CHAPTER 4

Characterization of an intact phycoerythrin and its cleaved 14 kDa functional subunit from Phormidium sp. A27DM

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4.1 Introduction

Cyanobacteria harvest light energy for photosynthesis with macromolecular antennae complexes, termed phycobilisomes (PBS), studded as large particles with a regular array on the outer surface of the photosynthetic lamellae (Gingrich et al., 1982; Kahn et al., 1997). These light-harvesting complexes increase the efficiency of photosynthetic cyanobacteria growing in low-light regimes (Wilk et al., 1999). They act as antennae, capturing photons over a broad frequency spectrum (500 – 680 nm) and transferring energy to membrane-bound reaction centres (Wilk et al., 1999) containing chlorophyll a, the final acceptor of energy. Phycobilisomes consist of two types of proteins: the phycobiliproteins (PBP) and the linker polypeptides.

Phycobiliproteins are oligomeric proteins, assembled from chromophore-appended dissimilar polypeptide chains belonging to two families (α and β) probably originating from a common ancestor but apparently diverged in evolution (Klotz et al., 1986; Thomas and Passaquet, 1999). These two polypeptides are present in equimolar amounts (Bernard et al., 1992; Wilk et al., 1999). The major phycobiliproteins, based on their spectral properties, are phycoerythrin (PE, λmax – 540-570 nm), phycocyanin (PC, λmax – 610-620 nm) and allophycocyanin (APC, λmax – 650-655 nm) (Bernard et al., 1992). These diverse spectral properties are due to various bilins, linear tetrapyrroles, covalently linked to specific cysteinyl residues of the polypeptide chains by means of (one or less frequently two) thioether bonds (Thomas and Passaquet, 1999). Several linker polypeptides, each specifically associated with different phycobiliprotein complexes, contribute to maintain the physical integrity and the structure of the phycobilisomes and to optimize its light-harvesting and energy transfer capability (Bernard et al., 1992).

High phycoerythrin content is a distinguishing adaptive feature of marine cyanobacteria (Wilbanks et al., 1991). PE absorbance and fluorescence properties are a consequence of interactions between covalently bound bilin chromophores and the PE apoprotein (Anderson and Grossman, 1990). PE extends the light-harvesting capability of the phycobilisome into the green region of the visible light spectrum, and its location at the phycobilisome periphery reflects the position of PE in the sequential
energy transfer pathway PE→PC→APC→Chl a (Anderson and Grossman, 1990; Wilbanks et al., 1991).

In photosynthetic organisms, the pigment content is modulated by many environmental factors such as nutrient availability, temperature and light (Mazel et al., 1986). Our understanding of the regulation and specificity of proteolysis \textit{in vivo} is limited. Properly folded proteins are stable because proteolysis-sensitive domains are hidden whereas damaged, misfolded or unassembled proteins are rapidly degraded (Gottesman and Maurizi, 1992; Hill et al., 1993). Protein folding is vital for a living organism because it adds flesh to the gene skeleton. A small error in the folding process results in a misfolded structure, which can sometimes be lethal. There are two different aspects of the protein stability. One is the chemical stability of the covalent structure, which involves covalent changes and is usually irreversible. The other is the conformational stability of the folded states, in the absence of covalent changes (Tanford, 1970; Pace, 1975; Privalov, 1979; Pace, 1990). Spectroscopic methods are widely used to determine protein stability and to follow structural transitions such as unfolding and refolding under a variety of conditions. It is the simplest method available for measuring how much more stable is the folded conformation of a protein than its unfolded conformations.

Environmental factors can strongly influence protein stability. Cyanobacteria deprived of essential macronutrient such as N, S, C or P (high level of stress), exhibit approx 6-fold increase in the basal rate of proteolysis (Collier and Grossman, 1994). The increased rate of proteolysis may affect most cellular proteins or a specific subset of them. It may result in complete lysis of the protein(s) or lysis of only a part of protein leading to the formation of a domain which may also be functional.

Cyanobacteria exhibit a suite of responses during nutrient limited growth that are either specific responses triggered by the depletion of a single nutrient or general responses that occur in medium that lacks any one of a number of different nutrients (Allen and Smith, 1969; Allen, 1984; Simon, 1987; Reithman et al., 1988). Degradation of the PBS could provide the cell with amino acids used for the synthesis of proteins important for the acclimation process (Wyman et al., 1985). These amino
acids may also be converted into carbon skeletons and used to produce other cellular constituents. The use of phycobiliproteins as amino acid storage molecules may be important for marine cyanobacteria, since nitrogen is frequently limiting in marine environments (Wyman et al., 1985).

