When cyanobacteria are starved for nitrogen, expression of the NblA protein increases and thereby induces proteolytic degradation of phycobilisomes, light-harvesting complexes of pigmented proteins. Phycobilisome degradation leads to a color change of the cells from blue-green to yellow-green, referred to as bleaching or chlorosis. As reported previously, NblA binds via a conserved region at its C terminus to the α-subunits of phycobiliproteins, the main components of phycobilisomes. We demonstrate here that a highly conserved stretch of amino acids in the N-terminal helix of NblA is essential for protein function in vivo. Affinity purification of glutathione S-transferase-tagged NblA, expressed in a Nostoc sp. PCC7120 mutant lacking wild-type NblA, resulted in co-precipitation of ClpC, encoded by open reading frame \( \text{alr2999} \) of the Nostoc chromosome. ClpC is a HSP100 chaperone partner of the Clp protease. ATP-dependent binding of NblA to ClpC was corroborated by \textit{in vitro} pull-down assays. Introducing amino acid exchanges, we verified that the conserved N-terminal motif of NblA mediates the interaction with ClpC. Further results indicate that NblA binds phycobiliprotein subunits and ClpC simultaneously, thus bringing the proteins into close proximity. Altogether these results suggest that NblA may act as an adaptor protein that guides a ClpC:ClpP complex to the phycobiliprotein disks in the rods of phycobilisomes, thereby initiating the degradation process.

Nitrogen deficiency causes a dramatic color change of cyanobacterial cultures from blue-green to yellow-green, a phenomenon described as “nitrogen chlorosis” nearly 100 years ago (1). This bleaching is mainly due to the degradation of the cyanobacterial antenna pigment complexes called phycobilisomes (2, 3). Phycobilisomes (PBS) are giant protein complexes consisting of chromophorylated phycobiliproteins and non-pigmented linker peptides. Each PBS is composed of a core, which is anchored to the thylakoid membrane, and peripheral rods building the typical hemidiscoidal structure (4–6). Phycobiliproteins can constitute up to half of the total soluble protein of a cyanobacterial cell (7). Their degradation during nitrogen deprivation is part of a complex acclimation process that is thought to fulfill two functions: preventing photo damage under stress and providing substrates for synthesis of proteins required for the adaptation. PBS degradation proceeds in an ordered manner, beginning with the successive loss of the peripheral rods (trimming) and ending with the degradation of the core components (8, 9). It is reversible at any time. As soon as a nitrogen source is available again, the PBS are resynthesized.

The detailed mechanism of PBS degradation is not yet understood. However, screening for non-bleaching (nbl) mutants of \textit{Synechococcus elongatus} PCC7942 revealed several genes that are involved in the degradation process (10–14). Among the proteins encoded by these “non-bleaching” genes, one (nblA encoding protein NblA) is directly involved in PBS degradation and appears to play the key role in this process (10). Inactivation of orthologs of \textit{nblA} led to non-bleaching phenotypes in several other cyanobacterial species (15–17). In the filamentous, diazotrophic cyanobacteria \textit{Nostoc} sp. PCC7120 (Nostoc 7120) and \textit{Anabaena variabilis} ATCC 29413 this non-bleaching phenotype is most obvious in heterocysts, cells specialized for fixing \( N_2 \) under aerobic conditions, which usually contain only low levels of phycobiliproteins (18, 19). Upon nitrogen step-down, transcription of \textit{nblA} is highly up-regulated (10, 15–17, 20–22). Increase of \textit{nblA} mRNA is considered as characteristic for nitrogen deprivation.

