Properties of Allophycocyanin II and its α - and β -Subunits from the Thermophilic Blue-Green Alga *Mastigocladus laminosus*

By Jürg R. GYSI* and Herbert ZUBER

Institut für Molekularbiologie und Biophysik, Eidgenössische Technische Hochschule Zürich-Hönggerberg, 8093 Zürich, Switzerland

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Purified allophycocyanin II and its subunits have been examined with respect to spectroscopic properties, sedimentation, reconstitution and isoelectric behaviour. In 0.02 M-potassium phosphate buffer, pH 8.0, and at a concentration of 0.25 mg/ml, allophycocyanin II and its α - and β -subunits show visible absorption maxima at 650, 615 and 615 nm respectively, whereas the fluorescence emission maxima were determined to be at 662, 640 and 630 nm respectively. The absorption difference spectrum (dilution difference) of allophycocyanin II displays maxima at 650 and 590 nm with a minimum at 610 nm. The c.d. spectrum of allophycocyanin II showed only one positive-ellipticity band at 635 nm, and a major negative-ellipticity band at 340 nm. Oxidation of allophycocyanin II, low- and high-pH solutions (pH 3.0 and 11.0), various ethanol concentrations as well as dialysis against distilled water induce a spectral change leading to phycocyanin-like characteristics. In most cases these shifts are reversible. Allophycocyanin II is thermostable over a period of 60 min at temperatures up to 60°C. The isoelectric points of allophycocyanin II and its α - and β -subunits are 4.65, 4.64 and 4.82 respectively. Estimated molecular weights from sedimentation-equilibrium analyses were 102 500 for allophycocyanin II, 16 000 for the α - and 31 500 for the β -subunit. Recombination of α - and β -subunits leads to allophycocyanin II, which is indistinguishable from native allophycocyanin with respect to its spectral form, to its gel-filtration and to its electrophoretic behaviour.

The photosynthetic apparatus of red (Rhodophyta), blue-green (Cyanophyta) and cryptomonad (Cryptophyta) algae contains phycobiliproteins, intensely coloured accessory pigments (Ó hEocha, 1965; O'Carra & Ó hEocha, 1976). Their brilliant colours are due to linear tetrapyrrole prosthetic groups covalently linked to the polypeptide chains. Phycoerythrobilin is the chromophore of the phycoerythrins, whereas phycocyanobilin is the chromophore for phycocyanins (Chapman *et al.*, 1967; Bennett & Siegelman, 1979). Both subunits of allophycocyanin as well as the α -subunits of phycoerythrin and phycocyanin each carry one chromophore per polypeptide chain, whereas the β -subunits of phycoerythrin and phycocyanin carry two (Bennett & Bogorad, 1971; Glazer & Fang, 1973). Biliproteins (phycoerythrin and phycocyanins) form high-molecular-weight aggregates that appear as discrete structures (phycobilisomes) on the outer surfaces of thylakoid membranes (Gantt & Conti, 1966; Gantt *et al.*, 1968; Gantt & Conti, 1969), except in cryptomonads, where they are apparently intrathylakoidal (Gantt *et al.*, 1971). The main function of the phycobiliproteins is light-harvesting. Photosynthetic action spectra and

excitation spectra of chlorophyll *a* fluorescence have demonstrated that allophycocyanin is the most efficient biliprotein for energy transfer (Lemasson *et al.*, 1973; Gantt *et al.*, 1976). Since it is the least abundant of all phycobiliproteins in the cell, and despite the fact that it is universally present in blue-green and red algae, it cannot play a quantitatively important role in light-energy capture. Fluorescence studies on isolated intact phycobilisomes have shown that allophycocyanin is the key pigment in funnelling excitation energy from the other biliproteins into the chlorophyll of the photosystem (Gantt *et al.*, 1976, 1977). Gantt *et al.* (1976) proposed a model for phycobilisomes in which allophycocyanin forms a central core to which energy is funnelled from an outer layer of phycoerythrin via a surrounding layer of phycocyanin.

