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The proteolysis adaptor, NblA, is essential for degradation of the core pigment of the cyanobacterial light-harvesting complex

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SUMMARY

The cyanobacterial light-harvesting complex, the phycobilisome, is degraded under nutrient limitation, allowing the cell to adjust light absorbance to its metabolic capacity. This large light-harvesting antenna comprises a core complex of the pigment allophycocyanin, and rod-shaped pigment assemblies emanating from the core. NblA, a low-molecular-weight protein, is essential for degradation of the phycobilisome. NblA mutants exhibit high absorbance of rod pigments under conditions that generally elicit phycobilisome degradation, implicating NblA in degradation of these pigments. However, the vast abundance of rod pigments and the substantial overlap between the absorbance spectra of rod and core pigments has made it difficult to directly associate NblA with proteolysis of the phycobilisome core. Furthermore, lack of allophycocyanin degradation in an NblA mutant may reflect a requirement for rod degradation preceding core degradation, and does not prove direct involvement of NblA in proteolysis of the core pigment. Therefore, in this study, we used a mutant lacking phycocyanin, the rod pigment of *Synechococcus elongatus* PCC7942, to examine whether NblA is required for allophycocyanin degradation. We demonstrate that NblA is essential for degradation of the core complex of the phycobilisome. Furthermore, fluorescence lifetime imaging microscopy provided *in situ* evidence for the interaction of NblA with allophycocyanin, and indicated that NblA interacts with allophycocyanin complexes that are associated with the photosynthetic membranes. Based on these data, as well as previous observations indicating interaction of NblA with phycobilisomes attached to the photosynthetic membranes, we suggest a model for sequential phycobilisome disassembly by NblA.

Keywords: phycobilisome, disassembly, phycocyanin, allophycocyanin, proteolysis, adaptor, *Synechococcus elongatus* PCC 7942.

INTRODUCTION

Photosynthetic organisms adjust the amount and composition of their light-harvesting pigments in response to environmental cues. These acclimation responses are required to allow sufficient photon absorbance for phototrophic metabolism, while under particular conditions, light harvesting is minimized to prevent photoinhibitory damage consequent to surplus excitation (Vass and Aro, 2007; Bailey and Grossman, 2008; Li *et al.*, 2009; Nixon *et al.*, 2010; Nowaczyk *et al.*, 2010; Kirilovsky and Kerfeld, 2012; Muramatsu and Hihara, 2012; Tikkanen *et al.*, 2012). Phycobilisomes, the protein pigment antennae that mediate light harvesting in cyanobacteria, red algae, cryptophytes and glaucophytes, are water-soluble complexes that are anchored to the photosynthetic membranes. These large complexes, which may reach 4 MDa in size, largely comprise pigment binding proteins (phycobiliproteins), and additional linker proteins, the majority of which are not chromophorylated. The core of the phycobilisome consists of the protein pigment allophycocyanin (APC, absorbance maximum 650 nm), whereas the rod-like structures that emanate from the core comprise phycobiliproteins with shorter absorbance maxima, e.g. phycocyanin (PC) and phycoerythrin (approximately 620 and 565 nm, respectively). A specialized linker protein, the core–membrane lin-

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ker, anchors the entire phycobilisome to the photosynthetic membrane (Glazer, 1985; Gantt, 1994; Ting *et al.*, 2002; Tandeau, 2003; Adir, 2005; Six *et al.*, 2007; Mullineaux, 2008; Su *et al.*, 2010; Watanabe and Ikeuchi, 2013; Overkamp *et al.*, 2014).

The composition of rod phycobiliproteins depends on the specific organism, and, in particular cases, is also determined by the spectral composition of the light reaching the organism (Glazer, 1985; Gantt, 1994; Grossman *et al.*, 1995; MacColl, 1998; Adir, 2005; Kehoe, 2010; Watanabe and Ikeuchi, 2013). In the cyanobacterium *Synechococcus elongatus* PCC7942, the model organism used in this study (hereafter *S. elongatus*), phycobilisome rods comprise phycocyanin.

