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Decomposition of cyanobacterial light harvesting complexes: NblA-dependent role of the bilin lyase homolog NblB

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SUMMARY

Phycobilisomes, the macromolecular light harvesting complexes of cyanobacteria are degraded under nutrient-limiting conditions. This crucial response is required to adjust light excitation to the metabolic status and avoid damage by excess excitation. Phycobilisomes are comprised of phycobiliproteins, apo-proteins that covalently bind bilin chromophores. In the cyanobacterium *Synechococcus elongatus*, the phycobiliproteins allophycocyanin and phycocyanin comprise the core and the rods of the phycobilisome, respectively. Previously, NblB was identified as an essential component required for phycocyanin degradation under nutrient starvation. This protein is homologous to bilin-lyases, enzymes that catalyze the covalent attachment of bilins to apo-proteins. However, the *nblB*-inactivated strain is not impaired in phycobiliprotein synthesis, but rather is characterized by aberrant phycocyanin degradation. Here, using a phycocyanin-deficient strain, we demonstrate that NblB is required for degradation of the core pigment, allophycocyanin. Furthermore, we show that the protein NblB is expressed under nutrient sufficient conditions, but during nitrogen starvation its level decreases about two-fold. This finding is in contrast to an additional component essential for degradation, NblA, the expression of which is highly induced under starvation. We further identified NblB residues required for phycocyanin degradation *in vivo*. Finally, we demonstrate phycocyanin degradation in a cell-free system, thereby providing support for the suggestion that NblB directly mediates pigment degradation by chromophore detachment. The dependence of NblB function on NblA revealed using this system, together with the results indicating presence of NblB under nutrient sufficient conditions, suggests a rapid mechanism for induction of pigment degradation, which requires only the expression of NblA.

Keywords: Phycobilisome, phycocyanin, allophycocyanin, pigment degradation, bilin lyase, starvation, cyanobacteria, *Synechococcus elongatus* PCC 7942.

INTRODUCTION

Photosynthetic organisms, including cyanobacteria, modulate their light harvesting capacity in response to environmental cues (Kehoe and Gutu, 2006; Vass and Aro, 2007; Bailey and Grossman, 2008; Nowaczyk *et al.*, 2010; Chen and Blankenship, 2011; Muramatsu and Hihara, 2012; Allahverdiyeva *et al.*, 2015; Erickson *et al.*, 2015; Kirilovsky and Kerfeld, 2016; Montgomery, 2016). Nutrient limitation of cyanobacteria elicits regulated proteolysis of the phycobilisomes, the macromolecular pigment complexes that harvest light for photosynthesis (Allen and Smith, 1969; Grossman *et al.*, 1995, 2001; Schwarz and Forchhammer, 2005). Resuscitation from starvation involves pigment synthesis as part of an organized recovery programme (Klotz *et al.*, 2016). Phycobilisomes are comprised of phycobiliproteins, apo-proteins that covalently bind linear tetrapyrrole (bilin) chromophores (Glazer, 1985; Gantt, 1994). The core of the phycobilisome consists of allophycocyanin (APC), whereas different pigments including phycocyanin (PC) and phycoerythrin comprise the rods that emanate from the core, depending on the particular organism and the spectral composition of the light environment (Grossman *et al.*, 1993; MacColl, 1998; Adir, 2005; Kehoe, 2010; Watanabe and Ikeuchi, 2013). In addition to their role as light harvesting components of the photosynthetic apparatus, bilin chromophores are also employed by photoreceptors such as plant phytochromes and bacterial photosensory proteins (Lamparter *et al.*, 2004; Rockwell *et al.*, 2006; Gomelsky and Hoff, 2011; Wiltbank and Kehoe, 2016; Rockwell and Lagarias, 2017).

Bilin-lyases catalyze chromophore attachment to apo-proteins via formation of a thioether bond (Dammeyer and

Frankenberg-Dinkel, 2008; Scheer and Zhao, 2008). This large family of enzymes includes several types of lyases:

1. The E/F type catalyze binding of the chromophore phycocyanobilin to α-subunits of the apo-protein (Zhou *et al.*, 1992; Tooley *et al.*, 2001), as well as binding of phycoerythrobilin, albeit with reduced kinetics (Fairchild and Glazer, 1994).
2. The S/U type enzymes are characterized by low specificity for the chromophore and for the apo-protein (Zhao *et al.*, 2006, 2007a; Saunee *et al.*, 2008; Shen *et al.*, 2008; Kronfel *et al.*, 2013).
3. The T-type enzymes catalyze attachment of phycocyanobilin to different β-apo-proteins (Shen *et al.*, 2006; Zhao *et al.*, 2007b; Zhang *et al.*, 2012; Zhou *et al.*, 2014).