Despite its importance, allophycocyanin still remains the least thoroughly studied phycobiliprotein. Only very little was known about the subunits of allophycocyanin until we reported their separation on a preparative scale (Gysi & Zuber, 1974). Since then, some properties such as isoelectric points, type and number of bilin chromophores, amino acid compositions and *N*-terminal sequences have been described (Gysi & Zuber, 1974; Bryant *et al.*, 1976; Glazer

* Present address: Department of Biology, University of California, Los Angeles, CA 90024, U.S.A.

et al., 1976; Brown & Troxler, 1977). We (Gysi & Zuber, 1976) also reported the properties of allophycocyanin I, a second allophycocyanin from the blue-green alga *Mastigocladus laminosus*. Glazer & Bryant (1975) described yet another cyanobacterial allophycocyanin for which the name 'allophycocyanin B' has been proposed. Our previous work (Gysi & Zuber, 1974; Gysi *et al.*, 1977) has shown that allophycocyanin II as well as three other biliproteins of *Mastigocladus laminosus* have a $(\alpha\beta)_n$ -type subunit structure common to many phycobiliproteins from other organisms (Bryant *et al.*, 1976; Glazer, 1976; Cohen-Bazire *et al.*, 1977). For one allophycocyanin form from *Nostoc sp.*, Zilinskas *et al.* (1978) reported the possible presence of an additional β -subunit or a high-molecular-weight polypeptide as in B-phycoerythrin (Gantt & Lipschultz, 1974; Glazer & Hixson, 1977).

The present paper describes spectral, biochemical and biophysical properties of allophycocyanin II and its individual α - and β -subunits from the thermophilic filamentous cyanophyte *Mastigocladus laminosus*.

Experimental

Organism, culture conditions and purification of phycobiliproteins

Mastigocladus laminosus is an N_2 -fixing photoautotroph capable of tolerating high temperature (48–62°C) and moderate pH (6.8–9.5 ranges). The temperature optimum is at 55°C. *M. laminosus* was recovered from, and grown in, a specifically designed culturing channel in the water of an alkaline hot spring near Reykjavik, Iceland (Binder *et al.*, 1972). Immediately after harvesting the alga was frozen and then shipped frozen in light-tight containers.

Preparation of allophycocyanin II and its α - and β -subunits was carried out as described by Gysi & Zuber (1974).

Gel electrophoresis and isoelectric focusing

Polyacrylamide-gel electrophoresis was performed on 7.5% (w/v) polyacrylamide gels by the method of Ornstein & Davis (1964). The Tris/barbiturate buffer system of Williams & Reisfeld (1964) was used for gels at pH 7.5. Isoelectric focusing was carried out as described in the LKB 8100 Ampholine Instruction Manual. The column volume was 110 ml, an ampholyte (LKB Instruments, Bromma, Sweden) with the pH range of pH 3.0–5.0 being used.

Sedimentation equilibrium

Average-molecular-weight values were obtained by the high-speed sedimentation-equilibrium method of

Yphantis (1964) at 4 and 20°C, in a MSE analytical ultracentrifuge type MK 2 by using solution columns of 3 mm height. Evaluations were done by u.v. spectroscopy with a photoelectric scanner. Determinations were performed at protein concentrations of 0.25 mg/ml and 0.5 mg/ml at pH 8, the alkalinity at which all the purification procedures were carried out.

Spectroscopic measurements

Electronic absorption spectra were recorded on a Bausch and Lomb UV 200 spectrophotometer, whereas the dilution difference spectrum of allophycocyanin II was measured on a Cary model 17 spectrophotometer and the fluorescence spectra on a Baird Atomic Fluorispec SF 1 spectrophotofluorimeter. The c.d. spectrum was determined on a Cary 61 spectropolarimeter calibrated with a (+)-camphor-10-sulphonic acid standard (Eastman Kodak Co., Rochester, NY, U.S.A.). The measurement was performed at 27°C in 0.02 M-potassium phosphate buffer, pH 8.0, at a concentration of 5 mg/ml. The slit width was 1 mm, except that below 530 nm an automatic slit was used. Mean residue ellipticity $[\theta]$ in degrees $\cdot$ cm² $\cdot$ dmol⁻¹ was determined by using a mean residue weight of 100 for allophycocyanin II. The mean residue weight was calculated from the amino acid composition of allophycocyanin II (Gysi & Zuber, 1974).

