Phormidium phycoerythrin forms hexamers in crystals: a crystallographic study

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The crystallographic analysis of a marine cyanobacterium (*Phormidium* sp. A09DM) phycoerythrin (PE) that shows distinct sequence features compared with known PE structures from cyanobacteria and red algae is reported. *Phormidium* PE was crystallized using the sitting-drop vapour-diffusion method with ammonium sulfate as a precipitant. Diffraction data were collected on the protein crystallography beamline at the Indus-2 synchrotron. The crystals diffracted to about 2.1 Å resolution at 100 K. The crystals, with an apparent hexagonal morphology, belonged to space group *P*1, with unit-cell parameters $a = 108.3$, $b = 108.4$ Å, $c = 116.6$ Å, $\alpha = 78.94$, $\beta = 82.50$, $\gamma = 60.34^\circ$. The molecular-replacement solution confirmed the presence of 12 $\alpha\beta$ monomers in the *P*1 cell. The *Phormidium* PE elutes as an $(\alpha\beta)_3$ trimer of $\alpha\beta$ monomers from a molecular-sieve column and exists as $[(\alpha\beta)_3]_2$ hexamers in the crystal lattice. Unlike red algal PE proteins, the hexamers of *Phormidium* PE do not form higher-order structures in the crystals. The existence of only one characteristic visual absorption band at 564 nm suggests the presence of phycoerythrobilin chromophores, and the absence of any other types of bilins, in the *Phormidium* PE assembly.

1. Introduction

The phycobilisome (PBS), found on the outer thylakoid membrane of cyanobacteria and red algae, is made up of pigmented phycobiliproteins (PBPs), namely phycoerythrin (PE; absorption $\lambda_{\max} = \sim 540$ –570 nm), phycocyanin (PC; absorption $\lambda_{\max} = \sim 610$ –620 nm) and allophycocyanin (APC; absorption $\lambda_{\max} = \sim 653$ nm), along with unpigmented linker proteins (MacColl, 1998). PBS harvests sunlight and exhibits efficient energy transfer to photosystems I and II in the direction PE→PC→APC→chlorophyll (Gantt & Lipschultz, 1973; Liu *et al.*, 2013). PE is believed to be the latest evolving component of PBS, expanding its absorbance capacity across the blue region of the solar spectrum (Six *et al.*, 2007).

Crystal structures have revealed remarkable similarities between the structural organizations of all PBPs, despite divergence in their amino-acid sequences (Anderson & Toole, 1998). A heterodimer ($\alpha\beta$ monomer) of two homologous α and β polypeptides of PE, PC and APC proteins is the building block of the PBS. In each of the PBPs, heterodimers associate into disc-shaped trimers, which have often been observed to form hexamers that arrange into higher-order assemblies such as rods and core cylinders (Anderson & Toole, 1998; MacColl, 2004). The polypeptide chains contain covalently attached noncyclic tetrapyrrole chromophore(s) enabling the PBPs to absorb and emit light within a specific wavelength range across

Table 1
Optimized parameters for PE crystallization.

Method	Sitting-drop vapour diffusion
Plate type	Intelli-Plates
Temperature (K)	298
Protein concentration (mg ml ⁻¹)	9.2
Buffer composition of protein solution	10 mM Tris-HCl pH 7.2
Composition of reservoir solution	1.25 M ammonium sulfate, 0.075 M Tris-HCl pH 8.5, 20% (w/v) glycerol
Volume and ratio of drop	Drop volume 4 µl with protein and buffer in a 1:1 ratio
Volume of reservoir (µl)	100

Table 2
Data collection and processing.

Values in parentheses are for the outer shell.

Diffraction source	PX-BL21, Indus-2
Wavelength (Å)	0.97947
Temperature (K)	100
Detector	MAR CCD
Crystal-to-detector distance (mm)	170
Rotation range per image (°)	1.0
Total rotation range (°)	189
Exposure time per image (s)	30
Space group	<i>P</i> 1
<i>a</i> , <i>b</i> , <i>c</i> (Å)	108.3, 108.4, 116.6
α , β , γ (°)	78.94, 82.50, 60.34
Mosaicity (°)	0.17
Resolution range (Å)	30–2.1 (2.2–2.1)
Total No. of reflections	512671 (74226)
No. of unique reflections	245717 (35529)
Completeness (%)	96.1 (95.1)
Multiplicity	2.1 (2.1)
$\langle I/\sigma(I) \rangle$	9.9 (3.2)
R_{meas}	0.115 (0.397)
Overall <i>B</i> factor from Wilson plot (Å ²)	23.29

