ALLOPHYCOCYANIN I–A SECOND CYANOBACTERIAL ALLOPHYCOCYANIN? ISOLATION, CHARACTERIZATION AND COMPARISON WITH ALLOPHYCOCYANIN II FROM THE SAME ALGA

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1. Introduction

In addition to chlorophyll and carotinoids blue-green algae contain phycocyanin, allophycocyanin and frequently phycoerythrin [1,2]. Action spectra for photosynthetic oxygen evolution and for the excitation of chlorophyll a fluorescence demonstrate that the phycobiliproteins function as accessory pigments [3,4]. Generally it has been assumed that the energy absorbed by phycoerythrin is passed via phycocyanin to chlorophyll a [5–7]. The role played by allophycocyanin has remained unclear mainly because of its low concentration in the cell and since its major absorption band (650 nm) is masked by those of phycocyanin and chlorophyll a. Recently it has been demonstrated for intact phycobilisomes from red [8] and blue-green algae [9] that excitation with light absorbed by phycoerythrin leads to a fluorescence of a much longer wavelength than that emitted by phycocyanin. This means that the energy is transferred from phycoerythrin through phycocyanin to allophycocyanin. The last-named compound seems to be the key pigment from which the energy is passed to chlorophyll a with high efficiency.

During our studies of biliproteins from the blue-green alga *Mastigocladus laminosus* [10,11] we observed the presence of very small amounts of an undescribed biliprotein with an absorption spectrum resembling that of allophycocyanin. According to its early elution from an ion exchange column we named this new biliprotein allophycocyanin I. In this report we describe the isolation and partial characterization of allophycocyanin I from the thermophilic blue-green alga *Mastigocladus laminosus*. The results are compared with those obtained for allophycocyanin II [11].

2. Materials and methods

(All reagents were of analytical grade.) Ampholytes were obtained from LKB, Sweden, and the cellogel-electrophoresis strips from Chemetron, Milan, Italy.

M. laminosus cells were cultured in the water of a hot spring near Reykjavik, Iceland [12].

All purification steps were performed routinely at 4°C in the dark. Extraction and preliminary purification procedures were the same as described elsewhere [11]. The 20% ammonium sulfate fractionation step after the Sephadex G-150 gel filtration was omitted. The following DEAE-Sephadex A-50 column was equilibrated in 0.02 M phosphate buffer, pH 8.0. Elution was performed with a linear gradient of 0.18–0.4 M NaCl in the same buffer (total volume 2.1 liters). The collected fractions of allophycocyanin I were concentrated and dialyzed for 24 h against 2 liters of phosphate buffer. Further purification was achieved by either one or other of the following two methods: (1) Preparative ion focussing in the pH range of pH 4.4–5.1 (this gradient was prepared using an ampholyte in the pH range of pH 4–6). (2) DEAE-Sephadex A-50 ion exchange chromatography in 0.02 M phos-

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phate buffer pH 8.0. The column (2.5 × 40 cm) was eluted with 250 ml 0.1 M KCl followed by a linear KCl-gradient (0.2–0.45 M, total volume 800 ml) in phosphate buffer. Allophycocyanin I fractions obtained by both methods were pooled, concentrated, dialyzed against phosphate buffer pH 8.0 and passed through a 2.5–90 cm Sephadex G-75 column. Again the fractions containing Allophycocyanin I were pooled, dialyzed for 48 h against 5 liters of distilled water and lyophilized.

To control the purity of the protein, polyacrylamide gel-electrophoresis at pH 7.5 was carried out according to Ornstein and Davies [13]. The Tris–barbiturate buffer system of Williams and Reisfeld [14] was used for urea disc gels at pH 7.5. The protein was treated with 8 M urea, 0.01 M β-mercaptoethanol (2 h, 37°C).

