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Structure of the novel 14 kDa fragment of α-subunit of phycoerythrin from the starving cyanobacterium *Phormidium tenue*

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ABSTRACT

The rod-like phycobilisome (PBS) in cyanobacterium is the light-harvesting complex of phycoerythrin (PE), phycocyanin (PC) and allophycocyanin (APC). The orderly degradation of PBS was observed under starvation conditions. A 14 kDa truncated fragment of α-subunit of PE (F-αPE) was identified from the degraded product. F-αPE was purified to homogeneity, sequenced and crystallized. The merohedrally twinned crystals with a twinning factor of approximately 0.5 were obtained. The crystal structure of F-αPE was determined with molecular replacement method using detwinned data and refined to an R_{cryst} factor of 23.2% (R_{free} = 27.6%). The structure consisted of two crystallographically independent molecules in the asymmetric unit. The two molecules were designated as molecules A and B with a buried area of 200 Å^2 at the interface. The structure of F-αPE consists of seven α-helices A, B, E, F, F′, G and H. The first 31 N-terminal residues that fold into parallel α-helices X and Y in other PEs are not present in the amino acid sequence of F-αPE. Both molecules, A and B contain two chromophore ligands, PEB1 and PEB2 in each. These are covalently linked to the polypeptide chain through Cys82 and Cys139, respectively. The superimposition of C^{α} tracings of molecules A and B shows an r.m.s. shift of 1.0 Å indicating that the structures of two independent molecules are very similar. The degradation of phycobilisome proteins under starvation stress seems to occur to supplement the requirement of amino acids for protein synthesis and to reduce the absorption of light energy.

1. Introduction

The light-harvesting system in cyanobacterium is formed by the rod-like phycobilisomes (PBS). This blue-colored multiprotein assembly is arranged in a certain order where phycoerythrin (PE) is located at the tip of the rod, while phycocyanin (PC) is situated in the middle and allophycocyanin (APC) is placed at the core which is attached to the thylakoid membrane (Glazer, 1982). The purpose of this assembly is to increase the efficiency of photosynthetic organism growing in low light regions (MacColl, 1998). Both APC and PC have covalently bound phycocyanobilin (PCB) prosthetic groups while PE has covalently attached phycoerythrobilin (PEB) groups whereas in the case of red algal phycocyanin (R-PC), found in certain red algae, both PCB and PEB chromophores are found (Wedemayer et al., 1992). The energy transfer in the organism proceeds successively in the direction from PE → PC → APC → chlorophyll *a*, with an overall efficiency of almost 100% (Gantt and Lipschultz, 1973). All the known phycobiliproteins are composed of two different α- and β-subunits. These are further arranged in the form of dimers of trimers $(\alpha\beta)_3$ forming a hexamer, $(\alpha\beta)_6$. These trimers and hexamers form discs and assemble themselves into rod-like organization (Glazer and Hixson, 1977). Although these essential multiprotein assemblies form a stable arrangement, they undergo degradation when the organisms are starved for nutrition (Lichtlé et al., 1996). The process of degradation can be observed visually due to a change in the color from blue-green to yellow-green. However, the molecular basis of degradation and the role of surviving polypeptides is unclear. Presumably, degradation occurs to reduce the absorption of excess light energy under the stress conditions and also to provide amino acids to sustain the limited protein synthesis. In order to characterize the surviving polypeptides for gaining insights into the nature of molecular assembly and the mode of association and possibly the role of the polypeptide fragment of αPE during the state of starvation, the crystal structure of F-αPE has been determined.

Abbreviations: APC, allophycocyanin; C-PE, cyanobacterial-phycoerythrin; PEB, phycoerythrobilin; R-PC, red algae phycoerythrin; C-PC, cyanobacterial-phycocyanin.

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The amino acid sequences of PEs from a number of species are known (Broughton et al., 2006; Ting et al., 2001; Zhang et al., 2006). PE consists of two subunits, α and β. The α-subunit is comprised of 164 amino acid residues while β-subunit contains 177 amino acid residues corresponding to molecular weights of approximately 19 and 20 kDa, respectively. The sequence identities between α- and β-subunits is of the order of 20% in PEs from different species (Chang et al., 1996; Contreras-Martel et al., 2001) while α- and β-subunits share the sequence identities of 65% and above with the corresponding subunits of PEs from various species (Chang et al., 1996; Contreras-Martel et al., 2001). The crystal structures of phycoerythrin from a few species, B-phycoerythrin from *Griffithsia monilis* (Ritter et al., 1999), R-PE from *Gracilaria* (Contreras-Martel et al., 2001), and *Polysiphonia urceolata* (Chang et al., 1996), cryptophyte phycoerythrin from *Rhodomonas* sp. CS24 and cryptophyte phycoerythrin 545 from *Rhodomonas* sp. CS24 (Doust et al., 2004; Wilk et al., 1999) are already known. Out of these the polypeptide chains of phycoerythrin from cyanobacterium and algal phycoerythrin show high sequence identity (≈85%). The basic structural unit of the most of phycoerythrins is a heterodimer of α- and β-subunits (Glazer, 1979; Glazer and Hixson, 1977). In the intact assembly, an additional polypeptide of variable molecular mass is also present (γ-polypeptide) which functions as a linker protein (Huber, 1989). In the case of R-PE and B-PE, this linker protein (γ-polypeptide) is pigmented, has a molecular mass of about 30 kDa, and is tightly bound to α- and β-subunits. This yields a stoichiometry of $(\alpha\beta)_6\gamma$ which corresponds to a molecular mass of about 240 kDa.

All PBPs carry different numbers of chromophores which are open-chain tetrapyrroles, linked via thioether bonds to cysteine residues and classified by spectral differences as caused by PEB, PCB, PXB and PUB. The structure of PEB released from PEs has been determined (Chapman et al., 1968) and confirmed by synthesis (Engel et al., 1991). The structure of polypeptide-bound PEB has also been determined for the prosthetic groups of B-PE (Lundell et al., 1984). Of the five PEB groups linked to the α- and β-subunits of PBP, four of them attached to the polypeptide through a single thioether bond from a cysteinyl residue to ring A of the bilin, and the fifth PEB is attached through two such thioester bonds to rings A and D (Schoenleber et al., 1984).