Degradation of the phycobilisome is a prominent acclimation response to nutrient limitation (Allen and Smith, 1969; Grossman *et al.*, 1993, 2001; Schwarz and Forchhammer, 2005). A mutant of *S. elongatus* led to identification of a small protein that is essential for the degradation process (Collier and Grossman, 1994). The protein was named NblA, to indicate the non-bleaching phenotype of this mutant, which remains blue-green under starvation, in contrast to the wild-type, which turns yellowish in color. The crystal structure of NblA suggests that this protein functions as a dimer (Bienert *et al.*, 2006; Dines *et al.*, 2008). Experimental support for the presence of a functional heterodimer of NblAI/NblAII in the cyanobacterium *Synechocystis* sp. PCC6803 was recently provided (Baier *et al.*, 2014).

Orthologs of NblA of *S. elongatus* have been identified in a variety of cyanobacteria and red algae (Baier *et al.*, 2001, 2004; Richaud *et al.*, 2001; Li and Sherman, 2002; Luque *et al.*, 2003; de Alda *et al.*, 2004; Kawakami *et al.*, 2009). The transcript abundance of *nblA* increases under nutrient limitation, particularly under nitrogen starvation, and numerous studies have revealed intricate and tight regulatory mechanisms that control transcription of the *nblA* gene (Schwarz and Grossman, 1998; Sauer *et al.*, 1999; Luque *et al.*, 2001; van Waasbergen *et al.*, 2002; Osanai *et al.*, 2005; Sendersky *et al.*, 2005; Lahmi *et al.*, 2006; Salinas *et al.*, 2007; Zabulon *et al.*, 2007; Kato *et al.*, 2008, 2011; Ruiz *et al.*, 2008; Leganes *et al.*, 2009; Klotz *et al.*, 2015). Of note, homologs of NblA have been identified in the genomes of cyanophages (Yoshida *et al.*, 2008; Gao *et al.*, 2012; Yoshida-Takashima *et al.*, 2012; Nakamura *et al.*, 2014; Voorhies *et al.*, 2015). The function of these viral *nblA* genes has not been elucidated to date; however, it was suggested that the phage may elicit host phycobilisome degradation for recruitment of amino acids for synthesis of phage proteins.

A study of the cyanobacterium *Synechocystis* PCC6803 demonstrated that phycobilisome degradation under starvation affects the pool size of many amino acids (Kiyota *et al.*, 2014). This observation supports the idea that proteolysis of the light-harvesting pigments under nitrogen and sulfur starvation provides amino acids for the synthesis of proteins required for acclimation to these particular nutrient-limited conditions. Another study demonstrated that an *nblA* mutant of the rapidly growing cyanobacterium *Synechococcus elongatus* UTEX 2973 grows more slowly and reaches lower maximal cell density compared to the wild-type. These data implicate NblA in biomass accumulation under nutrient-replete conditions (Yu *et al.*, 2015).

Affinity purification experiments were performed to address the role of NblA in phycobilisome degradation. These *in vitro* experiments demonstrated an interaction of NblA with rod phycobiliproteins (Luque *et al.*, 2003; Bienert *et al.*, 2006) as well as with ClpC (Karradt *et al.*, 2008), a chaperone subunit of the Clp protease. These interactions support a role for NblA in tagging rod phycobiliproteins for degradation (Karradt *et al.*, 2008). The role of NblA in degradation of the phycobilisome core was not addressed. Here, using a genetic approach, we show that NblA is essential for degradation of APC, the phycobiliprotein comprising the core of the phycobilisome. Additionally, using GFP-tagged NblA, we demonstrate *in situ* that NblA preferentially interacts with APC localized to the photosynthetic membranes. Based on these results, as well as our recent observations suggesting that NblA functions in phycobilisome disassembly (Sendersky *et al.*, 2014), we propose a model for decomposition of this large pigment complex.