Figure 1
Silver-stained and zinc-acetate-stained 15% SDS-PAGE of purified PE from *Phormidium* sp. A09DM. Protein molecular-mass standards (43, 29, 20.1 and 14.3 kDa from the top downwards) are shown in lane M. The manifestation of only two bands at around 20 kDa corresponding to the α and β subunits on the silver-stained purified PE gel indicated the absence of a linker peptide in the purified PE complex. The visualization of the same bands on zinc-acetate-stained SDS-PAGE indicated the presence of chromophore(s) in both PE subunits. Nearly 10 mg of PE protein was loaded in each lane.

the solar spectrum. The conformation and configuration of the bound chromophores or their interactions with the surrounding protein residues (chromophore-contact residues) are thought to influence their optical spectrum and the efficiency of energy transfer (MacColl, 1998; Gaigalas *et al.*, 2006; Camara-Artigas *et al.*, 2012; Singh *et al.*, 2015). For instance, phycoerythrobilin (PEB, the chromophore attached to PE) shows absorption at around 535 nm in a conformation bearing a high deviation from planarity ($>50^\circ$), whereas it shows a peak at 560 nm in a conformation with a low deviation from planarity ($<20^\circ$) (Gaigalas *et al.*, 2006).

Figure 2
Phormidium PE exists as a trimer of $\alpha\beta$ monomers in solution. The Superdex 200 gel-filtration profile of the *Phormidium* protein is shown. The PE protein elutes at a volume corresponding to an oligomer of 125 kDa. The elution volumes of the gel-filtration markers (carbonic anhydrase, 29 kDa; ovalbumin, 44.3 kDa; bovine serum albumin, 67 kDa; Apoferritin, 440 kDa; bovine thyroglobulin, 669 kDa) used for column calibration are also marked.

Figure 3
Spectroscopic characterization of *Phormidium* PE. The UV-visible absorption spectrum (red line) of purified PE showed a major band at 564 nm along with a shoulder peak at 540 nm. The fluorescence emission spectrum (blue line) of purified PE was measured upon excitation at 545 nm and an emission maximum is observed at about 581 nm. The peak at about 550 nm in the fluorescence emission spectrum is owing to Rayleigh scattering of the incident beam.

The crystal structures of PE from a number of red algae [*Porphyridium cruentum* (PDB entry 3v57; Camara-Artigas *et al.*, 2012), *Polysiphonia urceolata* (PDB entry 1lia; Chang *et al.*, 1996), *Griffithsia monilis* (PDB entry 1b8d; Ritter *et al.*, 1999) and *Gracilaria chilensis* (PDB entry 1eyx; Contreras-Martel *et al.*, 2001)], from the cyanobacterium *Gleobacter violaceus* (PDB entry 2vjh) and from cryptophytes have been reported. Additionally, structures of phycoerythrocyanin (PEC) from the thermophilic cyanobacterium *Mastigocladus laminosus* (PDB entries 2c7j and 2j96; Schmidt *et al.*, 2006, 2007) have been reported. The cyanobacterial and red algal PE proteins show poor sequence identity (<40%) to cryptophyte PE proteins and the cyanobacterial PEC protein. The PE proteins from cyanobacteria and red algae share significant

structural similarity and nearly 69% sequence identity. However, they differ in the type of bilin attached to the protein as well as in the microenvironment of the bilin chromophores. For instance, four different types of bilins (phycoerythrobilin, phycourobilin, phycocyanobilin and biliverdine) are found in known PE structures of cyanobacterial and red algal origin.