Phycobilisomes confer cyanobacteria with their typical blue-green colour. Starvation for either nitrogen or sulfur triggers rapid phycobilisome degradation, with the cells turning yellow-green (Allen and Smith, 1969; Grossman *et al.*, 1995, 2001; Schwarz and Forchhammer, 2005). The loss of this light harvesting complex under nutrient-limiting conditions provided a simple visual screen for isolating mutants that are compromised in the acclimation process. Such screening procedures led to the identification of several components that are involved in the regulation of phycobilisome degradation, including the kinase sensor homolog, NblS (van Waasbergen *et al.*, 2002), and the response regulator homolog, RpaB (Kato *et al.*, 2011). In addition, the response regulator homolog, NblR, is essential for phycobilisome degradation (Schwarz and Grossman, 1998); however, in contrast to other response regulators, its activation is phosphorylation independent, a cognate sensor kinase was not identified, and its mode of activation is yet to be resolved (Kato *et al.*, 2008; Ruiz *et al.*, 2008; Espinosa *et al.*, 2012). In addition to the regulatory components, genetic screens revealed the requirement for the proteins NblA and NblB, which were assigned a direct role in the mechanism of phycobilisome decomposition (Collier and Grossman, 1994; Dolganov and Grossman, 1999).

The *nblA* gene encodes a small protein that is essential for phycobilisome degradation (Collier and Grossman, 1994; Li and Sherman, 2002; de Alda *et al.*, 2004; Baier *et al.*, 2004; Kawakami *et al.*, 2009). Based on *in vitro* interaction of NblA with phycobiliproteins and ClpC, a chaperone subunit of the clp protease, it was suggested that this small protein serves as an adaptor of proteolysis that introduces the pigment to the degradation machinery (Karradt *et al.*, 2008). Furthermore, the rapid degradation of the chimeric protein, NblA::-green-fluorescent protein (GFP) compared with 'free' GFP protein demonstrated the function of NblA as a degradation tag *in vivo* (Sendersky *et al.*, 2014). Additionally, the interaction of NblA with phycobilisomes attached to thylakoid membranes supports a role for NblA in the sequential disassembly of this large pigment complex (Sendersky *et al.*, 2014, 2015). Genes encoding homologues of NblA were identified in several cyanophages – a finding suggesting that stimulation of phycobilisome degradation during infection is beneficial for the phage lifecycle (Yoshida *et al.*, 2008; Gao *et al.*, 2012; Yoshida-Takashima *et al.*, 2012; Nakamura *et al.*, 2014; Ou *et al.*, 2015; Voorhies *et al.*, 2016).

The NblB protein is essential for degradation of PC, the rod-pigment of phycobilisomes of the cyanobacterium *S. elongatus* (Dolganov and Grossman, 1999). NblB exhibits sequence similarity to bilin-lyases, enzymes that catalyze the covalent attachment of bilin chromophores to apo-protein subunits. Inactivation of *nblB*, however, does not affect the synthesis of phycobiliproteins, but rather impairs PC degradation. Therefore, it was proposed that the homology of NblB to the bilin-lyases underlies its ability to interact with bilin chromophores, and that this recognition is crucial for chromophore detachment and further protein pigment degradation (Dolganov and Grossman, 1999). The role of NblB, however, was not further characterized. Here, we demonstrated that NblB is essential for degradation of APC, the core pigment of the phycobilisome. In addition, we identified NblB residues that were involved phycobilisome degradation. Importantly, we demonstrated NblB-dependent degradation of PC in a cell-free system, thereby providing support for the suggestion that NblB directly mediates pigment degradation. Furthermore, the requirement of NblA for NblB function provided further insight into the mode of NblB activity and the mechanism of phycobilisome degradation.

RESULTS AND DISCUSSION

NblB is required for degradation of APC

Phycobilisomes of *S. elongatus* are comprised of two types of phycobiliproteins: APC, which is assembled into the core structure; and PC, comprising rod structures that emanate from the core. The similar absorbance maxima of PC and APC (620 and 650 nm, respectively) and the vast abundance of PC make it difficult to determine whether APC is degraded in the *nblB*-mutant (NblBΩ) during starvation. Additionally, current models support sequential progression of phycobilisome degradation, from the complex periphery to the core. Thus, lack of APC degradation in NblBΩ may only reflect the absence of rod degradation, and thus does not indicate direct involvement of NblB in breakdown of the core. To test the requirement of NblB for APC degradation, we employed a PC-deficient mutant (Olive strain; Sendersky *et al.*, 2015), in which *nblB* was inactivated (Olive/NblBΩ). Absorbance spectra of water-soluble pigments from the Olive and Olive/NblBΩ strains grown under nutrient-replete conditions are characterized by an absorbance maximum at 650 nm, typical of APC

(Figure 1, t_0). This absorbance maximum disappears in nitrogen-starved cultures of the Olive strain (Figure 1, –N 48 h), indicating APC degradation. In contrast, the Olive/NblBΩ strain is impaired in degradation of APC under nitrogen starvation (Figure 1, –N 48 h), suggesting the involvement of NblB in this process. Using a similar approach, we previously demonstrated the requirement of NblA for APC degradation (Sendersky *et al.*, 2015).

