16mg/ml. Phycocyanin was chromatographed twice at a concentration of 0.45mg/ml.

† This allophycocyanin sample was eluted from a brushite column in 0.1M-phosphate buffer, pH 7.0, and contained approx. 20% phycocyanin.

Gel-filtration chromatography

The results of gel-filtration chromatography of *C. caldarium* phycobiliproteins on calibrated Sephadex G-200 columns at pH 7.0 (0.05M-phosphate buffer) are shown in Table 1. The estimated mol.wt. of allophycocyanin at three different concentrations (1.6–0.16mg/ml) was 196000. Phycocyanin at a concentration of 0.45mg/ml was eluted as a single component with estimated mol.wt. 228000. On one occasion a partially purified allophycocyanin was eluted from the Sephadex G-200 column with apparent mol.wt. 660000.

Spectral measurements

Purified allophycocyanin and phycocyanin were dialysed against 0.05M-potassium phosphate buffer, pH 7.0, at 4°C and the absorption and c.d. spectra were determined. Purified allophycocyanin displayed absorption bands at 650, 618 (shoulder), 350 and 275 nm; positive-ellipticity bands occurred at 658 and 630 nm, and a major negative-ellipticity band was observed at 340 nm (Fig. 1). The absorption and c.d. maxima of phycocyanin were the same as described previously (Brown *et al.*, 1975).

Fig. 1. *Absorption (b) and c.d. (a) spectra of allophycocyanin and phycocyanin*

Both phycobiliproteins were dialysed against 0.05M-potassium phosphate buffer (pH 7.0). The concentration of protein was adjusted to 0.1mg/ml for the absorption and 1.1 mg/ml for the c.d. determinations. The values of $[\theta]$ at wavelengths from 260 to 700 nm have been multiplied by 10. ----, Allophycocyanin; —, phycocyanin.

Fig. 2. Absorption spectrum of allophycocyanin. The absorption spectra after denaturation with 8M-urea, pH 3.0, and exhaustive dialysis against water (—) and after incubation for 30 min in 0.05M-potassium phosphate buffer, pH 7.0 (●), are shown. The allophycocyanin concentration used in these experiments was 0.085 mg/ml.

The secondary structure of allophycocyanin and phycocyanin was evaluated by computer analysis of the c.d. spectrum at selected wavelengths between 207 and 243 nm (see the Experimental section). The results of linear least-squares fitting of the phycobiliprotein c.d. curves to ellipticity coefficients $[\theta]$ at selected wavelengths of a protein containing 100% α -helix or 100% β -pleated sheet indicated that both allophycocyanin and phycocyanin had approx. 42% α -helix and 58% β -structure.

For determination of the chromophore composition of allophycocyanin, equivalent amounts of allophycocyanin and phycocyanin were placed in 9M-urea, pH 3.0. Under these conditions, the visible spectra of allophycocyanin and phycocyanin resemble one another, with maxima occurring at 660 and 350 nm. The A_{660} of allophycocyanin (area under the peak) was 67% of the A_{660} of phycocyanin. Since the phycocyanin monomer is known to contain three residues of phycocyanobilin (Troxler *et al.*, 1975; Glazer *et al.*, 1976), these data indicate that allophycocyanin has two bile-pigment chromophores per monomer (i.e. the monomer = $1\alpha + 1\beta$ subunit).

C. caldarium allophycocyanin which was denatured with 8M-urea, pH 3.0, and subsequently dialysed against water displayed the characteristic spectral behaviour observed with allophycocyanins

from two species of blue-green algae (Erokchina & Krasnovskii, 1974; Brown *et al.*, 1975). The absorption spectrum of allophycocyanin in water after acid/urea treatment and dialysis resembled that of phycocyanin (Fig. 2). The absorption spectrum characteristic of the original allophycocyanin returned on addition of potassium phosphate buffer, pH 7.0 (final concentration 0.05M). *C. caldarium* phycocyanin treated similarly displayed an absorption spectrum nearly identical with that of phycocyanin not treated with acid/urea.