In the present paper, we report the preliminary crystallographic analysis of a marine cyanobacterial (*Phormidium* sp. A09DM) phycoerythrin. The *Phormidium* protein harbours an insertion of nine residues in the β -subunit and several substitutions of the highly conserved chromophore-contact residues compared with structures from a cyanobacterium and red algae. Its crystal structure could provide new insight into

Figure 4

Multiple sequence alignment of the α (A) and β (B) subunits of phycoerythrin orthologues from cyanobacteria and red algae with those of known structure. *Phormidium*, *Phormidium* sp. A09DM; 2VJH, *Gleobacter violaceus*; 1B8D, *Griffithsia monilis*; 3V57, *Porphyridium cruentum*; 1EYX, *Gracilaria chilensis*; 1LIA, *Polysiphonia urceolata*. Multiple sequence alignment was achieved with *Clustal Omega* (Sievers *et al.*, 2011). Residues which constitute the microenvironments of the PEB chromophores of *P. cruentum* PE were detected with the *LigPlus* suite (Laskowski & Swindells, 2011). These chromophore-contact residues are highly conserved and are shown in red boxes. Nonconserved chromophore-contact residues of *Phormidium* PE are shaded. This figure was prepared using *Jalview* (Waterhouse *et al.*, 2009).

the structure–function relationship of related cyanobacterial proteins. The protein crystallizes in the triclinic space group *P1* with 12 copies of an $\alpha\beta$ monomer. These constitute two independent hexamers, with the overall quaternary structure being similar to that of other known PEs. However, unlike red algal PE proteins, the hexamers of the *Phormidium* protein do not assemble as rods.

2. Materials and methods

2.1. Production, purification and characterization of phycoerythrin

The cyanobacterium *Phormidium* sp. A09DM was used as a natural source for the production of PE. PE was purified from the marine cyanobacterium *Phormidium* sp. A09DM as described previously (Sonani, Singh, Kumar *et al.*, 2014; Sonani, Singh, Asawthi *et al.*, 2014; Sonani *et al.*, 2015; Anwer *et al.*, 2015). The purity, integrity and homogeneity of PE were analysed by SDS–PAGE analysis as described in Singh *et al.* (2012). The oligomeric status of *Phormidium* PE in solution was estimated by loading it onto a Superdex 200 10/300 GL column pre-equilibrated with buffer consisting of 10 mM Tris–HCl pH 8.0, 100 mM NaCl. The Superdex 200 column was calibrated with molecular-mass markers (carbonic anhydrase, 29 kDa; ovalbumin, 44.3 kDa; bovine serum albumin, 67 kDa; apoferritin, 440 kDa; bovine thyroglobulin, 669 kDa). The oligomeric status of the PE was estimated by comparing its elution profile with those of the molecular-mass markers and using the experimental molecular masses of the α and β subunits determined by MALDI–TOF.

2.2. Sequence determination

The genes for the *Phormidium* PE α and β subunits (*cpeA* and *cpeB*, respectively) were amplified by polymerase chain reaction (Bio-Rad iCycler version 4.006, Bio-Rad, California, USA) using the sets of primers listed in Supplementary Table S1. The primers were designed on the basis of the conserved

nucleotide sequences of the *cpeA* and *cpeB* genes available in the UniProt database and the peptide sequence deduced from the electron-density maps during the crystal structure analysis. Amplicons of ~ 0.5 kb were sequenced with an 3730xl DNA Analyzer using BigDye Terminator v3.1 sequencing chemistry (Applied Biosystems, Foster City, California, USA). Partial sequences of *cpeA* and *cpeB* were submitted to the European Nucleotide Archive (ENA), EMBL–EBI with the accession numbers LN827613 and LN827614, respectively.

2.3. Crystallization of PE

Crystallization of *Phormidium* PE (9.2 mg ml^{-1}) was attempted using formulated crystallization screens, including the PEG and Cryo Kit crystallization screens from Sigma, with the sitting-drop vapour-diffusion method. The protein yielded small crystals in a number of conditions containing PEG 6000 (16–20%) at a pH of about 8.0 from the PEG screen, as well as with a condition consisting of 1.5 M ammonium sulfate, 0.075 M Tris–HCl pH 8.5, 25%(w/v) glycerol from the Cryo Kit crystallization screen (reagent A4). Diffraction-quality crystals of the protein were obtained using Intelli-Plate (Art Robbins Instruments) crystallization plates and an optimized condition consisting of 1.25 M ammonium sulfate, 0.075 M Tris–HCl pH 8.5, 20%(w/v) glycerol at 298 K (Table 1). The crystals grew to dimensions of about $200 \times 200 \times 60 \text{ }\mu\text{m}$ in 15 d.

