

Degradation of Phycobilisomes in *Synechocystis* sp. PCC6803 EVIDENCE FOR ESSENTIAL FORMATION OF AN NblA1/NblA2 HETERODIMER AND ITS CODEGRADATION BY A Clp PROTEASE COMPLEX[§]

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Background: In cyanobacteria, starvation-induced phycobilisome degradation is caused by NblA.

Results: *Synechocystis* expresses two NblA proteins that form a heterodimer. The heterodimer binds phycobiliproteins and ClpC and is degraded by a ClpC-ClpP1ClpR protease *in vitro*.

Conclusion: NblA1/NblA2 is an adaptor protein that mediates degradation of phycobilisomes by the ATP-dependent protease ClpC-ClpP1ClpR.

Significance: These findings improve our understanding of the mechanisms by which cyanobacteria adapt to changing environmental conditions.

When cyanobacteria acclimate to nitrogen deficiency, they degrade their large (3–5-MDa), light-harvesting complexes, the phycobilisomes. This massive, yet specific, intracellular degradation of the pigmented phycobiliproteins causes a color change of cyanobacterial cultures from blue-green to yellow-green, a process referred to as chlorosis or bleaching. Phycobilisome degradation is induced by expression of the *nblA* gene, which encodes a protein of ~7 kDa. NblA most likely acts as an adaptor protein that guides a Clp protease to the phycobiliproteins, thereby initiating the degradation process. Most cyanobacteria and red algae possess just one *nblA*-homologous gene. As an exception, the widely used “model organism” *Synechocystis* sp. PCC6803 expresses two such genes, *nblA1*₆₈₀₃ and *nblA2*₆₈₀₃, both of whose products are required for phycobilisome degradation. Here, we demonstrate that the two NblA proteins heterodimerize *in vitro* and *in vivo* using pull-down assays and a Förster energy-transfer approach, respectively. We further show that the NblA proteins form a ternary complex with ClpC (the HSP100 chaperone partner of Clp proteases) and phycobiliproteins *in vitro*. This complex is susceptible to ATP-dependent degradation by a Clp protease, a finding that supports a proposed mechanism of the degradation process. Expression of the single *nblA* gene encoded by the genome of the N₂-fixing, filamentous cyanobacterium *Nostoc* sp. PCC7120 in the *nblA1/nblA2* mutant of *Synechocystis* sp. PCC6803 induced phycobilisome degradation, suggesting that the function of the NblA heterodimer of *Synechocystis* sp. PCC6803 is combined in the homodimeric protein of *Nostoc* sp. PCC7120.

Cyanobacterial cultures growing in a nutrient-rich medium exhibit a typical blue-green color due to the photosynthetic pigments chlorophyll *a* and phycobiliproteins. Together with mostly uncolored linker proteins, phycobiliproteins form the

phycobilisome (PBS).² The PBS is a large (3–5 MDa) multiprotein complex associated with the cytoplasmic side of the thylakoid membranes (reviewed in Refs. 1–4). In addition to the non-pigmented linker proteins, the complex is composed of the chromophorylated phycobiliproteins phycocyanin (PC; $\lambda_{\max} \approx 620$ nm), allophycocyanin ($\lambda_{\max} \approx 650$ nm), and, in some cyanobacteria, phycoerythrin ($\lambda_{\max} \approx 565$ nm) or phycoerythrocyanin ($\lambda_{\max} \approx 590$ nm). The linker proteins are necessary for the assembly and stabilization of the PBS structure through their interactions with phycobiliprotein subunits. They also play a role in energy transfer from the PBS to photosystem II and, in some cyanobacteria, to photosystem I as well.

PBSs are classified according to their morphology. The most common PBS structure is the hemidiscoidal type, which is made up of a core complex that is anchored in the thylakoid membrane and peripheral rods (1, 3, 5). Size, composition, and position of PBSs on the thylakoid membrane vary according to light quality and quantity so as to optimize light absorption (6–9). Nearly complete degradation of PBSs can be observed in unicellular, non-diazotrophic cyanobacteria upon nitrogen starvation and, in some species, also upon sulfur depletion. Degradation begins with the sequential loss of the peripheral rods and terminates with degradation of the core complex (10, 11). Because phycobiliproteins constitute up to 50% of the total soluble protein of a cyanobacterial cell (2), a considerable amount of cellular protein becomes degraded within the first 24 h of nitrogen deprivation. The loss of phycobiliproteins results in a color change of cyanobacterial cultures from blue-green to yellow-green, a process referred to as bleaching or chlorosis (12). PBS degradation is not essential for acclimation to nutrient deprivation but is rather thought to prevent photo damage under stress and to provide substrates for the *de novo*

[§]This article contains supplemental Table S1 and Fig. S1.

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²The abbreviations used are: PBS, phycobilisome; *Nostoc* 7120, *Nostoc* sp. PCC7120; *Synechocystis* 6803, *Synechocystis* sp. PCC6803; *Synechococcus* 7942, *Synechococcus elongatus* PCC7942; PCC, Pasteur Culture Collection; PC, phycocyanin; IPTG, isopropyl β -D-1-thiogalactopyranoside; Cer, cerulean; CFP, cyan fluorescent protein; Ni-IDA, nickel-iminodiacetic acid; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

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synthesis of proteins required for the acclimation process (11, 13).

The small protein NblA (non-bleaching A) plays a key role in the degradation process (14). Cyanobacterial mutants lacking NblA degrade virtually none of their PBS under nitrogen starvation and thus exhibit a non-bleaching phenotype (14–17). The exact function of NblA has been the subject of numerous studies. Binding experiments have shown that NblA interacts with the α -subunits of phycobiliproteins (18, 19). In pull-down experiments, NblA was found to bind to ClpC, an HSP100 chaperone partner of a Clp protease, in an ATP-dependent manner (20). This result led to a proposed model of PBS degradation in which NblA acts as a so-called adaptor protein of a Clp protease (20). Clp degradation complexes consist of two functional elements: a cylinder-like proteolytic core of two heptameric rings and an AAA+ chaperone (21, 22). The hexameric chaperone ring is responsible for substrate recognition, unfolding, and threading of the extended polypeptide chain through a narrow pore into the protease compartment, where the proteolytically active sites are sequestered from the cytoplasm (23, 24). The substrate specificity of a Clp protease is determined by its chaperone partner. Substrate recognition occurs by binding to the chaperone partner of the Clp protease or is mediated by adaptor proteins (25, 26). Adaptor proteins simultaneously bind the substrate and the chaperone, thereby forming a ternary complex that causes degradation of the substrate. In other words, adaptor proteins modulate the substrate specificity of a protease, enabling the degradation of particular proteins at the proper time. This means that substrate degradation is solely regulated by the expression of the adaptor protein. This is precisely what is observed in PBS degradation, which is induced by the expression of the small protein NblA (14). Because the Clp proteases require NblA to recognize phycobiliproteins as substrates, strains lacking NblA degrade the PBS at a low rate, even under conditions of nitrogen starvation (14, 15, 17).

Most phycobiliprotein-containing cyanobacteria and red algae possess *nblA* genes. The NblA proteins are rather small, consisting of ~60 amino acids. Sequence alignment of NblA proteins reveals a low homology (about 30% sequence identity on average) (27). Short stretches of highly conserved amino acid residues located near the N and C termini of the proteins are involved in phycobiliprotein and ClpC binding (19, 20). NblA proteins from *Nostoc* sp. PCC7120 (*Nostoc* 7120), *Thermosynechococcus vulcanus*, and *Synechococcus elongatus* sp. PCC7942 (*Synechococcus* 7942) have been crystallized (19, 27). All three NblA proteins share a similar structure. They are homodimers that consist of two α -helices: one shorter N-terminal helix and a longer C-terminal helix. The biologically active form of NblA is thus a four-helix bundle.

Cyanobacteria and red algae usually possess one *nblA* gene. However, two genes have been found in *Nostoc* 7120 and *Synechocystis* sp. PCC6803 (*Synechocystis* 6803). Whereas only one of the two *nblA* genes is expressed upon nitrogen deprivation in *Nostoc* 7120 and is sufficient for PBS degradation (17), both genes are expressed in *Synechocystis* 6803, and both are necessary for induction of the degradation process (15, 16).

In this study, we investigated why the two NblA proteins, NblA1₆₈₀₃ and NblA2₆₈₀₃, are necessary for PBS degradation in

Synechocystis 6803. We present evidence that NblA1₆₈₀₃ and NblA2₆₈₀₃ act as a heterodimer in which NblA1₆₈₀₃ mediates the binding to ClpC₆₈₀₃ and phycobiliproteins. Both NblA proteins are degraded by a Clp protease *in vitro*, a finding that strongly supports the proposed model of the role of NblA in PBS degradation. We further show that expression of the single *nblA* gene from *Nostoc* 7120 complements the non-bleaching phenotype of the *nblA1/nblA2* double mutant of *Synechocystis* 6803.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Growth Conditions—Strains are listed in supplemental Table S1. Unless stated otherwise, sources of plasmids, antibiotics, medium, restriction enzymes, and chemicals were as described previously (20).

Synechocystis 6803 and its mutants were grown photoautotrophically under constant illumination essentially as described before (17) except that the growth temperature was 28 °C, and the internal diameter of the culture vessels was 3.5 cm. Whole-cell absorbance spectra were recorded from 550 to 750 nm on a Specord® 200 PLUS spectrophotometer (Analytik Jena, Jena, Germany) and were corrected for cell scattering at 750 nm.

Strains of *Escherichia coli* were grown under standard conditions (28). When appropriate, antibiotics were added to the medium to final concentrations of 50 $\mu\text{g ml}^{-1}$ ampicillin, 50 $\mu\text{g ml}^{-1}$ kanamycin, or 25 $\mu\text{g ml}^{-1}$ chloramphenicol, respectively.

