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Purification, Crystallization and Preliminary X-ray Diffraction Studies of C-Phycocyanin and Allophycocyanin from *Spirulina platensis*

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Abstract

C-phycocyanin and allophycocyanin from the green alga *Spirulina platensis* were isolated and crystallized by gel-acupuncture techniques. A novel two-step chromatographic procedure was used for purification. Blue hexagonal crystals were obtained by diffusing magnesium chloride into the protein solution for a week, followed by diffusion of PEG 6000 in order to complete the reduction of the solubility of the protein in the capillary tube used as a growth cell. In the case of allophycocyanin, crystals with a size of $0.4 \times 0.3 \times 0.3$ mm were characterized by X-ray diffraction. They belong to space group $P6_322$ with unit-cell parameters $a = b = 102.04$, $c = 131.22$ Å. The crystals of C-phycocyanin belong to either space group $P6$ or $P6_3$ with unit-cell constants $a = b = 182.38$, $c = 60.87$ Å, $\alpha = \beta = 90$, $\gamma = 120°$. The crystals diffract beyond 2.4 and 2.5 Å resolution, respectively, using a rotating anode as an X-ray source.

1. Introduction

Biliproteins are proteins with covalently attached open-chain tetrapyrrole pigments. They form light-harvesting supramolecular complexes called phycobilisomes (PBS), that are attached to the outer surface of thylakoid membranes of cyanobacteria (blue–green algae), red algae and cryptomonads. The major biliproteins are: the phycoerythrins which have the red chromophore phycoerythrobilin as the major prosthetic group; the phycocyanins (C-PC from cyanobacteria) which contain either a mixture of the phycocyanobilin and phycoerythrobilin chromophores or just phycocyanobilin, depending on the species of origin; the phycoerithrocyanin (PEC) which have both phycocyanobilin and a phycobiliviolinoid tetrapyrrole chromophore; and allophycocyanin (APC) with phycocyanobilin as prosthetic group. All PBS consist of a core of two or three cylinders composed of APC aggregates and a number of rods made up of discs of trimeric or hexameric units of either the same or different types of biliproteins connected in a hemidiscoidal array (for reviews, see Glazer, 1985, 1988, 1989; Scheer, 1982; Cohen-Bazire & Bryant, 1982; Zuber, 1987, 1993).

The spatial arrangement of the components makes the phycobilisome an ideal light transducer with a quantum efficiency of approximately 100% (Sauer, 1975). Time-resolved fluorescence emission spectra of intact cells show the energy-transfer pathways from the periphery of the PBS to APC, and eventually to chlorophyll *a* (Yamazaki *et al.*, 1984).

The introduction of biliproteins as fluorescent tags of cells and molecules (Oi, Glazer & Stryer, 1982) was followed by widespread application of these macromolecules in cell sorting, cell analyses, flow cytometry and immunoassay (Glazer & Stryer, 1984). APC and C-PC have been shown to be particularly useful using either tunable dye lasers or higher wavelength helium–neon lasers (Shapiro, Glazer, Christenson, Williams & Strom, 1983; Parks, Hardy & Herzenberg, 1984). The fact that the excitation and emission maxima for APC lie in the red is particularly important because of the lack of interfering emissions from most biological materials in this region of the spectrum. However, the widespread use of APC has been somewhat limited by high cost of the purified biliprotein.

On the other hand, antenna systems of some cyanobacteria, *i.e. Spirulina platensis*, are composed of only C-PC and APC at an approximate 10:1 ratio. Moreover, *S. platensis* grows easily in waters along the Mediterranean coast, serving as an inexpensive source material of these biliproteins.