Genes homologous to \textit{nblA} are present in most phycobiliprotein-containing cyanobacteria and in red algae. Of the 26 \textit{nblA} orthologs found in databases, 20 are from cyanobacterial strains, 5 from chloroplasts of red algae, and 1 from a cyanophage. However, \textit{nblA} seems to be absent from \textit{Prochlorococcus} strains, from marine \textit{Synechococcus} subclusters MC-A and MC-B, and from \textit{Gloeobacter violaceus} PCC7421 (24).
The NbIA proteins are rather small, consisting of around 60 amino acid residues corresponding to a molecular mass of ~7 kDa. Although the homology between different NbIA sequences is not very high (about 30% sequence identity on average), they seem to share similar structures. The crystal structure of NbIA from *Nostoc 7120* demonstrates that the NbIA polypeptide consists of two α-helices, a shorter N-terminal and a longer C-terminal one. Two NbIA molecules form a homodimer in which the C-terminal helices are involved in dimerization (25). Crystallographic data of NbIA from *Thermosynechococcus vulcanus* suggest a similar structure for this protein (26). Sequence and structure of NbIA show no significant similarity to proteins with known function, and the molecular mechanism of NbIA action in PBS degradation is not clear. *In vitro* studies showed that NbIA interacts with the α-subunits of phycobiliproteins (22, 25). Furthermore, results of binding experiments with variants of NbIA carrying amino acid substitutions at various positions provided evidence that the interaction with phycobiliproteins is mediated via conserved amino acid residues near its C terminus (25). However, the most highly conserved stretch of amino acids of the protein is located near its N terminus. In this study we show that NbIA interacts with a chaperone, the HSP100/Clp protein ClpC, via a highly conserved motif located at the beginning of its N-terminal helix.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Growth Conditions**—Strains are listed in supplemental Table S1. *Nostoc 7120* and its mutants were grown at 28 °C under constant illumination (cool-white fluorescent lamps, or in BG110, which lacks sodium nitrate (27). Liquid cultures were bubbled with 2% (v/v) CO2 in air. For growth on plates, the medium was solidified with separately autoclaved 0.6% (w/v) agar (Difco Laboratories, Detroit, MI). Mutants were grown in the presence of 150 µg ml−1 neomycin, 4 µg ml−1 spectinomycin, and 1 µg ml−1 streptomycin, respectively.

For nitrogen starvation experiments, cyanobacterial cultures exponentially growing in BG11 medium were collected by filtration through a nylon net filter (11 µm pore size, Millipore Corporation, Bedford, MA), washed twice with BG11p, resuspended in this medium to a concentration of about 5 µg of chlorophyll ml−1 and grown further.

Chlorophyll content was estimated in methanolic extracts according to Tandeau de Marsac and Houmard (28). Whole cell absorbance spectra were recorded in the wavelength range from 550 to 750 nm on a Shimadzu UV-2401PC spectrophotometer equipped with an integrating sphere and were corrected for residual cell scattering at 750 nm.

Strains of *Escherichia coli* (*E. coli*) were grown under standard conditions. When appropriate, antibiotics were added to the medium to final concentrations of 50 µg ml−1 ampicillin, 50 µg ml−1 kanamycin sulfate, 25 µg ml−1 chloramphenicol, or 50 µg ml−1 spectinomycin, respectively.

**Plasmid and Mutant Constructions**—Plasmids and oligonucleotides are listed in supplemental Table S1. For reversion of the *Nostoc 7120* ΔnbIA mutant to the wild-type phenotype, a 1239-bp fragment, bearing the chromosomal nbIA gene (asr4517 (kazusa.or.jp/cyano/Nostoc/index.html) (29) together with upstream and downstream sequences, was amplified by PCR using primers 2.11 (XbaI site inserted) and 2.12 (Xhol site inserted), and 2.16 (Xhol site inserted) and 2.17 (EcoRV and BglII site inserted), respectively. The fragments were ligated into the plC20R vector (73) to yield plasmid plC20RnbIA-T. For details of construction see supplemental Fig. S1A. Using plasmid plC20RnbIA-T as template, primers were designed to produce site-specific mutations, and mutagenesis was performed using the QuikChange® site-directed mutagenesis kit (Stratagene, La Jolla, CA). All DNA constructs were confirmed by DNA sequencing. The fragments harboring the nbIA gene or its mutated variants, flanked by the promoter and terminator regions, were then excised by EcoRI digestion and ligated into the self-replicating plasmid pRL1049 (74). Transfer of plasmids between the ΔnbIA mutant of *Nostoc 7120* and *E. coli* was achieved by conjugation using *E. coli* strain J53 bearing RP4 and cargo strain HB101, bearing helper plasmid pRL528, in triparental matings (30). Exconjugants were selected on BG11 agar plates containing 150 µg ml−1 neomycin, 4 µg ml−1 spectinomycin, and 1 µg ml−1 streptomycin.