For further investigations purified allophycocyanin I (final concentration 0.2 mg/ml) was dissolved in a 0.1 M EDTA solution and left standing at room temperature and at 40°C for several hours. Oxidation of allophycocyanin I (final concentration 0.15 mg/ml) with 7.5% hydrogen-peroxide was performed in 0.02 M phosphate buffer pH 5.9. For both experiments spectral changes were measured at intervals.

Subunit separation, electrophoresis on cellogel strips and molecular weight determinations in the presence of sodium dodecyl sulfate (SDS) were performed as described previously [11]. Amino acid analyses of acid hydrolysates (6 N HCl, 0.005% phenol, 110°C, 24, 48, 72 h) were carried out on a Beckman amino acid analyzer 121 C [15]. All visible and UV spectra were measured at room temperature on a Bausch and Lomb UV 200 spectrophotometer.

3. Results and discussion

The amount of crude allophycocyanin I eluting from the first ion exchange chromatography column (DEAE-Sephadex (fig.1a)) was increased when the preceding 20% ammonium sulfate fractionation described earlier was omitted. By preparative ion focussing in a narrow pH range allophycocyanin I was separated from several other blue components (fig.1b). If this method was replaced by a second ion exchange chromatography on DEAE-Sephadex, the corresponding allophycocyanin I fractons eluted even at 0.1 M KCl (fig.1c). The following gradient elution revealed two additional blue components (fig.1c). The first one (APC I_f) had a fairly strong red fluorescence, exhibited the same absorption spectrum as allophycocyanin I (with a slight intensification of the 600 nm shoulder) (fig.2) and was inhomogeneous on poly-

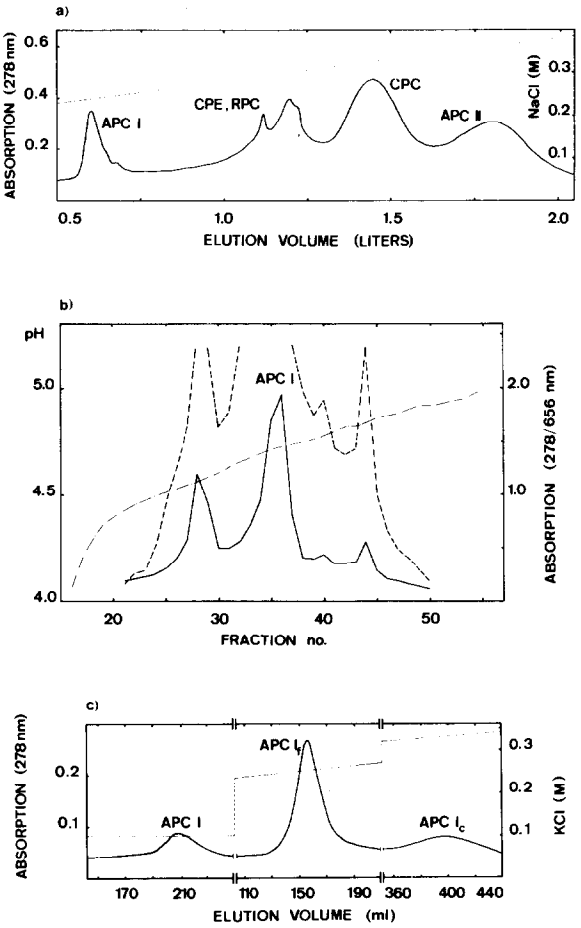

Fig.1. Purification of allophycocyanin I. (a) Chromatography on DEAE-Sephadex A-50 (5 × 100 cm) of the biliprotein fraction (after Sephadex G-150, 0.02 M phosphate buffer pH 8.0, linear NaCl gradient 0.18–0.40 M, total volume 2,1 liters). APC I: Allophycocyanin I. CPE: C-phycoerythrin. RPC: R-phycocyanin. CPC: C-phycocyanin. APC II: Allophycocyanin II. (b) Preparative ion focussing of the crude allophycocyanin I fraction from DEAE-Sephadex (110 ml column, ampholyte pH 4.4–5.1, 93 h, 4°C, 980 V). (c) Ion exchange chromatography on DEAE-Sephadex A-50 (2.5 × 40 cm) of the crude allophycocyanin I fraction from fig.1a (0.02 M phosphate buffer pH 8.0, 250 ml 0.1 M KCl followed by a linear gradient 0.2–0.45 M KCl, total volume 800 ml).