RESULTS

NblA is essential for degradation of allophycocyanin

Cyanobacterial strains in which *nblA* is inactivated maintain high absorbance of rod pigments under conditions that generally elicit degradation (Schwarz and Forchhammer, 2005), supporting the requirement of NblA for degradation of these phycobilisome pigments. Nevertheless, these previous experiments did not demonstrate a role for NblA in APC degradation, as the vast abundance of PC and the close absorbance maxima of PC and APC (620 and 650 nm, respectively) do not enable changes in the APC level to be followed spectrophotometrically. Additionally, if rod degradation is a prerequisite for degradation of the core, lack of APC degradation in an *nblA*-inactivated strain may merely be a consequence of impaired PC degradation. Thus, to examine the direct involvement of NblA in degradation of the core of the phycobilisome, we constructed a strain that does not produce phycocyanin.

A phycocyanin-less strain ('Olive strain') was obtained by deletion of the entire gene cluster encoding components of the phycobilisome rod in *S. elongatus*, and insertion of a gentamycin resistance cassette (Figure 1a,b). PCR analyses confirmed complete chromosome segregation in the mutant strain (Figure 1c). Thus, the Olive mutant does not exhibit the prominent absorbance maximum of PC at

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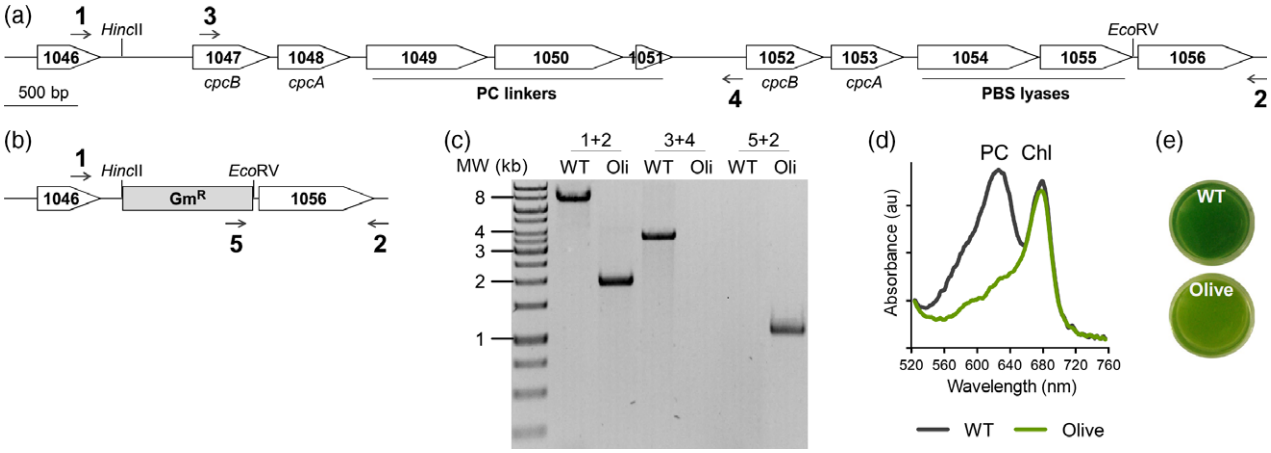


Figure 1. Deletion of the phycobilisome rod gene cluster to construct the Olive strain.
(a, b) Physical map of the gene cluster encoding components of the phycobilisome rod in *S. elongates* (a), and the genomic region of the Olive strain, in which the fragment between the *Hin*cII and *Eco*RV restriction sites was deleted and replaced with a gentamycin resistance cassette (Gm^R) (b). Arrows indicate specific primers used for PCR analysis (see Table S1).
(c) PCR analysis of genomic DNA of wild-type (WT) and the Olive strain (Oli). The numbers above the lanes indicate the specific primer pairs shown in (a) and (b). Molecular weight (MW) markers are indicated in kb.
(d, e) Absorbance spectra (d) and cultures (e) of WT and Olive strains grown in nutrient-replete medium. The absorbance maxima of phycocyanin (PC) and chlorophyll (Chl) are indicated.