Gel electrophoresis

The electrophoretic behaviour of *C. caldarium* phycobiliproteins on sodium dodecyl sulphate/polyacrylamide gels is shown in Fig. 3. Electrophoresis of allophycocyanin on calibrated gels, run by the procedure of Weber & Osborn (1969), revealed a single band with estimated mol.wt. 16000 (Fig. 3a, gel 1), whereas phycocyanin was separated into a more rapidly migrating α -subunit and a more slowly migrating β -subunit with estimated mol.wts. of 17000 and 19500 respectively (Fig. 3a, gel 2). A 4-fold decrease in the ionic strength and sodium dodecyl sulphate concentration of the electrophoresis buffer (modified electrophoresis buffer) enabled separation of allophycocyanin into a more rapidly migrating and a more slowly migrating subunit (Fig. 3b, gels 1–3). When allophycocyanin and phycocyanin were examined electrophoretically (modified electrophoresis buffer) on the same gel, a narrow band at the position of the more slowly migrating subunit and a very broad band at the position of the more rapidly migrating subunit were observed (Fig. 3b, gel 5).

One explanation for the failure of allophycocyanin to separate into two bands on the sodium dodecyl sulphate/polyacrylamide gels shown in Fig. 3(a) (gel 1) is that the procedure used to denature the sample before electrophoresis incompletely separated the subunits from one another. Therefore subunits that had been separated from one another by ion-exchange chromatography on Bio-Rex 70 columns (see below) were separately examined electrophoretically by the procedure of Weber & Osborn (1969). The separated subunits examined on the same or separate gels migrated as a single band with the same mobility (Fig. 4a, gels 1–3). Thus the behaviour of allophycocyanin on gels run by the method of Weber & Osborn (1969) cannot be ascribed to incomplete separation of subunits during sample pretreatment. To exclude the possibility that the different mobility of the allophycocyanin subunits in gels run with the modified electrophoresis buffer (see Fig. 3c, gels 1 and 2) might be explained by the fact that one subunit was a monomer (gel 1) and the other subunit was a dimer (gel 2), the protein standards were examined on gels by using the modified electro-

Fig. 3. Electrophoresis of *C. caldarium* allophycocyanin and phycocyanin on sodium dodecyl sulphate/polyacrylamide gels (a) Normal electrophoresis buffer (Weber & Osborn, 1969): 1, allophycocyanin, 10 μ g; 2, phycocyanin, 10 μ g. (b) Modified electrophoresis buffer (see the Experimental section): 1, allophycocyanin, 5 μ g; 2, allophycocyanin, 10 μ g; 3, allophycocyanin, 25 μ g; 4, phycocyanin, 10 μ g; 5, allophycocyanin and phycocyanin, 5 μ g each. (c) Modified electrophoresis buffer (see the Experimental section): 1, β -type allophycocyanin subunit from Bio-Rex 70 column, 10 μ g; 2, α -type allophycocyanin subunit from Bio-Rex 70 column, 10 μ g. The anode was at the lower end of the gels as shown.

phoresis buffer. A plot of $\log(\text{molecular weight})$ against mobility was slightly curved. In addition, the separated (Bio-Rex 70) subunits examined on the same gel separated into two bands (Fig. 4b, gel 5). The apparent mol.wts. for the subunits obtained from the curve for standards were 16300 and 17900. These results are not compatible with the premise that subunit dimers arise under the conditions of sample pretreatment and electrophoresis used.

Subunit preparation

The α - and β -subunits of phycocyanin from several algae have been resolved by ion-exchange chromatography on Bio-Rex 70 columns (Glazer & Fang, 1973; Troxler *et al.*, 1975). A modification of this procedure was used to separate the *C. caldarium* allophycocyanin subunits.

One subunit, corresponding to the more rapidly migrating polypeptide chain on sodium dodecyl sulphate/polyacrylamide gels in the modified electrophoresis buffer (Fig. 3c, gel 1), was eluted in 7.5M-urea, pH 3.0. The other subunit was eluted in 8M-urea, pH 3.0, and corresponded to the more slowly migrating subunit (Fig. 3c, gel 2). The ratio of total A_{660} (area under the peak) of the subunit eluted in 7.5M-urea to the total A_{660} of the subunit eluted in 8M-urea was 1:1.1.

Amino acid analysis

The amino acid composition of the two subunits of allophycocyanin is indicated in Table 2. Total amino acid residues were calculated by assuming a mol.wt. of 16000 for each subunit and 32000 for allophycocyanin.

