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X-ray crystallographic studies on C-phycocyanins from cyanobacteria from different habitats: marine and freshwater

L. Satyanarayana,[a] C. G. Suresh,[a]* Anamika Patel,[b] Sandhya Mishra[b]* and Pushpito Kumar Ghosh[b]

[a]Division of Biochemical Sciences, National Chemical Laboratory, Pune 411008, Maharashtra, India, and [b]Central Salt and Marine Chemicals Research Institute, G. B. Marg, Bhavnagar 364002, Gujarat, India

Correspondence e-mail: cgsuresh@ncl.res.in, smishra@csmcri.org

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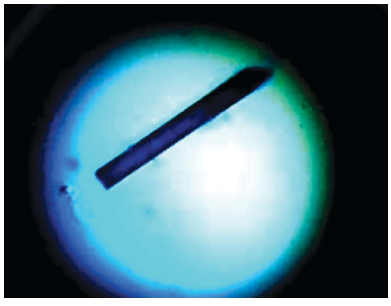


C-phycocyanins from three cyanobacterial cultures of freshwater and marine habitat, *Spirulina*, *Phormidium* and *Lyngbya* spp., were purified to homogeneity and crystallized using the hanging-drop vapour-diffusion method. Blue-coloured crystals in different crystal forms, monoclinic and hexagonal, were obtained for the three species. The crystals took 1–12 weeks to grow to full size using polyethylene glycols of different molecular weights as precipitants. The amino-acid sequences of these proteins show high similarity to other known C-phycocyanins from related organisms; however, the C-phycocyanins reported here showed different biochemical and biophysical properties, *i.e.* molecular weight, stability *etc.* The X-ray diffraction data were collected at resolutions of 3.0 Å for the monoclinic and 3.2 and 3.6 Å for the hexagonal forms. The unit-cell parameters corresponding to the monoclinic space group $P2_1$ are a = 107.33, b = 115.64, c = 183.26 Å, β = 90.03° for *Spirulina* sp. C-phycocyanin and are similar for crystals of *Phormidium* and *Lyngbya* spp. C-phycocyanins. Crystals belonging to the hexagonal space group $P6_3$, with unit-cell parameters $a = b$ = 154.97, c = 40.35 Å and $a = b$ = 151.96, c = 39.06 Å, were also obtained for the C-phycocyanins from *Spirulina* and *Lyngbya* spp., respectively. The estimated solvent content is around 50% for the monoclinic crystals of all three species assuming the presence of two hexamers per asymmetric unit. The solvent content is 66.5 and 64.1% for the hexagonal crystals of C-phycocyanin from *Spirulina* and *Lyngbya* spp. assuming the presence of one $\alpha\beta$ monomer per asymmetric unit.

1. Introduction

The major light-harvesting capacity of prokaryotic cyanobacteria and eukaryotic red algae is associated with large antennae complexes called phycobilisomes, which are located on the surface of the photosynthetic thylakoid membranes (Glazer, 1985; MacColl, 1998). The phycobilisomes are composed of rods and a core, which are highly organized, consisting of various phycobiliproteins and linker polypeptides. C-phycocyanins have α- and β-subunit polypeptides, which exhibit a high affinity for each other and associate into ($\alpha\beta$) monomers, which in turn aggregate into $(\alpha\beta)_3$ trimers and $(\alpha\beta)_6$ hexamers (Glazer, 1989; Glazer & Melis, 1987). The macromolecular light-harvesting complexes are an *in vivo* self-assembly of the hexameric phycobiliproteins and the corresponding linker peptides.

Phycobiliproteins contain different types and numbers of chromophores, which are open-chain tetrapyrroles linked to cysteine residues in the protein *via* thioester bonds. The chromophores are classified as phycoerythrobilin (PEB), phycocyanobilin (PCB), phycoviolobilin (PVB) or phycourobilin (PUB) (Bryant, 1991; Glazer, 1985). The phycobiliproteins are divided into three major classes: phycoerythrins, phycocyanins and allophycocyanins. The rods in phycobilisome normally include phycocyanin, but also contain phycoerythrin or phycoerythrocyanin in some species, which are located at the tips of the rods. The phycobilisome core is composed of allophycocyanins and linker polypeptides. The hexameric phycocyanins, the major component of the rods, not only absorb light energy but also transfer the absorbed energy from phycoerythrins to allophycocyanins in the core. The energy is finally transferred to the

photosynthetic reaction centre. This energy transfer from the phycobilisomes to the photosynthesis reaction centres located within the thylakoid membrane is a very fast and effective process (Gantt, 1990).