It is believed that phycobiliproteins cannot be assembled from only one type (α or β) of subunit. There are many reports available wherein complete phycoerythrin from both cyanobacteria and red algae have been characterized (Schoenleber, 1984; Ong and Glazer, 1991; Swanson et al., 1991; Federspiel and Scott, 1992; Wilbanks and Glazer, 1993; MacColl et al., 1996; Rossano et al., 2003). Conversely, no report is available wherein cyanobacterial phycoerythrin composed of single subunit is characterized, although there is one report describing red alga phycoerythrin composed of only β subunit (Thomas and Passague, 1999). The present study is one of its own kinds, describing the changes in the subunit content of phycoerythrin and the degradation of an intact phycoerythrin to only fragmented α-subunit in the marine cyanobacterium *Phormidium* sp. A27DM. The complete experimental analyses of how and why this intact phycoerythrin (with α and β) is degraded to stable and functional 14 kDa fragment of α-subunit under starvation has been described in the present study. Denaturation studies, using guanidinium chloride as a denaturant, were carried out to corroborate the stability of both intact and truncated phycoerythrin.

### 4.2 Materials and methods

#### 4.2.1 Chemicals

Sephadex G-150 powdered matrix (bead-diameter 20 – 300 μm and fractionation range 5 – 300 kDa) was purchased from GE Healthcare UK Limited (Little Chalfont, Bulkinghamshire, UK); protein molecular mass standard from Bangalore Genei (Bangalore, Karnataka, India); sodium dodecyl sulphate, electrophoresis grade acrylamide and bisacrylamide from Merck (Darmstadt, Hesse, Germany), guanidinium chloride (GdmCl) from Schwarz/Mann Biotech (Cleveland, OH, USA), sodium salt of cacodylic acid and monohydrate citric acid were also obtained from Sigma Chemicals (Riedstr, Steinheim, Germany). All other chemicals were of molecular biology grade and were used without further purification. Dialysis tubing
(molecular weight cut off 6000-8000) were purchased from Spectrum Medical Industries Inc (Sion, Mumbai, Maharashtra). Millipore filters (pore size 0.45μm) were bought from millipore Corporation (Billerica, MA, USA), Whatman filter paper no. 1 was obtained from Whatman Laboratories (Maidstone, Kent, UK) and parafilm was procured from American National Co. (Chicago, USA).

4.2.2 Growth conditions

*Phormidium* sp. A27DM was cultivated in ASN III medium (NaCl, MgCl₂·6H₂O, KCl, NaNO₃, K₂HPO₄·3H₂O, MgSO₄·7H₂O, CaCl₂·2H₂O, citric acid stock and trace metal mix) (Waterbury and Stanier, 1981) with 12:12 light:dark (L:D) cycles at 27°C ± 2°C and illumination of 36 W white fluorescent lamps at a flux density of 130 μmol photons.m⁻².s⁻¹ at the surface of the flask (Shah et al., 2001).

4.2.3 Extraction and purification of phycoerythrin

*Phormidium* sp. A27DM was harvested and the cell mass was washed with 1 M Tris-Cl buffer (pH 8.1) containing 3 mM sodium azide. One volume of washed cell mass was resuspended in five volumes of the same buffer and subjected to repeated freeze-thaw cycles of -30°C and 4°C for the release of phycoerythrin. Purification was done by two-step ammonium sulphate precipitation (20% and 70%) followed by single step gel permeation chromatographic step using Sephadex G-150 column (150 cm x 1.5 cm, bed height 105 cm) pre-equilibrated and eluted with 10 mM Tris-Cl buffer (pH 8.1). The flow rate was maintained at 60 ml h⁻¹ using peristaltic pump (Model P1, Pharmacia, Uppsala, Sweden).