The order and arrangement of PBPs in PBS is known (Conley et al., 1986) although structure of the intact assembly is not yet determined. Therefore, the exact molecular associations and mechanisms of energy transfer through the assembly are not yet fully understood. As observed, under normal conditions of nutrition, the system thrives and PBS works normally. However, under the stress conditions PBS assembly breaks down and the proteins are degraded. Though some reports are available that describe the phycobilisome degradation in nutrient deprived conditions (Collier and Grossman, 1994), but the mechanism and the mode of degradation are not known. We report here a detailed structure analysis of a surviving non-linker protein which is a fragment of αPE containing residues from 32 to 164 with a molecular weight of 14 kDa. This fragment was isolated from the crude mass obtained from the cultures under the starving state. The 14 kDa protein was also obtained from the stored samples of 19 kDa protein of C-PE.

2. Materials and methods

2.1. Isolation and purification

The strains of blue-green algae *Phormidium tenue* were collected from sea weeds. The weeds were cultivated in artificial seawater nutrients (ASN III) medium in a photoperiod of 12 h irradiation and 12 h darkness under 36 W white fluorescent lamps (130 μmol photon $m^{-2}s^{-1}$) at 27 ± 2 °C and purged with 0.22 μ filter sterilized air. The exponentially growing cyanobacterial cells at 15 days growth, after 60 days when growth retarted and after 90 days when growth completely stopped were harvested by centrifugation at 3000*g* for 15 min (Eppendorf, Hamburg, Germany, rotor F-34-6-38 07168) at 20 °C. The cell pellets were washed with 20 mM Tris–HCl buffer, pH 8.1. One volume from each sample of washed cell mass was resuspended in five volumes of the same buffer and subjected to repeated freeze–thaw cycles from −25 to 4 °C temperature shocks for the release of PBPs. The cell debris were removed by centrifugation at 17,000*g* for 10 min. The supernatants were pooled and labeled as crude extracts. The entire purification procedure was carried out in dark at 4 °C unless specified. All buffers and solutions were prepared in mili-Q water supplemented with 0.01% sodium azide. These extracts were precipitated with 80% ammonium sulfate. The precipitant was dissolved in 10 mM Tris–HCl, pH 8.1, and subsequently dialyzed against same buffer. All the three samples obtained from (i) 15 days growth (ii) when growth retarded after 60 days and (iii) when growth stopped after 90 days were preserved at 4 °C. These three samples were examined on sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE) for protein contents (Fig. 1). PE with two bands for α- and β-subunits as indicated in lane (b) in Fig. 1 was further purified. This sample was loaded on an ion exchange column (40 × 12 mm) packed wih DEAE-cellulose. It was eluted with 0.2 M NaCl. The eluents was collected and subjected to gel permeation chromatography on Sephadex G-50 (350 × 10 mm). The samples containing PE fractions were collected and the purity of protein was determined with SDS–PAGE. Similarly, the samples from lanes c and d of Fig. 1 were also used for the purification of the truncated 14 kDa fragment of PE (F-αPE). These were also loaded on the separate ion exchange columns (40 × 12 mm) packed wih DEAE-cellulose. Both samples were eluted with 0.2 M NaCl. The eluents were collected and subjected to gel permeation chromatography on Sephadex G-25 (350 × 10 mm). The samples containing F-αPE fractions were collected and the purities were determined with SDS–PAGE. When the isolated purified PE with two bands (Fig. 2A, lane b) was stored for a month under normal buffering conditions, an additional band was observed at 14 kDa (Fig. 2A, lane c). However, when it was allowed to stay in the same conditions for more than two months, only one band was observed at 14 kDa (Fig. 2A, lane d). On the other hand, when the glacial acetic acid was added for adjusting the pH of the solution of PE to 2.5, it did not suffer degradation and original two bands of 19 and 20 kDa were observed even after 4 months (Fig. 2B). All the steps were carried out at 4 °C in dark to protect the protein samples from denaturation. Both proteins PE and F-αPE were identified by determining their N-terminal sequences for the first 15 residues.

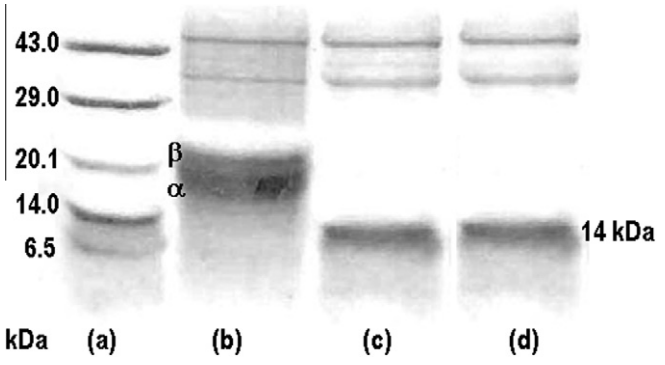

Fig. 1. The SDS–PAGE showing (a) molecular weight markers, (b) proteins from the standard growth condition under normal nutritional inputs, (c) at the time when growth stops under starvation condition and (d) under severe starvation condition.