NblB is expressed under nutrient sufficient conditions

To follow the levels of NblB expression under nutrient-replete conditions or nitrogen starvation, this protein was tagged with human influenza hemagglutinin (HA) at its C-terminus (Figure 2a, and see Table S1 for cloning details). The gene encoding the tagged protein was introduced into NblBΩ, and the resulting strain (NblB–HA) was examined for its pigmentation under starvation. The NblB–HA strain degraded its PC under nitrogen starvation in a manner similar to the control strain, as evidenced by decreased absorbance maxima at 620 nm, in contrast with NblBΩ (Figure 2b). This analysis indicated that the HA-tag does not interfere with NblB function, and thus the NblB–HA strain was used for further analyses.

Western blot analysis using anti-HA antibodies was performed on the soluble fraction of the cell lysate, and revealed a protein of ~25 kDa in cells grown under nutrient-replete conditions (Figure 2c), in agreement with the calculated MW of NblB–HA (24.68 kDa). Upon nitrogen starvation, the amount of NblB–HA protein decreased to about half the level present in non-starved cells (Figure 2c), in agreement with reduced levels of the *nblB* transcript following starvation (Dolganov and Grossman, 1999). Reduction in the amount of NblB under starvation suggests an active signal for proteolysis of this protein when the level of phycobilisome pigments decreases. In contrast, the *nblA* gene, encoding a small protein essential for phycobilisome

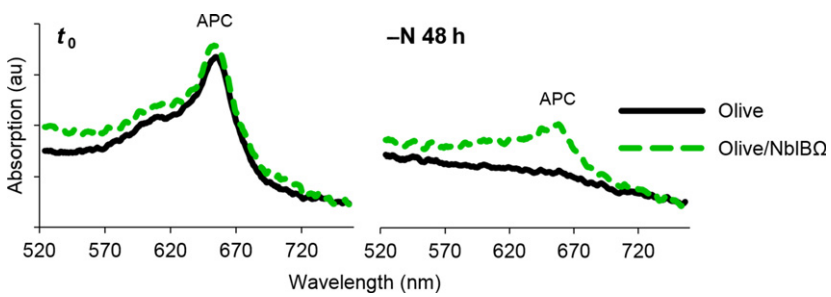

Figure 1. NblB is required for degradation of the core of the phycobilisome.
Absorbance spectra of water-soluble pigments from cultures grown under nutrient-replete conditions (t_0), or starved for nitrogen for 48 h. The absorbance maximum at 650 nm represents allophycocyanin (APC). Strains analyzed: Olive — phycocyanin-deficient mutant; Olive/NblBΩ – Olive strain in which *nblB* is inactivated. [Colour figure can be viewed at wileyonlinelibrary.com].