C-PC from cyanobacteria have been crystallized and characterized in several species. The three-dimensional structures of C-PC from *Mastigocladus laminosus* (Schirmer, Bode, Huber, Sidler & Zuber, 1985; Schirmer, Bode & Huber, 1987; Duerring, Huber & Bode, 1988), *Agmenellum quadruplicatum* (Schirmer *et al.*, 1986, 1987) and *Fremyella diplosiphon* (Duerring, Schmidt & Huber, 1991) were determined by high-resolution X-ray crystallography. Crystallization and preliminary X-ray diffraction studies of C-PC from the red alga *Porphyra tenera* were also reported (Miki *et*

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al., 1990). However, C-PC from *S. platensis* has been neither crystallized or X-ray characterized. With regard to APC, until Brejc *et al.* (Brejc, Ficner, Huber & Steinbacher, 1995) isolated, crystallized and analyzed the crystal structure of APC from *S. platensis*, little was known of the crystals of APC, perhaps because of the poor availability of the raw material from which it is isolated or because of the poor crystallization methods used. The methods used by these authors are different to the simpler one described in this paper. Moreover, their crystals are smaller than those obtained by us.

Here we report the purification of APC and C-PC from *S. platensis* by two chromatographic steps (Bermejo, 1994), the crystallization by a gel-acupunture technique and preliminary X-ray diffraction studies at 2.4 and 2.5 Å resolution, respectively.

2. Materials and methods

2.1. *Chemicals*

All chemicals were purchased from Sigma Chemical Company and used without further purification. Sephadex G 100 from Pharmacia. DEAE–cellulose DE-52 from Whatman. Standard molecular weight markers (14 400–94 000) were from LKB.

2.2. *Biliproteins purification*

Cells of *S. platensis* were grown off the south east coast of Spain and generously provided by Imade SL as frozen cells. For purification of biliproteins, cyanobacterial cells stored at 253 K have been used as starting material. Frozen cells were removed from the growth medium by centrifugation. In a typical experiment 50 g (wet weight) were resuspended in 50 ml of 1 *M* sodium acetate, pH 5.0. The slurry was sonicated for 10 min with stirring, transferred to ultracentrifuge tubes, and centrifuged at 80 000*g* for 1 h. The procedure was repeated again with the pellets, and the supernatants from both centrifugations pooled. Solid $(NH_4)_2SO_4$ was added to 70% of saturation, allowed to stand for 2 h, and then centrifuged at 20 000*g* for 15 min. The pellets were resuspended in 1 ml of 100 m*M* sodium acetate, pH 5.0, and dialyzed overnight against 2 l of the same buffer.

The dialyzed biliprotein-containing solution was applied to a column (2.5 × 60 cm) of Sephadex G-100, pre-equilibrated with the same acetate buffer, eluted at a flow rate of 50 ml h^{-1} until the eluate became blue, and collected in fractions of 3 ml. The blue fractions were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) and UV–vis absorption spectroscopy. The first seven fractions were C-PC rich, then three fractions in which the two biliproteins appear mixed and the last four fractions were APC-rich solutions.

The C-PC- and APC-rich tubes were pooled separately and brought to 70% of saturation with $(NH_4)_2SO_4$, and allowed to stand for 1 h prior to centrifugation at 20 000*g* for 15 min. The pellets were resuspended in 5 m*M* phosphate buffer, pH 7.0, and then dialyzed overnight against 4 l of the same phosphate buffer. Dialyzed solutions analyzed by SDS–PAGE showed additional impurity bands together with those typical of biliprotein subunits. For the C-PC-rich solution an A_{620}/A_{277} ratio of 4 was obtained. In the APC-rich solution both an A_{650}/A_{280} ratio of 3.5 and a constant A_{620}/A_{650} ratio of 0.72 have been obtained.

Each dialyzed solution was applied to a column (2.5 × 15 cm) of DEAE–cellulose DE-52 pre-equilibrated with 5 m*M* phosphate buffer, pH 7.0. After washing with 1 bed volume of starting buffer, both columns were developed with 0.29 *M* phosphate buffer, pH 7.0. C-PC is eluted from the column with the C-PC-rich solution. On the other column, the 0.29 *M* eluate was rejected, and immediately after APC was eluted with 0.40 *M* phosphate buffer, pH 7.0. The flow rate was 70 ml h^{-1}. The peak blue tubes from each biliprotein were pooled, brought to 70% saturation with $(NH_4)_2SO_4$ and left to stand overnight in the dark at 277 K before centrifugation. The pellets of purified biliproteins were resuspended in small volumes of 5 m*M* phosphate buffer, dialyzed overnight at 277 K against