2.2. Sequence determination

The sequence of the first 15 amino acid residues of F-αPE was determined using the protein sequencer PPSQ20 (Shimadzu, Japan). In order to obtain the complete amino acid sequence information of the 14 kDa polypeptide chain obtained from the samples prepared under the starvation conditions, the total RNA was extracted from *Phormidium tenue* by the phenol/chloroform method (Chomczynski and Sacchi, 1987). The poly (A^+) mRNAs were isolated from the total RNA using an oligo (dT) cellulose column (Amersham Pharmacia Biotech, Piscataway, USA). The small syringe column packed with oligo (dT) cellulose was washed with 10 ml of high salt buffer (1 M NaCl), 1 mM Na_2–EDTA, 40 mM Tris–HCl. The total RNA was mixed with an equal volume of salt buffer and warmed to 65 °C and cooled immediately by placing it in the ice. The chilled RNA was passed through the column packed with oligo (dT) cellulose. The column was washed with 3 ml of low salt buffer (0.1 M NaCl, 1 mM Na_2–EDTA). The reaction with Moloney Murine Leukemia Virus (MMLV) – reverse transcriptase polymerase chain reaction (RT-PCR) was used for amplification of the gene. The conserved nucleotide sequences from other PBPs and the N-terminal sequence of F-αPE were used for the design of primers. The degenerate primers were used to amplify the gene. The PCRs were carried out with Taq polymerase (Promega, Medicine, USA) using an MJ Research thermal cycler model PTC-100. The complete nucleotide sequence determination was carried out on the cloned double-stranded DNA (pGEM-T) using automatic sequencer model ABI-377. The complete nucleotide and deduced amino acid sequences have been deposited in the gene bank with an Accession No. EF414443.

2.3. Dynamic light scattering measurements

In order to determine the size of the protein molecule as well as the state of oligomerization, the samples of 14 kDa protein were prepared in the same buffers as that used for crystallization. Three dilutions of 0.1 mg/ml, 0.5 mg/ml and 1 mg/ml were used for the measurements. All the three samples were separately filled into three microcuvettes. The measurements were made using the instrument SpectroScatter – 201 (RiNA GmbH, Berlin, Germany). The values of hydrodynamic radii were obtained for all the three samples.

2.4. Crystallization

The purified samples of the fragment of α-subunit (F-αPE) were used for crystallization with hanging drop vapor diffusion method. The single drop contained 10 μl of protein with 100 mM sodium cacodylate buffer, pH 6.0. It was equilibrated against the reservoir solution containing 100 mM sodium cocodylate buffer, pH 6.0, and 15% PEG-4000. Small crystals in the form of clusters were obtained. These were transferred to the sitting drop vapor diffusion set up with identical conditions. The crystals grew to dimensions of $0.4 \times 0.3 \times 0.3\ mm^3$ and were found suitable for X-ray intensity data collection.

2.5. X-ray intensity data collection and processing

A suitable crystal measuring $0.4 \times 0.3 \times 0.3\ mm^3$ was used for data collection at 278 K. The intensities were measured using a 345 mm diameter MAR research dtb Imaging plate scanner mounted on a Rigaku RU-300 rotating anode X-ray generator operating at 50 kV and 100 mA. The Osmic blue confocal optics was used for focusing CuKα radiation. The crystal diffracted to 2.6 Å resolution. An examination of diffraction data indicated that the crystals were twinned. Therefore, data were required to be indexed in two space groups, orthorhombic space group P222 and monoclinic space group $P2_1$ and scaled separately using the programs DENZO and SCALEPACK (Otwinowski and Minor, 1994). Both data sets were analyzed using CCP4 programs (1994). Based on the cumulative intensity distribution ⟨E⟩, ⟨E3⟩ and ⟨E4⟩, Stanley Factor $\langle I2\rangle/\langle I\rangle^2$ and Wilson's diffraction distribution (Stokes and Wilson, 1944), the crystals were found to be pseudomerohedrally twinned with $\beta \approx 90°$. The unit cell parameters were found to be $a = 57.3$ Å, $b = 83.7$, $c = 62.5$ and $\beta = 90.2°$. The twinning fraction, α was estimated to be 0.49. The final data collection statistics is given in Table 1.

2.6. Structure determination and refinement

The structure was determined by molecular replacement method using the program AMoRe (Navaza, 1994) from CCP4 suite (1994). The atomic coordinates of the corresponding fragment of R-PE (Ducret et al., 1994) (PDB code: 1LIA) were used as the search model. The rotation function was calculated using diffraction data in the resolution range of 10.0–4.0 Å with a *Patterson* radius of 14 Å. The solution gave two distinct peaks indicating the presence of two molecules in the asymmetric unit in the space group $P2_1$. The structure was initially refined using intensities (Chandra et al., 1999) in CNS (Brunger et al., 1998) with twinned data which yielded the R_{cryst} and R_{free} factors of 0.335 and 0.385, respectively. The twinning fraction was estimated from the statistical comparison of twinning-related reflections (Kerfeld et al., 1997) which gave a twinning factor, α of 0.49. The inclusion of twinning fraction using twinning operator (h, $-k$, $-l$) and NCS restraints, the refinement was carried out using SHELXL (Uson et al., 1997) with TWIN and BASF options. In order to calculate R_{free} factor, the test reflections were selected in thin resolution shells. The refinement calculations were interleaved with several rounds of manual model

Table 1
Data collection and refinement statistics of C-phycoerythrin from *Phormidium tenue.*

PDB accession number	3MWN
Space group	$P2_1$
Resolution range (Å)	20.0–2.6
Unit cell dimensions(Å)	
a	57.3
b	83.7
c	62.5
β (°)	90.2
V_m ($Å^3$/Da)	5.1
Solvent content (%)	73.0
Number of molecules in the asymmetric unit	
Number of reflections collected	1,20,456
Number of unique reflections	18,133
Overall completeness (%)	85.0 (92.1)
Overall R_{sym} (%)	13.6 (55.0)
Overall I/σ	5.0 (2.0)
R_{Cryst} (for all data) (%)	23.1
R_{free} (5.0% data) (%)	27.8
Refined twinning fraction	0.49
Protein atoms	989
Chromophore atoms 4(43)	172
Water molecules	52
R.m.s.d. in bond lengths (Å)[a]	.02
R.m.s.d. in bond angles (°)	1.8
R.m.s. deviations in dihedral angles (°)	20.6
From Wilson plot	36.0
Average B factor for main-chain atoms ($Å^2$)	29.2
Average B factor for side-chain atoms and waters ($Å^2$)	35.3
Average B factor for all atoms ($Å^2$)	33.5
Residues in the most favored regions (%)	84.1
Residues in the additionally allowed regions (%)	14.2
Residues in the generously allowed regions (%)	1.7

