Ph.D. Thesis

Phycobilisome assembly in *Synechocystis* sp. strain PCC6803

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Introduction

Cyanobacteria are prokaryotes that perform oxygenic photosynthesis. Photosynthesis starts with the absorption of light energy by specialized macromolecular complexes, called antennae. In cyanobacteria and red-algae the phycobilisome (PBS) harvests light energy for photosynthesis. The PBS is a huge protein complexes (5-10 x 10^6 Da), twice as big as the ribosome. The light energy is used for the generation of NADPH and ATP. Energy transfer in PBS is unidirectional and its efficiency approaches 95 %. This supramolecular complex is composed of two main structural elements: a core substructure, and the peripheral rods. The main part of the PBS consists of the chromophore-containing phycobiliproteins, which are attached by smaller amount of color-less linker polypeptides. Phycobiliproteins are soluble proteins containing open-chain tetrapyrroles, known as phycobilins. The fundamental assembly units of PBS are trimeric or hexameric aggregates of phycobiliporteins. Two main questions arise regarding the structure and function of the PBS. One is how this huge protein complex assembles, the other is how its structure allows such efficient energy transfer. It is believed that the linker polypeptides have important function in these processes. The linker polypeptides induce face-to-face aggregation of phycobiliprotein trimers and cause the tail–to–tail joining of hexamers. Linker polypeptides modulate the spectral properties of the phycobiliproteins, and these small spectral changes may support the unidirectional transfer of excitation energy from the rod periphery to the core of the PBS.

The PBS of the cyanobacterium *Synechocystis* PCC6803, which was the target of our investigations, consists of a three-cylindrical core from which six rods radiate. Each rod is composed of three stacked phycocyanin (PC) hexamers. In this strain two independent genes, *cpcG1* and *cpcG2*, encode the rod-core linker (LRC) that binds the proximal PC-hexamer to the core. The remaining rod-subunit-encoding genes are clustered in the *cpc* operon. This operon contains five genes, *cpcB* and *cpcA* (encoding the β^{PC} and α^{PC} subunits respectively), and *cpcC2*, *cpcC1* and *cpcD* (encoding the rod linkers LR30, LR33 and LR10, respectively).

The ferredoxin-NADP(H)-oxidoreductase (FNR) catalyzes the electron transfer between ferredoxin and $NADP^+$ in the last steps of photosynthesis. Two isoforms of the FNR have been detected in *Synechocystis* PCC6803 cells: a 46.3 kDa FNR that is associated to the PBS and a soluble 34.3 kDa FNR. It is widely accepted that the 34.3 kDa isoform is produced by the proteolysis of the 46.3 kDa isoform of the FNR. Since the N-terminal amino-acid sequence of the 34.3 kDa FNR begins with methionine, which could be alternative translational event. The FNR participates in important physiological processes such as respiration and photosynthesis, and it is essential for the cells. The exact role of the two

isforms in the different processes is not known. The N-terminal domain of the 46.3 kDa FNR is similar to LR10, therefore it is believed to share the same binding site on the core-distal end of the rods.

The aims of this work

Various linker polypeptides are associated with phycobiliproteins in the PBS: they are essential for stabilizing the PBS structure and for optimizing the efficiency of energy transfer. The aim of the present work was to study PBS assembly in *Synechocystis* PCC6803.
The main questions addressed by our study were the following:

i. How do LR33 and LR30 participate in rod assembly following the attachment of the first hexamer by the LRC?
ii. What is the structural function of LR10 in the PBS?
iii. Does the absence of rod-linker polypeptides affect PC accumulation?
iv. Does the insertion of the commonly used Km and Ω cassettes affect the transcription of the *cpc* operon?
v. Are both LRC-encoding genes, *cpcG1* and *cpcG2* expressed? If so, are their products both functional in PBS rod biogenesis?
vi. The amino-acid sequence of the 34.3 kDa FNR begins with a methionine. Could it be a translational product starting from an alternative initiation site?
vii. The N-terminal part of the 46.3 kDa FNR is similar to LR10. Do they share the same binding site on the PBS rod? Would the FNR be capable of binding to the PBS core?