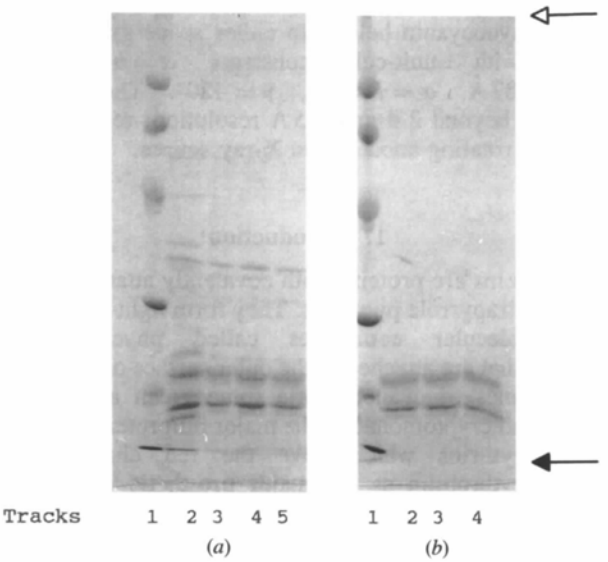


Fig. 1. SDS–PAGE of C-PC from gel permeation (Sephadex G-100) and ion-exchange (DEAE–cellulose DE-52) chromatography. (*a*) Gel permeation. From left to right, the tracks are: 1 marker proteins and 2–5 C-PC-rich solutions. (*b*) Ion exchange. From left to right, the tracks are: 1 marker proteins and 2–4 purified C-PC. The gel origin is shown by the open arrow and the front by the filled-in arrow. The molecular weight of the known markers from top were: phosphorylase *b* (94 000), albumin (67 000), ovalbumin (43 000), carbonic anhydrase (30 000), trypsin inhibitor (20 100) and α-lactalbumin (14 400).

the same buffer, and freeze dried for storage until utilization. Typically, only the bands corresponding to the α and β subunits are detected on analysis of purified biliproteins by SDS-PAGE, indicating that the linker polypeptides were effectively removed in the purification procedure. Fig. 1 contains the electrophoretic results for C-PC.

2.3. *SDS-PAGE*

Electrophoresis was carried out according to Laemmli (1970) using a 12.5%(*w/v*) polyacrylamide slab gel, 1.5 mm thick, containing 0.1%(*w/v*) SDS with a stacking gel of 4% acrylamide and 0.1% *bis*-acrylamide. Samples were preincubated with 2%(*w/v*) SDS, 10%(*v/v*) glycerol, 4.5%(*v/v*) β-mercaptoethanol, 0.025%(*w/v*) bromophenol blue, and 60 m*M* Tris, pH 6.8, for about 5 min at 368 K. Gels were run at room temperature, and visualized by staining for 12 h with 0.1%(*w/v*) Coomassie Brilliant Blue R-250, 40%(*v/v*) methanol with 7%(*v/v*) acetic acid and destained in dilute acetic acid. For calibration, marker proteins purchased from LKB were used.

2.4. *Absorption and fluorescence spectroscopy*

Absorption and fluorescence spectra, were recorded using a Perkin Elmer Lambda 16 spectrophotometer and a Shimadzu RF 5001 spectrofluorometer, respectively. Both were equipped with a controlled temperature cell holder with stirring system and computerized data acquisition. To determine molar absorption coefficients an accurately measured amount of freeze-dried protein was solubilized in 20 m*M* phosphate buffer, pH 7.0 and the spectrum recorded. The band widths of the excitation and emission monochromators were both equal to 1.5 nm.