4.2.4 Experimental analyses

The purified phycoerythrin from *Phormidium* sp. A27DM was characterized by UV-Visible Spectrophotometer (Analytik Jena AG Specord® 210, Jena, Germany) and Fluorescence Spectrophotometer (Shimadzu Spectrofluorophotometer, RF-5301 PC, Kyoto, Japan). The amount of PE used for fluorescence studies was approximately 0.75 mg. The excitation and emission slits were 1.5 nm and the fluorescent measurements were taken at 24°C. The purified PE was characterized in terms of homogeneity by native-PAGE and in terms of the molecular masses of the subunits by SDS-PAGE. Non-denaturing and denaturing polyacrylamide gel electrophoresis were
carried out as described in Parmar et al. (2010) using 10% and 15% polyacrylamide slab gels respectively, loading 5 μg of protein in each lane and the same were visualized by silver staining according to Garfin (1990). For calibration, marker proteins ranging from 6 kDa to 43 kDa (Aprotinin - 6 kDa, Lysozyme - 14.3 kDa, Soyabean Trypsin Inhibitor - 20.1 kDa, Carbonic Anhydrase - 29 kDa and Ovalbumin - 43 kDa) were used.

For all the experiments, the inoculum was taken from a freshly grown culture of Phormidium sp. A27DM (having intact phycoerythrin). The purified phycoerythrins from all the experiments were characterized in terms of subunit content by SDS-PAGE. The protein contents at each step were determined by the method of Lowry et al. (1951) with BSA as the standard. Phycoerythrin content was estimated using the equations of Bennet and Bogorad (1973).

4.2.4.1 Effect of growth conditions on PE subunit content
Two litre medium was inoculated with the culture of Phormidium sp. A27DM and was allowed to grow in static conditions. Culture medium of 100 ml was withdrawn and harvested at 5 days interval and replenished with fresh 100 ml sterile medium. A control flask was kept in which 100 ml culture was withdrawn and harvested at a regular interval of 5 days but without replenishment of the sterile medium.

Two litre medium was inoculated with the culture of Phormidium sp. A27DM and was purged with 0.22 μm filter sterilized air at the rate of 80 ml/min (sparging conditions). Similar to static growth experiment, 100 ml culture medium was withdrawn and harvested at 5 days interval and replenished with fresh 100 ml sterile medium each time. A control flask was kept in which 100 ml culture was withdrawn and harvested at a regular interval of 5 days but without replenishment of the sterile medium.

4.2.4.2 Effect of pH on PE subunit content
Culture of Phormidium sp. A27DM was inoculated in different flasks containing 200 ml medium of varying pH ranging from 2 - 10. The pH of the medium was adjusted by 0.5N HCl and 0.5N NaOH. The cultures were harvested after 50 days of growth.
4.2.4.3 Effect of absence of nitrogen source on PE subunit content

Culture of *Phormidium* sp. A27DM was inoculated in 500 ml medium without sodium nitrate. The culture was harvested at a regular interval of 3 days till 12 days.

4.2.4.4 In-vivo and in-vitro effect of proteases on PE subunit content

For *in-vivo* analyses, flasks containing 200 ml medium were inoculated with the culture of *Phormidium* sp. A27DM. They were allowed to grow for 15 days and then 0.25% of various proteases (trypsin, chymotrypsin and pepsin) were added and the cultures were then harvested after 48 h and checked for subunit content. For *in-vitro* analyses, purified PE was incubated with 0.25% of trypsin, chymotrypsin and pepsin for 24 h and checked for subunit content.

4.2.4.5 Effect of protease inhibitors on PE subunit content

Flasks containing 200 ml medium were inoculated with the culture of *Phormidium* sp. A27DM. Different filter sterilized protease inhibitors (DTT, sodium azide, EDTA, iodoacetamide and iodoacetic acid sodium salt) were added at a concentration of 1 mM at a regular interval of 10 days. The cultures were harvested after 60 days of growth.

4.2.4.6 Effect of glacial acetic acid on purified phycoerythrin

Glacial acetic acid (5% and 15%) was added to intact phycoerythrin and incubated at 4°C for 4 months and subsequently subunit content was analysed.

4.2.5 GdmCl-induced denaturation studies

4.2.5.1 Preparation of dialysis tubings

The dialysis tubings (5-6 inches) were prepared according to the procedure recommended by McPhie (1972). The tubings were soaked in 50% ethanol for one hour at 60°C and then thoroughly washed with solution (10 mM NaHCO3 and 1 mM EDTA). The tubings were then washed thoroughly with distilled water. This treatment removes all the impurities from the cellophane tubes which contain glycerine and plasticizers traces of sulphurous compounds and heavy metals (McPhie, 1972). These were thoroughly washed with dialysate before putting protein solution in them. Since the wet tubings are susceptible to attack by cellulolytic microorganism, they were
stored at low temperature. To avoid alteration of pore size due to shrinkage, the tubing were kept soaked in distilled water and stored at low temperature.