Reconstitution procedures

Portions (2.5 mg each) of the α - and β -subunits of allophycocyanin II were dissolved in 10 ml of 0.02 M-potassium phosphate buffer, pH 8.0. The solutions were mixed in a 1:1 ratio and allowed to stand for 16 h at room temperature in the dark. Electronic absorption spectra were recorded at intervals. The mixture was then centrifuged for 30 min at 27000g, concentrated in a Diaflo ultrafiltration cell (Aminco Instruments, Lexington, MA, U.S.A.) with a type UM 20 membrane and passed through a column (1.5 cm $\times$ 100 cm) of Sephadex G-100 (Pharmacia Fine Chemicals, Uppsala, Sweden) equilibrated with 0.02 M-potassium phosphate. The blue fraction eluting with the same volume as allophycocyanin II was collected, dialysed against water and freeze-dried.

Results

Isoelectric points

The pI values of the α - and β -subunits of allophycocyanin II are 4.64 and 4.82 respectively. This is in agreement with previous work (Gysi & Zuber, 1974), which established that the α -subunit has a higher ratio of acidic to basic amino acid residues than the β -subunit.

Sedimentation equilibrium

Allophycocyanin II and its α - and β -subunits, prepared as described by Gysi & Zuber (1974), appeared to be homogeneous in the ultracentrifuge over the protein concentration range of 0.25–0.5 mg/ml in 0.02M-potassium buffer, pH 8.0. Under these conditions the estimated molecular weight of allophycocyanin II was 102 500, whereas it was 16 000 for the α -subunit and 31 500 for the β -subunit, which apparently had dimerized (see the Discussion section).

Spectroscopic properties

Purified freeze-dried allophycocyanin II and its α - and β -subunits were dissolved in 0.02M-potassium phosphate buffer, pH 8.0, at a concentration of 5 mg/ml. Portions of these solutions were diluted to 0.25 mg/ml and the electronic absorption and fluorescence spectra were determined (Figs. 1a and 1b). The fluorescence emission spectra were determined by exciting at 650 nm for allophycocyanin II and at 615 nm for both subunits. Allophycocyanin II and its

α - and β -subunits show visible absorption maxima at 650 (shoulder at 625), 615 and 615 nm respectively, whereas the fluorescence emission maxima appear at 662, 640 and 630 nm respectively.

The absorption difference spectrum of allophycocyanin II was recorded with 2.0 mg of protein/ml in a 0.1 cm-pathlength cuvette in the sample chamber and 0.02 mg of protein/ml in a 10 cm-pathlength cuvette in the reference chamber. Allophycocyanin II displays maxima at 650 nm and 590 nm with a minimum at 610 nm (Fig. 2a).

The c.d. spectrum of an undiluted sample of allophycocyanin II (5 mg/ml) revealed a positive-ellipticity band occurring at 635 nm and a major negative ellipticity band at 340 nm (Fig. 2b).

The temperature-dependence of the absorption spectrum of allophycocyanin II was measured in the range 30–70°C over a period of 60 min (Fig. 3). At 30, 40 and 50°C the spectrum remains unchanged during a 60 min period. At 60°C there is a 18% decrease in A_{650} , whereas at 70°C the decrease is 68%.

The pH-dependence of the absorbance at the visible-absorption maxima of allophycocyanin II and its α - and β -subunits in 0.02M-potassium phosphate buffer (H_3PO_4 titrated with KOH for the entire pH range of pH 3.0–11.0) is shown in Fig. 4. When measured for a period of 8 days (in the dark at 4°C) the absorbance values of allophycocyanin II and its β -

Fig. 1. Absorption (a) and relative fluorescence (b) spectra of allophycocyanin II and its α - and β -subunits

Spectra were determined in 0.02M-potassium phosphate, pH 8.0, at concentrations of 0.25 mg/ml. —, Allophycocyanin II; ----, α -subunit; -.-., β -subunit

Fig. 2. Absorption difference (a) and c.d. (b) spectra of allophycocyanin II in 0.02M-potassium phosphate, pH 8.0

Protein concentrations were 2.0 mg/ml in a 0.1 cm-pathlength cuvette in the sample chamber and 0.02 mg/ml in a 10 cm-pathlength cuvette in the reference chamber for (a) and 5 mg/ml for (b).