Plasmid and Mutant Constructions—Plasmids and oligonucleotides are listed in supplemental Table S1 and Fig. S1.

Construction of Plasmids for Protein Overexpression in *E. coli*—Vectors for expression of GST-tagged NblA1₆₈₀₃ and NblA2₆₈₀₃ in *E. coli* were constructed as follows. The plasmid pGEX-2TK/NdeI (20) was digested with NdeI and SmaI in order to obtain the fragment encoding GST, the fragment was ligated into the expression vector pACYCDuet-1, which was digested with NdeI and EcoRV prior to ligation. The resultant plasmid pACYC/GST was digested with NdeI and, after dephosphorylation, was ligated to a fragment harboring *nblA1*₆₈₀₃ (*ssl0452*) or *nblA2*₆₈₀₃ (*ssl0453*), respectively, yielding plasmids pACYC/A1_GST and pACYC/A2_GST. Fragments encoding *nblA1*₆₈₀₃ and *nblA2*₆₈₀₃ were generated by PCR from genomic *Synechocystis* 6803 DNA using primers nbl7 and nbl28 (amplification of *nblA1*₆₈₀₃) or nbl24 and nbl31 (amplification of *nblA2*₆₈₀₃). Each primer sequence contained an NdeI restriction site for cloning. The primers nbl28 and nbl31 additionally contained a sequence encoding the PreScission™ protease recognition sequence, enabling cleavage of the NblA1₆₈₀₃/NblA2₆₈₀₃-GST fusion protein. For coexpression of His-tagged NblA1₆₈₀₃ and GST-tagged NblA2₆₈₀₃, the *nblA1*₆₈₀₃ gene was amplified by PCR using primers nbl32 (EcoRI site incorporated) and *nblA*-4 (HindIII site incorporated) and was ligated into plasmid pACYC/A2_GST to yield plasmid pACYC/His_A1 and A2_GST. For simultaneous expression of His-tagged NblA2₆₈₀₃ and GST-tagged NblA1₆₈₀₃, *nblA2*₆₈₀₃ was amplified by PCR using primers nbl33 (HindIII site inserted) and nbl34 (EcoRI site inserted). The PCR fragment was cloned into the plasmid pACYC/A1_GST, generating plasmid pACYC/His_A2 and A1_GST.

For coexpression of NblA₂₆₈₀₃ and GST-tagged NblA1₆₈₀₃, the *nblA2*₆₈₀₃ gene was amplified by PCR using the primer nbl14 (NcoI site incorporated) and nbl21 (EcoRI site incorporated). The resulting PCR fragment was ligated into plasmid pACYC/A1_GST, generating plasmid pACYC/A2 and A1_GST. For binding experiments, the ClpC₆₈₀₃ protein was expressed without any affinity tag. For construction of the expression plasmid, *clpC*₆₈₀₃ (*sll0020*) was amplified by PCR from total *Synechocystis* 6803 DNA using primers clpC_3 (NdeI site inserted) and clpC_5 (BamHI site inserted). The PCR fragment was digested with NdeI (partial digestion) and BamHI and cloned into plasmid pET11a, yielding plasmid pET11/ClpC. For degradation experiments, the ClpC₆₈₀₃ protein was expressed with an N-terminal His tag. ClpC₆₈₀₃ was amplified by PCR with clpC_3 (NdeI site inserted) and clpC_5 (BamHI site inserted) and ligated into plasmid pET22b, resulting in plasmid pET22b/His-ClpC₆₈₀₃. For expression of the phycocyanin α -subunit N-terminally fused to GST, the chromosomal gene *cpcA* (*sll1578*) from *Synechocystis* 6803 was amplified by PCR using primers cpcA_1 (BamHI site inserted) and cpcA_2 (EcoRI site inserted) and cloned into plasmid pGEX-2TK via BamHI and EcoRI to yield plasmid pGEX/cpcA. For expression of HisClpP1 and ClpR, the *clpP1* (*slr0542*) gene was first amplified with primers clpP1_1 (NcoI site incorporated) and clpP1_2 (NcoI site incorporated) from total *Synechocystis* 6803 DNA. The PCR product was digested with NcoI cloned into plasmid pACYCDuet-1, yielding the plasmid pACYC/HisClpP1. The *clpR* (*slr0164*) gene was then PCR-amplified with primers clpR_1 (NdeI site inserted) and clpR_2 (XhoI site inserted). The PCR fragment was digested with NdeI and XhoI and cloned into the plasmid pACYC/HisClpP1, resulting in the plasmid pACYC/HisClpP1, ClpR.

All DNA constructs were checked by DNA sequencing. All plasmids and oligonucleotides are listed in [supplemental Table S1](#).

PBS Isolation from *Synechocystis* 6803 Crude Extract—The PBS preparation was performed as described before (29).

Coexpression and Purification of HisClpP1 and ClpR and Expression of His-tagged ClpC₆₈₀₃—After induction with isopropyl β -D-1-thiogalactopyranoside (IPTG) for 3 h at 30 °C, cells were pelleted and resuspended in buffer A (10 mM Tris-HCl buffer (pH 7.5), 75 mM KCl, 1 mM DTT). Cells were disrupted by sonication with a homogenizer (Sonopuls) for 2 \times 2 min (settings: KS76, pulsation 2, 55% power), and the extract was clarified by centrifugation and incubated with Protino[®] Ni-IDA resins (Macherey-Nagel). Bound proteins were purified according to the manufacturer's protocol and eluted with buffer A containing 250 mM imidazole, followed by buffer change and concentration with a centrifugal concentrator (Vivaspin[®] 500, molecular weight cut-off 10,000) in buffer A.

Coexpression and Purification of NblA2₆₈₀₃ and GST-tagged NblA1₆₈₀₃—After induction with IPTG overnight at 18 °C, cells were pelleted, resuspended in buffer B (10 mM Tris-HCl buffer (pH 7.5), 200 mM KCl, 20 mM NaCl, 20 mM MgCl₂, 10% glycerol), cell-free extracts were prepared as described above. The soluble crude extract was incubated with glutathione-agarose (Macherey-Nagel). After washing with buffer B, the bound proteins were eluted using Pre-Scission[™] protease according to

the manufacturer's protocol (GE Healthcare). The NblA1₆₈₀₃ and NblA2₆₈₀₃ proteins were further purified by size exclusion chromatography on a Superdex 75[™] column (GE Healthcare).

Coexpression and Purification of the NblA1₆₈₀₃ and NblA2₆₈₀₃ Heterodimers—GST-NblA1₆₈₀₃ and His-NblA2₆₈₀₃ or His-NblA1₆₈₀₃ and GST-NblA2₆₈₀₃ were coexpressed after induction with IPTG (overnight, 18 °C). Crude extracts were divided; one half was incubated with glutathione-agarose (Macherey-Nagel) for binding of GST-tagged NblA, and the other half was incubated with Protino[®] Ni-IDA resins (Macherey-Nagel) for binding of His-tagged NblA. Purification was performed as described above. Eluted proteins were immediately analyzed by Tricine-SDS-PAGE with 6 M urea (30).

Protein Expression, Purification, and *In Vitro* Binding Assays Using GST-tagged PC or GST-tagged NblA1₆₈₀₃/NblA2₆₈₀₃—The N-terminally GST-tagged α -subunit of PC (GST-PC) was expressed after induction with IPTG for 3 h at 30 °C and purified according to the manufacturer's protocol (GE Healthcare). Expression of the C-terminally GST-tagged NblA1₆₈₀₃ (NblA1₆₈₀₃-GST) was induced with IPTG for 80 h at 18 °C. NblA2₆₈₀₃-GST was expressed with IPTG overnight at 18 °C. GST fusion proteins were purified as described above. GST-free NblA1₆₈₀₃ and NblA2₆₈₀₃ were obtained by on-column cleavage with PreScission[™] protease (GE Healthcare). The NblA1₆₈₀₃-GST and His-tagged NblA2₆₈₀₃ were purified as described above. The expression of ClpC₆₈₀₃ was induced by IPTG for 3 h at 30 °C. The soluble fraction of the crude extract containing overexpressed ClpC₆₈₀₃ without affinity tag was used for the *in vitro* binding assays.

In vitro binding assays were started by adding glutathione-agarose to the soluble cell extract containing the overexpressed GST-PC fusion protein. After incubation for 30 min at room temperature with gentle agitation, unbound proteins were removed by three washes with five column volumes of buffer B. The PC-loaded agarose was divided into four equal aliquots and incubated with crude extract containing overexpressed ClpC₆₈₀₃. Additionally ATP, NblA1₆₈₀₃, NblA2₆₈₀₃, or NblA1₆₈₀₃ and His-NblA2₆₈₀₃ were added in surplus. After incubation for 30 min at room temperature with gentle agitation, unbound proteins were removed by washing with buffer B containing 1 mM ATP as described above. Bound proteins were eluted by the addition of 40 mM glutathione in 50 mM Tris-HCl (pH 8.0). Eluted proteins were directly analyzed by Tricine-SDS-PAGE with 6 M urea.

***In Vitro* Degradation of α -Casein, PBS, NblA1₆₈₀₃ and NblA2₆₈₀₃ by ClpC₆₈₀₃-ClpP1-ClpR**—Each purified protein was used in a final concentration of 2 μ M in buffer C (50 mM Tris-HCl (pH 7.5), 75 mM KCl, 1 mM DTT, 5 mM MgCl₂). All reactions were performed at 37 °C with an ATP-regenerating system consisting of 20 ng of pyruvate kinase (Roche Applied Science), 4 mM phosphoenolpyruvate, and 2 mM ATP. Degradation of α -casein (Sigma-Aldrich), the NblA1₆₈₀₃/NblA2₆₈₀₃ heterodimers, and the PBSs was analyzed by Tricine-SDS-PAGE in the presence of 6 M urea.