4.2.5.2 Preparation of protein stock solutions
Protein solutions were dialyzed extensively against 0.1 M KCl at pH 7.0 at around 4°C. Protein stock solutions were filtered using 0.45 µm millipore filter paper. The concentration of the protein stock solution was determined using molar absorption coefficient, $\varepsilon (\text{M}^{-1} \text{cm}^{-1})$ at 567 nm.

4.2.5.3 Preparation of GdmCl stock solutions
Concentrated solution of GdmCl containing 0.1 M KCl was prepared. Refractive index measurements were made in an Abbe refractometer after filtering the solution through Whatman filter paper no. 1. This was done in order to determine the concentration of the buffered stock solution of GdmCl using tabulated values of the solution refractive indices (Nozaki, 1972). The stock solution of GdmCl was stored at 4°C.

4.2.5.4 Analysis of GdmCl-induced denaturation curves
GdmCl-induced denaturation was followed by measuring changes in $[\theta]_{222}$ at 25°C. It should be noted that each solution was kept for adequate time to allow equilibration for denaturation by GdmCl. Assuming that the Gdn-induced denaturation process is reversible and proceeds through two-state mechanism, the free energy change ($\Delta G_D$) for folding $\leftrightarrow$ unfolding reaction was calculated using the relation:

$$G_D = -RT \ln \left\{ \frac{y - y_N}{y_D - y} \right\}$$

where $y$ is the measured optical property at the particular pH, temperature and denaturant concentration, while $y_N$ and $y_D$ are the properties of the native and denatured states, respectively. The values of $y_N$ and $y_D$ for any point in the transition region are obtained by linear extrapolation of the pre- and post transition baselines, which is generally obtained by least-squares analysis (Ahmad et al., 1992) under the same experimental conditions in which $y$ has been observed. The variation in these values with the change in denaturant concentration, outside the transition region is known as solvent perturbation and the functions are commonly referred as solvent effect. $\Delta G_D (-1.3 \leq \Delta G_D (\text{kcal mol}^{-1}) \leq 1.3)$ was plotted against [GdmCl], the molar
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concentration of the denaturant, and least-squares analysis was used to fit the \((\Delta G_D, [\text{GdmCl}])\) data to the relation:

\[
G_D = \Delta G_D^\circ - m[d]
\]

where \(\Delta G_D^\circ\) is the value of \(\Delta G_D\) in the absence of denaturant and \(m\) is the slope of the line i.e., \((\partial \Delta G_D / \partial [d])_{T,P}\) and \([d]\) is molar denaturant concentration. The midpoint of transition curve, \(C_m\) was calculated from \(C_m = \Delta G_D^\circ / m\).

4.3 Results and discussion

On the basis of sequence homology and structural similarities, it appears that phycobiliproteins have evolved from globin-like protein (Pastore and Lesk, 1990). The specialization of \(\alpha\)- and \(\beta\)- subunits were a critical step in evolution of phycobiliproteins. The \(\alpha\)- subunit evolved initially with 133 amino acid residues and gene duplication subsequently gave rise to the polypeptide chain with 164 amino acid residues (Soni et al., 2010). This study presents the biochemical evidence of the cleavage of an intact phycoerythrin with \(\alpha\) and \(\beta\) subunits to phycoerythrin with only fragmented \(\alpha\)-subunit in the unicellular marine cyanobacterium \textit{Phormidium} sp. A27DM when grown for a prolonged period (under nutrient depletion). A series of experiments were carried out to yield the clues for understanding the rationale for this phenomenon. Intact and truncated phycoerythrins were also characterized in terms of their stability by GdmCl-induced denaturation studies.

4.3.1 Experimental analyses

The purification protocol yielded PE with a purity ratio of 3.9 and 3.6 for intact and truncated PE, respectively (Table 4.1). A comparative SDS-PAGE gel profile of intact PE and truncated PE is depicted in Fig. 4.1. The intact PE has two subunits i.e. \(\alpha\) and \(\beta\) corresponding to 19 and 21 kDa (Fig. 4.1), respectively whereas truncated PE has only one subunit corresponding to 14 kDa (Fig. 4.1). This truncated PE is fragmented \(\alpha\)-subunit as determined from sequencing and structure determination and is stable and functional as long as stored at 4°C (Soni et al., 2010).