Materials and Methods

Growth conditions

Wild-type and mutant strains of the *Synechocystis* PCC6803 were grown photoautotrophically in an illuminated orbital incubator at 32 °C in a CO_2-enriched (~5 %) atmosphere under continuous light (40 μmol m^{-2} s^{-1}). We used a modified BG11 medium containing: 30 μM ferric citrate, 3 μM EDTA-diNa, 30 mM sodium nitrate, 250 μM potassium phosphate, 250 μM magnesium sulphate, 250 μM calcium chloride, 200 μM sodium carbonate, 10 mM sodium bicarbonate and microelements as in BG11. All chemicals were purchased from Sigma. For growth on Petri dishes, the above medium was supplemented with 1.5 % (w/v) Difco Bacto-agar and 2-5 mM sodium thiosulfate. Petri dishes

were incubated at 30 °C under continuous light (30 μmol m^{-2} s^{-1}). When appropriate, media were supplemented with 25 to 50 μg/ml kanamycin, 25 to 50 μg/ml spectinomycin and 5 to 10 μg/ml streptomycin.

Wild-type and mutant Synechocystis strains used in the PBS related work

Strains	**Properties**
WT	Wild-type strain of *Synechocystis* PCC6803
30f	Δ*cpcC2*::*aphI* -deletion of 68 % of *cpcC2* (codon 21 to 208), *aphI* insertion (Km^r)
30r	Δ*cpcC2*::*aphI* -as above but *aphI* in the opposite orientation (Km^r)
Δ30D3	Δ*cpcC2, cpcD*$^-$::Ω -deletion of 83 % of *cpcC2* (codon 2 to 230) and termination of the *cpc* operon transcription before *cpcD* (Sp^r, Sm^r)
D3	*cpcD*$^-$::Ω - Ω insertion between *cpcC1* and *cpcD*, transcription termination before *cpcD* (Sp^r, Sm^r)
33	Δ*cpcC1*::*aphI* -deletion of 50 % of *cpcC1* (codon 77 to 220), *aphI* insertion (Km^r)
CB	Δ*cpcC2C1*::*aphI* -total deletion of *cpcC2* and *cpcC1* (codon 2 of *cpcC2* to the last codon of *cpcC1*), *aphI* insertion. (Km^r)
33c	33 transcomplemented with *cpcC1* in the *psbAII* locus (Km^r, Sp^r, Sm^r)
Cbc	CB transcomplemented with *cpcC1* in the *psbAII* locus (Km^r, Sp^r, Sm^r)
D4	*cpcD*$^-$::Ω - Ω insertion in *Hinc*II of *cpcD*, transcription termination within *cpcD* (Sp^r, Sm^r)
WD	WT plus *cpcD* in the *psbAII* locus (Km^r)
D3D	D3 plus *cpcD* in the *psbAII* locus (Km^r, Sp^r, Sm^r)
D4D	D4 plus *cpcD* in the *psbAII* locus (Km^r, Sp^r, Sm^r)
CK	Δ*cpc* operon :: aphI, sacB – deletion of 85 % of *cpc* operon, *aphI* and *sacB* insertion (Km^r, $Sucrose^s$)
G1	*cpcG1*$^-$ :: Ω - Ω insertion in *Nru*I of *cpcG1* (Sp^r, Sm^r)
G2	*cpcG2*$^-$ ::*aphI* insertion in *Bsp*1407I of *cpcG2* (Km^r)

Synechocystis mutant strains used in the FNR related work

Strains	**Properties**
I	Point mutation changing M 113 of FNR, to I, Ω casette insertion
V	Point mutation changing V 102 of FNR, to non-initiating V, Ω casette insertion
H	Ω casette insertion
FS1	C insertion, codon 110 of the FNR is a stop codon
FS2	C deletion, codon 113 of the FNR is a stop codon