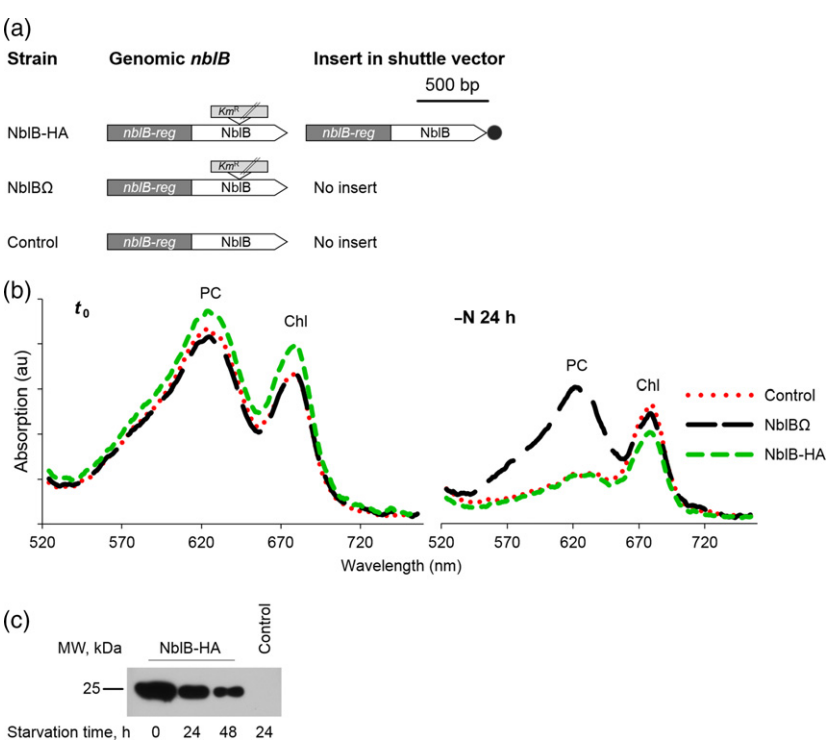

Figure 2. NblB–HA restores phycocyanin degradation to the *nblB*-inactivated strain (NblBΩ).
(a) Strains used to characterize NblB: NblB–HA–NblB tagged with human influenza hemagglutinin (HA) at its C-terminus (black circle) was introduced into the *nblB*-inactivated strain (NblBΩ). A shuttle vector without an insert was introduced into wild type (control) and NblBΩ strain cells, and cultures were grown in the presence of spectinomycin, similarly to NblB–HA. *nblB-reg*; the genomic region (611 nucleotides) immediately upstream of the start codon of NblB.
(b) Absorbance spectra of cultures grown in nutrient-replete medium (t_0) or starved for nitrogen for 24 h (–N 24 h). Absorbance maximum at 620 nm indicates phycocyanin (PC), the major component of the light harvesting complex in *S. elongatus*. Absorbance maximum at 680 nm represents the chlorophyll (Chl) level. Absorbance is shown in arbitrary units (au).
(c) Western blot analysis using anti-HA. Cells were grown under nutrient sufficient conditions (starvation time 0), or starved for nitrogen for 24 h or 48 h. [Colour figure can be viewed at wileyonlinelibrary.com].

degradation, is highly induced under nutrient starvation (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; Leganes *et al.*, 2009; Klotz *et al.*, 2015).

Sequence motifs required for NblB function

NblB proteins are characterized by distinct sequence motifs, some of which are homologous to conserved motifs of various bilin-lyases, enzymes that catalase covalent bond formation between bilin chromophore and their cognate apo-proteins (Dolganov and Grossman, 1999), and to CotB, which is required for the activation of green light-induced genes during chromatic adaptation in *Fremyella diplosiphon* (Balabas *et al.*, 2003). Several amino acids that are shared between NblB motifs, and motifs of particular bilin-lyases were mutated to examine to what extent they are required for NblB function (Figure 3a). Specifically, R26 (RpcE motif), Y50 (RpcF motif), W113 (RpcG motif), and three residues within the CpcF-I motif (D174, W175 and R178) were changed to alanine. The modified NblB proteins were expressed with a C-terminus HA-tag to allow detection of the modified proteins.

Mutation of either R26, W113 or W175 substantially affected PC degradation compared with the NblB–HA control (Figure 3b, left panel). Replacing either Y50 or D174 with alanine, however, had only a minor effect on PC degradation following 24 h nitrogen starvation (Figure 3b, right panel); upon longer starvation, PC levels in these mutants were indistinguishable from those of the NblB–HA strain (Figure S1). Western analysis of soluble fractions