The success of purification was revealed by the UV-Visible overlay spectrum (Fig. 4.2a, b) which is clearly showing an increase in the 562 nm wavelength peak at each step of purification. Application of two step ammonium sulphate precipitation and gel
permeation chromatography techniques have been found effective for the purification of phycoerythrin from *Phormidium* sp. A27DM. The precipitate obtained after the treatment of crude extract with 70% ammonium sulphate (w/v) helped in achieving around 80% of PE content (Table 4.1) with a purity ratio of around 1.5 for both intact and truncated phycoerythrin. Fractionation of this PE enriched 70% ASC with Sephadex G-150 further increased the purification level of PE as reflected from the purity ratio of 3.9 and 3.6 (Table 4.1) for intact and truncated phycoerythrin of *Phormidium* sp. A27DM, respectively.

The absorption and the emission spectra of the purified PE of both intact and truncated were compared for better understanding. The absorption spectra of the purified PE in its intact form and truncated form showed variations in the $\lambda_{\text{max}}$ (absorption maxima). Intact PE with both subunits showed a sharp absorption peak at 562 nm. However, truncated PE with only $\alpha$-subunit fragment showed a shoulder peak at 542 nm along with an absorption peak at 562 nm (Fig. 4.2c). On the other hand, when the same were compared for their emission wavelength, no difference was found. Both the PEs emitted light at 580 nm (Fig. 4.2d). PE generally emits light at 580 nm (Rigbi et al., 1980) and as both intact and truncated PEs purified from *Phormidium* sp. A27DM showed an emission wavelength at 580 nm, thus proving them to be functional.

Table 4.1 Data of PE purification from *Phormidium* sp. A27DM.

<table>
<thead>
<tr>
<th>PE</th>
<th>Total PE content after each purification step (mg)</th>
<th>Yield of PE (%)</th>
<th>Purity Ratio ($A_{562}/A_{280}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact</td>
<td>Crude 70 % Gel Permeation Chromatography</td>
<td>62.6</td>
<td>3.9</td>
</tr>
<tr>
<td></td>
<td>15.90  12.78 9.95</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Truncated</td>
<td>Extract ASC</td>
<td>68.2</td>
<td>3.6</td>
</tr>
<tr>
<td></td>
<td>6.60   5.30 4.50</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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**Fig. 4.1:** Silver stained 15% SDS-PAGE. Lanes: (1) Protein molecular mass standard in kDa, (2) Intact PE with two subunits, (3) Truncated PE with single subunit.

**Fig. 4.2:** (a) UV-Visible absorption overlay spectra of intact PE with both the subunits at each step of purification, (b) UV-Visible absorption overlay spectra of truncated PE with single subunit fragment at each step of purification [Steps for (a) and (b): crude extract ← 70% ASC ← purified PE ←], (c) UV-Visible absorption overlay spectra of purified intact PE ← and purified truncated PE ← and (d) Fluorescence emission overlay spectra of purified intact PE ← and purified truncated PE ←.
4.3.1.1 Effect of growth conditions on PE subunit content

The culture when grown in static condition and harvested at regular interval of 5 days with the replenishment of the sterile medium produced an intact PE, with $\alpha$ and $\beta$ subunit corresponding to 19 and 21 kDa till 50 days. However, after 50 days, the culture started producing PE with only one fragmented subunit corresponding to 14 kDa (Fig. 4.3a). A similar kind of phenomenon was observed in control i.e. without replenishment, but at an earlier stage. The culture produced complete PE, with $\alpha$ and $\beta$ subunit corresponding to 19 and 21 kDa till 40 days and thereafter which only truncated PE is produced (Fig. 4.3b). This proved that nutrient replenishment was helping to maintain protein integrity to some extent.

The next experiment was set up in the sparging conditions to achieve a faster growth rate by supplying artificial air. When the cells grew faster, nutrients would deplete faster. As anticipated, the culture in sparging conditions, both with and without replenishment of the sterile medium, resulted in truncated PE (only $\alpha$ subunit fragment) formation much earlier, before 20 days of growth (Fig. 4.3c, d). Whether static or sparging conditions, the culture started producing truncated PE after a certain period of time. Thus we concluded that the growth conditions were not the direct reason for cleavage of PE.