Cloning and Expression of Fluorescent Proteins in *Synechocystis* 6803—For FRET measurements, the donor cerulean (Cer) and the acceptor YFP were used. Due to the spectral overlap between donor (Cer) emission (excitation/emission maxima,

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433/475 nm) and acceptor (YFP) absorbance (excitation/emission maxima, 514/527 nm), excitation of the donor molecule leads to emission from the acceptor molecule when the proteins are in close proximity (1–10 nm). For labeling, we used the monomeric forms of YFP and Cer. These forms were generated by a single amino acid exchange (A206K) in order to eliminate self-association of the proteins and thus avoid false positive FRET signals (31).

For expression of fluorescent NblA proteins in *Synechocystis* 6803, a ~1480-bp fragment containing *nblA1*₆₈₀₃ and *nblA2*₆₈₀₃, flanked by putative promoter and terminator regions, was amplified using primers nbl11 (XhoI incorporated) and nbl27 (XbaI incorporated). The PCR product was digested by the restriction enzymes XbaI and XhoI and ligated into the XbaI-XhoI-digested plasmid pET22b, yielding plasmid pET/A1 and A2, which served to introduce an NcoI site at the 3'- or 5'-end of the *nblA1*₆₈₀₃ coding region and an NdeI site at the 3'- or 5'-end of the *nblA2*₆₈₀₃ coding region. The restriction sites were successively generated by mutagenesis (QuikChange® site-directed mutagenesis kit; Stratagene) using primers nblA1QCM1 and nblA1QCM2 (3'-end) or nblA1QCM3 and nblA1QCM4 (5'-end) as well as nblA2QCM1 and nblA2QCM2 (3'-end) or nblA2QCM3 and nblA2QCM4 (5'-end). Mutagenesis at the 3'-end of the *nblA1*₆₈₀₃ gene resulted in the change of the base triplet for Leu⁶¹ to a methionine residue and the concomitant loss of the base triplet for one amino acid (Gly⁶²). The amino acid sequence of the NblA2₆₈₀₃ protein was altered by site-directed mutagenesis from Leu⁵⁹ to a histidine residue and from Pro⁶⁰ to a methionine residue, whereby the mutagenesis of both 5'-ends did not result in any changes. The resultant plasmids were restricted with NcoI or NdeI and, after dephosphorylation, ligated to a PCR fragment harboring the coding region for *yfp* or *mCerulean*, yielding the plasmids pET22/A1_Y and A2, pET22/A1 and A2_C, pET22/Y_A1 and A2, and pET22/A1 and A2_C. The *yfp* gene was amplified by PCR using primers YFP1 (NcoI site inserted) and YFP2 (NcoI site inserted) and plasmid pEmYFP as a template. The fragment coding for *mCerulean* was PCR-amplified using primers CFP1 (NdeI site inserted) and CFP2 (NdeI site inserted); here the plasmid pEmCer served as a template. Using the described cloning strategy, we also constructed the plasmids pET22/A1_Y and A2_C and pET22/A1_Y and A2_C for coexpression of NblA1₆₈₀₃ and NblA2₆₈₀₃ C-terminally or N-terminally fused to different fluorophores in *Synechocystis* 6803 cells.

For the FRET controls, each of the two fluorophores used in this study, mCerulean and YFP, was C-terminally linked to an NblA2₆₈₀₃ molecule. To this end, the pET22/A1_Y and A2_C constructs described above were used to generate a second NcoI restriction site at the 5'-end of the *nblA1*₆₈₀₃ coding region by QuikChange® mutagenesis using primers nblA1QCM3 and nblA1QCM4. The resultant plasmid pVZ/Y and A2_C was partially restricted with NcoI, dephosphorylated, and ligated to a PCR fragment harboring the coding region of *nblA2*₆₈₀₃, yielding plasmid pET22/A2_Y and A2_C. The *nblA2*₆₈₀₃ fragment was amplified with the primers nbl14 and nbl37, whereby the inserted NcoI site at the 3'-end led to a change from Leu⁵⁹ to a proline residue and from Pro⁶⁰ to a tryptophan residue. In addition to the A2_Y and A2_C control,

we also used the yielded construct pVZ/Y and A2_C as a control.

All DNA constructs were confirmed by DNA sequence analysis. Finally, the plasmids were restricted with XbaI and XhoI, obtaining DNA fragments for expression of fluorophore-tagged *nblA1*₆₈₀₃ and *nblA2*₆₈₀₃ together with the promoter and terminator region, which were then ligated into the conjugative, self-replicating plasmid pVZ321. The plasmids pVZ/A1_Y and A2, pVZ/A1 and A2_C, pVZ/A1_Y and A2_C, pVZ/Y_A1 and A2, pVZ/A1 and C_A2, pVZ/Y_A1 and Cer_A2, and pVZ/A2_Y and A2_C were transferred to the *nblA1/nblA2* double mutant of *Synechocystis* 6803, and the plasmid pVZ/YFP and A2_Cer was transferred to $\Delta nbl2$ mutant cells of *Synechocystis* 6803 by conjugation (32). Exconjugants were selected on BG11 agar containing 50 $\mu\text{g ml}^{-1}$ kanamycin and 14 $\mu\text{g ml}^{-1}$ chloramphenicol.

FRET Measurements—The FRET signal was detected with a fluorescence spectrometer (FluoroMax-4-Horiba) in a suspension of living *Synechocystis* 6803 cells with an A_{750} of ~0.3. Cer expressed in the cells was excited by monochromatic 458-nm light, and fluorescence emission spectra were recorded in the wavelength range from 500 to 600 nm.

Fluorescence Lifetime Measurements—Fluorescence lifetime measurements were performed using a FluoTime 200 instrument (PicoQuant) in suspension of living *Synechocystis* 6803 cells with an A_{750} of ~0.3 as described before (33).

Complementation Experiments with NblA of Nostoc 7120—The plasmid for expression of *nblA* from *Nostoc* 7120 was constructed as follows. A 669-bp fragment, bearing upstream sequences of *nblA1*₆₈₀₃ (*ssl0452*) and *nblA2*₆₈₀₃ (*ssl0453*) and a 417-bp fragment containing downstream sequences of *nblA1*₆₈₀₃ and *nblA2*₆₈₀₃, was amplified by PCR from total *Synechocystis* 6803 DNA using primers nbl11 (XhoI site inserted) and nbl13 (NcoI site inserted) and primers nbl26 (NdeI site inserted) and nbl27 (XbaI site inserted), respectively. The fragments were cloned into the plasmid pET22b, yielding plasmid pET22/P₆₈₀₃T₆₈₀₃. Subsequently, the plasmid pET22/P₆₈₀₃T₆₈₀₃ was digested with NcoI and NdeI and ligated to a fragment harboring the *nblA* gene from *Nostoc* 7120, which was amplified by PCR from total *Nostoc* 7120 DNA using primers nblana.2.23 (NcoI site inserted) and nblana.2.24 (NdeI site inserted), yielding plasmid pET22/P₆₈₀₃NblA₇₁₂₀T₆₈₀₃. The fragment harboring the *nblA* gene from *Nostoc* 7120, flanked by the putative promoter and terminator regions of *nblA1*₆₈₀₃ and *nblA2*₆₈₀₃ from *Synechocystis* 6803, was excised by digestion with XbaI and XhoI and inserted into the conjugative, self-replicating plasmid pVZ321 (32), replacing the plasmid's Km resistance cassette, yielding plasmid pVZ/P₆₈₀₃NblA₇₁₂₀T₆₈₀₃. The plasmid pVZ/P₆₈₀₃NblA₇₁₂₀ was transferred to *nblA1/nblA2* double mutant cells by conjugation, and exconjugants were selected on BG11 agar containing 50 $\mu\text{g ml}^{-1}$ kanamycin and 14 $\mu\text{g ml}^{-1}$ chloramphenicol.

Zinc-induced Fluorescence—For zinc-induced fluorescence, the Tricine-SDS-PAGE was run with 1 mM zinc acetate in cathode buffer (30). The fluorescence was visualized by a UV transilluminator (Bio-Rad).

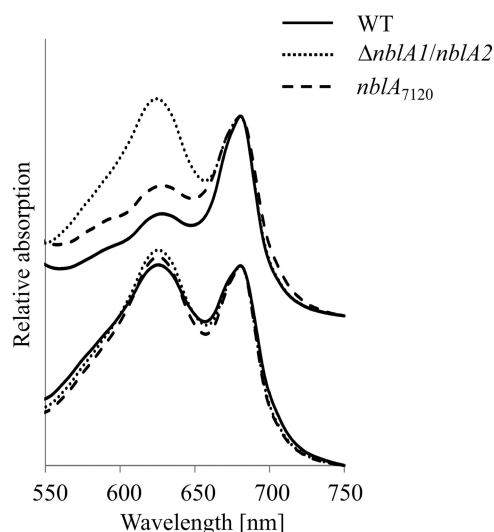


FIGURE 1. **Complementation experiment.** Absorbance spectra of the *Synechocystis* 6803 wild type, the *nblA1/nblA2* double mutant of *Synechocystis* 6803, and the *nblA1/nblA2* mutant of *Synechocystis* 6803 complemented *in trans* with *nblA* from *Nostoc* 7120, either grown in nitrogen-replete medium (+N) or starved of combined nitrogen for 24 h (–N), are shown.