The structures of C-phycocyanins from *Mastigocladus laminosus* (Schirmer *et al.*, 1985), *Agmenellum quadruplicatum* (Schirmer *et al.*, 1986), *Fremyella diplosiphon* (Duerring *et al.*, 1991), *Spirulina platensis* (Wang *et al.*, 2001; Padyana *et al.*, 2001) and *Thermosynechococcus vulcanus* (Adir & Lerner, 2003) have been studied using X-ray crystallography. In this paper, we report the preliminary X-ray crystallographic analysis of C-phycocyanins from the Indian cyanobacteria *Spirulina* (freshwater), *Phormidium* (marine) and *Lyngbya* (marine). The sequences of these proteins have high similarity to the other C-phycocyanins from other related organisms, but differ in their biochemical and biophysical properties. For example, the estimated molecular weights of the purified C-phycocyanins from *Spirulina*, *Phormidium* and *Lyngbya* spp. were 112, 131 and 81 kDa, respectively. SDS–PAGE of pure C-phycocyanin yielded two bands corresponding to the α- and β-subunits. This result shows that the β-subunits have the same molecular weights (24.4 kDa) in all three cyanobacterial species, whereas the molecular weight of the α-subunit varies between the organisms: 17 kDa for *Spirulina* sp., 19.1 kDa for *Phormidium* sp. and 15.2 kDa for *Lyngbya* sp. Thus, the C-phycocyanin has been characterized as a trimer $(\alpha\beta)_3$ in *Spirulina* and *Phormidium* spp., but has been identified as a dimer $(\alpha\beta)_2$ in *Lyngbya* sp. (Patel *et al.*, 2005). No previous report of the crystallization of C-phycocyanin from *Phormidium* sp. or *Lyngbya* sp. has been found; we have also studied the stability of the C-phycocyanin hexamers in the presence of various denaturing agents such as urea or guanidium hydrochloride and under various conditions of temperature, ionic strength and pH. It is found that of the three C-phycocyanin hexamers studied, that from *Lyngbya* sp. has the highest stability, followed by that from *Phormidium* sp. which shows intermediate stability and that from *Spirulina* sp. which possess the lowest stability (unpublished work). Our objective in crystallizing these proteins and in subsequent three-dimensional structure determination is the study of the structural basis of these functional variations.

Table 1
Optimized crystallization conditions for growing single crystals of various C-phycocyanins from different organisms.

Source	Crystal form and space group	Composition of the well solution	Time for crystal growth (weeks)
Spirulina sp.	Hexagonal, $P6_3$	0.05 *M* sodium phosphate buffer pH 7.0, 20%(*w*/*v*) PEG 4000	3–4
Spirulina sp.	Monoclinic, $P2_1$	0.01 *M* sodium phosphate buffer pH 6.5, 0.72 *M* sodium formate, 13.5%(*w*/*v*) PEG 4000	1
Phormidium sp.	Monoclinic, $P2_1$	0.01 *M* sodium phosphate buffer pH 6.5, 0.72 *M* sodium formate, 9%(*w*/*v*) PEG 1000, 9%(*w*/*v*) PEG 8000	1
Lyngbya sp.	Hexagonal, $P6_3$	0.05 *M* sodium phosphate pH 6.0, 20%(*w*/*v*) PEG 4000	10–12
Lyngbya sp.	Monoclinic, $P2_1$	0.01 *M* sodium cacodylate buffer pH 6.5, 0.72 *M* sodium formate, 7.2%(*w*/*v*) PEG 20 000, 7.2%(*w*/*v*) PEG 550 MME	1