4.3.1.2 Effect of pH on PE subunit content

The other important factor that could be playing a role was pH of the medium. The culture was consequently grown in medium with different pH ranging from 2 to 10, to determine whether pH is playing any role in the cleavage of the complete phycoerythrin. The culture died in medium having pH 2 and 4. The growth was normal in pH 6, 8 and 10 and in all cases truncated PE with only $\alpha$ subunit fragment corresponding to 14 kDa was produced after 50 days of growth (Fig. 4.4). From this we concluded that pH of the medium was not leading to cleavage of the protein.
Fig. 4.3: Silver stained 15% SDS-PAGE of PE purified from *Phormidium* sp. A27DM grown in (a) static condition with medium replenishment (Lanes: 1, Protein molecular mass standard in kDa; 2, PE purified after 10 days; 3, PE purified after 20 days; 4, PE purified after 30 days; 5, PE purified after 40 days; 6, PE purified after 50 days; 7, PE purified after 60 days; 8, PE purified after 70 days), (b) static condition without medium replenishment (Lanes: 1, Protein molecular mass standard in kDa; 2, PE purified after 10 days; 3, PE purified after 20 days; 4, PE purified after 30 days; 5, PE purified after 40 days), (c) sparging condition with medium replenishment (Lanes: 1, Protein molecular mass standard in kDa; 2, PE purified after 10 days; 3, PE purified after 20 days; 4, PE purified after 30 days; 5, PE purified after 40 days; 6, PE purified after 50 days), (d) sparging condition without medium replenishment (Lanes: 1, Protein molecular mass standard in kDa; 2, PE purified after 10 days; 3, PE purified after 20 days; 4, PE purified after 30 days; 5, PE purified after 40 days).
Fig. 4.4: Silver stained 15% SDS-PAGE of PE purified from *Phormidium* sp. A27DM grown in medium of different pH. Lanes: (1) Protein molecular mass standard in kDa, (2) PE purified from medium with pH 6, (3) PE purified from medium with pH 8, (4) PE purified from medium with pH 10.

### 4.3.1.3 Effect of absence of nitrogen source on PE subunit content

The above experiments gave a strong indication that depletion of the nutrients leading to starvation conditions could be playing some role in the cleavage of the intact PE to the fragmented α-subunit. To confirm this, experiment was carried out by growing the culture in the medium lacking NaNO₃ (absence of nitrogen source). As hypothesized, only after 9 days of growth the culture produced cleaved phycoerythrin (Fig. 4.5). This proved that sooner the nitrogen depletion, sooner was the cleavage of intact PE. All the above experiments demonstrated that the cleavage of the protein was actually taking place due to nutrient depletion.
4.3.1.4 In-vivo and in-vitro effect of proteases on PE subunit content

Reports are available suggesting that during nitrogen or sulphur limited (nutrient depletion) growth, degradation of phycobilisomes takes place in an orderly process. The production of phycobiliproteins during nutrient-limited growth is blocked by both transcriptional and post-translational events. The levels of the mRNAs encoding the phycobiliproteins also decline during nutrient limited growth (De Lorimier et al., 1984). Studies have shown that nutrient depletion leads to expression of nblA gene and any condition that favours increased expression of nblA gene triggers the degradative process of PBS (Grossman et al., 1993). Reports suggest that it is likely to be the only gene whose activity directly increases (NblA itself may be involved in the activation of other genes) during sulfur or nitrogen stress. The small size of this protein and the lack of similarity to any known protease suggest that it is not a protease itself. There are few reported functions of this polypeptide that would explain the role of NblA in degradation of phycobilisomes. NblA may function to activate a protease (Wood and Haselkorn, 1980) or may trigger PBS degradation by interacting with the constituents of the complex and altering their susceptibility to
proteolysis (Hershko, 1988) or this small peptide may disrupt hydrophobic and/or ionic interactions among various constituents of the PBS, rendering them susceptible to degradation or may be involved in activating other genes that are directly responsible for causing PBS degradation (Grossman, 1993). Under nutrient depletion, \textit{nblA} gene might be getting over expressed and which in turn is activating other proteases which subsequently cleave phycoerythrin.

To confirm whether the cleavage is due to proteases, \textit{in-vivo} and \textit{in-vitro} experiments were carried out using different proteases and protease inhibitors. Trypsin, chymotrypsin and pepsin were added in actively growing cultures (complete PE with both the subunits) and were harvested after two days of incubation. Though trypsin and chymotrypsin did not have any effect, addition of pepsin resulted in production of PE with only one subunit corresponding to 14 kDa within 48 h (Fig. 4.6a). The same set of proteases were added to purified PE and incubated for 48 h to check for their \textit{in-vitro} effect. In all the cases, PE of 14 kDa subunit was produced (Fig. 4.6b). This led us to assume that the cleavage of the PE could possibly be due to some proteolytic activity.