RESULTS

Homodimeric NblA of Nostoc 7120 Complements the Non-bleaching Phenotype of the nblA1/nblA2 Double Mutant of Synechocystis 6803—Genomes of most cyanobacteria, such as *Synechococcus* 7942 and *Nostoc* 7120, encode just one *nblA* gene, whose expression suffices to initiate the proteolytic degradation of the PBS. The intensively studied unicellular strain *Synechocystis* 6803, however, simultaneously expresses two sequence-related *nblA* genes, *nblA1*₆₈₀₃ and *nblA2*₆₈₀₃ to induce PBS degradation (15, 16).

Complementation experiments were performed to determine whether the two NblA proteins from *Synechocystis* 6803 act in the same manner as NblA from *Nostoc* 7120 (20). To this end, the coding region of *Nostoc* 7120 *nblA* (ORF *asr4517*) and a DNA fragment encoding *Synechocystis* 6803 *nblA1*₆₈₀₃ and *nblA2*₆₈₀₃ were amplified by PCR. Both fragments were ligated into the self-replicating plasmid pVZ321 containing the predicted promoter and terminator regions of *Synechocystis* 6803 *nblA1*₆₈₀₃ and *nblA2*₆₈₀₃, which are cotranscribed from a common promoter (15). The generated plasmids were subsequently transferred to the *Synechocystis* 6803 *nblA1/nblA2* double mutant by triparental mating (34).

When grown in nitrate-containing medium, the pigmentation of the resulting exconjugants was indistinguishable from that of the wild type, as judged from whole-cell absorbance spectra (Fig. 1). After transfer to nitrogen-free medium, *nblA1*₆₈₀₃ and *nblA2*₆₈₀₃, expressed *in trans* in the *nblA1/nblA2* double mutant, complemented the mutant phenotype, visible in the absorbance spectra shown in Fig. 1 as a decrease in the PC absorbance peak at ~625 nm. A similar complementation effect was obtained with *nblA* from *Nostoc* 7120. This clearly demonstrates that the homodimeric NblA from *Nostoc* 7120 is able to replace the function of NblA1₆₈₀₃ and NblA2₆₈₀₃ in *Synechocystis* 6803.

Detecting the Interaction between NblA1₆₈₀₃ and NblA2₆₈₀₃ in Vivo by FRET—The biologically active form of NblA from *Nostoc* 7120 is a homodimer (19). Dimer formation seems to be a characteristic feature of NblA proteins (27) and is assumed to be essential for their function. To monitor the dimerization of the two NblA proteins during the PBS degradation process, we used a method based on FRET. As a donor-acceptor pair, we chose Cer (35), a variant of enhanced cyan fluorescent protein (CFP), and YFP.

Plasmids containing the monomeric forms of YFP and Cer (31), designated Y and C, respectively, were used for labeling. The NblA-fluorophore fusion proteins were expressed in the *nblA1/nblA2* double mutant under the control of the NblA promoter and terminator region in the self-replicating vector pVZ321. The following controls were used for evaluation of FRET measurements: (i) *nblA1/nblA2* double mutant transformed with plasmids expressing only a single fluorophore fused to NblA1₆₈₀₃ or NblA2₆₈₀₃; (ii) *nblA1/nblA2* double mutant transformed with plasmid pVZ/A2_Y and A2_C expressing two NblA2₆₈₀₃ proteins, one tagged with YFP and one tagged with Cer; (iii) *nblA2* single mutant (15) transformed with plasmid pVZ/Y and A2_C, which allows expression of the YFP protein and C-terminal Cer-tagged NblA2₆₈₀₃; and (iv) *nblA2* single mutant transformed with plasmid pVZ/A2_Y and A2_C (see above).

In preliminary experiments, we determined whether NblA-fluorophore fusion proteins were functional *in vivo*. Mutants grown in nitrogen-containing medium (BG11) were transferred to medium without combined nitrogen (BG11₀), and whole-cell absorbance spectra were recorded after 24 h (Fig. 2, A–C). PBS degradation after nitrogen deprivation took place in all strains expressing only a single fluorophore, regardless of whether the fluorophore was fused to the C or N terminus of the respective NblA protein (Fig. 2, A and B). Complementation was also observed in mutants expressing C-terminally tagged NblA1₆₈₀₃ and NblA2₆₈₀₃ (A1_Y and A2_C) (Fig. 2A). However, expression of N-terminally tagged NblA proteins (Y_A1 and C_A2) did not induce PBS degradation (Fig. 2B). This observation can be explained by our previous finding that NblA triggers PBS degradation by binding to ClpC via its N-terminal helix (20). Accordingly, fluorophores fused to the N terminus of NblA presumably sterically hinder the interaction with ClpC, thereby interfering with the *in vivo* function of NblA. In this context, it is remarkable that complementation occurred when only one of the two NblA proteins was N-terminally fused to a fluorophore, whereas it did not matter which NblA protein was labeled or what fluorophore it was labeled with (Fig. 2B). Furthermore, as shown in Fig. 2C, expression of YFP and C-terminally tagged NblA2₆₈₀₃ (Y and A2_C) and simultaneous expression of two NblA2₆₈₀₃ proteins with C-terminal YFP or Cer (A2_Y and A2_C) complemented the non-bleaching phenotype of the *nblA2*-deficient mutant.

On the basis of the results of complementation experiments, we used the mutant expressing functional C-terminally tagged NblA1₆₈₀₃ and NblA2₆₈₀₃ for our initial FRET measurements. To monitor possible interactions between NblA1₆₈₀₃-YFP and NblA2₆₈₀₃-Cer, we used fluorescence spectroscopy. We excited the donor molecule (Cer) by actinic light with a wavelength of

PBS Degradation Depends on NblA1/NblA2 and a Clp Protease

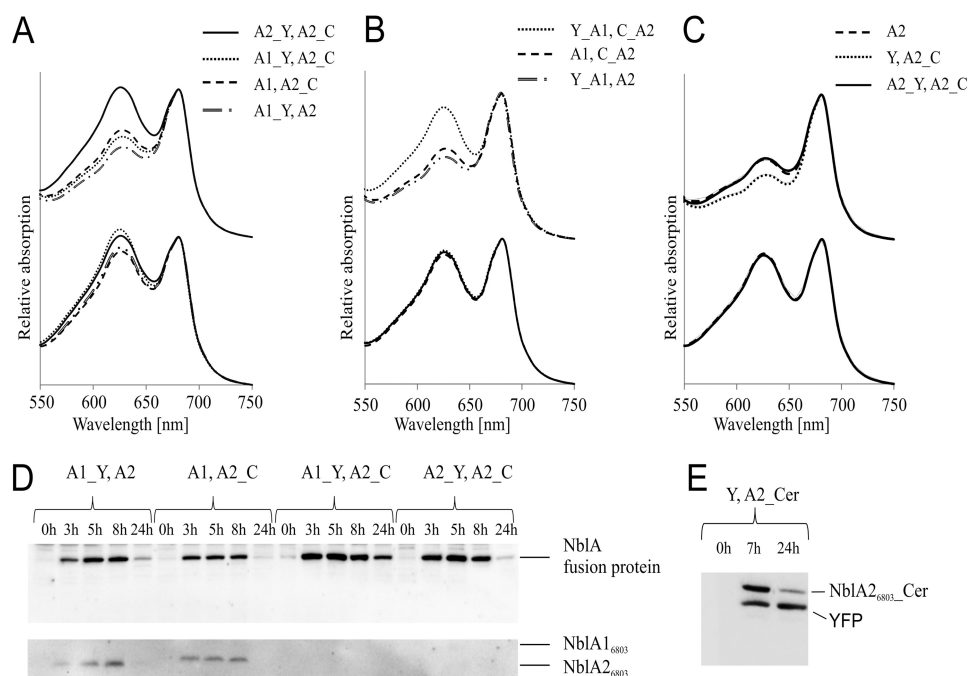


FIGURE 2. FRET constructs and their functionalities. Analyses of different NblA proteins, C- or N-terminally fused to the FRET donor Cer or FRET acceptor YFP, in *Synechocystis* 6803. **A**, NblA proteins C-terminally fused to the FRET donor Cer or FRET acceptor YFP complement the *nblA1/nblA2* double mutant of *Synechocystis* 6803. Absorbance spectra of mutants either grown in nitrogen-replete medium (+N) or starved of combined nitrogen for 24 h (–N) are shown. **B**, NblA proteins N-terminally fused to the FRET donor Cer and FRET acceptor YFP cannot complement the *nblA1/nblA2* double mutant of *Synechocystis* 6803. Mutants were either grown in nitrogen-replete medium (+N) or starved of combined nitrogen for 24 h (–N). A decrease in absorbance at 620 nm indicates degradation of PBS. **C**, expression of NblA2₆₈₀₃ proteins in the *nblA2*-deficient mutant of *Synechocystis* 6803 complements the non-bleaching phenotype. Mutants were either grown in nitrogen-replete medium (+N) or starved of combined nitrogen for 24 h (–N). A decrease in absorbance at 620 nm indicates degradation of PBS. **D**, immunoblot analysis of the NblA fusion proteins in crude extracts of *Synechocystis* 6803 immediately (0 h) and at various times after nitrogen step-down. Blots were probed with a mixture of NblA1₆₈₀₃ and NblA2₆₈₀₃ antisera. **E**, immunoblot analysis of YFP and A2_C proteins in crude extracts of *Synechocystis* 6803 mutants immediately (0 h) and at 7 and 24 h after nitrogen step-down. The blots were probed with a GFP antiserum.

458 nm and detected the emitted light in the range of 525–575 nm. YFP-specific emission was detected in the range of 500 and 600 nm, indicating the occurrence of FRET due to the interaction of fluorophore-tagged NblA1₆₈₀₃ and NblA2₆₈₀₃.