2.4. Data collection and processing

For diffraction experiments at 100 K, crystals were flash-cooled in liquid-nitrogen gas. As shown in Table 2, the crystals belonged to space group *P1* and diffracted to about $2.8 \text{ }\text{\AA}$

Figure 5
Photograph of *Phormidium* PE crystals.

Figure 6
Validation of crystal symmetry. The unmerged intensity data processed by *MOSFLM* and *autoPROC* do not show the presence of a nonprimitive lattice or rotational symmetry. The observed intensities in the *hk1* zone are shown.

resolution using a fine-focus laboratory X-ray source (Agilent Supernova). The crystals diffracted to a higher resolution (2.1 Å) on the protein crystallography beamline (PX-BL21) at the Indus-2 synchrotron source, where data were acquired at a wavelength of 0.97947 Å and were recorded using a MAR CCD detector. A total of 189 frames of 1.0° oscillation were collected with an exposure time of 30 s per frame. The data were processed using *MOSFLM* and *XDS/autoPROC* (Leslie & Powell, 2007; Vonrhein *et al.*, 2011). The initial phases for *Phormidium* PE crystals were obtained by the molecular-replacement method using *Phaser* (McCoy *et al.*, 2007) with a truncated model of an $\alpha\beta$ monomer of PE from the cyanobacterium *G. violaceus* (PDB entry 2vjh) as a search model. In the truncated model (poly-Ala model) all side-chain atoms beyond the C β atom, solvent and chromophore atoms were removed from the coordinate list. The initial phases from the molecular-replacement solution were reasonably accurate such that electron density for most of the side-chain atoms and covalently bound chromophores was clearly visible in the electron-density maps.

3. Results and discussion

3.1. Purification and characterization of *Phormidium* PE

The PE protein was purified to homogeneity from exponentially growing cell mass of *Phormidium* sp. A09DM. The SDS-PAGE profile of purified PE showed only two bands,

which corresponded to the α and β subunits, and also suggested the absence of any linker peptide in the purified PE complex (Fig. 1). The emission of orange fluorescence by identical bands upon UV illumination in zinc acetate-stained SDS-PAGE indicated the presence of chromophore(s) in both subunits (Fig. 1).

Two peaks at 18 891 and 21 173 Da were observed in MALDI-TOF experiments that corresponded to the α and β subunits of *Phormidium* PE (Supplementary Fig. S1). The molecular mass of the *Phormidium* PE oligomer was estimated to be 125 kDa based on its elution profile from a Superdex 200 molecular-sieve column (Fig. 2). With an observed molecular mass of 40 064 Da for an $\alpha\beta$ monomer, *Phormidium* PE is expected to form a trimer in low-salt buffer consisting of 10 mM Tris-HCl pH 8.0, 100 mM sodium chloride.

Phormidium PE shows an emission band centred at around 581 nm upon excitation at 545 nm (Fig. 3). The fluorescence emission spectrum of *Phormidium* PE indicates the presence of PEB chromophores in the purified PE protein. The absorption spectrum of PE showed one dominant peak at 564 nm along with a shoulder peak at ~540 nm (Fig. 3). These bands are owing to absorption by two types of PEB chromophores, which differ owing to a deviation from planarity of the conjugated tetrapyrrole (A–B) rings (Gaigalas *et al.*, 2006). The absence of an absorption band at ~490 nm also suggests that the *Phormidium* protein does not contain a phycourobilin (PUB) chromophore, unlike the only other available cyano-

Figure 7

Validation of the molecular-replacement solution. The fit of the atomic model (sticks) of a fragment of the α -subunit (F- α PE) and the PEB chromophore (red sticks) into the electron-density maps computed using molecular-replacement phases and $2mF_o - DF_c$ coefficients (cyan contours drawn at the 1σ level) or $mF_o - DF_c$ coefficients (ΔF map; red contours drawn at the 3σ level) is shown in stereo. The structure of the F- α PE fragment (PDB entry 3mwn; Soni *et al.*, 2010) was superposed onto the poly-Ala model resulting from molecular-replacement calculations. The coordinates of PEB were derived by superposition of the 2vjh structure onto the poly-Ala model. The electron density for the side-chain atoms of most of the residues of the F- α PE fragment and the chromophores is visible for all 12 of the $\alpha\beta$ monomers, although these atoms were not included in phase calculations. The S atoms of the Cys82 and Met90 residues are visible at a 3σ contour level in the ΔF map. This figure was prepared using the *Chimera* suite (Pettersen *et al.*, 2004).

bacterial PE structure from *G. violaceus*. Of the available structures, only *Porphyridium cruentum* PE harbours five PEB chromophores.