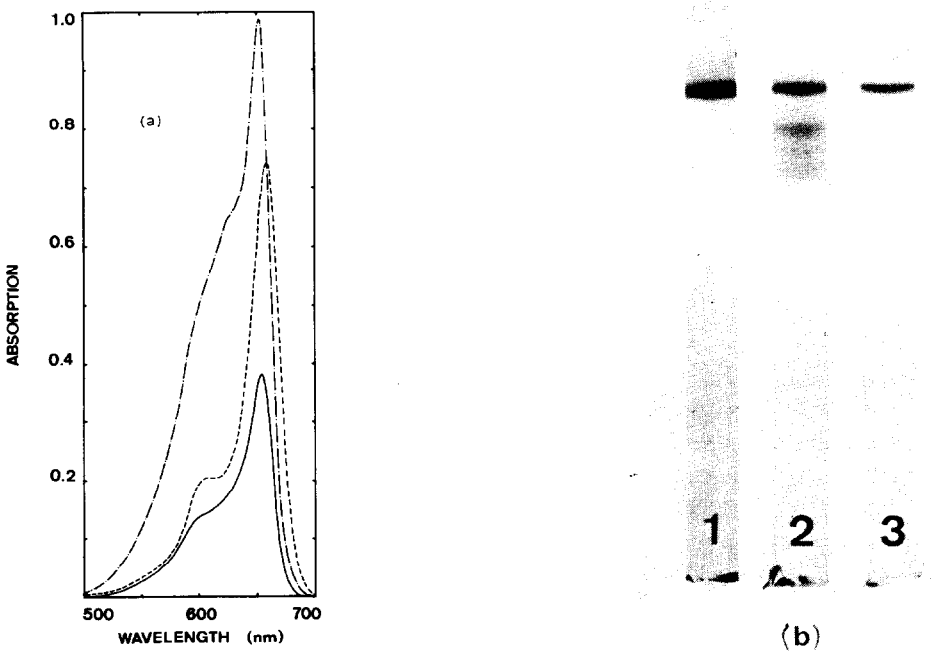

Fig.2. (a) Absorption spectra of allophycocyanin I (——), APC I_f (– – – –) and APC I_c (–.–.–.–) in 0.02 M phosphate buffer pH 8.0 at room temperature. (b) Polyacrylamide gels (7.5%, pH 7.5) of the same samples: (1) allophycocyanin I; (2) APC I_f; (3) APC I_c.

acrylamide gels (fig.2). The second component (APC I_c) did not fluoresce, showed the typical absorption spectrum of allophycocyanin II (fig.2) and only one single band on polyacrylamide gels (fig.2). Since crude allophycocyanin I cannot be contaminated with allophycocyanin II (fig.1a) this new fraction APC I_c has to originate from allophycocyanin I itself by loosing some positive or gaining some negative charge. Indeed we could observe that the ratio allophycocyanin I : APC I_c is dependent on the time of storage (–25°C) of crude allophycocyanin I. The longer such a sample is kept, the more APC I_c accumulates. Complete purification of allophycocyanin I was achieved by gel filtration on Sephadex G-75. The yield from 300 g algal cells (wet weight) was between 2 and 3 mg of pure allophycocyanin I.

Purified allophycocyanin I showed a single band on polyacrylamide disc gels in the absence and in the presence of SDS (fig.3). The R_f-values are identical with those of allophycocyanin II. The isoelectric point was determined during the last purification step but one. It was found to be at pH 4.73 which is higher than that of allophycocyanin II (4.65). Upon cellogel electrophoresis in urea as well as urea disc gel electrophoresis allophycocyanin I was separated into two subunits (fig.3) just like allophycocyanin II. Complete and preparative separation of the α- and β-subunit for both, allophycocyanin I and II, was achieved by ion exchange chromatography in the presence of 8 M urea and 0.01 M β-mercaptoethanol. The α- and β-subunits of both proteins are blue, indicating that each subunit contains at least one bilin chromophore.