Note: Values in parentheses are for the outermost resolution shell.
[a] R.m.s.d., root mean square deviation.

building with program 'O' (Jones et al., 1991). The structure was refined with conjugate gradient least squares refinement (CGLS) using SHELX that yielded the R_{cryst} and R_{free} factors of 0.281 and 0.334, respectively. The difference electron density $|F_o - F_c|$ Fourier map computed at this stage indicated the presence of two chromophores covalently linked to Cys82 and Cys139 in both molecules. The chromophores were modeled in the densities and were included in further cycles of CGLS refinements. The R_{cryst} and R_{free} factor, reduced further to 0.244 and 0.301. The omit map was calculated at this stage and 52 water molecules were added using $|2F_o - F_c|$ Fourier and $|F_o - F_c|$ difference Fourier maps. Further CGLS refinements were carried out using SWAT option which yielded final R_{crys} and R_{free} factors of 0.238 and 0.283, respectively, while the twinning fraction was $\alpha = 0.49$. The overall refinement statistics is summarized in the Table 1. The atomic coordinates for the refined structure of F-αPE have been deposited in the Protein Data Bank with an entry code of 3MWN.

3. Results

3.1. Effect of nutritional starvation

As seen from Fig. 1 (lane b), the predominant protein content corresponds to 19 and 20 kDa subunits of PE. However, under severe stress of nutritional starvation (Fig 1, lanes c and d), the phycoerythrin is reduced to a 14 kDa single band which has been identified as a truncated α-subunit of PE consisting of amino acid residues from 32 to 164. Although it was not clear as to what caused this specific degradation but there is a report that α-subunit is associated with the process of a systematic degradation (Baier et al., 2004). The degradation of the stored purified samples of PE containing α- and β-subunits to F-αPE of 14 kDa was also observed (Fig. 2A). It is however, intriguing to note that the α-subunit is degraded to a 14 kDa fragment while the β-subunit is completely digested (Fig. 2A). On the other hand, when glacial acetic acid was added to the purified samples of PE, no degradation was observed (Fig. 2B). These results are interesting but there are still no clear answers to many questions. It is noteworthy that the starvation-induced degradation product of F-αPE is a properly folded globular structure with two bound phycoerythrobilins, PEB1 and PEB2 as observed in the full length αPE subunit. Since phycoerythrobilins are normally bound to F-αPE, it may still have activity pertaining to the absorption of light energy. It appears that by retaining the minimum functional fragment of αPE with bound PEBs, the system tries to survive on the residual activity for as long as possible. The degradation of the phycobiliproteins appears to be a desperate process for making amino acids available for the required protein synthesis.

3.2. Sequence analysis

The N-terminal sequence of the F-αPE was determined for identifying the correct N-terminal residue. The first 15 amino acid residues, obtained using automated Edman degradation were Gln-Arg-Ala-Ala-Ala-Arg-Leu-Glu-Ala-Ala-Glu-Lys-Leu-Gly-Ser. The

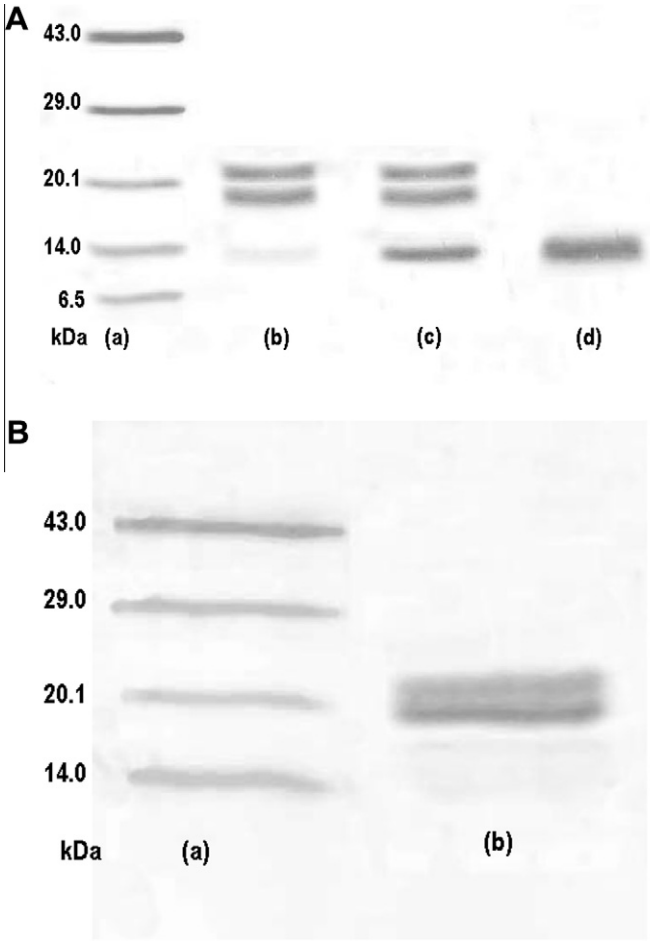

Fig. 2. (A) The SDS–PAGE for (a) molecular weight markers, (b) Two bands of purified PE at 19 and 20 kDa, (c) protein bands after storage for two months and (d) protein bands after storage for 4 months. (B) The SDS–PAGE showing (a) molecular weight markers and (b) after 4 months of storage in glacial acetic acid.

TremBl-BLAST search showed an exact match with α-subunit of cyanobacterial PE starting from residue, Gln32. It showed that the first 31 amino acid residues of αPE were absent in F-αPE. The full cDNA sequence of F-αPE has been determined (Accession No. EF414443). It was compared with the sequences of α PE from other sources (Apt et al., 1995; Contreras-Martel et al., 2001; Ducret et al., 1994; Hu et al., 1999; Jiang et al., 2001; Swanson et al., 1991; Ting et al., 2001; Wilk et al., 1999; Zhang et al., 2006). The PEs generally contain 164 amino acid residues. The present structure represents a truncated α-subunit with 133 amino acid residues including three cysteines, Cys59, Cys82 and Cys139 out of which two cysteine residues, Cys82 and Cys139 are involved in covalent linkages with chromophores phycoerythrobilins, PEB1 and PEB2, respectively. The side-chain of the third cysteine residue, Cys59 is free. F-αPE showed a sequence identity of more than 60% with the corresponding segments of PEs from other species (Fig. 3A). The residues that interact with ligands PEB1 and PEB2 are conserved in all these segments.