Expression of NblA proteins was induced by nitrogen depletion and monitored by immunoblot analysis (Fig. 2, *D* and *E*). PBS degradation was verified by recording emission spectra of whole cells immediately as well as 3, 5, 8, and 24 h after nitrogen deprivation. There were no significant differences in emission spectra among analyzed mutants grown on nitrate-containing medium (Fig. 3, *A* and *B*; 0 h); the emission peak at ~540 nm corresponds to *Synechocystis* 6803 autofluorescence. Mutants were then switched to nitrogen starvation conditions, and fluorescence emission was monitored for 24 h. The mutant expressing C-terminally tagged NblA1₆₈₀₃ and NblA2₆₈₀₃ (A1_Y and A2_C) showed an increase in the amplitude of acceptor (YFP) emission at ~530 nm over the first 8 h. After 24 h of nitrogen starvation, acceptor emission was still detected, albeit with a lower amplitude (Fig. 3*A*, 24 h).

Whole-cell emission spectra of control samples of mutants containing only Cer donor (A1 and A2_C) or YFP acceptor (A1_Y and A2) fluorophores showed no YFP emission at 530 nm following excitation at 458 nm (Fig. 3*A*), indicating the absence of FRET. Expression of the fluorophores was verified by immunoblot analysis (Fig. 2*D*). The emission spectra of control samples indicated that YFP is not directly excited by the donor (Cer) excitation wavelength and that the detected emission signal is YFP-specific and hence results from FRET. Our

attempts to measure Cer emission in cells expressing only Cer fused to NblA2₆₈₀₃ failed due to the strong autofluorescence of the photosynthetic pigments, which spectrally overlaps with the emission of Cer (475–525 nm; not shown).

As noted above, we also analyzed mutants expressing both fluorophores (YFP and Cer) together in one *Synechocystis* 6803 cell but not fused to interacting NblA proteins. Protein expression was once again monitored by immunoblot analysis with anti-NblA (Fig. 2*D*) and anti-GFP antibodies (Fig. 2*E*). Both fluorophores were presumed to be expressed, and FRET was measured as described above. The emission spectrum of the mutant expressing YFP and Cer coupled to NblA2₆₈₀₃ showed no FRET signal (Fig. 3*B*, Y and A2_C), and its spectrum did not differ significantly from that of mutants containing only a single fluorophore (Fig. 3, *A* and *B*). The A2_Y and A2_C mutant also showed no FRET signal during the first 8 h after nitrogen depletion (Fig. 3*A*). After 24 h, however, an increase in acceptor fluorescence indicative of FRET was detected, albeit to a lesser extent than in the A1_Y and A2_C mutant. Interestingly, immunoblot analyses revealed an induction of protein expression within the first hours after nitrogen step-down and a decrease in protein levels after 24 h, a pattern inconsistent with the temporal changes in the FRET signal (Fig. 2*D*).

In order to attribute the observable changes in steady-state fluorescence characteristics to changes in the donor fluorescence lifetime, we performed time-resolved measurements of the donor (Cer) lifetime in addition to measuring steady-state FRET. Donor fluorescence lifetime might decrease, for exam-

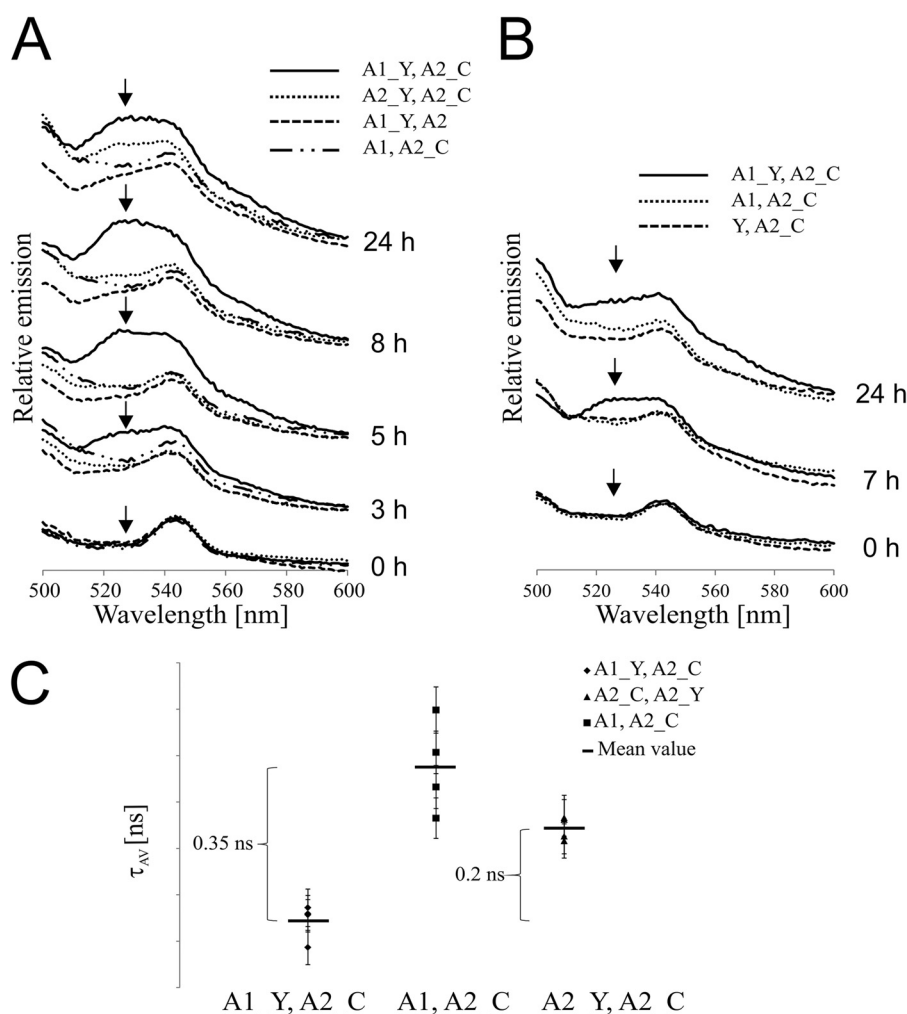


FIGURE 3. Formation of NblA1₆₈₀₃–NblA2₆₈₀₃ heterodimers in vivo. The *nblA1/nblA2* double mutant of *Synechocystis* 6803 was complemented in *trans*, under the control of the *nblA* promoter and terminator, with one NblA protein C-terminally fused to the FRET donor Cerulean (C) and the other fused to the FRET acceptor YFP (Y). For expression of NblA fusion proteins, the *Synechocystis* 6803 mutants were starved of combined nitrogen. **A**, fluorescence emission spectra of *Synechocystis* 6803 cells. Emission spectra of A1_Y, A2_C, A1_Y, A2, A1, A2_C, and A2_Y, A2_C mutants were recorded on cultures adjusted to an A_{750} of 0.3. Cerulean was excited at 458 nm, and emission of the FRET partner YFP was recorded between 500 and 600 nm after 0, 3, 5, 8, and 24 h of nitrogen depletion. **B**, fluorescence emission spectra of *Synechocystis* 6803 cells. Emission spectra of A1_Y and A2_C, A1 and A2_C, and Y and A2_C mutants were recorded at cell densities corresponding to an A_{750} of 0.3. Cerulean expressed in the cells was excited at 458 nm, and emission of the FRET partner YFP was recorded between 500 and 600 nm after 0, 7, and 24 h of nitrogen depletion. **C**, fluorescence lifetime spectroscopy of the A1_Y and A2_C, A2_C and A2_Y, and A1 and A2_C mutants. Cells at a density corresponding to an A_{750} of 0.3 were analyzed after 8 h of nitrogen starvation. Fluorescence intensity measurements were carried out in 1-cm cuvettes with excitation at 440 nm and emission of cerulean-based fluorescence decay recorded at 475 nm. Only a single photon every 100 laser pulses was registered (1 count); therefore, measurements were repeated for a total of 10,000 counts, and the measured time differences were sorted into a histogram. Each measurement was repeated seven times. The resulting decay curves were analyzed by fitting to the sum of two exponential terms using a non-linear least squares iterative procedure (FluoFit, PicoQuant). For each measurement, the amplitude-weighted lifetime (τ_{AV}) of cerulean was calculated. The mean values of τ_{AV} for each biological replicate are shown in the diagram, and the S.D. for each value is indicated. Furthermore the mean value of τ_{AV} for cerulean-based fluorescence of each mutant is shown.

ple, when the acceptor molecule is in close proximity to the donor molecule and absorbs energy through FRET. These measurements were performed 8 h after nitrogen step-down, the time at which the strongest FRET signal occurred (Fig. 3A). The average fluorescence lifetime of Cer fused to NblA2₆₈₀₃ was 2.29 ns (Fig. 3C). In the presence of YFP fused to NblA1₆₈₀₃, the fluorescence lifetime of Cer decreased to 1.94 ns, a difference ($\Delta\tau_{AV}$) of 0.35 ns. This faster decay of Cer fluorescence is attributable to the energy transfer (FRET) from Cer to YFP that occurred when the two fluorophores were brought close together by interactions between NblA1₆₈₀₃ and NblA2₆₈₀₃. When YFP was linked to NblA2₆₈₀₃ instead of NblA1₆₈₀₃, the fluorescence lifetime of Cer fused to another NblA2₆₈₀₃ mole-

cule also decreased, albeit to a lesser extent ($\Delta\tau_{AV} = 0.15$ ns; Fig. 3C). The possibility that the reduced fluorescence lifetime is due to nonspecific energy transfer from the donor to the acceptor cannot be excluded. However, the resulting difference in fluorescence lifetimes between Cer and Cer expressed together with YFP ($\Delta\tau_{AV} = 0.2$ ns) still indicates a clear FRET signal.