Expression of NbIA-GST Fusion Protein in *Nostoc 7120*—For expression of NbIA C-terminal fused to GST in ΔnbIA mutant cells the nbIA gene together with upstream sequences was amplified by PCR using primers 2.11 (XbaI site inserted) and 2.12 (Xhol site inserted) and ligated into the plC20R vector yielding plasmid plC20RnbIA. This plC20RnbIA vector was used to create an Ndel site at the 3’ end of the nbIA coding region. The Ndel site was generated by mutagenesis (QuikChange® site-directed mutagenesis kit; Stratagene) using plasmid plC20RnbIA as template and the primers QCM23for and QCM24rev. This mutagenesis resulted in the change from Thr65 to a histidine residue and the loss of the two amino acids, Pro64 and Ala65, of the NbIA protein. The resulting plasmid plC20RnbIA/Ndel was digested with BglIII and Ndel (partial digestion) and subsequently ligated to a fragment harboring the glutathione S-transferase (gst) gene excised from plasmid pGEX-2TK/Ndel (see “Construction of Plasmids for Expression of GST Fusion Proteins”) by Ndel and BamHI digestion. The resultant construct was restricted with EcoRV and, after dephosphorylation, ligated to a PCR fragment bearing the terminator region of the nbIA gene, amplified using primers 2.17 (EcoRV and BglII site inserted) and 2.20 (EcoRV site inserted). For schematic representation of construction see supplemental Fig. S1B. Plasmid plC20RnbAgst-T was verified by DNA sequencing. Restriction with EcoRI yielded a fragment encoding the GST-tagged NbIA protein with promoter and terminator regions that was ligated into the self-replicating plasmid pRL1049. The transfer of plasmids between the ΔnbIA mutant of *Nostoc 7120* and *E. coli* was attained as described above.

**Purification of the NbIA-GST Fusion Protein from *Nostoc 7120*—**Cultures of the ΔnbIA mutant expressing the GST-tagged NbIA protein, transferred to BG11p medium (which lacks an N-source) for 6, 8, and 15.5 h, respectively, were harvested by filtration through a nylon net filter (11 µm pore size, Millipore Corporation, Bedford, MA). The three pellets (each containing ~8 mg of chlorophyll) were collected and resuspended in ~30 ml of sodium/potassium phosphate buffer (20
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mm sodium/potassium phosphate (pH 7.6), 200 mm KCl, 20 mm NaCl, 2 mm MgCl₂, 10% (v/v) glycerol). The following steps were performed at 4 °C. Cells were disrupted in a bead beater (Hamilton Beach) using glass beads of 0.25–0.5 mm. Glass beads and unbroken cells were removed by centrifugation for 5 min at 4,000 × g. The insoluble fraction consisting of cells, cell fragments, and membranes was removed by two centrifugations (1.5 h, 164,000 × g) and the supernatant incubated with ~150 μl of Glutathione-Sepharose™ 4B (GE Healthcare) in sodium/potassium phosphate buffer overnight at 4 °C with slow agitation. Crude extracts of the ΔnblA mutant expressing the non-tagged NblA served as control. After washing with sodium/potassium phosphate buffer (2 times, 800 μl each), bound proteins were eluted 3 times with 200 μl of 40 mM glutathione in 50 mM Tris/HCl (pH 8.0). Eluates were concentrated by using Vivaspin 500 tubes (Membrane: 5,000 MWCO, Vivascience, New York) and analyzed by SDS-PAGE.