Fig. 3. Thermal stability of allophycocyanin II. The protein concentration was adjusted to 0.25 mg/ml in 0.02 M-potassium phosphate, pH 8.0. Absorbance is shown as a percentage of the initial A_{650} . ○, 30°C; △, 40°C; □, 50°C; ●, 60°C; ▲, 70°C.

subunit are highest at pH 6–9 and pH 5–6 respectively, whereas the α -subunit is only slightly influenced by pH. Allophycocyanin II at pH 3.0 and pH 11.0 displayed the characteristic spectral behaviour observed with other allophycocyanins after acid-urea treatment and dialysis against water (Erokhina & Krasnovskii, 1974; Brown *et al.*, 1975; Brown & Troxler, 1977), in that its absorption spectrum resembled that of phycocyanin (Fig. 5). The spectral change at pH 3.0 is irreversible, whereas after 10 days at pH 11.0 the absorption spectrum, characteristic of the original allophycocyanin, returned in a diminished form (Fig. 5). This spectral change of allophycocyanin II also occurred when phosphate from an undenatured sample was dialysed out against water. On re-addition of phosphate to the initial concentration (0.02 M) the original spectrum returned.

The influence of various ethanol concentrations on the absorbance at the visible-absorption maxima of allophycocyanin II and its subunits, in the dark at 4°C, is illustrated in Fig. 6. At 2% (v/v) ethanol the absorption spectrum of allophycocyanin II changes steadily over a period of time, until after 38 days it resembles the spectrum of phycocyanin. In 4 and 8% ethanol the absorption maximum also shifts to 615 nm but at these concentrations a secondary maximum remains at 650 nm. Ethanol concentrations of 16 and 40% immediately lead to a spectral change similar to the one observed at pH 3.0. These spectral shifts are partially reversible at concentrations of 4, 8 and 16% ethanol, but completely irreversible at the lowest (2%) and highest (40%) concentrations tested. In 2% ethanol, allophycocyanin II and its subunits 'bleach out' quite quickly, whereas at higher concentrations there is no apparent loss of colour. Ethanol at 40% concentration has practically no effect on the absorption spectrum of the α -subunit but with the β -subunit

Fig. 4. pH-dependence of the absorbances at the visible absorption maxima of allophycocyanin II (c) and its α - (a) and β -subunits (b).

Protein concentrations were 0.25 mg/ml in 0.02 M-potassium phosphate ($\text{H}_3\text{PO}_4/\text{KOH}$ for the entire pH range). ○, Immediately after dissolving freeze-dried protein in buffer; ▲, after 8 days.

it produces a pronounced decrease of the absorbance at 615 nm followed by a slow increase (Fig. 6).

Oxidation of allophycocyanin II *in vitro* by H_2O_2 [0.25 mg/ml, 0.02 M-potassium phosphate buffer, pH 5.9, 7.5% (v/v) H_2O_2 , 20°C] again produces a spectrum resembling that of phycocyanin. The addition of Na_2EDTA (0.01–0.1 M) to solutions of allophycocyanin II and its α - and β -subunits (0.25 mg/ml) has no effect on the absorption spectra.

Recombination of the α - and β -subunit

The reconstitution of allophycocyanin II from its subunits was monitored spectrally (Fig. 7a). After 16 h the A_{650} had reached 75% of the original value

Fig. 5. Absorption spectra of allophycocyanin II —, At pH 8.0; ----, at pH 3.0; - · - ·, at pH 11.0; · · · ·, at pH 11.0 after 10 days. The allophycocyanin II concentration used in these experiments was 0.25 mg/ml in 0.02 M-potassium phosphate ($\text{H}_3\text{PO}_4/\text{KOH}$ for the entire pH range).

found in native allophycocyanin II at the same concentration, and the A_{650}/A_{625} ratio was 1.1:1.0. The reconstituted allophycocyanin II, recovered in an overall yield of 74%, is spectrally and electrophoretically indistinguishable, both before and after gel filtration on Sephadex G-100, from native allophycocyanin II (Figs. 7a and 7b).