620 nm (Figure 1d), and appears greenish yellow rather than the blue-green of the wild-type strain (Figure 1e).

To determine whether NblA is required for APC degradation, we inactivated the *nblA* gene in the Olive strain to construct the Olive/NblAΩ strain (Table S1). Absorbance spectra of water-soluble pigments of this strain indicated the presence of APC under nutrient-replete conditions (Figure 2a), as well as after 24 or 48 h of nitrogen starvation (Figure 2b, c, respectively). In contrast, APC was degraded in the Olive strain, as indicated by the disappearance of an absorbance maximum at approximately 650 nm after 48 h starvation (compare Figure 2a and c). Taken together, these analyses demonstrate that NblA is essential for APC degradation.

NblA interacts with APC

Previously, using Förster resonance energy transfer (FRET) implemented through fluorescence lifetime imaging microscopy (FLIM) we demonstrated interaction of NblA with phycobilisomes attached to the photosynthetic membranes (Sendersky *et al.*, 2014). As the previous study used the wild-type strain of *S. elongatus*, which possesses PC as well as APC, we could not determine whether NblA specifically interacts with APC. Here, we characterized the Olive strain using FRET-FLIM to examine whether NblA interacts with the core of the phycobilisome. The FRET-FLIM technique identifies protein–protein interactions by means of changes in fluorescence lifetime: a shortened fluorescence lifetime of the donor indicates energy transfer to the acceptor, and hence proximity of the donor to the acceptor (see Experimental Procedures). Our previous FRET-FLIM measurements, which used a strain bearing the NblA::GFP fusion product, revealed a shorter fluorescence lifetime of GFP fused to NblA compared to the free GFP protein. These data support a scenario in which interaction of GFP-labeled NblA with the phycobilisome brings GFP in apposition to a pigment (phycocyanin or allophycocyanin). Further experimental details are provided by Sendersky *et al.* (2014).

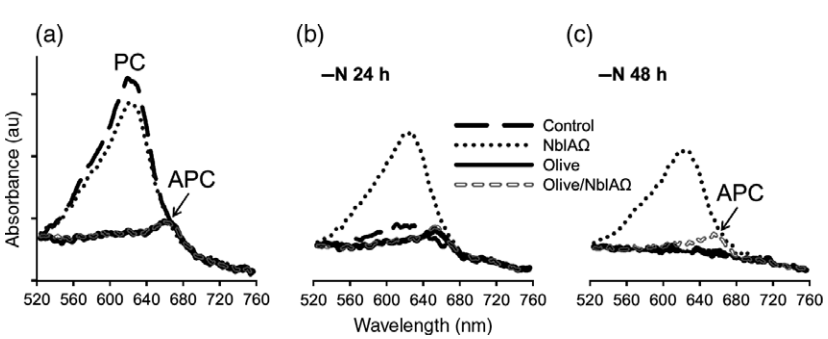


Figure 2. NblA is required for degradation of allophycocyanin (APC).
Absorbance spectra of water-soluble pigments from cultures grown under nutrient-replete conditions (a), or starved for nitrogen for 24 or 48 h (b and c, respectively). Strains analyzed: control, a wild-type strain that possesses a shuttle vector; NblAΩ, the *nblA*-inactivated strain; Olive, the phycocyanin-deficient mutant; Olive/NblAΩ, Olive strain in which *nblA* is inactivated (see Table S1 for further details). The absorbance maxima of PC and APC are indicated.