Fig. 4. Electrophoresis of separated subunits of *C. caldarium* allophycocyanin on sodium dodecyl sulphate/polyacrylamide gels (a) Normal electrophoresis buffer (Weber & Osborn, 1969): 1, β -type subunit, 10 μ g; 2, α -type subunit, 10 μ g; 3, β -type and α -type subunit, 5 μ g each; 4, protein standards: O, ovalbumin (mol.wt. 43000); C, carbonic anhydrase (mol.wt. 29000); M, myoglobin (mol.wt. 17200); L, lysozyme (mol.wt. 14300); 10 μ g each. (b) Modified electrophoresis buffer (see the Experimental section): 1, ovalbumin, 10 μ g; 2, carbonic anhydrase, 10 μ g; 3, myoglobin, 10 μ g; 4, lysozyme, 10 μ g; 5, β -type and α -type subunit which had been separated by ion-exchange chromatography on Bio-Rex 70 column (see the Experimental section), 10 μ g each. The anode was at the lower end of the gels as shown.

cyanin. Significant differences in the glutamic acid/glutamine, methionine and tyrosine composition of the two subunits were observed.

Automated sequence analyses

Allophycocyanin was subjected to automated sequence analysis. The first 10 residues at the *N*-terminus and the criteria for identification and recoveries at each sequencer cycle are shown in Table 3(a). Table 3(b) contains similar data for the β -type subunit. These results confirm that there are two distinct subunits in *C. caldarium* allophycocyanin and that the ratio of subunit polypeptides is 1:1. Of the first 10 residues at the *N*-terminus of the two subunits, three are identical, five can be accounted for by

a single base change in the codon for the amino acid and two show no homology.

Discussion

The present investigation has shown that allophycocyanin from the unicellular eukaryote *C. caldarium* has a subunit composition, chromophore content, absorption and c.d. spectrum (700–200 nm), spectral behaviour after denaturation with 8M-urea and amino acid sequence at the *N*-terminus which are remarkably similar to these properties for allophycocyanin from the filamentous cyanophyte *P. luridum* (Brown *et al.*, 1975). In addition, both allophycocyanins have proved more difficult to purify to homogeneity than the corresponding phycocyanins from these organisms.

Table 2. *Amino acid composition of C. caldarium allophycocyanin and of its subunits*

Amino acid	No. of residues/molecule			
	α -Type subunit*	β -Type subunit**†	$\alpha + \beta$ Subunits‡	Allophycocyanin§
Asp	14.5	14.1	28.6	27.7
Thr	6.8	9.7	16.5	15.8
Ser	11.3	13.8	25.1	21.6
Glu	17.2	11.6	28.8	28.2
Pro	4.9	2.7	7.6	9.3
Gly	14.8	14.1	28.9	26.3
Ala	15.4	17.6	33.0	30.5
‡Cys	N.D.	N.D.	—	3.3
Val	8.1	6.0	14.1	15.8
Met	1.3	4.4	5.7	7.1
Ile	9.1	8.3	17.4	18.0
Leu	12.2	12.5	24.7	23.0
Tyr	5.8	9.6	15.4	15.6
Phe	3.6	2.0	5.6	6.3
Lys	6.8	8.0	14.8	13.1
His	1.3	0.4	1.7	1.2
Arg	8.1	6.9	15.0	15.6
Sum	141.2	141.7	282.9	278.4

* Values based on a single 22h hydrolysate and a mol.wt. of 16000 minus the weight of phycocyanobilin, assuming one residue of chromophore per subunit. N.D., Not determined.

† The β -type subunit in this study refers to the subunit eluted from Bio-Rex 70 columns in 7.5M-urea and migrated the farthest on sodium dodecyl sulphate/polyacrylamide gels run in the modified electrophoresis buffer system (see the Experimental section).

‡ Values represent the arithmetic sum of the residues determined in separate analyses of the α - and β -subunit.

§ Values based on 24 h, 48 h and 72h hydrolysates each done in duplicate. The assumed mol.wt. of allophycocyanin was 32000 minus the weight of two residues of phycocyanobilin.