The molecular weight of the α- and β-subunit of allophycocyanin I as measured by gel electrophoresis in SDS is 17 200 each, the same as for the subunits of allophycocyanin II.

The visible absorption maximum of allophycocyanin I is at 656 nm (fig.2) (Allophycocyanin II: 650 nm). There is only one shoulder at 600 nm (Allophycocyanin II: 625 nm, 595 nm). The spectral ratio A 656 : A 600 is 2.65 compared to only 1.50 for A 560 : A 625 of allophycocyanin II. Poor solubility of both, the α- and

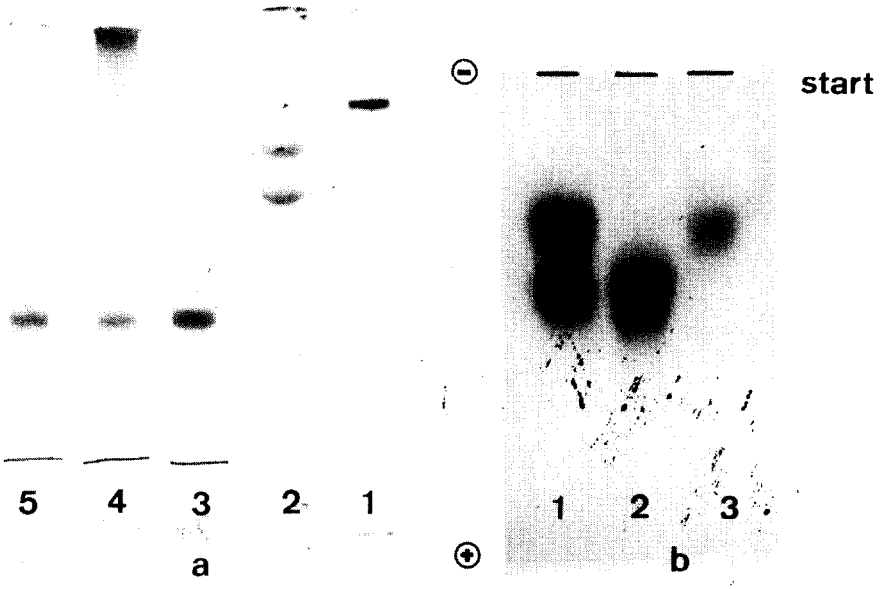

Fig.3. Electrophoresis of allophycocyanin I and the subunits. (a) Polyacrylamide gel electrophoresis: (1) allophycocyanin I, gel 7.5%, pH 7.5; (2) subunit separation, 8 M urea gel 7.5%, pH 7.5; (3) allophycocyanin I, 0.1% SDS, gel 10%, pH 7.0; (4) α-subunit, 0.1% SDS, gel 10%, pH 7.0; (5) β-subunit, 0.1% SDS, gel 10%, pH 7.0. (b) Cellogel electrophoresis in the presence of 6 M urea: (1) allophycocyanin I; (2) α-subunit; (3) β-subunit.

β-subunit of allophycocyanin I did not permit good spectra to be taken over the full range. However, the absorption maxima of both subunits could be determined to be at 615 nm. The α-subunit shows a red fluorescence while the β-subunit and native allophycocyanin I have only a very faint fluorescence. The same results were obtained for allophycocyanin II.

Treatment of allophycocyanin I with 0.1 M EDTA at room temperature leads to a drastic change of the visible absorption spectrum in the range from 500–700 nm (fig.4). The absorption maximum is shifted from 656–650 nm. A new shoulder appears at 625 nm and a faint one at 595 nm. The typical shoulder of allophycocyanin I at 600 nm disappears completely. The most striking fact is that the new spectrum is identical with that of allophycocyanin II. The same results were obtained at 40°C but at this temperature the shoulder at 625 nm was intensified. In contrast to that, the spectra of C-phycocyanin and allophycocyanin II were not affected (except for a minor drop in extinction) when these biliproteins were treated with 0.1 M EDTA (unpublished results).