The amino acid sequence of F-αPE was also aligned with the corresponding fragments of α-subunits of R-PEs whose crystal structures are known (Fig. 3B). It shows high sequence identities ranging from 75% to 80%. The most important residues in the dimerization of α- and β-subunits are Asp13 and Arg84. These two residues are conserved in both α- and β-subunits. These residues of α-subunit form strong ionic interactions with those of β-subunit which impart considerable stability to the α–β dimer. Since Asp13 is not present in F-αPE, it is unable to form a stable α–β dimer. Furthermore, aspartic acid residue at 13th position also plays a role in preventing β–β homodimerization by destabilizing the interaction of the N termini of identical β-subunits. The positive charge at the N-terminus of the α-subunits may similarly be unfavorable for the α–α association. The additional unique residues that were found in each subunit may probably be responsible for assuring the correct subunit assembly. Most of the protein residues that interact with chromophores PEB1 and PEB2 are conserved in phycoerythrins (Fig. 3B). However, out of two cysteines to which chromophores are attached, only Cys82 is conserved while Cys139 is not very conserved (Jiang et al., 1999).

3.3. Final model

Final model of F-αPE contains 989 protein atoms from polypeptide chains of two crystallographically independent molecules A and B. There are 172 atoms of chromophores, PEB1 and PEB2 and 52 water molecules. Inspite of a high solvent content of 73% in the crystals, the overall mean B factor has a reasonable value of

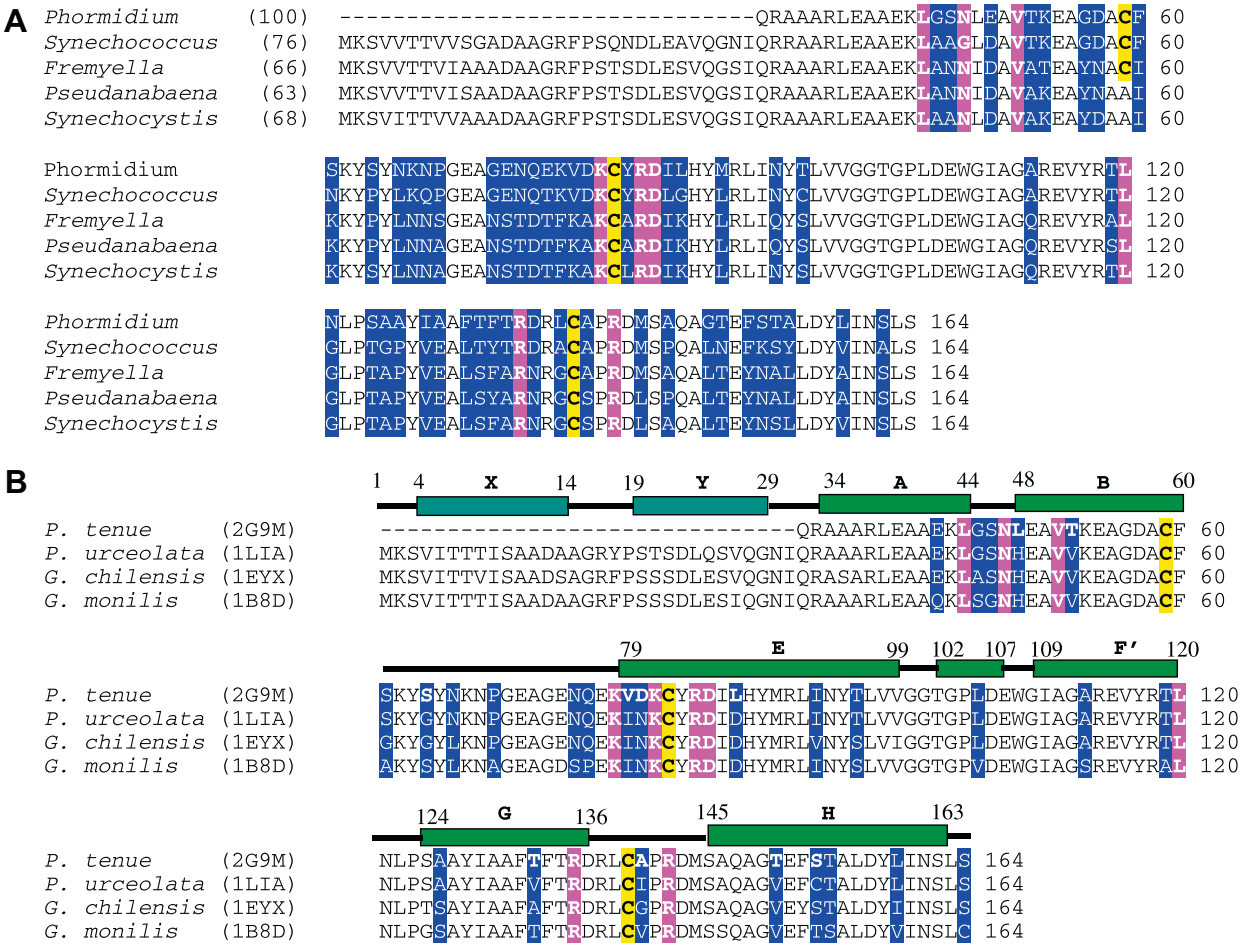

Fig. 3. (A) Sequence comparison of F-αPE from *Phormidium tenue* with those from other species such as *Synechococcus* sp. (WH7803), *Fremyella diplosiphon*, *Pseuoanabaena* sp. (PCC7409) and *Synechococystis* sp. (PCC6701). The sequence identities are given in parentheses. The residues that are different were indicated in blue color. Cysteines linking the chromophores covalently to the protein are shown in yellow color and the residues involved in non-covalent interactions with chromophores PEB1 and PEB2 are shown in red and green colors, respectively. (B) The sequence comparison of F-αPE from *Phormidium tenue* with R-αPEs from *Polysiphonia urceolata* (1LIA), *Gracilaria chilensis* (1EYX) and *Griffithsia monilis* (1B8D). The PDB codes are given in parentheses. The cylinders indicate helices and the bold lines represent loops. The first 32 residues are absent in F-αPE. The differences are shown in blue. The cysteines linked covalently with chromophores are in yellow color. The hydrogen bonded residues to chromophore are shown in red.