SDS-PAGE was performed in slab gels containing 12% (w/v) acrylamide:methylene bisacrylamide (29:1) in the buffer system of Laemmli (31). Following electrophoresis, protein bands were visualized by colloidal Coomassie Brilliant Blue G-250 staining (32). For protein identification, protein bands of interest were analyzed by matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) peptide mass fingerprinting as well as by MALDI-MS/MS (33). To this end, protein bands were excised from Coomassie Brilliant Blue G-250-stained gels and in-gel digested using porcine sequencing grade modified trypsin (Promega, Madison, WI). The resultant proteolytic peptides were desalted and concentrated using ZipTipμ-C₁₈ pipette tips (Millipore Corporation, Bedford, MA) and analyzed with a 4700 Proteomics Analyzer (Applied Biosystems, Foster City, CA) using α-cyano-4-hydroxy-cinnamic acid (Sigma) as a matrix. The MS spectra were recorded in the reflectron mode of the 4700 Proteomics Analyzer and calibrated using trypsin autolysis peaks as internal markers. The MS/MS spectra were acquired without applying collision-induced dissociation. The spectra were processed using the 4700 Explorer software version 3.0 (Applied Biosystems, Foster City, CA). The peak lists of the spectra were created by the Peak-to-Mascot script of the 4700 Explorer software using the following filter settings: mass range from 500 to 4,000 Da (MS); 60 Da to precursor mass – 20 Da (MS/MS); peak density, ≤10 peaks per 200 Da (MS), ≤20 peaks per 100 Da (MS/MS); minimal signal-to-noise-ratio, 20 (MS), 3 (MS/MS); minimal peak area, 1,000 (MS), 0 (MS/MS); maximal number of peaks per spectrum, 100 (MS, MS/MS). No smoothing was applied and the peaks were not de-isotoped. To allow protein identification, the peak lists of the MS and MS/MS spectra were compared against theoretical mass data deduced from the proteins stored in the Nostoc 7120 data base of The Institute for Genomic Research (Release 7.0, 6128 protein entries). To this end, the search algorithm Mascot version 2.0 (Matrix Science, Boston, MA) was used in conjunction with the Mascot Daemon tool version 2.0. The search parameters were as follows: search mode, MS/MS ions search; enzyme, trypsin/P; protein mass, unrestricted; peptide ion mass tolerance, ± 30 ppm; fragment mass tolerance, ± 0.3 Da; maximum number of missed cleavage sites, 1; variable protein/peptide modifications, acetylation of N termini of proteins, modification of cysteines by acrylamide (propionamide), and oxidation of methionines. The primary identification criterion was a significant (p < 0.05) Mascot search result with a total protein score ≥50. To increase the confidence in protein identification, the data base search results were manually validated, in particular MS/MS identifications based on few peptide assignments.

For immunoblotting, proteins were electrophoretically transferred to nitrocellulose membranes. ClpC and NblA-GST fusion protein were detected by specific antisera. Antibody-antigen complexes were visualized with a goat anti-rabbit IgG-peroxidase conjugate and SuperSignal West Pico (Pierce) as chemiluminescent substrate.

Construction of Plasmids for Expression of GST Fusion Proteins—The plasmid for expression of GST-tagged ClpC was constructed as follows: the chromosomal gene alr2999 (29) from Nostoc 7120 was PCR-amplified from total Nostoc 7120 DNA using primers clpC7 and clpC8, incorporating two BamHI sites for cloning. The PCR fragment was digested with BamHI and ligated into the expression vector pGEX-6P-1 (GE Healthcare) yielding plasmid pGEX-6P/ClpC.

For expression of different NblA-GST fusion proteins, the nblA gene or its variants were amplified by PCR from plasmid pIC20RnblA and pIC20RnblA variants using primers 2.5 and 2.15, Ndel sites incorporated. The fragments were cloned into plasmid pGEX-2TK/Ndel to express NblA or NblA variants containing a C-terminal GST tag. The plasmid pGEX-2TK/Ndel was generated by mutagenesis (QuikChange site-directed mutagenesis kit, Stratagene) using plasmid pGEX-2TK as template and the primers GEXN-For and GEXN-Rev.