Taken together, the results from time-resolved fluorescence measurements confirm our steady-state measurements, indicating that FRET occurred when Cer and YFP were connected to NblA1₆₈₀₃ and NblA2₆₈₀₃, respectively. The much weaker FRET signal that occurred when both fluorophores were coupled to NblA2₆₈₀₃ suggests a weak tendency of NblA2 to form homodimers.

PBS Degradation Depends on NblA1/NblA2 and a Clp Protease

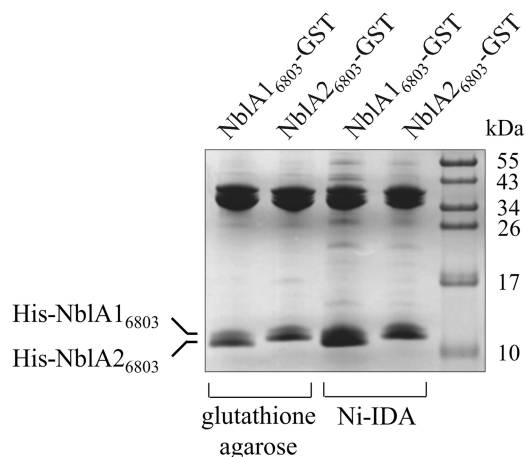


FIGURE 4. Coprecipitation of affinity-tagged NblA1₆₈₀₃ and NblA2₆₈₀₃. Crude extracts from *E. coli* coexpressing either GST-tagged NblA1₆₈₀₃ and His-tagged NblA2₆₈₀₃ or His-tagged NblA1₆₈₀₃ and GST-tagged NblA2₆₈₀₃ were incubated with glutathione-agarose and Protino Ni-IDA resins, respectively. Protein complexes bound to the agarose were eluted with glutathione- or imidazole-containing buffer, respectively, separated by Tricine-SDS-PAGE, and stained with Coomassie Blue R-250.

Detecting the Interaction between NblA1₆₈₀₃ and NblA2₆₈₀₃ in Vitro by Pull-down Experiments—To validate our *in vivo* results, we performed *in vitro* binding studies. We first cloned *nblA1₆₈₀₃* and *nblA2₆₈₀₃* into the expression vector pACYC-Duet-1, which is designed for coexpression of two target proteins. This vector was chosen to imitate expression in *Synechocystis* 6803, where *nblA1₆₈₀₃* and *nblA2₆₈₀₃* are adjacent cotranscribed genes (15).

Affinity purification of NblA1₆₈₀₃-GST invariably resulted in coprecipitation of HisNblA2₆₈₀₃ and *vice versa* (Fig. 4), clearly demonstrating an interaction between NblA1₆₈₀₃ and NblA2₆₈₀₃ *in vitro*. These findings corroborate the results obtained with *in vivo* binding studies, described above. Taken together, *in vivo* and *in vitro* experiments provide evidence for an interaction between NblA1₆₈₀₃ and NblA2₆₈₀₃, indicating that the biologically active form is a heterodimer.

NblA1₆₈₀₃ and NblA2₆₈₀₃ Heterodimers Interact with ClpC₆₈₀₃ and the α -Subunit of PC in Vitro—Previous investigations of NblA function in *Nostoc* 7120 revealed that formation of a ternary complex among NblA, phycobiliproteins, and ClpC₆₈₀₃, the chaperone partner of Clp proteases, is indispensable for PBS degradation (20). Here, we performed binding experiments with NblA1₆₈₀₃, NblA2₆₈₀₃, ClpC₆₈₀₃, and the apoprotein of the α -subunit of PC from *Synechocystis* 6803. The N-terminally GST-tagged apoprotein of the PC α -subunit was immobilized on glutathione-agarose. The loaded matrix was then divided into two equal aliquots and incubated with crude extracts of ClpC₆₈₀₃-overexpressing *E. coli* in the presence or absence of NblA1₆₈₀₃ and NblA2₆₈₀₃. As expected, copurification of ClpC₆₈₀₃ was observed only when both NblA1₆₈₀₃ and NblA2₆₈₀₃ were present (Fig. 5A). Similar results were obtained using another experimental approach. In this case, heterodimers consisting of NblA1₆₈₀₃ and His-tagged NblA2₆₈₀₃ were immobilized on nickel-nitrilotriacetic acid, incubated first with a crude extract of *E. coli* expressing ClpC₆₈₀₃ and then with a crude extract of *Synechocystis* 6803. As shown in Fig. 5 (B and

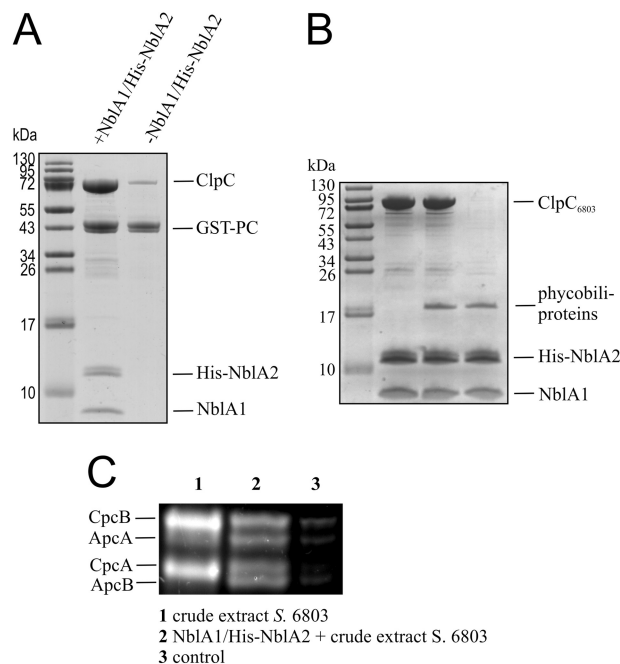


FIGURE 5. The NblA1₆₈₀₃-NblA2₆₈₀₃ heterodimer interacts simultaneously with phycobiliproteins and ClpC₆₈₀₃. A, the α -subunit of phycocyanin was expressed with an N-terminal GST tag, immobilized on glutathione-agarose, and incubated with soluble crude extract from ClpC₆₈₀₃-expressing *E. coli* cells in the presence or absence of NblA1₆₈₀₃ and His-tagged NblA2₆₈₀₃. After washing, bound protein complexes were eluted with glutathione, resolved by Tricine-SDS-PAGE, and stained with Coomassie Blue R-250. Experiments were performed in buffer B containing 1 mM Mg-ATP. B, the NblA1₆₈₀₃/His-NblA2₆₈₀₃ protein complex was immobilized on Protino Ni-IDA resin and incubated with crude extract from *E. coli* cells expressing ClpC₆₈₀₃. After washing, the matrix was incubated with *Synechocystis* 6803 crude extract. The bound protein complexes were eluted with imidazole-containing buffer, resolved by Tricine-SDS-PAGE, and stained with Coomassie Blue R-250. Experiments were performed in buffer B containing 1 mM Mg-ATP. C, NblA1 and His-NblA2 were immobilized on Protino Ni-IDA resin and incubated with *Synechocystis* 6803 crude extract (lane 2). As a control, HisClpC-loaded Protino Ni-IDA resin was incubated with *Synechocystis* 6803 crude extract (lane 3). The bound proteins were eluted as described above. Additionally, *Synechocystis* 6803 crude extract was loaded to visualize the phycobili subunits (lane 1) and resolved by Tricine-SDS-PAGE. The phycobiliproteins were detected by zinc-induced fluorescence after SDS-PAGE.

C), ClpC₆₈₀₃ and phycobiliproteins (PC and apophycocyanin) copurified with NblA1₆₈₀₃ and His-tagged NblA2₆₈₀₃.

NblA1₆₈₀₃ Mediates the Interaction of ClpC₆₈₀₃ with Phycobiliproteins—As shown previously, a highly conserved stretch of amino acids near the N terminus of NblA from *Nostoc* 7120 is involved in ClpC binding (20), whereas residues near the C terminus are involved in phycobiliprotein binding (19). Assuming that the biologically active form of NblA in *Synechocystis* 6803 is a heterodimer, we would expect that both NblA1₆₈₀₃ and NblA2₆₈₀₃ would be able to bind ClpC₆₈₀₃ and phycobiliproteins and that both NblA proteins would be essential for formation of a complex with ClpC₆₈₀₃ and phycobiliproteins. To verify this hypothesis, we first analyzed whether NblA1₆₈₀₃ and NblA2₆₈₀₃ interact with ClpC₆₈₀₃ *in vitro*. To this end, we immobilized C-terminally GST-tagged NblA1₆₈₀₃ and NblA2₆₈₀₃ on glutathione-agarose and incubated each with crude extracts of *E. coli* expressing ClpC₆₈₀₃. After washing, proteins were eluted and separated by Tricine-SDS-PAGE (Fig. 6A). ClpC₆₈₀₃ copurified only with GST-tagged NblA1₆₈₀₃. There was no interaction between ClpC₆₈₀₃ and NblA2₆₈₀₃