34.2 Å^2. The quality of electron density is generally excellent except the side-chains of a few residues in the loop regions. A Ramachandran plot of the main chain torsion angles (Φ, Ψ) (Ramachandran and Sasisekaran, 1968), as defined in the PROCHECK (Laskowski et al., 1993) shows that 82.8% of the residues fall in the most favored regions while only 2.2% of the residues belong to generously allowed regions of the map.

3.4. Overall molecular structure

The overall structural organization of F-αPE in the asymmetric unit is shown as ribbon diagrams in Fig. 4. The two structurally similar molecules, A and B are oriented with respect to each other at 165° along the long molecular axis. They have a short interface region which is stabilized by two ionic interactions and two hydrogen bonds together with a few van der Waals contacts. The conformations of molecules A and B are very similar with an r.m.s. shift of about 1.0 Å for the C^{α} atoms when the C^{α} tracings of molecules, A and B were superimposed (Fig. 5A). However, the superimpositions of ligands PEB1 and PEB2 from molecules A and B indicate a few interesting facts. The r.m.s. shifts when PEB1 of molecule A is superimposed on PEB1 of molecule B and that of PEB2 of molecule A on PEB2 of molecule B are of the order of 1.3 Å (Fig. 5B). Further, the superimpositions of PEB1 of F-αPE over PEB1 of *P. urceolata* R-PE and PEB2 of F-αPE over PEB2 of R-PE (*P. urceolata)* (Fig. 5C) gave significantly large deviations of propionate chains of rings A and D. The substituents of ring B also deviate considerably although the pyrrole rings are well superimposed. On superimpositions of PEB1 over PEB1 and PEB2 over PEB2 of molecules A and B (Fig. 5D), the side-chains of ring D and ring B deviate equally in PEB1. It is important to note that in the absence of first 31 residues, the truncated polypeptide chain of αPE does not form conventional dimmer with βPE. It is also noteworthy that F-αPE contains two PEB binding sites whereas other known PEs posses three PEB binding sites out of which two sites are identical whereas the third site is not present in cynobacterial αPE.

3.5. Binding of PEB1 and PEB2

The structure of F-αPE contains two covalently linked phycoerythrobilin molecules PEB1 and PEB2. PEB1 is covalently linked to the protein through Cys82 (Fig. 6A) while PEB2 (Fig. 6B) forms a covalent bond with Cys139. In addition to covalent linkages both PEB1 and PEB2 also form several other hydrogen bonds (Table 2). An examination of non-covalent interactions of PEB1 and PEB2 with protein molecule shows that PEB1 is held more tightly than PEB2 (Table 2). As seen from Fig. 4 PEB1 is more favorably placed in the binding pocket formed by helices E and F′. The similar binding characteristics were observed in the heme pocket of globins (de Sanctis et al., 2004). In contrast, PEB2 is found on the surface in the proximity of N-terminus and α-helices B and G. The PEB1 propionate groups of the central pyrrole rings A and D are connected through salt bridges with Arg84 and Lys78. The corresponding groups in PEB2 also form salt linkages with Arg142 and Arg137, respectively. It may be noted that Arg84, Arg137 and Arg142 are conserved in C-PE and R-PE across various species whereas Lys78 is not conserved among C-PEs from different species. It shows that the binding of PEB1 in C-PE is more similar to that of R-PE than those in F-αPE from other species. The nitrogen atoms of central pyrrole rings A and D of PEB1 are linked to Asp85 while the corresponding nitrogen atoms of PEB2 are not involved in such interactions because the corresponding Asp143 at that site is not oriented favorably with respect to the nitrogen atoms of two central pyrrole rings. Instead, Asp143O^1 interacts with Arg142N and Cys139N. Due to dissimilar binding environments, the planarity of pyrrole rings of PEB1 and PEB2 are significantly different in the two ligands. The pyrrole rings, A, B, C and D of PEB1 are considerably non-planar while in PEB2 only A and C rings are non-planar while the pyrrole rings B and D are essentially planar. A similar feature was observed in molecule B. The extensive interactions between protein and chromophores are facilitated by unique structural features in which α-helix, G (residues 125–143) breaks at residues 138–139 and then forms a single turn α-helix with residues 140–143 and