Gel-filtration chromatography of *C. caldarium* allophycocyanin showed that this phycobiliprotein had apparent mol.wt. 196000. Previously reported values for the mol.wt. of allophycocyanins from red and blue-green algae range from 96000 to 155000 (Hattori & Fujita, 1959a; Noland & O'hEocha, 1967; Craig & Carr, 1968; Glazer & Cohen-Bazire, 1971; Gantt & Lipschultz, 1974; MacColl *et al.*, 1974; Brown *et al.*, 1975). Differences in the apparent molecular weight of allophycocyanins may result from the conditions used during purification, the method of molecular-weight determination, the pH, the choice of buffer and ionic strength, the concentration of protein and also species differences (Berns, 1971; Brown *et al.*, 1975). The presence of an allophycocyanin hexamer at protein concentrations from 0.16 to 1.6 mg/ml and the finding of even higher aggregates (mol.wt. 660000) in samples also containing phycocyanin are compatible with the structural requirements for the proposed core of allophycocyanin in the phycobilisome (Gantt *et al.*, 1976), and suggest that *C. caldarium* allophycocyanin could

serve as a point of departure for the study of phycobilisome assembly *in vitro*.

Allophycocyanin has visible absorption bands at 650 and 620 nm (shoulder), yet the linear tetrapyrrole phycocyanobilin is the only chromophore associated with this phycobiliprotein (Chapman *et al.*, 1967). When allophycocyanin is placed in 8M-urea (pH 3.0), disrupting chromophore-protein interaction, a single absorption band is observed at 660 nm which corresponds to phycocyanobilin in the hydrochloride form still covalently linked to protein. Thus the allophycocyanin absorption bands at 650 and 620 nm and the corresponding positive-ellipticity bands at 658 and 620 nm can be attributed to induced asymmetry of phycocyanobilin due to chromophore-protein interaction.

The spectral properties of allophycocyanin after denaturation and removal of the denaturant by dialysis are particularly interesting, because the resulting allophycocyanin has an absorption spectrum comparable with that of phycocyanin. Allophycocyanins from *P. luridum* and *Anacystis nidulans* also

Table 3. *Sequential degradation of the N-terminus of C. caldarium allophycocyanin and of the β -type subunit*
 X signifies an unknown amino acid; parentheses indicate a suspected residue. N.D., Not done; N.C., not calculated but identifiable. For g.l.c., the packing material in the columns was SP100. For amino acid analysis, as indicated in the Experimental section, HCl/0.25% SnCl₂ hydrolysis was used for allophycocyanin; HI hydrolysis was used for the β -type subunit. See the Discussion section for rationale for designation of subunits as α and β .

Step	Deduced residues	G.l.c.		Amino acid analysis	
		Residue	Content (nmol)	Residue	Content (nmol)
(a) Allophycocyanin					
1	Met + Met	Met	N.C.	Met	9.04
2	Ile + Glx	Leu/Ile	11.6	Ile + Glx	9.7+19.7
3	Val + Asx	Val	8.4	Val + Asx	7.7+10.9
4	Thr + Ala	Ala	44.2	Thr* + Ala	N.C.+18.5
5	Lys + Ile	Leu/Ile	35.5	Lys + Ile	7.9 + 8.2
6	(Thr) + Thr	Gly/Pro†	N.C.	Thr*	N.C.
7	Ile + Ala	Ile + Ala	8.0+17.2	Ile + Ala	9.0+19.2
8	Val + Val	Val	22.5	Val	19.3
9	Asx + Ile	Leu/Ile	N.D.	Asx + Ile	10.2+5.4
10	Ala + Asx	Ala	8.9	Ala + Asx	11.0+11.5
(b) β -Type subunit of allophycocyanin					
1	Met	Met	N.C.	N.D.	—
2	Glx	X	—	Glx	14.1
3	Asx	X	—	Asx	13.5
4	Ala	Ala	27.2	Ala	20.3
5	Ile	Ile	11.9	Ile	15.0
6	Thr	X	—	Thr*	N.C.
7	Ala	Ala	16.6	Ala	21.8
8	Val	Val	10.1	Val	12.7
9	Ile	Leu/Ile	7.5	Ile	N.C.
10	Asx	X	—	Asx	N.C.

* Threonine was determined as α -aminobutyric acid by amino acid analysis after hydrolysis of threonine phenylthiohydantoin (see the Experimental section).