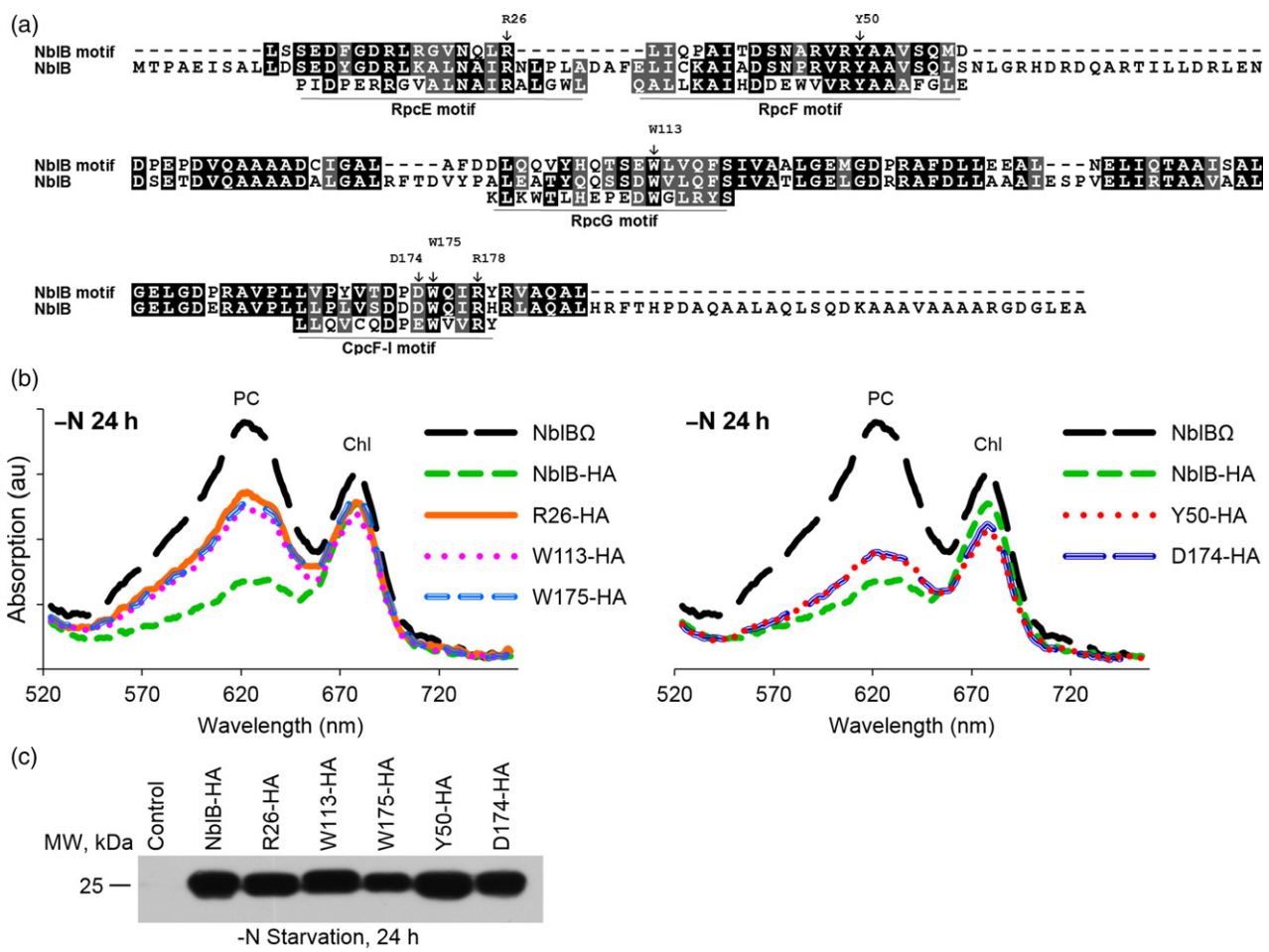

Figure 3. Mutation of either R26, W113 or W175 strongly affects NblB function, whereas changing either Y50 or D174 only slightly retards PC degradation.
(a) Amino acid sequence of NblB of *S. elongatus* aligned with motifs of the NblB-family, and with different bilin lyase enzymes. Amino acids mutated to alanine as part of this study are indicated by an arrow. Numbering is according to NblB of *S. elongatus*. Motifs are derived from the web-based tool 'CyanoLyase' (Bretaudeau *et al.*, 2013).
(b) Absorbance spectra of nitrogen-starved cultures (–N 24 h). See legend to Figure 2(b) for further details.
(c) Western blot analysis, using anti-HA, of the soluble fraction of lysates from cells starved for nitrogen for 24 h. [Colour figure can be viewed at wileyonlinelibrary.com].

using anti-HA antibodies indicated similar expression levels of the mutated proteins compared to NblB–HA, except for the W175 strain, which had approximately two-fold reduced levels (Figure 3c). If the higher PC level in the W175 strain represents merely the lower expression of NblB in this mutant, longer starvation of this strain should have resulted in decreased intracellular PC. High levels of PC, however, were observed in strains R26, W113 and W175 following 72 h nitrogen starvation (Figure S1). Thus, these results demonstrated that each one of these mutations substantially impaired NblB function. Probably, bilin-lyases and NblB proteins differ in their catalytic mechanisms, and thus sites of sequence conservation between these proteins do not represent amino acids crucial for catalysis. Therefore, we suggested that amino acids conserved between bilin-lyases and NblB proteins, the mutation of which substantially impaired NblB function, affect interaction with the bilin chromophore.

A recent study determined the crystal structure of the heterodimer of CpcE/F from *Nostoc* sp. PCC7120 and used CpcE as a template to model NblB of this cyanobacterium (Zhao *et al.*, 2017). Given the sequence conservation between NblB proteins of *Nostoc* and *S. elongatus* (51% identity and 67% similarity), we used *Nostoc* CpcE as a template to model NblB of *S. elongatus*, and mapped the residues whose mutation strongly affected NblB activity (Figure S2). This modelling revealed that R26 and W175 point inwards towards a cavity in the structure, whereas W113 points outwards (Figure S2). Of note, modelling of a chromophore into the structure determined for CpcE/F from *Nostoc* together with a mutational approach supported the suggestion that the chromophore resides in a cavity formed between CpcE and CpcF (Zhao *et al.*, 2017). Whether NblB functions in a monomeric form or as a dimer, it is possible that R26 and W175 directly affect the chromophore binding cavity, whereas mutation of W113 elicits a structural change that indirectly affects NblB activity.