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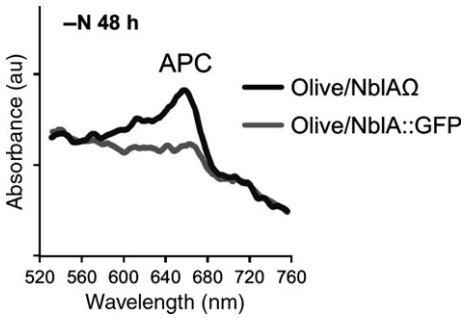


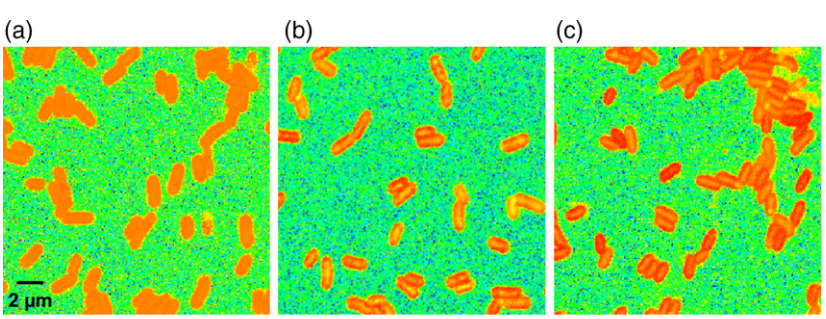
Figure 3. Introduction of NblA::GFP to the Olive/NblAΩ strain restores allophycocyanin (APC) degradation.
Absorbance spectra of water-soluble pigments from cultures starved for nitrogen for 48 h. The strains analyzed comprised an Olive strain in which the *nblA* gene was inactivated (Olive/NblAΩ), and the same strain into which NblA::GFP was introduced (Olive/NblA::GFP). See Table S1 for details. The absorbance maximum of APC is indicated.

The requirement of NblA for degradation of APC (Figure 2) suggests interaction of NblA with this pigment. Therefore, we analyzed the Olive strain by the FRET-FLIM approach to examine whether NblA closely associates with APC. To this end, we transformed the strain Olive/NblAΩ with NblA::GFP, and first tested whether the fusion protein confers APC degradation to the parental Olive/NblAΩ strain. The absorbance spectrum of the Olive/NblAΩ/NblA::GFP strain (hereafter Olive/NblA::GFP) demonstrated lower APC levels after 48 h of nitrogen starvation compared with the Olive/NblAΩ strain (Figure 3), indicating that NblA::GFP is functional in APC degradation.

Next, we compared the fluorescence lifetime for the Olive/NblA::GFP and NblA::GFP strains. Fluorescence lifetime images demonstrated a uniform distribution of lifetime pixels in the free GFP strain (Figure 4a), whereas regions of shortened fluorescence lifetimes in the NblA::GFP strain (Figure 4b) and the Olive/NblA::GFP strain (Figure 4c) correspond with the region of the photosynthetic membranes, which lie underneath the cell membrane. In 87% of the NblA::GFP-expressing cells and 92% of the Olive/NblA::GFP cells, we observed a pattern of fluorescence lifetimes that delineated the photosynthetic membranes enclosing the cell interior (Figure 4d). In both cases, the shorter fluorescence lifetime was apparent predominantly at the photosynthetic membranes (see further details below). In contrast to these strains, only 6% of the cells expressing free GFP exhibited a lifetime pattern delineating the photosynthetic membranes (Figure 4d); the majority of the cells showed an even distribution of lifetime pixels (Figure 4a).

The mean fluorescence lifetime obtained for the region of the photosynthetic membranes in NblA::GFP was significantly shorter than the mean fluorescence lifetime of free GFP (2.46 and 2.83 nsec, respectively) (Figure 5a,b). The short fluorescence lifetime of NblA::GFP indicates energy transfer between GFP and pigments of the phycobilisome due to interaction of NblA with the phycobilisome, and consequent placement of GFP in close vicinity to its pigments (Sendersky *et al.*, 2014). Importantly, the mean fluorescence lifetime characterizing the region of the photosynthetic membranes in the Olive/NblA::GFP strain was also significantly shorter compared to the free GFP strain (2.15 versus 2.83 nsec, respectively) (Figure 5a,c). As the Olive/NblA::GFP strain does not express PC, the shorter fluorescence lifetime in this case indicates interaction between NblA and APC. Additionally, the preferential localization of the short-lifetime pixels to the region of the photosynthetic membranes in NblA::GFP and Olive/NblA::GFP cells (Figure 4b–d) indicates that NblA intimately associates with pigment complexes while anchored to the photosynthetic membranes, and supports a role for the NblA protein in disassembly of the phycobilisome (see Discussion).