4.3.1.5 Effect of protease inhibitors on PE subunit content

To corroborate with the above studies, another experiment was performed by addition of different protease inhibitors (sodium azide, DTT, EDTA, iodoacetamide and iodoacetic acid sodium salt) that would inhibit the proteolytic activity. Initial concentration chosen for inhibitors was 10 mM, but the culture died at such high concentration. Consequently the concentration of the protease inhibitors was reduced to 1 mM. The culture still died in iodoacetamide and iodoacetic acid. Cultures with sodium azide, DTT and EDTA were harvested after 60 days of growth and it was observed that all of them produced intact PE with both subunits i.e. both \(\alpha\) and \(\beta\) subunits (Fig. 4.6c). This strongly indicated that the degradation/cleavage could be due to proteolytic action, enhanced under nutrient depletion (Grossman, 1993).

4.3.1.6 Effect of glacial acetic acid on purified phycoerythrin

Purified intact PE when stored at 4°C gets cleaved to 14 kDa fragment. This observation suggests that PE might be getting cleaved by self splicing mechanism.
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However, astonishingly when glacial acetic acid was added to purified PE, no cleavage was observed even after four months of storage (Fig. 4.6d).

![Fig. 4.6: Silver stained 15% SDS-PAGE](image)

(a) Silver stained 15% SDS-PAGE of PE purified from Phormidium sp. A27DM grown in medium with different proteases (Lanes: 1, Protein molecular mass standard in kDa; 2, PE purified from medium with trypsin; 3, PE purified from medium with chymotrypsin; 4, PE purified from medium with pepsin; 5, Control - PE purified from medium without any proteases), (b) of PE after incubation with different proteases (Lanes: 1, Protein molecular mass standard in kDa; 2, purified PE incubated with trypsin; 3, purified PE incubated with chymotrypsin; 4, purified PE incubated with pepsin), (c) of PE purified from Phormidium sp. A27DM grown in medium with different protease inhibitors (Lanes: 1, Protein molecular mass standard in kDa; 2, PE purified from medium with sodium azide; 3, PE purified from medium with DTT; 4, PE purified from medium with EDTA), (d) of PE incubated with glacial acetic acid (Lanes: 1, Protein molecular mass standard in kDa; 2, PE with 5% glacial acetic acid; 3, PE with 15% glacial acetic acid; 4, Control - PE with no glacial acetic acid).
The findings are interesting, however there are many more facts to be explored for complete understanding. The events indicate that production of the truncated PE is meant for continuing the absorption of light energy. The exact molecular mechanism is still vague and can possibly be understood by structural analysis. The structure of 14 kDa fragmented phycoerythrin has been described in detail in chapter 6.

4.3.2 GdmCl-induced denaturation studies

Proteins absorb and emit light in the UV range of the spectrum. The absorbance is caused by the peptide groups, by the aromatic amino acids, and to a small extent, by disulphide bonds. Fluorescence emission originates from the aromatic amino acids. The fluorescence of the exposed amino acids of a protein depends on the solvent conditions even in the absence of conformational changes. This probe is important for the evaluation of fluorescence difference spectra between the native and unfolded proteins. Guanidinium chloride (GdmCl) is one of the commonly used unfolding agent/denaturant which exerts a significant influence on the fluorescence of tyrosine and tryptophan.

If the conformational stability of a protein is defined as the Gibbs energy required to convert the native protein in water (or dilute buffer) from its folded conformation into a structureless polypeptide chain (random coil), its evaluation is then connected to the study of reversible unfolding by denaturants that give rise to a random coil. The reason for this is that the equilibrium between the native (N) state and denatured (D) structureless conformation can be established and studied only in the presence of denaturant, but not in its absence. Yet it is the value of Gibbs energy which is a quantity of fundamental interest in nearly all aspects of protein structure and dynamics.

To determine the stability of truncated and intact phycoerythrins from *Phormidium* sp. A27DM, denaturation studies were carried out and monitored by three different probes - absorbance at 567 nm, fluorescence emission at 320 nm and fluorescence emission at 573 nm. Fig. 4.7 represents the absorption spectra of phycoerythrins, both intact (Fig. 4.7a) and truncated (Fig. 4.7b), from *Phormidium* sp. A27DM in its native and in the unfolded states at 25°C. It shows that the proteins get denatured at
6.0 M GdmCl. Fig. 4.8 shows absorbance studies at 567 nm as a function of GdmCl concentration at pH 7.0 and 25°C of intact (Fig. 4.8a) and truncated (Fig. 4.8b) C-PE. The 567 nm absorbance probe was taken because PE absorbs maximally in the range of 560-570 nm.