2. Materials and methods

2.1. Protein purification

The cultures of *Spirulina* sp., *Phormidium* sp. and *Lyngbya* sp. were grown at 300 K for 15 d and the biomass was collected after centrifugation. The lyophilized biomass was then lysed by soaking in

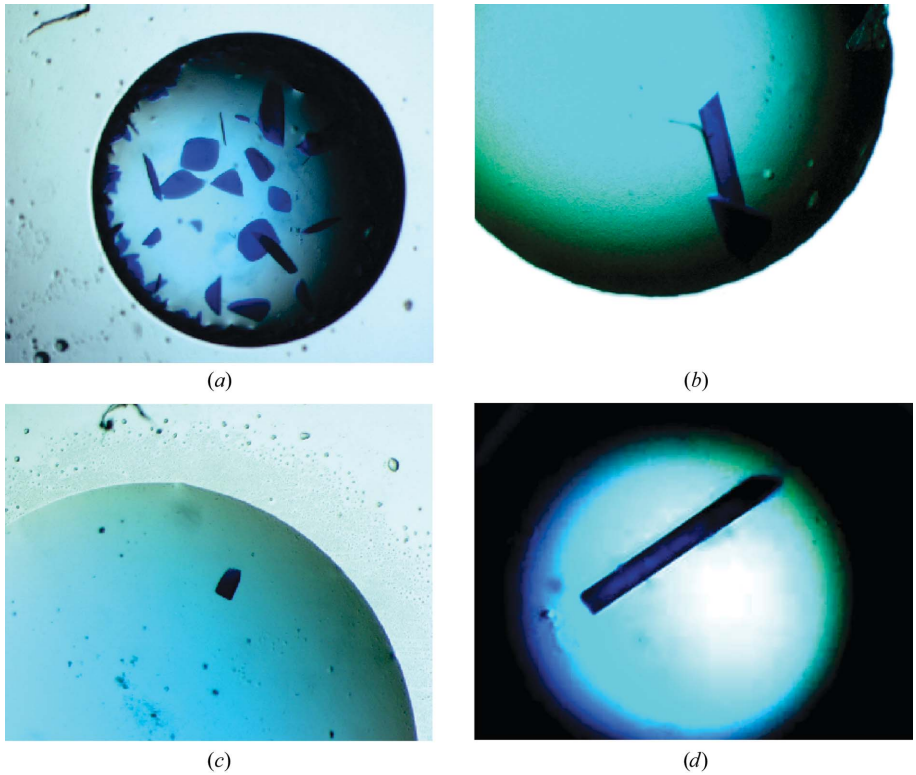


Figure 1
(*a*) Crystals (0.3 × 0.17 × 0.1 mm) of C-phycocyanin (C-PC) from *Phormidium* sp. (monoclinic). (*b*) Crystals (0.4 × 0.2 × 0.1 mm) of C-PC from *Spirulina* sp. (hexagonal). (*c*) Crystals (0.15 × 0.15 × 0.1 mm) of C-PC from *Lyngbya* sp. (hexagonal). (*d*) Crystals (0.4 × 0.2 × 0.1 mm) of C-PC from *Lyngbya* sp. (monoclinic).

Table 2
Data-collection statistics.

Values in parentheses are for the last shell.

Organism	*Spirulina*	*Spirulina*	*Phormidium*	*Lyngbya*	*Lyngbya*
Crystal system	Hexagonal	Monoclinic	Monoclinic	Hexagonal	Monoclinic
Space group	$P6_3$	$P2_1$	$P2_1$	$P6_3$	$P2_1$
Unit-cell parameters (Å, °)	$a = b = 154.97$, $c = 40.35$	$a = 107.33$, $b = 115.64$, $c = 183.26$, $\beta = 90.03$	$a = 107.87$, $b = 115.76$, $c = 183.54$, $\beta = 90.3$	$a = b = 151.96$, $c = 39.06$	$a = 107.45$, $b = 115.33$, $c = 183.36$, $\beta = 90.08$
Resolution range (Å)	40.0–3.2 (3.26–3.2)	40.0–3.0 (3.11–3.0)	40.0–3.0 (3.11–3.0)	50.0–3.6 (3.73–3.6)	25.0–3.0 (3.05–3.0)
Total No. of reflections	92421	277752	235483	201951	217963
Unique reflections	9451	86909	82067	10380	86811
Data completness (%)	99.6 (100.0)	96.6 (93.8)	96.6 (93.4)	95.1 (90.2)	96.4 (92.7)
Average $I/\sigma(I)$	13.1 (6.54)	9.19 (3.90)	5.27 (2.46)	4.85 (3.66)	6.78 (3.65)
$R_{\rm merge}$† (%)	11.6 (33.8)	9.2 (20.6)	12.7 (29.7)	13.1 (23.4)	9.1 (20)
Unit-cell volume (Å^3)	839348	2273080	2292598	781006	2272527
Matthews coefficient $V_{\rm M}$ (Å^3 Da^{-1})	3.68	2.50	2.51	3.42	2.49
Solvent content (%)	67	51	51	64	51