The α-subunits of phycocyanin (PC) and phycocerythrocyanin (PEC), respectively, were expressed N-terminal fused to a GST tag. For the construction of the expression plasmids, we amplified chromosomal genes cpcA (alr0529, α-subunit of PC) and pecA (alr0524, α-subunit of PEC) by PCR from total Nostoc 7120 DNA using primers pecGX1 (BamHI site inserted) and pecGX2 (EcoRI site inserted) or cpcGX1 (BamHI site inserted) and cpcGX2 (EcoRI site inserted), respectively. After digestion with BamHI and EcoRI, the PCR fragments were cloned into plasmid pGEX-2TK. All DNA constructs were confirmed by DNA sequencing. The constructs described in this paragraph are depicted in supplemental Fig. S1, C–G.

Protein Overexpression, Purification, and in Vitro Binding Assays Using N-terminal GST-tagged NblA—Expression, purification, and site-directed mutagenesis of N-terminal GST-tagged NblA was performed as described earlier (25). GST-NblA or its variants were bound to Glutathione-Sepharose 4B columns and incubated with soluble crude extracts from Nostoc 7120, prepared as described (17). After extensive washing, proteins bound to the resins were eluted with 20 mM glutathione in 50 mM Tris/HCl (pH 8.0) and analyzed by SDS-PAGE and immunoblotting. SDS-PAGE and immunoblots were performed as described above. PC subunits and GST were detected by specific antisera as described before (25).

Protein Overexpression, Purification, and in Vitro Binding Assays Using C-terminal GST-tagged NblA or Its Variants—The C-terminal GST-tagged NblA or its variants were expressed in E. coli after induction with isopropyl 1-thio-β-d-galactopyranoside for 2–3 h at 30 °C. Fusion proteins were
purified according to the manufacturer’s protocol (GE Healthcare). The GST–ClpC fusion protein was expressed after induction with isopropyl-1-thio-β-D-galactopyranoside overnight at 18 °C. ClpC containing an N-terminal GST tag was bound to Glutathione-Sepharose 4B in sodium/potassium phosphate buffer (composition see above) containing 0.5 mM ATP, AMPPNP, ATP, ADP, or GTP (each at 5 mM), and subsequently bound to Glutathione-Sepharose 4B overnight at 4 °C. After washing with the sodium/potassium phosphate buffer containing 0.5 mM ATP, AMPPNP, ATPγS, ADP, or GTP, proteins were eluted with 40 mM glutathione in 50 mM Tris/HCl (pH 8.0) containing 0.5 mM ATP, AMPPNP, ATPγS, ADP, or GTP, concentrated using deoxycholate/tri-chloroacetic acid precipitation (35), and analyzed by Tricine-Tris SDS-PAGE with 6 M urea as described (36). The in vitro binding assays using the different NblA-GST variants and ClpC were performed by the same procedure except that the incubation buffer contained 5 mM ATP and the washing buffer 0.5–1 mM ATP. The polyclonal anti-ClpC serum was purchased from Agrisera (Agrisera, Vännäs, Sweden, product number AS01 001).

**Protein Overexpression, Purification, and in Vitro Binding Assays Using GST-tagged PEC and PC—** The N-terminal GST-tagged α-subunits of PC and PEC were expressed after induction with isopropyl-1-thio-β-D-galactopyranoside overnight at 18 °C and purified according to the manufacturer’s protocol (GE Healthcare). The GST-ClpC fusion protein was expressed and purified as described above. NblA expression and purification was performed as described earlier (25). Protein concentrations were determined according to Bradford (34).

GST–PEC or GST–PC was incubated with ClpC for 3 h at 4 °C in the presence or absence of NblA (each protein at 3 μM) in sodium/potassium phosphate buffer (composition see above) containing 5 mM ATP. Affinity purification of GST-PEC or GST-PC complexes using Glutathione-Sepharose was performed as described above with 0.5 mM ATP in the washing and elution buffers. The eluted proteins were analyzed by Tricine-Tris SDS-PAGE in the presence of 6 M urea as described (36).