Discussion

The present study has shown that allophycocyanin II from the thermophilic filamentous blue-green alga *M. laminosus* has spectral properties quite similar to those observed for allophycocyanin from *Phormidium luridum* (Brown *et al.*, 1975), *Nostoc* sp. (Gray & Gantt, 1975), *Anacystis nidulans* (Erokhina & Krasnovskii, 1974) and *Cyanidium caldarium* (Brown & Troxler, 1977).

Sedimentation-equilibrium studies of *M. laminosus* allophycocyanin II indicated that the apparent molecular weight of this phycobiliprotein was 102 500. This value and the calculated molecular weight of the trimer of 103 200, based on a monomer mol.wt. of 34 400 (Gysi & Zuber, 1974), are in good agreement, indicating that the trimer is indeed the predominant aggregate in this preparation. Glazer & Cohen-Bazire (1971) and MacColl *et al.* (1974) reported allophycocyanins with corresponding molecular weights of 96 000 and 103 000, the latter originating from two strains of the thermophilic blue-green alga *Synechococcus lividus*. However, previously reported molecular weights for allophycocyanins [120 000 (Gantt &

Fig. 6. Influence of various ethanol concentrations on the absorbances at the visible-absorption maxima of allophycocyanin II (c) and its α - (a) and β -subunits (b)

Protein concentrations were 0.25 mg/ml. Absorbance is shown as a percentage of the initial A_{650} (c) and A_{615} (a and b) in distilled water. $\circ$, Immediately after dissolving freeze-dried material; Δ , after 10 days; $\square$, after 38 days.

Lipschultz, 1974), 134 000 (Hattori & Fujita, 1959), 138 000 (Nolan & Ó hEocha, 1967), 155 000 (Brown *et al.*, 1975), 196 000 (Brown & Troxler, 1977)] indicate tetra- or hexa- rather than tri-meric structures. Possible reasons for these discrepancies have been discussed by Brown *et al.* (1975) and Brown & Troxler (1977). The results of sedimentation-equilibrium analyses on the α -subunit are in agreement with the values found after sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Gysi & Zuber, 1974). The β -subunit (monomer mol.wt. 17 200) gave a weight-average mol.wt. of 31 500. This indicates that the β -subunit dimerizes. A similar tendency has been observed for both subunits of C-phycocyanin

Fig. 7. Reconstitution of allophycocyanin II from its subunits

The subunit concentrations were 0.25 mg/ml in 0.02 M-potassium phosphate, pH 8.0. (a) Spectra: ----, immediately after mixing in a 1:1 ratio; - · - · -, after 1 h; —, after 16 h. (b) Polyacrylamide-gel electrophoresis (7.5%, pH 7.5) of allophycocyanin II, and its α - and β -subunits and of the reconstituted allophycocyanin II. 1, Allophycocyanin II; 2, α -subunit; 3, β -subunit; 4, reconstituted allophycocyanin II. The anode was at the lower end of the gels.

from *Synechococcus* sp. (Glazer *et al.*, 1973). Since two cysteine residues are present in the β -subunit of allophycocyanin II (Gysi & Zuber, 1974) and only one of them seems to be involved in chromophore linkage, a dimerization could possibly occur through cysteine residues. The presence of only one cysteine residue in the α -subunit would explain the apparent lack of dimerization in this subunit.

Spectrally the α - and β -subunits are strikingly different from the intact allophycocyanin II and from each other. The remarkable features of the visible absorption spectra of the subunits are the complete disappearance of the long-wavelength maximum in allophycocyanin II (650 nm), together with the appearance of single absorption maxima at 615 nm and the general similarity of these spectra to that of