Genetic transformation

3 ml of a *Synechocystis* PCC6803 culture ($OD_{580nm} \approx 1.0$) was harvested by centrifugation. The cell pellet was washed with 3 ml modified BG11 and resuspended in 150 μl of modified BG11. Approximately 2 μg of plasmid DNA was added to the cells. The mixture was incubated 6 to 12 hours under dim light then plated on modified BG11 Petri dishes. 12 to 24 hours later the antibiotic containing solution (250 μl) was layered under the agar.

Northern blot

The RNA samples after denaturation with glyoxal and dimethyl sulphoxide (DMSO) were loaded with glyoxal/DMSO gel-loading buffer into the wells of a 0.8 % agarose gel containing sodium phosphate buffer and sodium iodoacetate.

The gel was run at 45 V for 6.5 h in 10 mM sodium phosphate buffer (pH 7.0). To maintain the pH during the migration the buffer was circulated.

After electrophoresis the gel was soaked in 200 ml 0.025 M NaOH solution for 15 min, rinsed with distillated water then soaked in 200 ml 20 x SSC for 15 min. The RNA was transferred from the gel to a nitrocellulose filter by capillarity blot using 20 x SSC solution and Millipore ImmobilonTM-Ny+ Transfer membrane. After capillarity blotting the RNA was linked to the membrane surface using Stratalinker UV Crosslinker Model 2400 with 1200 μJ (x 100). The hybridization procedure was as recommended by Millipore. To label the probes we used the Amersham Biosciences Ready-To-Go-DNA labeling (-dCTP) kit. The blot was hybridized for 16 hours at 68 °C. After removing the hybridization solution the membrane was washed with Wash Solution I (1 x SSC and 0.1 % w/v SDS) and twice with Wash Solution II (0.2 x SSC and 0.1 % w/v SDS). Radioactivity was detected by Molecular Dynamics STORM 860 Phosphorimager.

Control hybridization of the filter was carried out with constitutively-expressed *rnpB* gene.

For the Northern blot analysis we used probes hybridizing

- with each of the three transcript of *cpc* operon;
- with the two larger transcripts of the *cpc* operon;
- with the *aphI* gene containing transcripts;
- with the *cpcD* containing transcripts;
- with *cpcG1* transcripts;
- with *cpcG2* transcripts, respectively.

PBS isolation

500 ml of cyanobacterial culture at OD_{580nm} = 0.8-0.9 were harvested by centrifugation at room temperature (6500 g for 5 min). The cells were washed with 0.8 M potassium/sodium phosphate buffer (pH 8.0) and then the pellet was frozen at –20 °C. The cells were thawed at room temperature and resuspended in Complete™ (Roch) protease inhibitor containing 0.8 M potassium phosphate buffer. The mixture was wortexed 6 times (1 min each) with glass beads to break the cells. The solution was centrifuged at room temperature (1500 g for 2 min) to remove the glass beads and any unbroken cells. The supernatant was transferred to a 15 ml tube and Triton-100 was added to a final concentration of 2 % (v/v). The mixture was incubated for 20 min at room temperature in the dark, then it was transferred to a polycarbonate tube and centrifuged for 15 min (31000 g at 18 °C) in a Beckman JA-25.50 rotor. The supernatant was removed carefully with a 1 ml pipette from underneath the floating chlorophyll containing layer. Two ml supernatant aliquots were layered on a sucrose step gradient in a Beckman Ultra Clear™ Tube 14x89 mm, with the following sucrose molarities: 2.0 M (3 ml); 1.0 M (2 ml); 0.5 M (2 ml); 0.25 M (3 ml). The sucrose was dissolved in 0.8 M potassium/sodium phosphate buffer (pH 8.0) in all layers. After centrifugation (using SW41 rotor, with 35000 rpm at 18 °C for 16 h) the PBS containing blue bands were collected with syringe from the gradients in Eppendorf tubes.