2.5. *Crystallization*

Crystallization was performed using a typical gel-acupuncture technique set-up (García-Ruiz, Moreno, Viedma & Coll, 1993; García-Ruiz, Moreno, Parraga & Coll, 1995) (Fig. 2). We used a vessel 2.5 cm in diameter and 10 cm high. The silica gel layer was prepared by neutralization of sodium metasilicate solution (1.06 g ml^{-1}) with 1 *M* acetic acid to reach pH 7.0. The gel settled in about 4 h. X-ray capillary tubes with an internal diameter of 0.5 mm were filled by capillarity with a protein solution of concentration of 30 mg ml^{-1} prepared in buffer phosphate 100 m*M*, pH 7.0. The open part of the capillaries was carefully sealed using a green clay mounting (Hampton-HCMM). The capillary tubes were punctuated into the gel whilst trying to keep a penetration length less than 10 mm inside the gel layer. Subsequently, 6.0 ml of aqueous magnesium chloride solution 20%(*w/v*) were added onto the gel using a syringe. The system was closed to avoid evaporation and then we waited for a week to permit the slow diffusion of the magnesium solution through the protein solution filling the capillary tube. After that time, this solution was removed and replaced by 6.0 ml of PEG 6000 30%(*w/v*) (prepared in Tris-HCl pH 7.0 100 m*M*). 30 d later, small blue hexagonal crystals appeared along the capillary tube. The maximum crystal size was obtained after two months. All the experiments were carried out at laboratory temperature (293 ± 1 K).

2.6. *X-ray diffraction studies*

The crystals, grown inside the capillary tubes, were maintained with a trace of the mother liquor and cooled to 273 K. These crystals were analyzed using a MAR Research imaging-plate system with Cu $K\alpha$ radiation generated by an Enraf-Nonius rotating-anode generator, operated at 40 kV and 100 mA with a fine-focus filament. The crystal-to-film distance was set to 100 cm.

3. Results and discussion

Sonication and $(NH_4)_2SO_4$ addition to 70% of saturation results in a precipitate which contains C-PC and APC. In preliminary studies, we have tested columns with different bed volumes, 2.5 × 20, 40, 60 and 80 cm in length and different flow rates in the range 30–90 ml h^{-1}. Fractions of 3 ml were collected. These fractions were analyzed by UV-vis spectroscopy and SDS-PAGE. The better resolution was obtained with 60 cm long columns. Likewise, the optimum flow rate was 50 ml h^{-1}. In these conditions, SDS-PAGE showed that three fractions contain biliproteins mixture of roughly 50%. The other coloured fractions showed enriched C-PC or APC solutions, though other contaminant proteins appear in all fractions. For example Fig. 1 shows the electrophoretic results for C-PC.

The results obtained by size-exclusion chromatography indicate that purification of biliproteins from *S.*

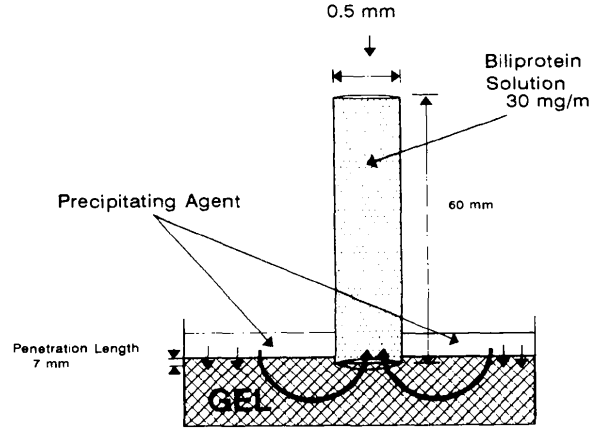


Fig. 2. Experimental set-up for biliprotein crystal growth.

platensis is not reached with a chromatographic process only but also needs an ion-exchange process. Before proceeding with the ion-exchange, the pH and the initial ionic strength (I) have to be selected by a preliminary test with a continuous and simultaneous gradients of both pH and I, and with the simplest continuous gradient of I only. At pH 7 we found that C-PC elutes at 0.29 M phosphate buffer concentration and APC at 0.4 M approximately. The method has been improved using two independent columns and different departure solutions. For C-PC purification, C-PC-rich fractions from Sephadex G-100 were pooled, added into the column and eluted with 0.29 M phosphate buffer. Similar steps were carried out for APC purification, with APC-rich tubes and 0.4 M phosphate buffer. This procedure has the advantage that the column development can be accomplished with discontinuous ionic strength gradients.

Both biliproteins were homogeneous as determined by SDS–PAGE yielding two bands (Fig. 1) of closely similar amounts. By comparison with standards, the molecular weights were 21 500 and 19 000 Da, corresponding to the α and β subunits of C-PC, and 19 600 and 17 700 Da for α and β subunits of APC, respectively.