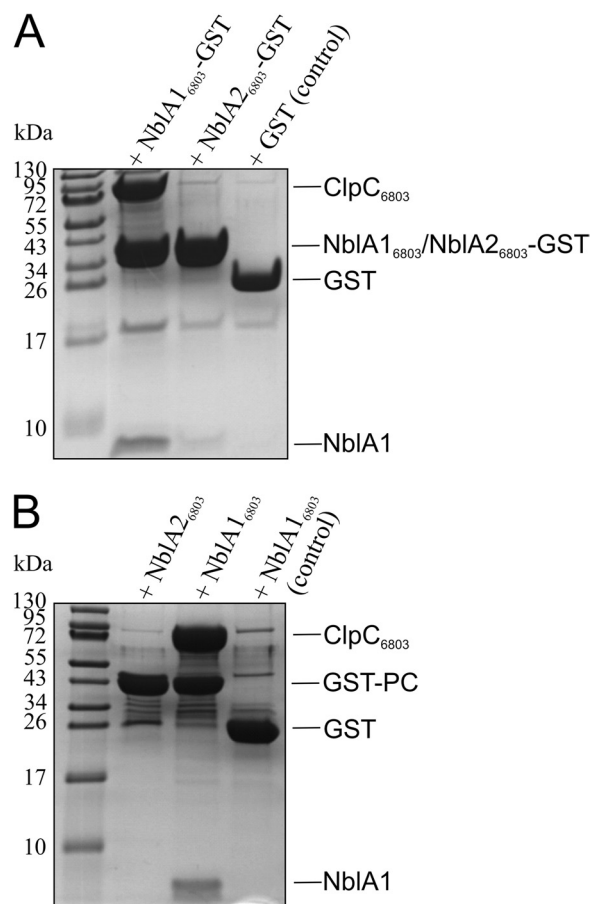


FIGURE 6. NblA1₆₈₀₃ mediates binding to ClpC₆₈₀₃ and phycobiliproteins. A, binding of ClpC₆₈₀₃ to GST-tagged NblA1₆₈₀₃ *in vitro*. NblA1₆₈₀₃ and NblA2₆₈₀₃ were expressed with a C-terminal GST tag, immobilized on glutathione-agarose, and incubated (in the presence of 5 mM Mg-ATP) with soluble crude extract from *E. coli* cells expressing ClpC₆₈₀₃. After washing, NblA-GST complexes were eluted with glutathione, resolved by Tricine-SDS-PAGE, and stained with Coomassie Blue R-250. GST protein served as control. B, NblA1₆₈₀₃ binds simultaneously to ClpC₆₈₀₃ and the GST-tagged apoprotein of phycocyanin. The α -subunit of phycocyanin was expressed with an N-terminal GST tag and preincubated with NblA1₆₈₀₃ or NblA2₆₈₀₃. After the addition of glutathione-agarose, proteins were incubated with soluble crude extract from ClpC₆₈₀₃-expressing *E. coli* cells in the presence of 5 mM Mg-ATP. After washing, GST-PC complexes were eluted with glutathione, separated by Tricine-SDS-PAGE, and stained with Coomassie Blue R-250. GST protein served as a control.

(lane 2). We conclude that the ClpC₆₈₀₃ binding site on the heterodimer is located on NblA1₆₈₀₃.

To demonstrate that NblA1₆₈₀₃ is sufficient to induce formation of the ternary complex with ClpC₆₈₀₃ and phycobiliproteins *in vitro*, we first immobilized the GST-tagged apoprotein of α -PC on glutathione-agarose. We then incubated the protein-agarose conjugate with a crude extract of ClpC₆₈₀₃-expressing *E. coli* in the presence of NblA1₆₈₀₃ or NblA2₆₈₀₃. As expected, given the inability of NblA2₆₈₀₃ to interact with ClpC₆₈₀₃ (Fig. 6A), ClpC₆₈₀₃ did not copurify with NblA2₆₈₀₃ (Fig. 6B). Notably, NblA2₆₈₀₃ itself also did not copurify with GST-tagged PC, whereas NblA1₆₈₀₃ bound simultaneously to the PC apoprotein and ClpC₆₈₀₃ (Fig. 6B). These results indicate that the presence of NblA1₆₈₀₃ is sufficient for *in vitro* formation of the ternary complex required for PBS degradation.

Heterologously Coexpressed ClpP1 and ClpR Form a Proteolytic Complex—Clp proteases of cyanobacteria were first identified in *Synechococcus* sp. PCC7942 (36). In this organism, the Clp machinery consists of two chaperones (ClpC and ClpX), three different proteolytic subunits (ClpP1, ClpP2, and ClpP3), and the ClpP variant ClpR, which lacks the typical catalytic triad of serine-type proteases. In *Synechococcus* 7942, two Clp proteases could be identified by gel filtration and immunoblot analyses. Each protease is a complex composed of a unique proteolytic core, containing ClpP1 and ClpP2 or ClpP3 and ClpR, and a particular chaperone partner: ClpC in the case of ClpP3ClpR and ClpX in the case of ClpP1ClpR. In addition to these two soluble Clp proteases, a third, membrane-associated proteolytic complex consisting of ClpP1 and ClpR is assumed.

Homologs of the entire Clp machinery, including the ClpP1-ClpR complex, can be found in *Synechocystis* 6803. This protease has been suggested to interact with ClpC and, as shown by cross-linking studies, probably binds to the PBS (36, 37), making it a logical candidate for subsequent *in vitro* studies in *Synechocystis* 6803. Here, we expressed the proteolytic core complex composed of ClpP1 and ClpR using the pACYCDuet-1 vector. Expecting that ClpP1 and ClpR would spontaneously associate with each other, we used only one affinity tag, a His tag fused to the C terminus of ClpP1, for purification of the core complex. At 25.2 and 24.8 kDa, respectively, HisClpP1 and ClpR have nearly the same molecular mass; thus, they cannot be separated from each other by Tricine-SDS-PAGE (Fig. 7A). To determine whether ClpR copurifies with HisClpP1, we performed size-exclusion chromatography³ and native PAGE.³ The apparent molecular mass of the protein complex was 200 kDa, indicating that ~3–4 copies of ClpR and HisClpP1 assemble into an oligomeric structure.

Hydrolysis of α -Casein by the ClpP1-ClpR Core Complex Depends on ClpC₆₈₀₃ and ATP—To analyze the proteolytic activity of the recombinant ClpP1-HisClpR complex, we tested its capacity to degrade α -casein. Accordingly, we incubated the complex with α -casein, HisClpC₆₈₀₃, and ATP in the presence of an ATP-regenerating system (38). With numerous exposed hydrophobic amino acids on its surface (39), α -casein from cow's milk is a nonspecific substrate for Clp proteases (40, 41). ClpC₆₈₀₃ is the putative chaperone partner of the ClpP1-ClpR proteolytic core complex and is required for substrate recognition, ATP-dependent unfolding, and translocation into the proteolytic chamber. At intervals after combining reaction components, aliquots of the assay mixture were withdrawn, proteins were separated by Tricine-SDS-PAGE, and α -casein concentration was estimated by Coomassie Blue staining (Fig. 7A). The ClpC₆₈₀₃-ClpP1-ClpR complex completely degraded α -casein within 10 min (Fig. 7A). As expected, given the functional roles of ClpC₆₈₀₃, no degradation occurred in the absence of this putative chaperone (Fig. 7A, lanes 7 and 8). Moreover, no degradation of α -casein was observed upon incubation with ClpC₆₈₀₃ alone (Fig. 7A, lanes 9 and 10), demonstrating that all three proteins (ClpP1, ClpR, and ClpC₆₈₀₃) are required to form a proteolytically active Clp complex.

³ A. Baier, W. Winkler, T. Korte, W. Lockau, and A. Karradt, unpublished data.

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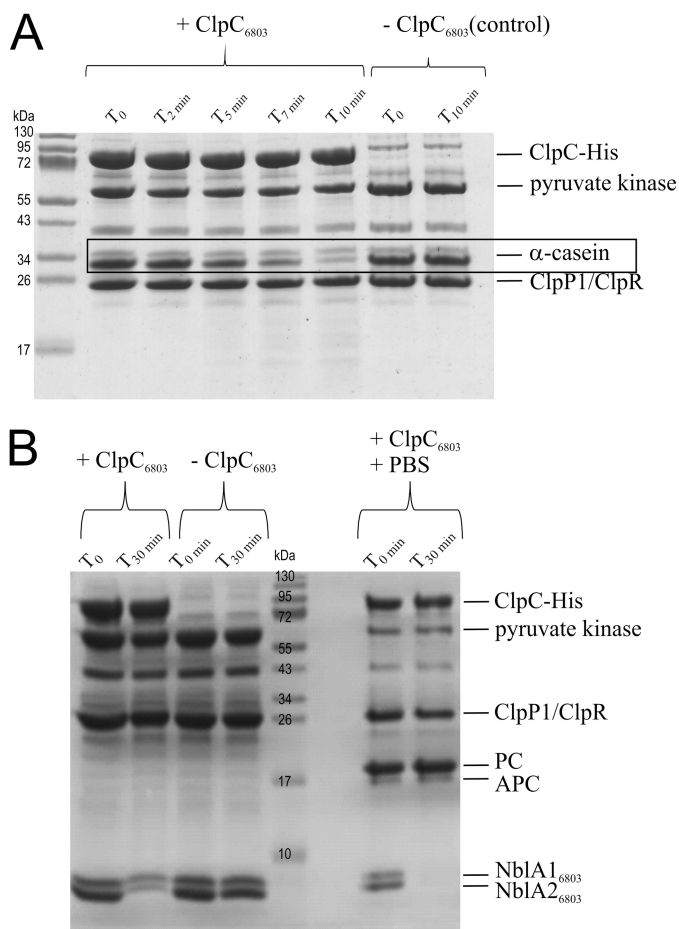


FIGURE 7. ClpC₆₈₀₃ forms an active proteolytic complex with ClpP1-ClpR and degrades the adaptor proteins NblA1₆₈₀₃ and NblA2₆₈₀₃. *A*, *in vitro* degradation of α-casein. α-Casein was incubated in the presence of pyruvate kinase (20 ng/μl), 4 mM phosphoenolpyruvate, 2 mM ATP, and purified ClpC₆₈₀₃ and ClpP1/R (2 μM each) at 37 °C. Samples were taken at the indicated time points, separated by SDS-PAGE, and visualized by Coomassie Blue R-250 staining. *B*, *in vitro* degradation of purified NblA1₆₈₀₃ and NblA2₆₈₀₃. NblA1₆₈₀₃/NblA2₆₈₀₃ (5 μM each) were incubated in the presence of pyruvate kinase (20 ng/μl), 4 mM phosphoenolpyruvate, 2 mM ATP, purified ClpC₆₈₀₃ and ClpP1-ClpR (2 μM each), and purified PBS from *Synechocystis* 6803 (29) at 37 °C. Samples were taken at the indicated time points, separated by SDS-PAGE, and visualized by Coomassie Blue R-250 staining.