† Degradation product of threonine on g.l.c. is Gly/Pro.

display this spectral behaviour (Brown *et al.*, 1975; Erokchina & Krasnovskii, 1974). One explanation for this result is that there is a region within urea-treated allophycocyanin where phycocyanobilin can assume a configuration comparable with that normally assumed in phycocyanin. The reversibility of this spectral anomaly on addition of phosphate buffer, pH 7.0, suggests that regeneration of the original allophycocyanin spectrum may be due to a conformation change occurring during subunit or monomer aggregation.

Previous reports have indicated that allophycocyanin is composed of one subunit (Bennett & Bogorad, 1971; Rice & Briggs, 1973; Gantt & Lipschultz, 1974; Gray & Gantt, 1975), two subunits (Glazer & Cohen-Bazire, 1971; Gysi & Zuber, 1974) or three subunits (Glazer & Cohen-Bazire, 1971). The present work has shown that *C. caldarium* allophycocyanin is composed of two polypeptide subunits with dissimilar amino acid compositions, chromatographic properties and N-terminal sequences, but with nearly identical molecular weights.

These results confirm more recent work which has shown that allophycocyanins comprise two distinct polypeptide subunits (Brown *et al.*, 1975; Glazer *et al.*, 1976), and that conventional electrophoresis in sodium dodecyl sulphate/polyacrylamide gels is not sufficient in itself for evaluation of the subunit composition of allophycocyanin. In addition, the data suggest that the basic unit of aggregation in all phycobiliproteins from red and blue-green algae is a monomer consisting of one α and one β polypeptide subunit in the 30000–35000-dalton range (Troxler *et al.*, 1975; Brown *et al.*, 1975; Glazer *et al.*, 1976).

A brief discussion of subunit nomenclature seems appropriate. The terms α - and β -subunit were originally applied to the more rapidly and the more slowly migrating bands respectively of phycocyanin on sodium dodecyl sulphate/polyacrylamide gels (Bogorad, 1975). When Glazer & Fang (1973) first resolved the subunits of *Synechococcus* sp. phycocyanin by ion-exchange chromatography on Bio-Rex 70 columns, it was found that the more rapidly

Table 4. *N-Terminal sequences of allophycocyanins*

Empty parentheses indicate that positive identification of the residue could not be made. A residue shown in parentheses is suspected but not definitely proved.

Organism		Sequence		
α -Type subunits		1	5	10
<i>C. caldarium</i> *	Rhodophyta	Met-Ile	-Val -Thr-Lys-(Thr)-Ile	-Val-Asx-Ala-
<i>Porphyridium cruentum</i> †	Rhodophyta	Ser -Ile	-Val -Thr-Lys-()-Ile	-Val-Asn-Ala-
<i>Phormidium luridum</i> ‡	Cyanophyta	Met-Ile	-Val -Thr-Lys-Thr	-Ile -Val-Asx-Ala-
<i>Mastigocladus aeruginosa</i> †	Cyanophyta	Ser -Ile	-Val -Thr-Lys-()-Ile	-Val-Asn-Ala-
<i>Anabaena variabilis</i> †	Cyanophyta	()-Ile	-Val-()-Lys-()-Ile	-Val-Asn-Ala-
β -Type subunits				
<i>C. caldarium</i>	Rhodophyta	Met-Glx-Asx-Ala-Ile	-Thr -Ala-Val-Ile	-Asx-
<i>P. cruentum</i> †	Rhodophyta	Met-Gln-Asp-Ala-Ile	-Thr -()-Val-Ile	-Asn-
<i>P. luridum</i>	Cyanophyta	Met-Glx-Asx-Ala-Leu-Thr	-Ile -Val-Ile	-Asx-
<i>M. aeruginosa</i> †	Cyanophyta	Met-Gln-Asp-Ala-Ile	-()-()-Val-Ile	-Asn-
<i>A. variabilis</i> †	Cyanophyta	Ala -Gln-Asp-Ala-Ile	-Thr -Ala-Val-Ile	-Asn-

* The sequence was deduced after determination of the *N*-terminus of the unseparated subunits and of the β -type subunit.

† Data from Glazer *et al.* (1976).