Absorption spectra

200 µl PBS containing samples collected from the sucrose gradients were diluted with 1800 µl 0.8 M potassium/sodium phosphate buffer (pH 8.0). Absorption spectra were recorded in 1 ml cuvettes, using a Varian Cary-5E double-beam spectrophotometer, with a data interval of 0.5 nm.

Protein gels

To analyze the protein composition of the isolated PBS, we used either 10-20 % Tris-Glycine gels were used or Invitrogene NuPAGE® Novex Bis-Tris Gels 4-12 %.
Thirty µl PBS sample with OD_{620nm}= 10 (with exception of PBS complex from the rod less mutants, when we used 7.5 µl with OD_{650nm}= 10) was precipitated on ice for 5 min after

adding an equal volume of 20 % TCA. This was followed by a 2 min centrifugation (at 21000 g at 4 °C) then the supernatant was removed and the pellet was resuspended in the loading buffer, heated for 5 min at 85 °C and finally loaded on the gel.

The gels were stained with Bio-SafeTM Coomassie G250 (BIO-RAD) as it was proposed in its manual. The NuPAGE® Novex Bis-Tris Gels were also colored with the Invitrogene SimplyBlue staining. After destaining the gels were photo-documented with a CCD camera. As the composition of wild-type PBS is well known, PBS bands from the mutants were identified by comparing their positions on the gel with those of the wild-type PBS protein bands. These were identified by their size compared to the molecular-weight marker.

Results

In order to study the roles of the rod linkers we generated and characterized interposon mutants in the three rod-linker genes located in the *cpc* operon of *Synechocystis* PCC6803. We studied the fluorescence emission spectra of the wild-type and mutant cells, and analyzed the absorption spectra and protein composition of their PBSs. With the characterization of the PBS assembly process in the mutants we were able to deduce the sequential order of PBS-rod biogenesis in *Synechocystis* PCC6803.

i. Our results clearly demonstrated that the rod linkers are incorporated in a precise order during the biogenesis of the PBS rods; i. e. following the attachment of the core-proximal PC-hexamer by the LRC, only LR33 can bind the intermediary PC-hexamer, whereas only the LR30 attaches the distal PC-hexamer. Our data strongly suggest that the function of LR33 and LR30 are not interchangeable. Our model is in a good agreement with results obtained upon nitrogen starvation, where sequential loss of PC and associated linkers occurs, and LR30 disappears first from the PBS during the initial steps of nitrogen starvation.

ii. We found that LR10 was present in PBSs lacking either the core-distal or both the intermediary and the core-distal PC-hexamers, however in smaller amounts. The reduced level of LR10 was first attributed only to the polar effect of the Km cassette on the expression of LR10 encoding *cpcD* in these strains. The loss of LR10 did not affect PBS assembly or function. Over-expression of this linker polypeptide in mutant strains in which *cpcD* was under the control of the highly active *psbAII* promoter indicate that when LR10 is produced *in trans* proteolysis prevents its association to the PBS. It is known, that linker polypeptides are highly sensitive to proteolysis when they are not associated to phycobiliproteins.

Our results demonstrated that LR10 has a higher affinity for PC-hexamers containing LR30 than LR33 containing PC-hexamers. Earlier LR10 was proposed to be associated with the distal end of the rods, and our results indicated that the LR30 binds the core-distal PC-hexamer.

iii. Fluorescence emission spectra of whole cells and the sucrose-gradient pattern of the PBS preparations indicated the presence of free PC in the rod-linker deficient strains. These observations demonstrate that under our culture condition, PC biosynthesis was not down-regulated in the absence of the rod-linker polypeptides.