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$.

double-distilled water and subjected to two or three cycles of freezing and thawing. The crude extract was treated with 50% saturated ammonium sulfate. The precipitate collected after centrifugation (0.2 g) was redissolved, dialyzed and loaded onto a DEAE Sepharose column equilibrated with 0.05 *M* sodium phosphate buffer pH 7.0. The bound protein (0.07–0.075 g) was eluted using 0.20 *M* NaCl. The purity was checked using SDS–PAGE and also by measuring the absorption ratio between 620 and 280 nm, the value of which was observed to be greater than 4.4.

2.2. Crystallization

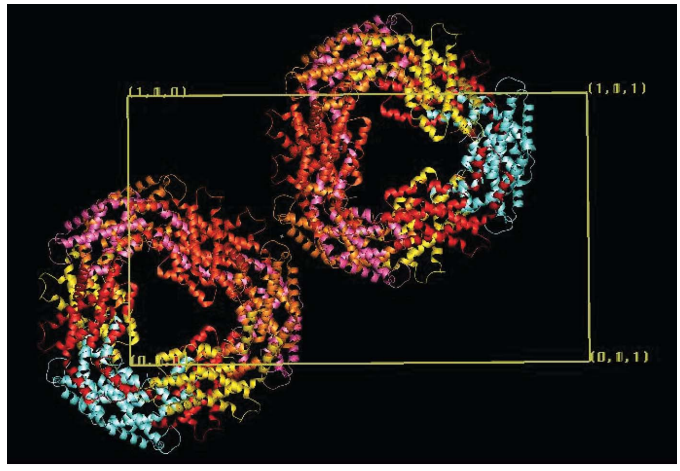
Screening for crystallization conditions was carried out by the hanging-drop vapour-diffusion method using sparse-matrix screen solutions (Hampton) at 295 K. The hanging drops were prepared by mixing 1 µl reservoir solution with 1 µl protein solution in water (20 mg ml^{-1}) and were equilibrated against 1 ml reservoir solution. Blue crystals of C-phycocyanin appeared and grew to full size in 1–12 weeks depending on the conditions.