The mean fluorescence lifetimes in the cell interior region of the NblA::GFP and the Olive/NblA::GFP strains (2.76 and 2.73 nsec, respectively) were shorter compared with the free GFP strain (2.83 nsec, see Figure 5). These differences, although smaller compared to the differences



(d)

Strain	% cells depicting the region of the PS membranes	Number of cells analyzed
Free GFP	6	169
NblA::GFP	87	149
Olive/NblA::GFP	92	123

Figure 4. Fluorescence lifetime images of *S. elongatus* strains expressing GFP.
(a–c) Cells were nitrogen-starved for 48 h (free GFP and NblA::GFP) or 24 h (Olive/NblA::GFP). Each pixel represents fluorescence lifetime. The cell images indicate uniform distribution of lifetime pixels in the free GFP strain, whereas the images of the NblA::GFP and the Olive/NblA::GFP strains exhibit distinct fluorescence lifetimes in the region of the cell interior versus the photosynthetic membrane. Images were obtained using the PicoQuant Microtime 200 system. The strains analyzed were free GFP (a), NblA::GFP (b), and the Olive/NblA::GFP (c).
(d) Quantitative analysis of fluorescence lifetime patterns (see Experimental Procedures). PS, photosynthetic membrane.

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between the lifetimes representing the region of photosynthetic membranes and free GFP, are statistically significant (Table S2). The shorter fluorescence lifetimes in the cell interior may represent small pigment assemblies associated with NblA after their detachment from phycobilisomes or from phycobilisome core complexes (see Discussion).

DISCUSSION

NblA is essential for degradation of the core pigment of the phycobilisome

The vast abundance of phycobilisome rod pigments compared to the core pigment APC makes it difficult to determine from absorbance spectra whether inactivation of NblA impairs APC degradation. Furthermore, previous studies, which indicated that degradation of the phycobilisome progresses from its periphery to the core (Collier and Grossman, 1992), support a scenario in which rod degradation precedes core proteolysis. Therefore, impaired APC degradation in NblAΩ may be due to the absence of PC degradation, and does not directly implicate NblA in APC degradation. Thus, it was not known whether NblA is involved in degradation of the core pigments of the phycobilisome. The phycobilisome of *S. elongatus* comprises a sole rod pigment, PC, and, similarly to other cyanobacteria, the core is comprised of APC (Grossman *et al.*, 1993). Here, using a PC-less mutant (the Olive strain), we demonstrated that NblA is essential for degradation of APC. Inactivation of the *nblA* gene in the Olive strain resulted in a mutant that maintained high levels of APC under nitrogen starvation, in contrast to the Olive strain, which exhibited APC degradation under these conditions (Figure 2). Additionally, using the FRET-FLIM approach, we provide direct evidence for interaction of NblA with APC. The short mean fluorescence lifetime characterizing the Olive/NblA::GFP strain compared to the free GFP strain supports close association of the fusion protein with APC, allowing energy transfer between NblA and the core pigment.

Model of phycobilisome disassembly by NblA

After its identification, NblA was suggested to tag pigments of the phycobilisome for degradation (Collier and Grossman, 1994). More recent studies, which demonstrated that NblA interacts with rod phycobilisome pigments and with ClpC, a chaperone of the Clp protease, supported a role for NblA as an adaptor of proteolysis, i.e. it interacts with phycobiliproteins and introduces them to the proteolysis machinery (Bienert *et al.*, 2006; Karradt *et al.*, 2008). Degradation of a giant antenna such as the phycobilisome may involve an as yet unidentified factor that dissociates the phycobilisome, complex, releasing small pigment assemblies that interact with NblA. However, the data presented in this study, together with our recent observations, which indicated interaction of NblA with phycobilisomes anchored to the photosynthetic membranes (Sendersky *et al.*, 2014), do not support such a scenario. First, the shortest fluorescence lifetimes detected in the NblA::GFP and Olive/NblA::GFP strains were observed at the region of the photosynthetic membranes (Figures 4 and 5). These data indicate close association of NblA with phycobiliproteins, which are still associated with phycobilisomes (or phycobilisome cores), and suggest that these