**RESULTS**

The Conserved N-terminal Region of NblA Is Relevant for in Vivo Function—We have previously shown that NblA specifically binds to the α-subunits of PC and PEC via amino acid residues in a short conserved motif at the end of the C-terminal helix (Fig. 1C and Ref. 25). However, the highest conserved stretch of amino acids among all known NblA proteins is located at the beginning of the N-terminal helix. A random mutation in this N-terminal region of the *Synechococcus* 7942 NblA protein (Ser9 to Phe) caused a non-bleaching phenotype in this strain (10), and this observation also suggested that these conserved residues have important roles in protein function. Hence, we investigated the capability of several NblA variants mutated in this region for their function.
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for mutagenesis that are highly conserved in orthologs of NbIA, not involved in dimerization, and expected to be accessible for putative interaction partners (for further details and an alignment of various NbIA proteins see supplemental Fig. S2 and Ref. 25). The experiments were carried out with the following NbIA variants generated by site-directed mutagenesis: L7A, S8A/L9A, E10A/Q11A, and Q12A/F13A with mutations located in the highly conserved stretch in the N-terminal helix (Fig. 1A, α1), as well as variants Q49A, E50A, L51A, and K53A with mutations in the conserved region at the end of the C-terminal helix (Fig. 1A, α2). Gene nblA or its variants, together with 570 bp of the 5′ untranslated region and 471 bp of the putative 3′ terminator region, respectively, were ligated into the self-replicating plasmid pRL1049 and transferred to the Nostoc 7120 ΔnblA mutant by conjugation, as described under “Experimental Procedures.” When grown on nitrate-containing medium, the resulting exconjugants showed no significant differences in whole cell absorbance spectra in the wavelength region where phycobiliproteins and chlorophyll absorb (Fig. 1B, left). After nitrogen step-down, wild-type NbIA expressed in trans in the NbIA-deficient mutant complemented the non-bleaching phenotype (Fig. 1B). None of the NbIA variants with amino acid exchanges in the N-terminal helix was able to complement the mutant phenotype. As is visible in the absorbance spectra shown in Fig. 1B, left, the amplitude of the PC absorbance peak at ~625 nm remained essentially unchanged after the nitrogen step-down. Expression of the nblA mRNA under conditions of nitrogen deficiency was proven in all strains by Northern blot analysis (data not shown). NbIA variants with amino acid exchanges located near the C terminus complemented the ΔnblA mutant to various degrees, but never completely (Fig. 1B, right). We also performed pull-down assays with heterologously expressed NbIA and its just mentioned variants, respectively, N-terminal fused to GST, to show the effects of amino acid exchanges on phycobiliprotein binding. The NbIA variants with amino acid exchanges in the N-terminal region interacted with phycobiliproteins like the NbIA wild-type protein (Fig. 1C, left), two of the NbIA variants with amino acid exchanges in the C-terminal helix (L51A and K53A) bound no phycobiliproteins (Fig. 1C, right). NbIA variant K53A migrated somewhat faster in SDS-PAGE than wild-type NbIA and the other mutated variants. Determination of the molecular mass of the K53A variant by MALDI-MS gave an m/z value of 8443.7, which corresponds well with its calculated mass of 8443.7, which corresponds well with its calculated mass of 32398.

The NbIA variants with amino acid exchanges located in the highly conserved stretch in the N-terminal helix were anticipated to be involved in phycobiliprotein degradation. This polypeptide of about 90 kDa apparent molecular mass is encoded by open reading frame alr2999 of the Nostoc 7120 chromosome. As mentioned above, it is annotated as the “endopeptidase Clp ATP-binding chain ClpB” (bacteria.kazusa.or.jp/cyanobase/). However, a protein BLAST search with Alr2999 against the NCBI data base (www.ncbi.nlm.nih.gov/BLAST/) led to the conclusion that the gene alr2999 encodes in fact ClpC. Protein sequence alignments showed that the predicted gene product is 87% identical to ClpC from Synechococcus 7942, 77% identical to ClpC from Arabidopsis thaliana, and 61% identical to ClpC from Bacillus subtilis (see supplemental Fig. S4). Furthermore, Alr2999 possesses the conserved IGF motif combined with an upstream basic residue (Lys), which is involved in the interaction of ClpC with the proteolytic complex and which is absent in ClpB proteins (supplemental Fig. S4 and Refs. 37 and 38). These findings strongly suggest that alr2999 encodes ClpC of Nostoc 7120. For clarity, in the following this protein will be referred to as ClpCAlr2999.