Replacing R178 with alanine resulted in high PC levels under starvation similarly to NblBΩ (Figure S3a); further analysis revealed that this mutated protein is not expressed (Figure S3b). It is likely that this particular mutation affects the stability or solubility of NblB protein. Mutational analysis of the arginine residue of CpcF-I from *Nostoc* sp. PCC7120, which is conserved between NblB and CpcF-I motifs (Figure 3a), indicated its involvement in the binding of phycocyanobilin (Kupka *et al.*, 2009).

NblB allows PC degradation in vitro in an NblA-dependent manner

To further characterize the role of NblB, we examined its ability to elicit PC degradation *in vitro*. Soluble cell extract from a nitrogen-starved culture of NblBΩ served as the pigment source for the *in vitro* assay. Cells extracts were intentionally prepared under conditions that do not maintain whole phycobilisome structure, in order to provide small phycocyanin assemblies, probably trimers and hexamers. Additionally, cells were subjected to nitrogen starvation rather than growth under nutrient-replete conditions to allow the induction of NblA, in case it is required for pigment decomposition *in vitro*. Phycobilisome degradation, however, did not occur in these cultures due to inactivation of *nblB*. HA-tagged NblB was supplied to the assay from *E. coli* cells harbouring an inducible *nblB* gene. The combination of cyanobacterial extracts from NblBΩ, and lysates from *E. coli* expressing NblB resulted in reduced levels of PC compared with those observed following addition of a lysate from non-induced *E. coli* cells (Figure 4a, 72 h; grey versus black curves, respectively). Western blot analysis indicated the presence of NblB–HA upon induction, whereas it was undetectable in non-induced *E. coli* cells (Figure 4c). These results, which demonstrate regulated PC degradation in a cell-free system support a direct role for NblB in pigment degradation. Furthermore, free bilins absorb light poorly compared to bilins covalently bound to their cognate apo-protein (Schirmer *et al.*, 1986; Glazer, 1989). Therefore, high absorbance observed in the absence of NblB indicated that the chromophore remains attached to the apo-protein and supports a role for NblB in chromophore detachment.

To further substantiate the role of NblB in PC degradation in this cell-free system, *E. coli* cells expressing the NblB R26, NblB W113 and NblB W175 mutants were used (Figure 4c). These mutated NblB proteins, which exhibited substantially impaired PC degradation *in vivo* (Figures 3b and S1), did not support PC degradation in the cell-free assay (Figure 4b, R26 – orange; W113 – pink, and W175 – blue).

Interestingly, when starved cultures of the double mutant NblAΩ/NblBΩ were used as the pigment source, PC degradation was also not observed (Figure 4a, 72 h; green curve) regardless of the presence NblB (Figure 4c). Thus, using this cell-free system, we revealed that NblB function in PC degradation is dependent on NblA. This finding would not have been possible based on genetic studies, as inactivation of either *nblA* or *nblB* abolishes pigment degradation.

A recent attempt to detect NblB activity *in vitro* did not reveal phycocyanobilin detachment (Zhao *et al.*, 2017), in line with data reported here demonstrating dependence of NblB activity on the presence of NblA.

Model for phycobilisome degradation by NblA and NblB

Our previous studies, which employed fluorescence lifetime imaging microscopy, demonstrated the interaction of NblA with phycobilisomes anchored to the thylakoid membranes, and support a role for NblA in phycobilisome disassembly. We proposed that NblA intercalates into the rod