2.3. X-ray diffraction data collection

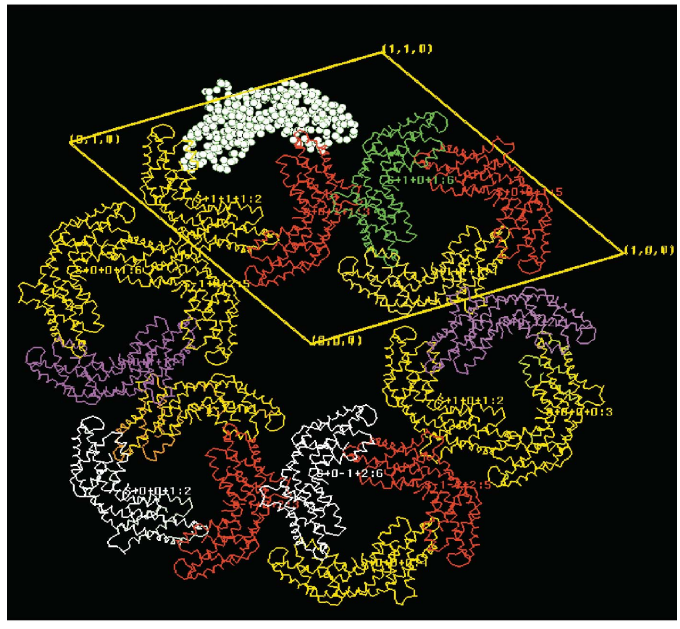
The cryoprotection of crystals for low-temperature data collection was carried out by serially transferring the crystals into synthetic mother liquors containing 5, 10 and 15%(*v*/*v*) PEG 400. The crystals were then mounted in cryoloops and flash-cooled by exposing them to a stream of liquid nitrogen at 100 K from an X-Stream system (Rigaku/MSC, USA). X-ray diffraction data were collected from single crystals using Cu $K\alpha$ radiation generated by a RU-H2R rotating-anode generator (Rigaku/MSC, USA) operated at 50 kV and 100 mA. X-rays were both monochromated and focused using confocal Blue optics mirrors (Osmic, MI, USA) and passed through a collimator with a 0.3 mm front-end pinhole slit. Intensities were recorded on an R-AXIS IV^{++} image-plate detector (Rigaku/MSC, Japan). The exposure time varied between 5 and 10 min depending on the extent of diffraction. The reflections were processed and scaled using *DENZO* and *SCALEPACK* from the *HKL* suite (Otwinowski & Minor, 1997).

3. Results and discussion

C-phycocyanins purified from the three cyanobacterial species using single-step purification were screened for crystallization using sparse-matrix Hampton screens. Small needle-shaped crystals grew under several conditions in this screening. Varying the concentrations of the



(*a*)



(*b*)

Figure 2
(*a*) Crystal packing of the two hexamers that form the asymmetric unit of C-phycocyanin in space group $P2_1$. (*b*) Crystal packing of the $\alpha\beta$ (one $\alpha\beta$ monomer per asymmetric unit is drawn in space-filling representation) monomers of C-phycocyanin from *Lyngbya* sp. packed into trimers in space group $P6_3$.

Table 3
Molecular-replacement parameters (Eulerian angles, translation, correlation coefficient and reliability factor).

Crystal	α (°)	β (°)	γ (°)	T_x	T_y	T_z	CC (%)	R factor (%)
Spirulina, hexagonal	50.1	162.9	234.5	0.514	0.867	0.000	76.3	34.6
Spirulina, monoclinic	267.6	92.4	130.9	0.186	0.471	0.082	67.9	39.3
Phormidium, monoclinic	267.4	92.1	131.4	0.186	0.472	0.082	67.2	38.0
Lyngbya, hexagonal	19.6	16.1	54.2	0.851	0.317	0.000	74.5	35.7
Lyngbya, monoclinic	267.7	92.0	131.1	0.186	0.472	0.082	66.7	39.2

ingredients and also the pH further refined the conditions obtained from initial screening. The best crystals (Figs. 1*a*, 1*b* and 1*c*) were grown by mixing 1 µl protein solution (20 mg ml^{-1} in distilled water) with an equal volume of reservoir solution and equilibrating against a 1 ml reservoir. The optimized conditions for growing the best crystals of the C-phycocyanins from the three different species and those used to obtain different crystal forms are listed in Table 1. The statistics of X-ray diffraction data collection are summarized in Table 2.

All the structures were solved using the molecular-replacement (MR) program *AMoRe* implemented in *CCP*4 (Navaza, 1994) using the coordinates of C-phycocyanin from *S. platensis* (PDB code 1gh0, chains *A*–*F*) as the input model for the monoclinic form and that from *T. vulcanus* (PDB code 1on7) for the hexagonal forms. The initial correlation factors and *R* factors obtained for MR solutions for all crystals are listed in Table 3. The differences between the packing of the two crystal forms of C-phycocyanin reported here are shown in Figs. 2(*a*) and 2(*b*). There are no packing differences in the monoclinic forms of the three C-phycocyanins and similarly there are no packing differences between the two C-phycocyanins crystallized in the hexagonal form. Further refinement and analysis of the structures are in progress.

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