To prove further that ClpCAlr2999 co-purifies with GST-tagged NbIA, we performed immunoblot analyses using an antiserum against ClpC (Fig. 2). As expected, ClpCAlr2999 was present in the eluate fraction from Nostoc 7120 cells expressing extracts and separated by SDS-PAGE. Mutant cells expressing non-tagged NbIA served as control. Twelve polypeptide bands that were present in the elution fraction but missing or less abundant in the control were analyzed by peptide mass fingerprinting. Besides NbIA the following proteins were identified: an unknown protein (All0454), which is 90.3% identical to a predicted transcriptional regulator of A. variabilis ATCC 29413, two hypothetical proteins (Alr1960, Asr3294) of unknown function, a GroEL-type chaperonin (Alr3662), a DnaK-type molecular chaperone (Alr1742), a translation elongation factor EF-G (All4338), the 1,4-α-glucan branching enzyme GlgB (All0713), the RNA polymerase α subunit RpoA (All4191), the fructose-1,6-bisphosphate aldolase FdA (All4563), an AhpC/TSA family protein (Alr4404), phycobilisome subunits PecB, ApcD, and ApcE (Alr0523, All3653, Alr0020), and an endopeptidase Clp ATP-binding chain (Alr2999) erroneously annotated as ClpB (see below).

Only one of the identified proteins was anticipated to be involved in phycobiliprotein degradation. This polypeptide of about 90 kDa apparent molecular mass is encoded by open reading frame alr2999 of the Nostoc 7120 chromosome. As mentioned above, it is annotated as the “endopeptidase Clp ATP-binding chain ClpB” (bacteria.kazusa.or.jp/cyanobase/). However, a protein BLAST search with Alr2999 against the NCBI data base (www.ncbi.nlm.nih.gov/BLAST/) led to the conclusion that the gene alr2999 encodes in fact ClpC. Protein sequence alignments showed that the predicted gene product is 87% identical to ClpC from Synechococcus 7942, 77% identical to ClpC from Arabidopsis thaliana, and 61% identical to ClpC from Bacillus subtilis (see supplemental Fig. S4). Furthermore, Alr2999 possesses the conserved IGF motif combined with an upstream basic residue (Lys), which is involved in the interaction of ClpC with the proteolytic complex and which is absent in ClpB proteins (supplemental Fig. S4 and Refs. 37 and 38). These findings strongly suggest that alr2999 encodes ClpC of Nostoc 7120. For clarity, in the following this protein will be referred to as ClpCAlr2999.

To prove further that ClpCAlr2999 co-purifies with GST-tagged NbIA, we performed immunoblot analyses using an antiserum against ClpC (Fig. 2). As expected, ClpCAlr2999 was present in the eluate fraction from Nostoc 7120 cells expressing...
NblA-GST but not in the control derived from cells expressing the non-tagged NblA. ClpC is the HSP100 chaperone partner of a Clp protease and responsible for substrate recognition, unfolding, and translocation into the proteolytic chamber (39–41). Thus, ClpC seems to be a likely candidate for interacting with NblA.

**Binding of ClpC to NblA Is ADP/ATP-dependent** — The interaction of ClpC with NblA was analyzed in detail by in vitro pull-down assays. To this end, NblA expressed with a C-terminal GST tag was incubated with purified ClpCAlr2999 under various conditions and subsequently bound to Glutathione-Sepharose, as described under “Experimental Procedures.” After washing, proteins were eluted with glutathione and separated by Tricine-SDS-PAGE. As control we used the GST protein incubated with ClpCAlr2999 under otherwise identical conditions. Reactions were performed in the presence of ATP, ADP, the non-hydrolysable ATP analogs AMPPNP or ATPγS, or GTP. Fig. 3A shows the result of such a binding experiment. ClpCAlr2999 interacted with NblA in the presence of ATP, ADP, and ATPγS (Fig. 3, A and B). When ATP was replaced by AMPPNP or GTP, no interaction between ClpCAlr2999 and NblA was observed (Fig. 3A). These results indicate that the ClpCAlr2999-NblA interaction depends on an appropriate nucleotide but not on its hydrolysis.