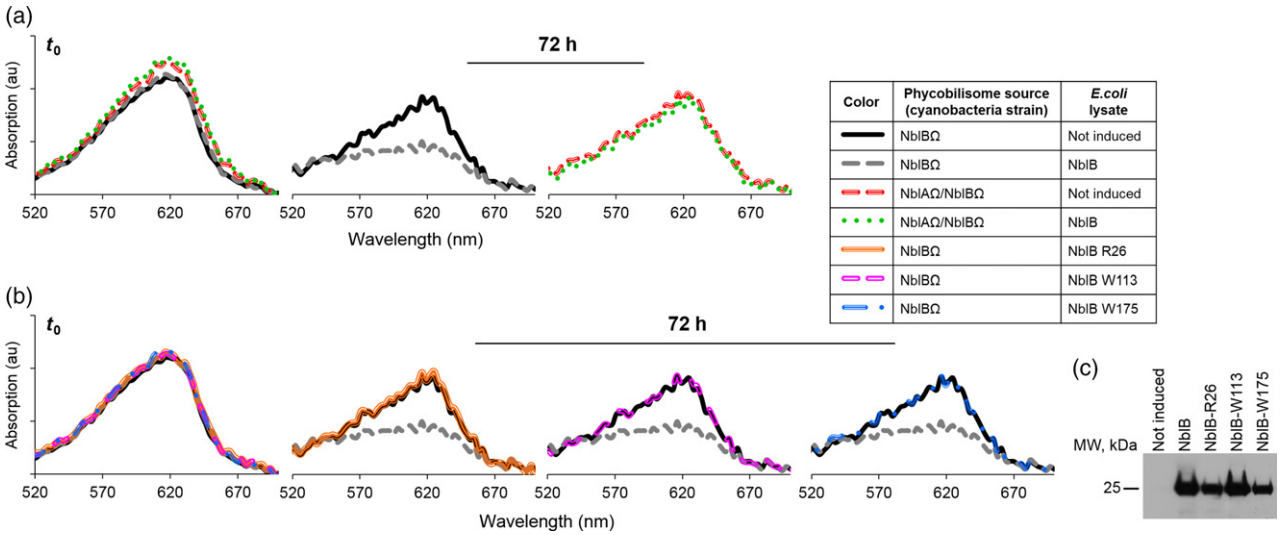

Figure 4. Phycocyanin degradation in a cell-free system is dependent on NblA and NblB.
(a) Absorbance spectra of soluble cyanobacterial pigments immediately following addition of *E. coli* lysates (t_0), and following 72 h incubation. Pigments were isolated either from NblBΩ or NblAΩ/NblBΩ strain, and *E. coli* cells were either induced or non-induced for expression of NblB (see inset table for details).
(b) Absorbance spectra representing *in vitro* pigment degradation as in (a). Assays included *E. coli* cells expressing native or mutated NblB proteins (see inset table for details).
(c) Western blot analysis using anti-HA on *E. coli* extracts. [Colour figure can be viewed at wileyonlinelibrary.com]

structure, thereby destabilizing the interaction between pigment assemblies (Dines *et al.*, 2008; Sendersky *et al.*, 2014, 2015). Additionally, studies by Nguyen *et al.* using chemical cross-linking, suggested that NblA disrupts the integrity of the rod structure (Nguyen *et al.*, 2017). Taking into account previous results, which support sequential degradation of the phycobilisome from the complex periphery to the core (Collier and Grossman, 1992), we suggest that NblA allows gradual detachment of pigment assemblies (e.g. hexamers) that may remain associated with NblA. As noted above, cyanobacterial cell extracts used as a pigment source for the cell-free degradation assay (Figure 4) were deliberately prepared under conditions that do not maintain stable whole phycobilisomes and thus, most likely, small pigment assemblies e.g. PC trimers and hexamers, were supplied as substrates for degradation. Therefore, the activity of NblA in mediating disassembly of whole phycobilisomes should not be required in this cell-free system. Yet, NblB-dependent PC degradation did not occur in the absence of NblA (Figure 4a, 72 h; green curve). We propose two conceivable scenarios explaining the NblA-dependent function of NblB. It is possible that NblB cannot detach chromophores from trimers or hexamers, and NblA is required for further disassembly of phycobilisome sub-complexes following their detachment from the whole phycobilisome. It is also possible that NblB evolved to interact with PC-assemblies associated with NblA. Such NblA-pigment assemblies are predicted to be produced when NblA inserts itself into the rod structure (Dines *et al.*, 2008) thereby leading to detachment of the peripheral pigment complexes. Conceivably, stable NblB-PC interaction and further catalysis requires a tripartite complex in which NblA participates. The two suggested modes of operation are not mutually exclusive; namely, NblA may promote disassembly of hexamers or trimers, and subsequently, NblB could interact with and detach the chromophore from PC monomers associated with NblA.

Our findings of NblA-dependent function of NblB and the presence of NblB under nutrient sufficient conditions support rapid initiation of phycobilisome degradation upon induction of NblA expression by nutrient starvation.

Gradual detachment of rod-pigments and PC degradation as described above, exposes the core of the phycobilisome, consequently enabling APC disassembly. As demonstrated by use of a PC-deficient mutant (Olive strain), NblB is required for degradation of APC, thereby allowing complete decomposition of the macromolecular pigment complex.

EXPERIMENTAL PROCEDURES

Strains, culture conditions and absorbance measurements

Synechococcus elongatus PCC 7942 and derived strains were grown as described previously (Parnasa *et al.*, 2016; Sendersky *et al.*, 2017). Starvation was induced and absorbance measurements were performed as described previously (Sendersky *et al.*, 2014). Absorbance spectra were normalized at 750 nm. To observe the absorbance peak of APC, chlorophyll was extracted, and the

absorbance of water-soluble pigments was measured as described (Sendersky *et al.*, 2015). 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.

Escherichia coli BL21 (DE3) (IBA) was used for expression of NblB. Details of cloning of native and mutated *nblB* in pASK-IBA3 under the *tet* promoter are provided in Table S1.