Fig. 4.7: Absorbance spectra of (a) intact and (b) truncated C-PE in the presence and absence of GdmCl at pH 7.0 and 25°C.
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Fig. 4.8: GdmCl-induced denaturation of (a) intact and (b) truncated C-PE. Absorbance studies at 567 nm as a function of GdmCl concentration at pH 7.0 and 25°C.

Two fluorescence probes were chosen for measuring emission, one at 320 nm (emission characteristic of aromatic rings when excited at 287 nm) (Fig. 4.9) and the
other at 573 nm (emission characteristic of chromophore groups when excited at 479 nm) (Fig. 4.10).

**Fig. 4.9:** GdmCl-induced denaturation of (a) intact and (b) truncated C-PE. Fluorescence intensity at 320 nm as a function of GdmCl concentration at pH 7.0 and 25°C.
Fig. 4.10: GdmCl-induced denaturation of (a) intact and (b) truncated C-PE. Fluorescence intensity at 573 nm as a function of GdmCl concentration at pH 7.0 and 25°C.
Normalized denaturation curves of phycoerythrins (Fig. 4.11) were calculated from the results of change in absorbance at 567 nm (Fig. 4.8) and fluorescence at 320 nm (Fig. 4.9) and 573 nm (Fig. 4.10) as a function of GdmCl concentrations at pH 7.0 and 25°C.

Fig. 4.11: Normalized denaturation curves of phycoerythin. Curves were calculated from the results of change in absorbance at 567 nm and fluorescence at 320 nm and 573 nm as a function of GdmCl concentrations at pH 7.0 and 25°C.
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The results of the GdmCl denaturation studies showed that the denaturation is uniform and there is no third candidate (any intermediate molecule during denaturation) other than native (N) and denatured (D) forms. The stability parameters of the intact (Table 4.2a) as well truncated PE (Table 4.2b) is almost similar. Since the pattern of denaturation is similar in both the cases (intact and truncated PE), it shows that there is no significant role played by first 32 amino acid residues (missing in the truncated PE) in the stability, denaturation and renaturation but in the folding of the protein.

Table 4.2a Calculation of stability parameters of intact C-PE by GdmCl induced denaturation.

<table>
<thead>
<tr>
<th>Probe</th>
<th>ΔG°</th>
<th>m (Kcal mol⁻¹ M⁻¹)</th>
<th>Cm (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F573</td>
<td>11.56</td>
<td>5.99</td>
<td>1.93</td>
</tr>
<tr>
<td>f320</td>
<td>11.77</td>
<td>6.13</td>
<td>1.92</td>
</tr>
<tr>
<td>A567</td>
<td>11.45</td>
<td>5.67</td>
<td>2.02</td>
</tr>
</tbody>
</table>

Table 4.2b Calculation of stability parameters of truncated C-PE by GdmCl induced denaturation.

<table>
<thead>
<tr>
<th>Probe</th>
<th>ΔG°</th>
<th>m (Kcal mol⁻¹ M⁻¹)</th>
<th>Cm (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>f573</td>
<td>10.16 (±0.27)</td>
<td>5.83 (±0.19)</td>
<td>1.74 (±0.04)</td>
</tr>
<tr>
<td>f320</td>
<td>10.42 (±0.16)</td>
<td>6.35 (±0.12)</td>
<td>1.64 (±0.02)</td>
</tr>
<tr>
<td>A567</td>
<td>10.11 (±0.40)</td>
<td>5.77 (±0.16)</td>
<td>1.75 (±0.04)</td>
</tr>
</tbody>
</table>

4.4 Conclusion

The present work is of great importance in understanding how phycobiliprotein plays a role in adapting cyanobacterium *Phormidium* sp. A27DM to starvation conditions. The truncated phycoerythrin with 14 kDa subunit is functional and continues to absorb light energy. However, the mechanism of proteolysis is not absolutely clear but the experimental analyses indicate that the truncated PE formation basically takes place for the future sustenance of the organism. Subsequently, the basic question that needs to be solved is that why and how the 14 kDa fragment is stable and the other obscurity is if this fragmented phycoerythrin is functional too than why does the cyanobacterium need the complete large phycoerythrin initially.
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4.5 References