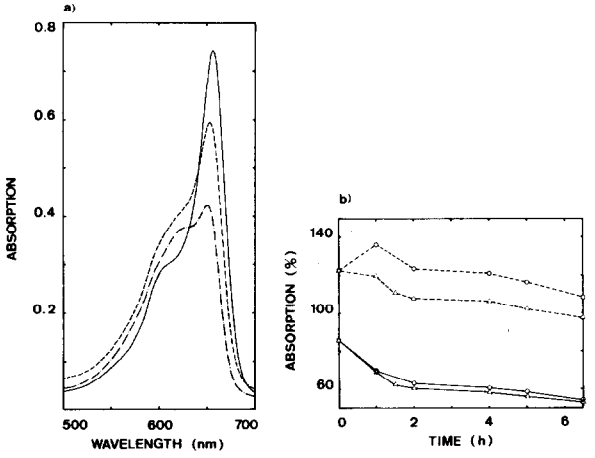

Fig.4. Influence of EDTA (0.1 M in water) on the absorption spectrum of allophycocyanin I (0.2 mg/ml) at room temperature and at 40°C. (a) Spectra: (——) allophycocyanin I in H_2O; (– – – –) allophycocyanin I in 0.1 M EDTA solution immediately after dissolving; (–.–.–.–) allophycocyanin I in 0.1 M EDTA solution after 90 min at room temperature (not shown: 1h, 2h, 4h, 5h, 6½ h). (b) Dependence of the absorption values at 650 nm and 625 nm at both temperatures (allophycocyanin I in H_2O = 100%). (△——△) 650 nm, room temperature; (△– – – –△) 625 nm, room temperature; (○——○) 650 nm, 40°C; (○– – – –○) 625 nm, 40°C.

Allophycocyanin I oxidized with hydrogen peroxide shows a completely different spectrum (fig.5). Both, the 656 nm peak and the shoulder at 600 nm have vanished while a new absorption maximum appears at 617 nm and a weak shoulder at 575 nm. This spectrum is practically identical with that of C-phycocyanin. Oxidation of allophycocyanin II leads to the same result (unpublished observations).

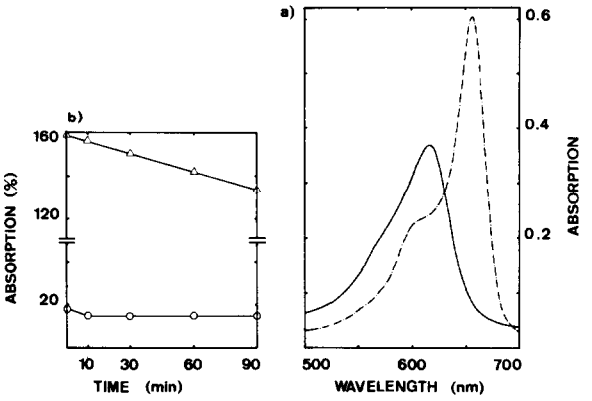

Fig.5. Influence of hydrogen peroxide oxidation on the absorption spectrum of allophycocyanin I (0.15 mg/ml, phosphate buffer 0.02 M, pH 5.9, 7.5% H_2O_2, room temperature). (a) Spectra: (–.–.–.–) allophycocyanin I in H_2O; (——) allophycocyanin I after 10 minutes exposure to H_2O_2 (not shown 30, 60, 90 min). (b) Dependence of the absorption values at 650 nm and 617 nm (allophycocyanin I in H_2O = 100%): (o——o) 650 nm; (△——△) 617 nm.