By comparing absorption and fluorescence spectra from selected pooled fractions with the published spectra of pure C-PC and APC (Oi *et al.*, 1982; Boussiba & Richmond, 1979; MacColl, Csatorday, Berns & Traeger, 1980) we observed an excellent fitting of the data. C-PC exists in a reversible equilibrium between monomer, trimer and hexamer (Glazer & Cohen-Bazire, 1971). The aggregation state of the protein is very sensitive to the pH and ionic strength of the medium, and the protein concentration. MacColl *et al.* (MacColl, Lee & Berns, 1971) and Saito *et al.* (Saito, Iso & Mizumo, 1974) showed that at pH 6.5–8.0, monomer and trimer of C-PC exist in equilibrium, but the protein is primarily in a monomeric form at concentrations less than 8 $\mu g\,ml^{-1}$. However, the absorption spectra of C-PC in hexameric and monomeric forms show that the spectra are slightly affected by the aggregation changes in the protein (Saxena, 1988). On the other hand, the dissociation of trimeric APC into monomeric APC causes the absorption maximum to shift from 650 to 615 nm and the emission maximum to shift from 660 to 641 nm (MacColl *et al.*, 1980; Huang, Berns & MacColl, 1987). The purified APC showed no absorbance and fluorescence peak other than that corresponding to the trimeric APC. Thus, the APC purified in our experiments retained its native trimeric structure.

The initial nucleation of both proteins was carried out by diffusion of 20% (w/v) magnesium chloride (Jancarik & Kim, 1991), application of this precipitating agent results in local supersaturation gradients. After a week, PEG 6000 was added. This addition affects the supersaturation value (García-Ruiz & Moreno, 1994) and usually results in sustained growth of small crystals obtained in the first step. Following this methodology of variation of the protein solubility properties, blue single crystals were obtained as shown in Figs. 3(*a*) and 3(*b*).

Once both biliproteins were crystallized, the crystals of C-PC and APC were taken out from the capillary tube followed by a careful cleaning with diluted solutions of PEG. After that procedure, the crystals were dissolved in 100 mM phosphate buffer pH 7.0, and their absorption and fluorescence spectra were recorded. All spectra have the same profiles and characteristic maxima as before crystallization, and agree well with those reported previously for purified biliproteins (Priestle, Ryhne, Salmon & Hackert, 1982).

X-ray photographs (Fig. 4*a*) showed that crystals of allophycocyanin, with an average size of 0.4× 0.3 × 0.3 mm belong to the hexagonal system space group $P6_322$. The unit-cell dimensions found were $a = b = 102.04$ and $c = 131.22$ Å. These results according very well to the obtained by Brejc *et al.* (1995) ($a = b = 101.9$, $c = 130.6$ Å).