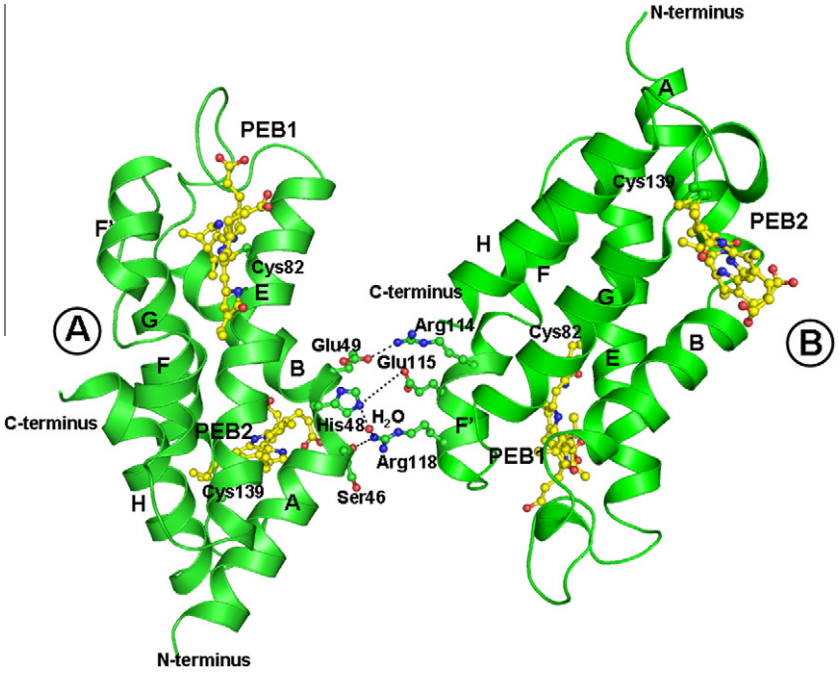

Fig. 4. Overall structure of the fragment F-αPE with two crystallographically independent molecules A and B drawn as a ribbon diagram using PYMOL. The dotted lines indicate ionic and hydrogen bonded interactions between molecules A and B. The covalently linked chromophores to protein via Cys82 and Cys139 are indicated in yellow color. The α-helices are named according to the standard scheme of nomenclature for phycobiliproteins.

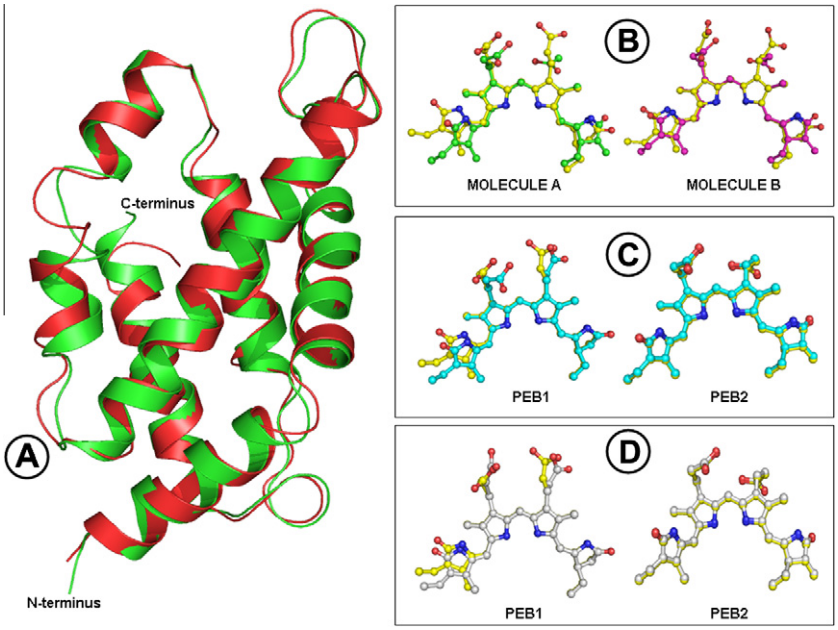

Fig. 5. (A) The superimposition of C^{α} traces of molecule A (green) on the C^{α} traces of molecule B (red) for F-αPE from *Phormidium tenue*. (B) The superimposition of PEB1 over PEB2 of molecule A and PEB1 over PEB2 of B, respectively. (C) The superimposition of PEB1 of F-αPE over PEB1 of *Polysiphonia urceolata* R-αPE and PEB2 of F-αPE over PEB2 of *Polysiphonia urceolata* R-αPE. (D) The superimposition of chromophores, PEB1 and PEB2 of molecule A on PEB1 and PEB2 of molecule B, respectively.

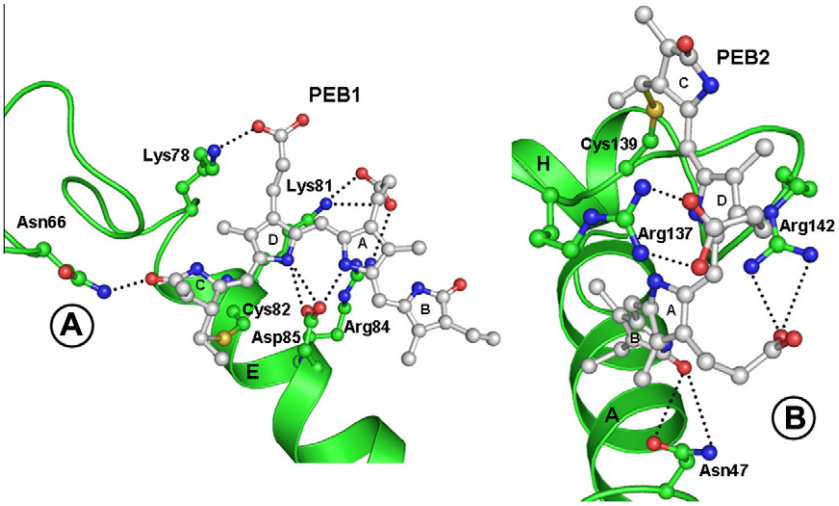

Fig. 6. Selected hydrogen bonded interactions between protein atoms and chromophore atoms: (A) PEB1 and (B) PEB2.

again breaks at residues 144–145 and then continues into next α-helix H (residues 146–163). Similarly, PEB1 is aligned in between helices E (residues 78–98) and F′ (residues 113–123) in such a way that residues Lys78, Lys81, Cys82, Arg84, Asp85 and His88 in helix E and residues Tyr117, Leu122 and Pro123 from helix F′ interact with PEB1 leading to a very stable binding of this chromophore.

3.6. Comparison with R-PE

The F-αPE from C-PE shows several interesting features when compared with the corresponding segments of various R-PEs (Chang et al., 1996; Doust et al., 2004; Ficner et al., 1992; Jiang et al., 1999; Ritter et al., 1999). This segment of R-PEs shows a sequence identity of more than 60% with F-αPE. The superposition of the C^{α} traces of the structure of F-αPE on the corresponding segment from the structure of R-PE (Jiang et al., 2001) shows an r.m.s shift of 0.9 Å (Fig. 7). Both structures are mainly α-helical consisting of seven similar helices named as A, B, E, F, F′, G and H. The helices X and Y are absent in this segment because they are part of the first 31 residues (Jiang et al., 2001).