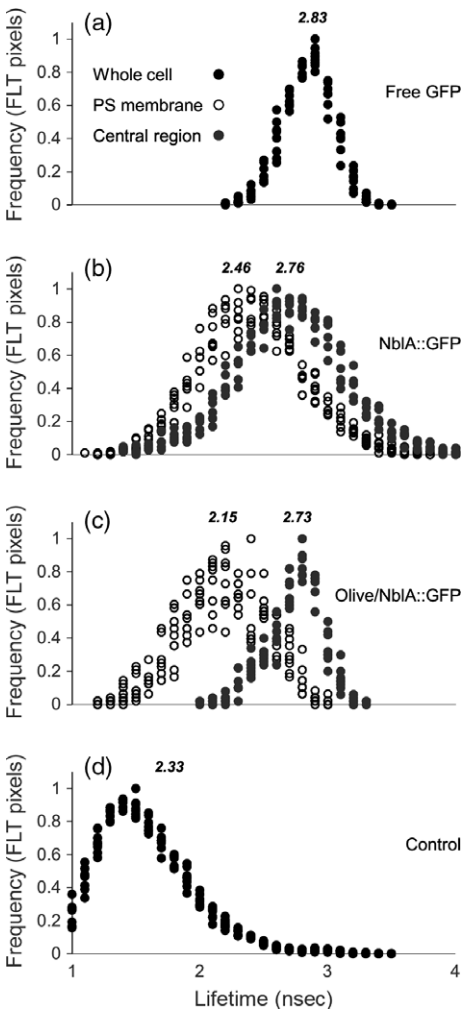


Figure 5. Histograms of the fluorescence lifetimes obtained for the following strains: free GFP (a), NblA::GFP (b), Olive/NblA::GFP (c) and the control strain (d).
Cultures were nitrogen-starved for 48 h, except for the Olive/NblA::GFP strain, which was starved for 24 h. Data were normalized to the maximal frequency. For strains NblA::GFP and Olive/NblA::GFP, data are presented for fluorescence lifetimes in the region of the photosynthetic (PS) membranes (open circles) and the central cell area (closed gray circles). Data obtained from whole cells (closed black circles) are shown for the free GFP and control strains. The mean fluorescence lifetime (nsec) is indicated above each histogram. These values were calculated from data obtained from three microscopic fields for each strain. ANOVA analysis with a Bonferroni *post hoc* test indicated significant differences between populations of the three strains (see details in Table S2).

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large pigment complexes remain anchored to the photosynthetic membranes at this stage. Furthermore, disassembly of phycobilisomes prior to interaction with NblA is expected to result in small pigment assemblies that are dispersed throughout the cell. If these were the initial substrates of interaction with NblA, an even distribution of lifetime pixels would be expected. As demonstrated here (Figures 4 and 5), this is not the case.

Based on these results, we propose the following model for progression of phycobilisome degradation. NblA initially interacts with phycobilisomes attached to the photosynthetic membranes (Figure 6a). Based on the crystal structure of NblA, as well as the model of the phycobilisome rod, there is a space between two hexamers that is large enough to accommodate the putative functional unit of NblA, an NblA dimer (Dines *et al.*, 2008). Such intercalation of NblA into the rod structure was suggested to undermine interactions stabilizing this sub-complex, and thus NblA enables release of a small pigment assembly, thereby acting as a 'disassemblase' (Dines *et al.*, 2008). We propose that, due to steric hindrance resulting from the fan-like arrangement of the phycobilisome, only the peripheral part of the rod is accessible to NblA, thus dictating consecutive degradation of the rod from the rod periphery towards the core, as suggested previously based on the sequential disappearance rod linker proteins (Collier and Grossman, 1992). As the process of sequential rod dissociation progresses, the core part of the phycobilisome is exposed, allowing interaction of NblA with APC and further detachment of these pigments from the core complex (Figure 6b). After detachment from the phycobilisome, PC or APC associated with NblA are introduced to the Clp protease.