The amino acid compositions of allophycocyanin I and II and their subunits are given in table 1. The total amino acid compositions of allophycocyanin I and its

Table 1
Amino acid composition of native allophycocyanin I and the α- and β-subunit[a)]

	α-subunit[b)] average integer			β-subunit[b)] average integer			Total α- and β-subunits (α+ β)		Native allophycocyanin I[c)] integer	
Lysine	7.31	7	(7)	8.24	8	(8)	15	(15)	16	(16)
Histidine[d)]	–	–	(–)	–	–	(–)	–	(–)	–	(–)
Arginine	9.71	10	(10)	7.77	8	(8)	18	(18)	18	(18)
Aspartic acid	16.37	16	(16)	16.43	16	(15)	32	(31)	31	(31)
Threonine[e)]	7.46	7	(7)	12.24	12	(12)	19	(19)	20	(19)
Serine[e)]	11.88	12	(12)	12.31	12	(12)	24	(24)	25	(24)
Glutamic acid	16.80	17	(17)	12.88	13	(12)	30	(29)	30	(30)
Proline	4.65	5	(5)	3.83	4	(4)	9	(9)	9	(12)
Glycine	14.80	15	(15)	12.96	13	(12)	28	(27)	27	(25)
Alanine	22.04	22	(22)	24.30	24	(24)	46	(46)	44	(46)
Valine[f)]	14.69	15	(14)	11.91	12	(12)	27	(26)	27	(26)
Methionine	3.41	3	(3)	4.38	4	(4)	7	(7)	7	(7)
Isoleucine[f)]	12.79	13	(13)	10.61	11	(11)	24	(24)	24	(24)
Leucine	11.81	12	(13)	14.61	15	(15)	27	(28)	28	(28)
Tyrosine	6.94	7	(8)	9.83	10	(11)	17	(19)	18	(19)
Phenylalanine	2.18	2	(3)	2.26	2	(2)	4	(5)	5	(5)
Tryptophane	n.d.	n.d.	(–)	n.d.	n.d.	(–)	n.d.	(–)	n.d.	(–)
Cysteic acid	n.d.	n.d.	(1)	n.d.	n.d.	(2)	n.d.	(3)	n.d.	(3)
Total		163	(166)		164	(164)	327	(330)	329	(333)

[a] The respective values for allophycocyanin II are listed in parentheses.
[b] Amino acid residues calculated per 17 200 molecular weight after 24, 48, 72 h of hydrolysis.
[c] Amino acid residues calculated per 34 400 molecular weight after 24, 48, 72 h of hydrolysis.
[d] Only in 2 out of 10 analyses of native APC I minor traces of Histidine were detectable. In the analyses of the subunits Histidine was never found.
[e] Extrapolated to zero time of hydrolysis
[f] Extrapolated to 120 h of hydrolysis

n.d. = Not determined

α- and β-subunits are, within the limits of experimental error, identical with those of allophycocyanin II and its subunits, respectively.

APC I_c was partly characterized and the results obtained were found to be identical with those of allophycocyanin II.

Allophycocyanin I and allophycocyanin II seem to be identical with respect to electrophoretic mobility, molecular weight, subunit and amino acid composition. However, according to the elution from an ion exchange column their net charge has to be distinctly different (allophycocyanin I is more positive than allophycocyanin II). This is in agreement with the higher isoelectric point of allophycocyanin I. The absorption spectra of allophycocyanin I and II resemble each other but still show some major differences. The conversion of allophycocyanin I to APC I_c during the purification and especially the spectral conversion of EDTA-treated allophycocyanin I, together with all the other results, lead to the following hypothesis:

The chromophore of allophycocyanin I contains an as yet unknown metal atom which is lost to a large extent during the extraction and purification procedures (low yield of allophycocyanin I). Allophycocyanin II is a metalfree form of allophycocyanin I with a therefore more acidic p*K*. The protein parts of both protein–pigment complexes are either entirely identical or have only very small differences.

At this time it cannot be said whether the cell actually contains allophycocyanin I and II or only allophycocyanin I from which allophycocyanin II is artificially formed after cell disruption.

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