iv. In *cpc* operon mutants, containing Km cassette insertion, the PBS contained smaller amount of the linker polypeptides encoded by the genes located downstream from the inactivated gene. Therefore we examined whether this was due to the absence of the inactivated gene product or to the polar effect of the insertion. The Km cassette is commonly used for gene inactivation, but it was not measured in cyanobacteria how its insertion affects expression of adjacent genes. By monitoring steady state RNA levels in the mutants, we demonstrated that the insertion affected the gene expression in its vicinity regardless of orientation. The wild-type *cpc* operon generates three transcripts from one transcription initiation site. The most abundant 1.6 kb transcript contains only *cpcB* and *cpcA*. Two larger, less abundant transcripts, contain in addition PC subunits genes, LR30 and LR33 encoding *cpcC2* and *cpcC1* or these plus the LR10 encoding *cpcD*, respectively. The sizes and relative abundance of these transcripts varied in the mutants. Their low amounts demonstrate that the insertion of the Km cassette destabilizes the rod-linker transcripts. This is the reason why we found weaker incorporation of the flanking genes products into the PBS. Using Km-specific probe we detected transcripts, which were the products of read-through transcription from the *aphI* promoter driving Km expression. When the Km cassette was inserted in an opposite orientation to the operon transcription, the presence of an anti-sense mRNA effect was demonstrated. In such a case complementary mRNA can anneal to the original transcript, thereby preventing the translation of upstream genes.

We constructed two LR10 less-strains. In one the Ω cassette was inserted into the LR10 encoding *cpcD* gene, while in the other insertion took place upstream of *cpcD*, destroying a stem-loop structure. The PBS of the latter mutant contained slightly lesser PC than the other LR10-less strain and the wild type, which could be due to the destabilization of the linker-encoding mRNA. This observation suggests that the Ω terminator does not provide a 3' stabilizing effect to the transcript.

v. We demonstrated by Northern-blot analysis and RT-PCR that both LRC encoding *cpcG1* and *cpcG2* genes are transcribed. In the PBS structure formation only the *cpcG1* encoded LRC participates, since inactivation of *cpcG1* prevented the PC attachment to the core, while inactivation of *cpcG2* did not alter the PBS composition.

vi. Site directed mutations of the *petH* gene, encoding the FNR, were constructed to identify the function of the two isoforms of the FNR. Analysis of the FNR encoding *petH* gene sequence indicates two other translational starts after the first methionine, valine 112 and methionine 113. In order to determine whether these sites are functional initiation sites, mutant strains I and V were constructed, in which methionine 113 (M113) was replaced by isoleucine, and valine 102 (V102) was changed to a non-initiating valine. SDS-PAGE analysis of the purified PBSs from these strains confirmed that they have assembled PBS with the FNR attached, similar to the PBS of the wild type. The FNR content of whole cell extracts from the mutant strains and wild-type cells was detected by Western-blot analysis using *Synechocystis* PCC6803 FNR antibodies. The I mutant strain contained only the 46.3 kDa isoform of the FNR. The lack of the 34.3 kDa protein in this strain supports the idea of an alternative initiation site corresponding to M113. On the other hand the replacement of the methionine to isoleucine may also prevent the proteolysis if the methionine is a specific site of the proteolysis. To confirm our hypothesis, we constructed two frame-shift mutant strains in which stop codons were created either at the codon 110 or 113, without the alteration of the ATG code of the original M113. In these strains translation from the first methionine is interrupted, but translation from methionine 113 is still allowed if it is a real initiation site. The frame-shift containing strains were able to segregate and they contained only the 34.3 kDa form of the FNR, suggesting that our hypothesis was correct. That means the 34.3 kDa form of the FNR, in contrast to the earlier assumptions, is not produced by the proteolysis of the 46.3 kDa form. We proved that the two isoforms of FNR are the products of different translation initiation sites.

vii. The PBS of the LR10-less mutant strains contained the same amount of FNR as the wild-type PBS, and the FNR did not co-purify with the core-complex of the PBS. Therefore we suggest that FNR and LR10 do not share the same binding site, and the FNR it is not able to bind directly to the core, but it is located in a core-proximal position on the rods.

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