4. Discussion

On the basis of sequence homology and structural similarities, it appears that PBPs might have evolved from the globin-like proteins (Pastore and Lesk, 1990). Furthermore, the specialization of α- and β-subunits was a critical step in the evolution of phycobiliproteins. The findings suggest that α-subunit evolved initially with 133 amino acid residues while the gene duplication subsequently gave rise to the polypeptide chain with 164 amino acid residues.

Table 2
Covalent and hydrogen bonded interactions of PEB1 and PEB2 with atoms of molecule A.

	Protein atoms	Distance (Å)
PEB 1		
CAC	Cys 82 S^{γ}	1.84
NA	Asp 85 $O^{\delta 1}$	3.25
	Asp 85 $O^{\delta 2}$	2.74
O1A	Arg 84 NH1	3.24
	Arg 84 NH2	3.27
O2A	Lys 81 N^{ζ}	2.57
OB	HOH119 O	2.99
HOH 119 O	Arg 84 NH2	2.67
ND	Asp 85 $O^{\delta 1}$	2.83
	Asp 85 $O^{\delta 2}$	3.25
O1D	Lys 78 N^{ζ}	2.61
O2D	Lys 78 N^{ζ}	3.08
PEB 2		
CAC	Cys 139 S^{γ}	2.07
O1A	Arg 142 NH2	2.84
O2A	Arg 142 NH2	3.26
O1D	Arg 137 NH2	2.90
O2D	Arg 137 NH1	2.93
OB	Asn 47 $O^{\delta 1}$	3.27
	Asn 47 $N^{\delta 2}$	3.08

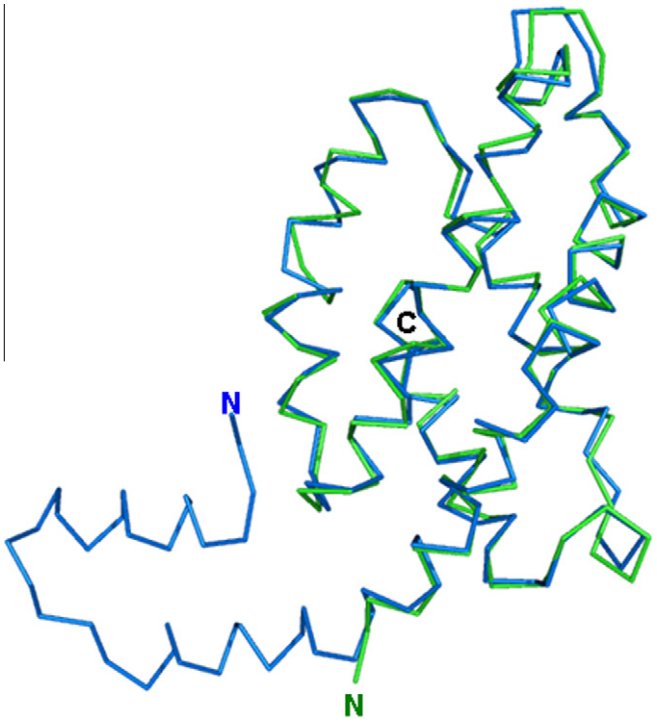

Fig. 7. The superimposition of C^{α} atoms of F-αPE from *Phormidium tenue* (green) on the C^{α} atoms R-αPE from *Polysiphonia urceolata* (blue). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

The loss of the first 31 residues from the N-terminus in αPE and the stable folding of the truncated chain with properly bound phycoerythrobilin ligands indicate that it may represent a viable functional unit for light harvesting although the association of subunits may not occur. Only when a high light through put energy transfer was required a gene duplication may produce the full length αPE for producing the dimeric structure with the help of a full length β-subunit. The first 31 residues are essential to make the dimer stable which may also improve the assembling of phycobilisome. An examination of inter subunit interactions involving α and β-subunits in R-PE revealed that out of 17 interactions that are observed between two subunits, 10 are contributed by the first 31 amino acid residues. It may also be noted that amino acid residues other than 31 N-terminal residues that are involved in dimerization are the ones which are least conserved in F-αPE and the corresponding fragment of R-PE (F-αRPE). In F-αRPE, there are a total of eight residues (Arg37, Asp89, Arg93, Asn96, Tyr97, Gly102, Thr104 and Asp108) which are involved in α–β interactions but only three (Arg37, Tyr97 and Gly102) are present in αC-PE. It indicates that C-PE is a relatively poor dimer than R-PE. It is also observed that the presence of only a few residues in the truncated α-subunit are able to induce a non-crystallographic molecular association between F-αPE molecules in the structure. However, the observed molecular association is relatively weak due to very few intermolecular contacts. In this regard, this is the first structure of a monomeric αPE with bound PEBs. This is also the first structure of a functionally viable truncated α-subunit of any phycobiliprotein. Both PEB1 and PEB2 are bound as in the native PEs with the distances between them are adequate (25 Å) for effective energy transfer. Furthermore, the observed non-planarity in the chromophores is compatible with the requirements for the energy absorption and transfer to reaction center in photosynthetic organism. Therefore, under the starvation conditions, the only source of energy available to the system is the truncated fragment of subunit αPE consisting of residues from 32 to 164.

The orderly protein degradation in the cynobacterial system under nutritional stress seems to occur for supplementing the requirements of amino acids for the synthesis of essential proteins for survival of the organism. It has been indicated that a small protein nbIA (7 kDa) is expressed during nutrient starvation and as the first step, it tags to the α-subunit of PE (Baier et al., 2004). Although the molecular mechanism of proteolytic action is not clear but our results suggest that the binding of nbIA to αPE might have provided an enzymatic capability to it because the purified PE when stored for a long period results in the production of 14 kDa fragment of αPE. Therefore, the events indicate that the proteolytic production of the truncated fragment F-αPE is meant for continuing the absorption of minimum light energy and the digestion of other proteins for producing amino acids for the bare minimum requirement of protein synthesis.

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