phycocyanin. The previously reported shoulder of the β -subunit at 650 nm (Gysi & Zuber, 1974) was eliminated by improving the denaturing conditions (increasing the denaturing temperature to 40°C) before subunit separation. Although completely purified α - and β -subunits have identical absorption maxima in the visible region and almost equal absorbances ($A_{1\text{cm}}^{1\%}$), their fluorescence-emission maxima (640 nm for the α - and 630 nm for the β -subunit), and especially their fluorescence intensities, are different. Despite the fact that the two subunits do not differ in their long-wavelength-absorption maxima, the theory of an 's'- and an 'f'-type chromophore (Teale & Dale, 1970) is still applicable under the assumption that the fluorescence properties of separated subunits do not markedly differ from their properties in native allophycocyanin. Since there is little direct fluorescence from the β -subunit, it therefore must carry the 's'-type chromophore, whereas the strongly fluorescing α -subunit carries the 'f'-type chromophore. The absorption difference spectrum of allophycocyanin II is identical with the one reported for allophycocyanin from the blue-green alga *Nostoc* sp. (Gray & Gantt, 1975), demonstrating that the absorbance at the absorption maximum is enhanced at higher protein concentrations. The c.d. spectrum of *M. lamosus* allophycocyanin II revealed only one positive-ellipticity band at 635 nm, whereas two bands at 655 nm and 625 nm and two bands at 658 nm and 630 nm were found for allophycocyanin from *P. luridum* and *Cyanidium caldarium* (Brown *et al.*, 1975; Brown & Troxler, 1977) respectively.

In its role as an accessory pigment in *M. lamosus*, allophycocyanin II obviously must be very thermostable. The fact that, even after extraction from the alga and subsequent purification, allophycocyanin II exhibits a distinct thermostability leads to the assumption that thermostability cannot solely be due to the environment in the cell, but rather is an inherent property of the polypeptide chain.

Low-acidic- and high-basic-pH values as well as high ethanol concentrations have a denaturing effect on allophycocyanin II that is manifested in a complete spectral change. The partial reversibility at pH 11.0 and at ethanol concentrations of 4, 8 and 16% suggest that the original chain conformation was not completely disrupted, thus allowing a partial reaggregation of α - and β -subunits. It is unclear why dilute ethanol (2%) has such a strong influence on allophycocyanin II and its subunits. The possibility that bacterial degradation or action contributed to the unexpected results with 2% ethanol can be discounted. The experiments were carried out at 4°C, which would retard bacterial growth, and there was no detectable opacity or suspension, indicative of bacterial growth, in the samples after 38 days. Previous reports have shown that urea-denatured allophycocyanin after removal of the denaturant exhibits a

phycocyanin-like spectrum that can be reversed on addition of phosphate buffer (Brown *et al.*, 1975; Brown & Troxler, 1977). Undenatured allophycocyanin II when dialysed into water and subsequently back into phosphate buffer displays the same spectral behaviour. This indicates that, under these conditions, a reversible partial unfolding of the polypeptide chains in the chromophore regions takes place and/or subunits or polymers readily dissociate and reaggregate.

Chemical oxidation of allophycocyanin II produces the same effect as oxidation of allophycocyanin I (Gysi & Zuber, 1976). In contrast with the findings for allophycocyanin I (Gysi & Zuber, 1976), however, the addition of Na₂EDTA to allophycocyanin II does not alter its spectral characteristics. This finding excludes the possibility that the Na₂EDTA-induced spectral change in allophycocyanin I could be due to a denaturing effect, thus strengthening the hypothesis that allophycocyanin I converts into allophycocyanin II by the loss of a metal atom (Gysi & Zuber, 1976).

In the present work we have demonstrated that allophycocyanin II can be easily reconstituted from separated subunits and that it is indistinguishable from native allophycocyanin II treated under the same conditions. The high yield of reconstituted allophycocyanin II (74%) clearly shows that α - and β -subunits do not randomly reaggregate but form predominantly monomeric $\alpha\beta$ units or polymers thereof. The only comparable data on reconstitution comes from C-phycocyanin (Glazer *et al.*, 1973), where the overall yield was much lower (24–26%).

The pI value of native allophycocyanin II is 4.65 (Gysi & Zuber, 1974) and thus is lower than the mean of the pI values of the α - and β -subunit (4.73). The same observation has been made for a number of biliproteins (Bryant *et al.*, 1976). One possible explanation for this result is that, in native allophycocyanin II, side-chain amino groups could interact between subunits through hydrogen bonding, which leads to a decrease in net charge.

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