3.2. Sequence determination and analysis

Phormidium PE is made up of α and β subunits of 164 and 184 amino-acid residues, respectively. The amino-acid sequences of the α and β subunits show ~69% identity to cyanobacterial and red algal PE proteins of known structure. The theoretical molecular masses of both the α and β subunits, determined from the deduced amino-acid sequences, matched the MALDI-TOF results well. We surprisingly found an insertion of nine amino-acid residues (151-AGGKLRMMG) and a deletion of two residues at position 19 in the β subunit of *Phormidium* PE during multiple sequence alignment with PE sequences for which structures have been reported in the PDB. Also interestingly, several of the conserved chromophore-contact residues of the *P. cruentum* PE protein were substituted (Fig. 4) in the β subunit of *Phormidium* PE. This indeed suggests divergence of the *Phormidium* PE protein from other known structures.

3.3. Crystallization, X-ray diffraction and structural characterization of PE

The protein crystals grown using ammonium sulfate precipitant with apparent hexagonal morphology (Fig. 5) belonged to space group $P1$, with unit-cell parameters $a = 108.3$, $b = 108.4$, $c = 116.6$ Å, $\alpha = 78.94$, $\beta = 82.50$, $\gamma = 60.34^\circ$. Three-dimensional diffraction intensity data from the crystals were acquired on the PX-BL21 beamline at Indus-2. The space group for the unmerged data processed by both *MOSFLM* and *autoPROC* was confirmed using the *POINTLESS* utility in *CCP4* (Evans, 2011; Winn *et al.*, 2011) with a confidence value of 1.0, as well as by visual examination of the symmetry of the diffraction pattern (Fig. 6). Data statistics are given in Table 2. Based on the observed molecular mass of 40 064 Da for the $\alpha\beta$ monomer and the volume of the asymmetric unit, Matthews parameter (Matthews, 1968) values of 2.65, 2.42, 2.24 and 2.08 Å³ Da⁻¹ corresponding to 11, 12, 13 and 14 copies of the $\alpha\beta$ monomer in the asymmetric unit of the triclinic cell, respectively, are within the acceptable range.

The normalized probabilities (P_{tot}) for 11, 12, 13 and 14 copies of the $\alpha\beta$ monomer in the triclinic cell were estimated to be 0.16, 0.21, 0.22 and 0.15, respectively. Molecular-

Figure 8

(a) Cartoon of the *Phormidium* PE hexamer shown in stereo. The α and β subunits of an $\alpha\beta$ monomer are shown in different shades of the same colour. This figure was prepared using the *Chimera* suite. (b) Arrangement of hexamers of $\alpha\beta$ monomers in the $P1$ cell. The alternate layers of the hexamers are displaced with respect to each other such that the hexamers do not form rods in the crystals. This figure was prepared by generating $2 \times 2 \times 2$ unit cells using the *Chimera* suite. The two hexamers of each unit cell are shown in the same colour.

replacement calculations using *Phaser* yielded a unique solution with 12 copies of the $\alpha\beta$ monomer in the *P1* cell. The poly-Ala model, without the side-chain atoms, chromophores and solvent molecules, shows a disagreement value (R_{work}) of about 0.46 against all of the observed data. Molecular-replacement phases are sufficiently accurate to show electron density for the side chains and covalently bound chromophores (Fig. 7). The *Phormidium* PE protein exists as a hexamer (Fig. 8a) of $\alpha\beta$ monomers, and two hexamers are found in the crystal structure. The hexameric status of PE oligomers is also observed in the PE structures from a cyanobacterium and various algae (PDB entries 2vjh, 3v57, 1b8d, 1lia and 1eyx). The hexamers of algal PE interact to form higher-order rod-like structures. In contrast, alternate layers of hexamers of the *Phormidium* protein are displaced with respect to each other such that they do not form rods (Fig. 8b). The *Phormidium* PE structure is thus likely to represent an interesting variant of PE compared with other known structures.

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