In summary, we suggest a model that assigns an additional function to the adaptor protein NblA. It initially acts as a disassemblase, and, introduces them to the degradation machinery.

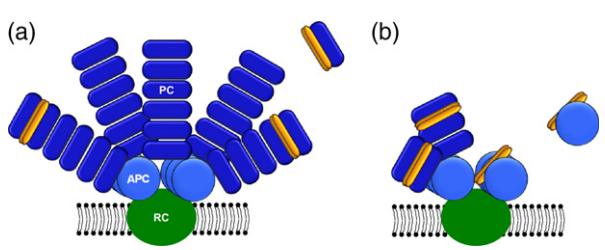


Figure 6. Model of phycobilisome disassembly by NblA.
(a) NblA intercalates into the rod structure, thereby undermining the association between adjacent pigment subunits, resulting in detachment of NblA associated with small pigment assemblies.
(b) As rod degradation progresses, the core pigments become accessible to NblA, and the phycobilisome core is disassembled.
The model shows the photosynthetic reaction center (RC, green), allophycocyanin (APC, light blue), phycocyanin (PC, blue) and dimers of NblA (orange).

EXPERIMENTAL PROCEDURES

Strains and culture conditions

Synechococcus elongatus PCC 7942 and derived strains were grown as described previously (Schatz *et al.*, 2013). The cells were grown in the presence of antibiotics, and starvation was induced as described previously (Sendersky *et al.*, 2014). The Olive strain was constructed essentially as described previously (Bhalerao *et al.*, 1995). Details of construction of this mutant, as well as additional strains used in this study, are provided in Table S1.

Absorbance measurements and cell imaging

Absorbance was measured as described previously (Sendersky *et al.*, 2014). Absorbance spectra were normalized at 750 nm. To clearly observe the absorbance peak of APC, chlorophyll was extracted, and the absorbance of water-soluble pigments was measured. Briefly, cell cultures were adjusted to an OD_{750} of 0.8; these cultures were acetone-extracted in 80% acetone, centrifuged (11 300 ***g***) for 3 min, the supernatant was discarded, and the pellet was suspended in double-distilled water.

For microscopic analyses, cells were starved for 48 h, except for the Olive/NblA::GFP strain, which was starved for 24 h. A shorter starvation time was used in the latter case as a very low amount of APC was present in these cells after 48 h starvation (Figure 3).

The FRET-FLIM approach (Sun *et al.*, 2011; Bakker *et al.*, 2012) was used to examine the interaction between NblA and phycobilisome pigments, as previously described (Sendersky *et al.*, 2014). For statistical analysis, data from three microscopic fields for each of the strains analyzed were used to obtain histograms of the time delays, and ANOVA analysis with the Bonferroni *post hoc* test was performed. To obtain separate fluorescence lifetime values for the region of the photosynthetic membranes and the region of the cell interior, these two cell regions were defined using fluorescence intensity-based images obtained by the PicoQuant Microtime 200 system (http://www.picoquant.com/products/category/fluorescence-microscopes/microtime-200-time-resolved-confocal-fluorescence-microscope-with-unique-single-molecule-sensitivity). A corresponding fluorescence lifetime image served to obtain data for each of these cell regions, using FIJI software (http://fiji.sc/Main_Page).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Table S1. Strains and cloning procedures.

Table S2. ANOVA analysis with the Bonferroni *post hoc* test was used for comparison of fluorescence lifetime data (shown in Figure 5).

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