A Clp Protease Composed of ClpC₆₈₀₃, ClpP1, and ClpR Degrades NblA1₆₈₀₃ and NblA2₆₈₀₃ in Vitro—Previous investigations of the interaction between NblA and ClpC from *Nostoc* 7120 showed that exchanging amino acids in the ClpC binding site of NblA results in accumulation of NblA *in vivo*.³ From this we inferred that NblA acts as an adaptor protein that is codegraded with its substrate as part of the overall regulatory mechanism. We therefore asked whether NblA proteins are degraded *in vitro* by recombinant *Synechocystis* 6803 Clp protease.

To test the hypothesis that NblA itself may be codegraded by the Clp protease, we performed degradation experiments with NblA1₆₈₀₃ and NblA2₆₈₀₃ essentially as described above. Degradation of NblA1₆₈₀₃ and NblA2₆₈₀₃ was monitored by Coomassie Blue staining of samples resolved on Tricine-SDS-polyacrylamide gels. Fig. 7*B* illustrates the simultaneous degradation of NblA proteins within 30 min. No degradation was observed in the absence of ClpC₆₈₀₃ or ATP.³ These results

demonstrate that the putative Clp protease ClpC₆₈₀₃-ClpP1-ClpR degrades NblA1₆₈₀₃ and NblA2₆₈₀₃ *in vitro* at similar rates. Attempts to degrade the GST-tagged PC apoprotein or PBS isolated from *Synechocystis* 6803 *in vitro* have not yet proved successful (see below).

DISCUSSION

Complementation of the Non-bleaching Phenotype of the nblA1/nblA2 Double Mutant of Synechocystis 6803 with the NblA Protein from Nostoc 7120—In this study, we solved part of the mystery of why *Synechocystis* 6803 possesses two *nblA* genes, which are both essential for PBS degradation (15), when other cyanobacteria accomplish this by expressing only one *nblA* gene (14, 17, 18).

The complementation experiment shown in Fig. 1 demonstrates that the NblA protein from *Nostoc* 7120 is capable of restoring the wild-type phenotype to the non-bleaching *nblA1/nblA2* double mutant of *Synechocystis* 6803, suggesting that the functions of NblA1₆₈₀₃ and NblA2₆₈₀₃ are combined in one protein in *Nostoc* 7120. A similar result was obtained by Dines *et al.* (27), who reported successful complementation of the non-bleaching phenotype of the *nblA* mutant of *Synechococcus* 7942 by expression of the *Nostoc* 7120 *nblA* gene. These results support the conclusion that NblA proteins are functionally interchangeable among different cyanobacteria. Consistent with this, the various NblA proteins contain highly conserved regions near the N and C termini that mediate binding to phycobiliproteins and ClpC₆₈₀₃ (19, 20), and the three existing crystal structures are very similar (19, 27).

Formation of NblA1₆₈₀₃/NblA2₆₈₀₃ Heterodimers—FRET measurements, fluorescence lifetime measurements, and *in vitro* pull-down experiments demonstrated that NblA1₆₈₀₃ and NblA2₆₈₀₃ form a heterodimer. In contrast, the tendency toward homodimer formation was observed only under conditions that do not reflect those that exist in the cell, where *nblA1₆₈₀₃* and *nblA2₆₈₀₃* are cotranscribed (15, 16). On the basis of our results, we postulate that the biologically active form is a heterodimer, and we assume that the structure of the heterodimer is similar to that of other NblA proteins that have been crystallized to date.

Heterologous Expression of the Putative Protease ClpC₆₈₀₃-ClpP1-ClpR and Degradation of NblA1₆₈₀₃ and NblA2₆₈₀₃—To provide evidence that PBSs are degraded by a Clp protease, we performed *in vitro* degradation assays. Investigations of Clp proteases in *Synechococcus* 7942 have demonstrated that cyanobacteria possess two distinct Clp proteases in the soluble fraction of the cell and probably a third that is associated with the thylakoids. This membrane-associated protease appears to be involved in PBS degradation because (i) the protease has been reported to interact with PBS (37), and (ii) ClpC, the putative HSP100 chaperone partner of this protease, interacts with NblA (20).

The membrane-associated protease consists of a unique proteolytic core complex, composed of the Clp subunits, ClpP1 and ClpR, and the HSP100 chaperone partner ClpC, as noted above. To heterologously express the protease, we first identified orthologs of ClpP1, ClpR, and ClpC in *Synechocystis* 6803.³ We then successfully cloned, expressed, and purified the corre-

sponding proteins. As expected, coexpression of the two proteolytic subunits, ClpR and ClpP1, enabled the purification of a multiprotein complex. The complex had a molecular mass of about 200 kDa,³ which is similar to that of the ClpR-ClpP3 proteolytic core complex of *Synechococcus* 7942 (41), and appeared to correspond to a single mixed heptameric ring of ClpR and ClpP1, as expected. The protease activity of the complex, determined by analyzing the degradation of α -casein, was also similar to that of the proteolytic core ClpP3-ClpR from *Synechococcus* 7942 (41). Both cyanobacterial proteases are thus much slower than the model protease ClpA-ClpP from *E. coli* (41). The lower activity of cyanobacterial Clp proteases compared with that of *E. coli* may be due, at least in part, to differences in the structures of the core complexes (41, 42). We further demonstrated that NblA1₆₈₀₃ and NblA2₆₈₀₃ are degraded by the recombinant Clp protease ClpC-ClpP1ClpR.

The Clp protease-mediated degradation of NblA1₆₈₀₃ and NblA2₆₈₀₃ represents an elegant NblA regulatory mechanism. During the first hours after nitrogen starvation, the heterodimer is highly expressed, forming a ternary complex with ClpC₆₈₀₃ and the PBS that leads to ClpC₆₈₀₃ and PBS degradation. When all PBSs are degraded, the remaining NblA proteins are also degraded by the protease, eliminating the need for further regulation. This finding that the protease degrades NblA reinforces previous reports by our group that NblA accumulates when the ClpC binding site on NblA is inactivated by mutagenesis.³ Collectively, these observations indicate that the NblA protein acts like other identified adaptor proteins that undergo protease-mediated degradation, even when they are not delivering a substrate (43, 44).

Despite the fact that all conditions necessary for PBS degradation were fulfilled, including demonstrable protease activity and formation of a ternary complex between NblA1₆₈₀₃/NblA2₆₈₀₃, phycobiliproteins, and ClpC, we were unable to demonstrate *in vitro* degradation of PBS isolated from *Synechocystis* 6803 or the GST-tagged PC apoprotein.³ In further attempts to measure PBS degradation, we used a crude extract of wild-type cells grown under nitrogen or harvested immediately after nitrogen deprivation and also tried adding recombinant ClpC, NblA1₆₈₀₃, NblA2₆₈₀₃, ClpP1-ClpR, ATP, and an ATP regeneration system. Both strategies failed to induce degradation. One possible explanation for these unexpected results might be that dephosphorylation of PBS linker proteins is necessary for the degradation process. In this context, Piven *et al.* (45) reported that nitrogen starvation and long term exposure to higher light intensities resulted in an increase in dephosphorylated linker proteins and partial disassembly of the PBS. Even studies in *Nostoc* 7120, which exhibit a bottom-to-top disassembly of the PBS in early heterocysts, suggest that dephosphorylation of linker proteins may act as a signal for degradation (46). Accordingly, one possible scenario could be that, when the V-shaped NblA-dimers penetrate the gap formed by four back-to-back-assembled PBS β -subunits (27), the PBS structure disassembles (27), enabling dephosphorylation of the linker proteins by a phosphatase and opening the door for subsequent degradation. However, this explanation cannot account for the observation that the PC apoprotein is not degraded *in vitro*³ despite binding to NblA1₆₈₀₃/NblA2₆₈₀₃ (Fig. 6B). It is also pos-

sible that a bilin lyase could be involved in the degradation process. Bilin lyases catalyze the covalent ligation of chromophores to specific binding sites of phycobiliproteins and also control subsequent detachment (47). In *Synechococcus* 7942, the bilin lyase NblB participates in the degradation of PBS (48); in contrast, the two homologs of NblB in *Synechocystis* 6803 are not essential for PBS degradation in this strain (16). Thus, it may be that another unidentified lyase remains to be found. A further possibility could be that NblA1₆₈₀₃ and NblA2₆₈₀₃ must be activated by phosphorylation or dephosphorylation. Examples in which adaptor proteins must be activated are known from other organisms, including *Bacillus subtilis*, where the tyrosine kinase MecB is activated by phosphorylation (49). In this context, it is conceivable that light could induce NblA1/NblA2 activation, perhaps through a light-dependent phosphatase like NblS (50, 51). Clearly, further biochemical analyses will be required to establish the relevance of the mechanisms discussed here and close out the topic of PBS degradation.

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