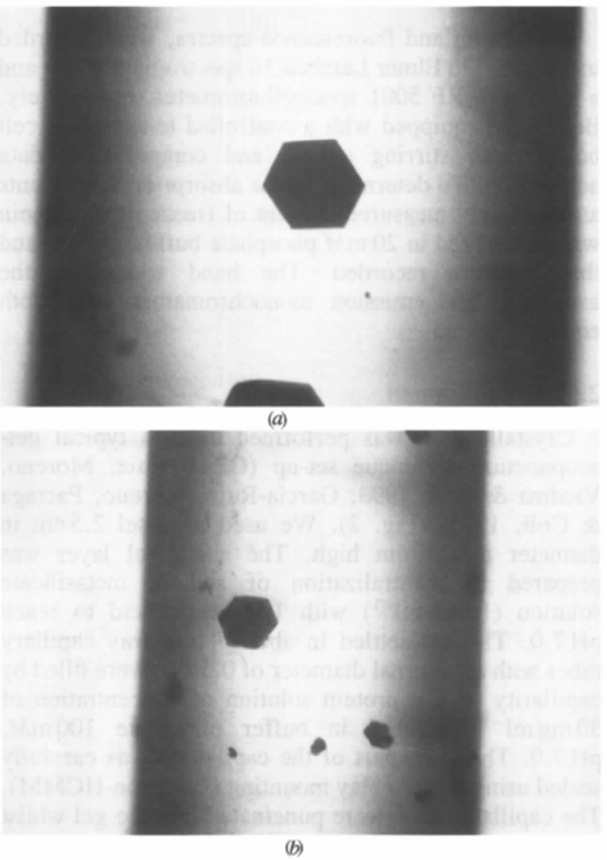
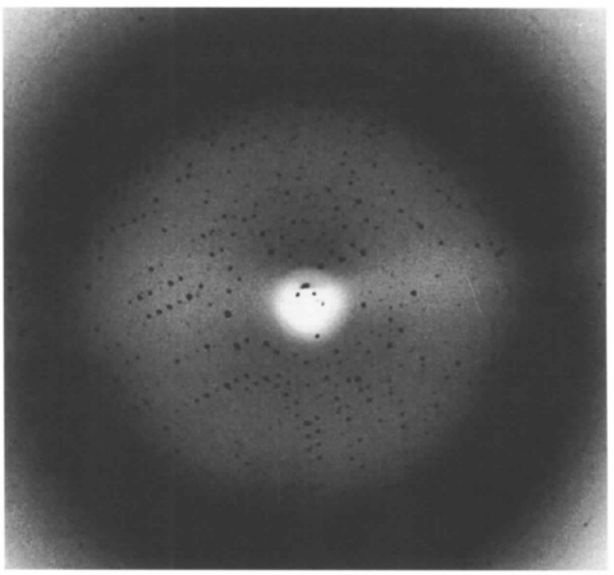
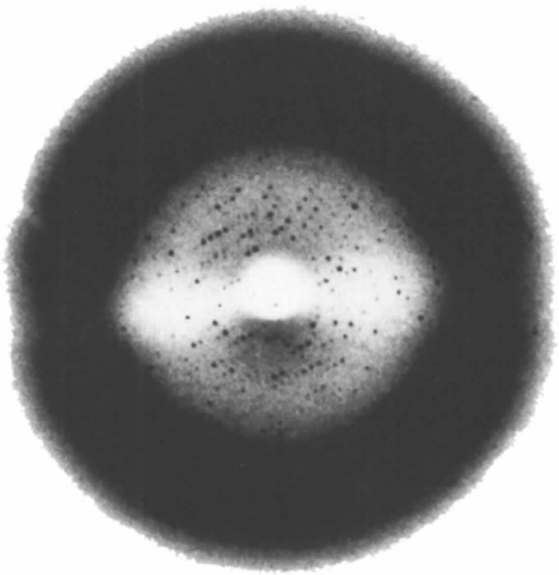


Fig. 3. Biliprotein single crystal in their growth environment. (*a*) allophycocyanin crystal, and (*b*) C-phycocyanin crystal. Inner diameter of the capillary fiber = 0.5 mm.

With regard to C-PC (Fig. 4*b*), X-ray diffraction studies showed that the crystals belong to the hexagonal system. The systematic absences of reflections indicate that the space group is either $P6$ or $P6_3$, with unit-cell constants $a = b = 182.38$, $c = 60.87$ Å, $\alpha = \beta = 90$, $\gamma = 120°$. Comparison of C-PC crystals from different cyanobacteria show that the unit-cell dimensions of C-PC crystals from *S. platensis* are between the values obtained for crystals from *F. diplosiphon* (Duerring *et al.*, 1991) and *A. quadruplicatum* (Schirmer *et al.*, 1986, 1987). Therefore, it is reasonable to assume that crystalline C-PC from *S. platensis* has a very similar three-dimensional structure to that of C-PC from those cyanobacteria. Moreover, since in the C-PC crystals of *F. diplosiphon* and *A. quadruplicatum* the molecules are associated into hexamers and the crystal unit cell contains three $(\alpha\beta)_6$ hexamers (Schirmer *et al.*, 1986, 1987; Duerring *et al.*, 1991), our results might also imply that the crystal unit cell of C-PC from *S. platensis* contains three $(\alpha\beta)_6$ hexamers. Probably this hexameric aggregation form is closely related to the discs of native PBS rods.



(*a*)



(*b*)

Fig. 4. (*a*) 1° X-ray diffraction pattern of APC crystal. (*b*) 1° X-ray diffraction pattern of C-PC crystal.

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