Cell-free system for analysis of PC degradation

Preparation of cyanobacterial crude cell extracts. Cultures of 50 mL of the NblBΩ or NblAΩ/NblBΩ strain were starved for nitrogen for 24 h and harvested by centrifugation (8000 ***g***, 4°C, 10 min). Pellets were resuspended in 1 mL 20 mM Tris pH 8 and 50 mM NaCl (Tris–NaCl buffer), transferred to a screw cap Eppendorf tube, centrifuged (11 300 ***g*** 4°C, 2 min), and the supernatant was discarded. The cell pellets were frozen in liquid nitrogen and preserved at −20°C for up to 4 days. Pellets were resuspended in 800 μL of Tris–NaCl buffer, and 0.2 g glass beads (Sigma, ≤106 μm, unwashed; Sigma Aldrich (Merck) - Merck KGaA, Darmstadt, Germany, https://www.sigmaaldrich.com/) were added. Cells were broken in screw cap Eppendorf tubes using Mini Bead-Beater-8 (BioSpec Products) for 15 sec × 4 cycles, followed by 10 min incubation on ice and additional breakage programme, as above. Broken cells were centrifuged (11 300 ***g*** 4°C, 2 min), the supernatant was transferred to a clean Eppendorf tube and centrifuged (11 300 ***g*** 4°C, 40 min). The supernatant from the latter step served as the pigment source for the *in vitro* PC degradation assay. The absorbance of this crude soluble extract was measured using NanoDrop ND-1000 (UV–vis) and adjusted to OD_{619} nm of 0.2 using Tris–NaCl buffer. Protein concentration in these soluble extracts was approximately 0.8 μg μl^{-1}, as determined by Bradford protein assay (Bio-Rad Laboratories Ltd., Rishon LeZion, Israel, http://www.bio-rad.com/).

Preparation of E. coli cell extracts. *E. coli* BL21 (DE3) were grown in Luria-Bertani (LB) growth medium supplemented with ampicillin (100 μg ml^{-1}) at 37°C (10 ml culture in 50 ml Falcon tube) under agitation (200 rpm). For expression of the recombinant NblB proteins, cultures were grown overnight, diluted 10-fold and grown for 4 h and diluted again to OD_{750} of 0.4. To induce expression of recombinant proteins, anhydrotetracycline (Clontech, Mountain View, CA, USA, http://www.clontech.com/US/About/Contact_Us) was added (0.2 μg ml^{-1}), and cultures were incubated with shaking at 200 rpm at 37°C for 17 h. For cell harvest 10 mL of culture (OD_{750} of 2.7) was pelleted by centrifugation (8000 ***g*** 4°C, 10 min) and the supernatant was discarded. The cell pellet was resuspended in 1 mL of Tris–NaCl buffer, and the cells were broken using Sonics Ultrasonic Processor Vibra Cell, Model VC 130PB for 10 sec × 4 cycles (setting: pulsed mode, 40% intensity). Broken cells were centrifuged (11300 ***g*** 4°C, 40 min), and the supernatant was transferred to a clean tube and served as a source of recombinant NblB. Protein concentration in this crude lysate was approximately 2.5 μg μl^{-1}, as determined by the Bradford protein assay (Bio-Rad Laboratories Ltd.).

In vitro NblB activity. To assess NblB activity in a cell-free system, 250 μL cyanobacterial extract was mixed with an equal volume of *E. coli* lysate and incubated in the dark at 37°C for 72 h. Absorbance was followed in the range of 520–700 nm using NanoDrop ND-1000 (UV–vis).

Western blot analysis

Preparation of cyanobacterial cell extracts for western blot analysis was performed essentially as described above; however, cells were resuspended in TE (10 mM Tris and 1 mM EDTA) supplemented with protease inhibitors (Sigma P8465, final concentration 0.95 mg mL^{-1}; Sigma Aldrich (Merck) - Merck KGaA). After cell breakage, an aliquot of 10–20 μl was removed for chlorophyll extraction as follows: TE was added up to 200 μl, acetone was added (800 μl) and extraction and measurement were performed as described (Sendersky *et al.*, 2017). Further steps to obtain soluble crude extract were performed as described above in 'Preparation of cyanobacterial crude cell extracts'. Western blot analysis was performed as described previously (Balint *et al.*, 2006) using mouse monoclonal anti-HA (Covance #MMS-101R) and anti-mouse IgG. For analysis of NblB in cyanobacterial soluble extracts, loading was normalized based on the chlorophyll amount in the crude extract (2 μg chlorophyll). For detection of NblB in *E. coli* extracts, each lane was loaded with 30 μg protein.

ACKNOWLEDGEMENTS

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

SUPPORTING INFORMATION

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

Figure S1. Absorbance spectra of cultures starved for nitrogen for 72 h (–N 72 h).

Figure S2. Structural model of *S. elongatus* NblB.

Figure S3. The R178-HA mutant does not produce a stable product.

Table S1. Strains and cloning procedures.

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