‡ Data from Brown *et al.* (1975). The sequences are proposed on the basis of homology with the other allophycocyanins. Sequence analysis was performed on unseparated subunits.

migrating (α) subunit in the gel system was eluted from the column in 8M-urea, and the more slowly migrating (β) subunit in 9M-urea. Nevertheless, this pattern of migration on gels and elution from columns need not follow with all phycobiliproteins. Therefore Glazer *et al.* (1976) have suggested that the subunits of phycobiliproteins be designated α or β by virtue of their homology with the α - and β -subunits of *Synechococcus* sp. phycocyanin.

We have adopted this convention in the present investigation, but have noted that the elution pattern and electrophoretic mobility of allophycocyanin subunits are the reverse of those observed for phycocyanin. Thus the allophycocyanin subunit which moved fastest on sodium dodecyl sulphate/polyacrylamide gels (modified electrophoresis buffer) and was eluted first (7.5M-urea) from Bio-Rex 70 columns was homologous to the β -subunit of *Synechococcus* sp. phycocyanin. The more slowly migrating allophycocyanin subunit which was eluted second (8.0M-urea) from the ion-exchange column was homologous with the α -subunit of *Synechococcus* sp. phycocyanin.

The sequences of the *N*-terminus of *C. caldarium* allophycocyanin subunits are compared with those of allophycocyanins from other red and blue-green algae in Table 4. It is obvious that major insights into phylogenetic relationship between blue-green algae and rhodophytan chloroplasts do not necessarily follow from these limited data. It is noteworthy that there is 90% conservation of sequence among α -subunits and among β -subunits at the *N*-terminus of rhodophytan and cyanophytan allophycocyanins.

These results could be marshalled to argue either for or against the endosymbiont theory (Margulis, 1970) for the origin of organelles in the eukaryotic cell. In addition, one could speculate that, allowing for conservative amino acid substitution, one allophycocyanin subunit might have given rise to the other by gene duplication.

Finally, the functional relationship of phycobiliproteins in the phycobilisome (Gantt *et al.*, 1976) and the homology between the subunits of allophycocyanin, phycocyanin and phycoerythrin (Harris & Berns, 1975; Troxler *et al.*, 1975; Brown *et al.*, 1975; Glazer *et al.*, 1976) are consistent with the possibility that allophycocyanin gave rise to phycocyanin and phycoerythrin during evolution of accessory pigments in the photosynthetic apparatus of red and blue-green algae.

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References

- Allen, M. B. (1959) *Arch. Mikrobiol.* **32**, 270-277
- Andrews, P. (1965) *Biochem. J.* **96**, 595-606
- Beckman Sequencer Manual (1969) Spinco Division of Beckman Instruments, Palo Alto, CA
- Bennett, A. & Bogorad, L. (1971) *Biochemistry* **10**, 3625-3636

- Bennett, A. & Siegelman, H. W. (1977) in *The Porphyrins* (Dolphin, D., ed.), Academic Press, New York, in the press
- Berns, D. S. (1971) in *Biological Macromolecules* (Timasheff, S. N. & Fasman, G., eds.), vol. 5A, pp. 105–148, Marcel Dekker, New York
- Bjorn, G. S. & Bjorn, L. O. (1976) *Physiol. Plant.* **36**, 297–304
- Bogorad, L. (1975) *Annu. Rev. Plant Physiol.* **26**, 369–401
- Bogorad, L., Mercer, F. V. & Mullens, R. (1963) in *Photosynthetic Mechanisms of Green Plants* (Kok, B. & Jagendorf, A. T., eds.), pp. 560–570, National Academy of Science, National Research Council
- Briggs, W. R. & Rice, H. (1972) *Annu. Rev. Plant Physiol.* **23**, 293–334
- Brown, A. S., Foster, J. A., Voynow, P. V., Franzblau, C. & Troxler, R. F. (1975) *Biochemistry* **14**, 3581–3588
- Chapman, D. J., Cole, W. J. & Siegelman, H. W. (1967) *Biochem. J.* **105**, 903–905
- Chen, Y. H., Yang, J. T. & Martinez, H. M. (1972) *Biochemistry* **11**, 4120–4131
- Craig, I. W. & Carr, N. G. (1968) *Biochem. J.* **106**, 361–366
- Diakoff, S. & Scheibe, J. (1973) *Plant Physiol.* **51**, 382–385
- Edman, P. & Begg, G. (1967) *Eur. J. Biochem.* **1**, 80–91
- Erokhina, L. G. & Krasnovskii, A. A. (1974) *Mol. Biol. (Kiev)* **5**, 651–662
- Foster, J. A., Bruenger, E., Gray, W. R. & Sandberg, L. B. (1973) *J. Biol. Chem.* **248**, 2876–2879
- Gantt, E. & Conti, S. F. (1966) *J. Cell Biol.* **29**, 423–434
- Gantt, E. & Lipschultz, C. A. (1973) *Biochim. Biophys. Acta* **292**, 858–861
- Gantt, E. & Lipschultz, C. A. (1974) *Biochemistry* **13**, 2960–2966
- Gantt, E., Lipschultz, C. A. & Zilinskas, B. (1976) *Biochim. Biophys. Acta* **430**, 375–388
- Glazer, A. N. & Cohen-Bazire, G. (1971) *Proc. Natl. Acad. Sci. U.S.A.* **68**, 1398–1401
- Glazer, A. N. & Fang, S. (1973) *J. Biol. Chem.* **248**, 659–662
- Glazer, A. N., Apell, G. S., Hixson, C. S., Bryant, P. A., Rimon, S. & Brown, D. M. (1976) *Proc. Natl. Acad. Sci. U.S.A.* **73**, 428–431
- Gray, B. H. & Gantt, E. (1975) *Photochem. Photobiol.* **21**, 121–128
- Gysi, J. & Zuber, H. (1974) *FEBS Lett.* **48**, 209–213
- Harris, J. & Berns, D. S. (1975) *J. Mol. Evol.* **5**, 153–163
- Hattori, A. & Fujita, Y. (1959a) *J. Biochem. (Tokyo)* **46**, 633–644
- Hattori, A. & Fujita, Y. (1959b) *J. Biochem. (Tokyo)* **46**, 903–909
- Haxo, F. T., O'hEocha, C. & Norris, P. (1955) *Arch. Biochem. Biophys.* **54**, 162–173
- Lazaroff, N. & Schiff, J. (1962) *Science* **137**, 603–604
- Lemasson, C., Tandeau de Marsac, N. & Cohen-Bazire, G. (1973) *Proc. Natl. Acad. Sci. U.S.A.* **70**, 3130–3133
- Lemberg, R. & Bader, G. (1933) *Justus Liebigs Ann. Chem.* **505**, 151–177
- Levin, O. (1962) *Methods Enzymol.* **5**, 27–32
- MacColl, R., Edwards, M. R., Mulks, M. R. & Berns, D. S. (1974) *Biochem. J.* **141**, 419–425
- Margulis, L. (1970) *Origin of Eukaryotic Cells*, Yale University Press, New Haven, CT
- Mendez, E. & Lai, C. Y. (1975) *Anal. Biochem.* **68**, 47–53
- Mercer, F. V., Bogorad, L. & Mullens, R. (1962) *J. Cell Biol.* **13**, 393–403
- Myers, J. (1971) *Annu. Rev. Plant Physiol.* **22**, 289–312
- Nolan, D. N. & O'hEocha, C. (1967) *Biochem. J.* **103**, 39p–40p
- O'hEocha, C. (1965) *Annu. Rev. Plant Physiol.* **16**, 415–434
- Pisano, J. J. & Bronzert, T. J. (1969) *J. Biol. Chem.* **244**, 5597–5607
- Rice, H. V. & Briggs, W. R. (1973) *Plant Physiol.* **51**, 927–938
- Scheibe, J. (1972) *Science* **176**, 1037–1039
- Siegelman, H. W. & Firer, E. M. (1964) *Biochemistry* **3**, 418–423
- Smithies, O., Gibson, D., Fanning, E. M., Goodfliesch, R. F., Gilman, J. G. & Ballantyne, D. L. (1971) *Biochemistry* **10**, 4912–4921
- Troxler, R. F. & Bogorad, L. (1966) *Plant. Physiol.* **41**, 491–499
- Troxler, R. F. & Lester, R. (1967) *Biochemistry* **6**, 3840–3846
- Troxler, R. F., Foster, J. A., Brown, A. S. & Franzblau, C. (1975) *Biochemistry* **14**, 268–274
- Weber, K. & Osborn, M. (1969) *J. Biol. Chem.* **244**, 4406–4412