The faint polypeptide band at ∼116 kDa visible in all lanes of Fig. 3A represents the GST-ClpCAlr2999 fusion protein. Recombinant ClpCAlr2999 used in these assays was expressed as a GST fusion protein in *E. coli*. Following affinity purification, the GST tag was removed by PreScission protease treatment (see “Experimental Procedures”) but cleavage was obviously not complete. This residual GST-ClpCAlr2999 apparently bound to Glutathione-Sepharose in the pull-down experiments. The observed binding of non-tagged ClpCAlr2999 could be explained by formation of oligomeric complexes between tagged and non-tagged versions of ClpCAlr2999, a phenomenon that is well known for other members of the Clp family (41–44).

**Mutations in the Conserved Region Near the N Terminus of NblA Affect ClpCAlr2999 Binding** — As described above, the highly conserved stretch of amino acid residues near the N terminus of NblA is essential for its in vivo function (Fig. 1), suggesting that ClpCAlr2999 binds to this region of the protein. Two NblA variants were used to test this hypothesis: S8A/L9A and Q12A/F13A, each with two amino acid exchanges in the conserved N-terminal region. In addition two other NblA variants were tested for their ability to interact with ClpCAlr2999. NblA variants L51A and K53A, bearing mutations in the C-terminal helix, and known to be affected in phycobiliprotein binding (Fig. 1 and Ref. 25). All four NblA variants were expressed with a C-terminal GST tag, and pull-down assays were performed as described under “Experimental Procedures.” Fig. 3C shows that variants S8A/L9A and Q12A/F13A did not bind ClpCAlr2999, whereas variants L51A and K53A retained the ability to interact. We conclude that the conserved region of NblA located near its N terminus is responsible for ClpC binding.

**The α-Subunits of PEC and PC Bind to ClpCAlr2999 Only in the Presence of NblA** — Assuming that a Clp protease is responsible for PBS degradation, the interaction between phycobiliproteins and ClpC should depend on NblA. To test this hypothesis, the apoproteins of the α-subunits of PEC and PC, respectively, were expressed as GST fusion proteins in *E. coli*, incubated with ClpCAlr2999 in the presence or absence of NblA and were then affinity-purified using Glutathione-Sepharose (for details see “Experimental Procedures”). As shown in Fig. 4, ClpCAlr2999 co-purified with the GST-tagged PEC or PC apoproteins only in the presence of NblA. This strongly suggests that the stress-inducible NblA protein mediates the interaction between phycobiliproteins.
and ClpC<sub>Alr2999</sub> as an adaptor protein and regulates PBS degradation in this manner. To get an idea of the binding stoichiometry of NblA (which is actually a homodimer; Ref. 25 and Fig. 5) to ClpC<sub>Alr2999</sub> we incubated the GST-tagged apoprotein of PC with NblA and increasing concentrations of ClpC<sub>Alr2999</sub> in the presence of ATPγS. After affinity purification, bound ClpC<sub>Alr2999</sub> was visualized by immunoblotting (Fig. 4B). The amount of bound ClpC<sub>Alr2999</sub> increased up to a concentration of 8 μM. Higher ClpC<sub>Alr2999</sub> concentrations (10 or 12 μM) did not lead to a further increase in ClpC<sub>Alr2999</sub> binding. Because the concentration of the NblA dimer was kept constant at 4 μM, the titration suggests a binding stoichiometry of the NblA dimer to ClpC<sub>Alr2999</sub> of 1:2. Calculated on a monomer basis the ratio would be 1:1.

**DISCUSSION**

Complementation of an NblA-deficient Mutant of Nostoc 7120 with Mutated Variants of the NblA Protein—As reported previously, conserved amino acid residues near the C terminus of NblA are involved in PBS binding (25). Because NblA itself has no detectable proteolytic activity, it was assumed that further protein(s) or cofactor(s) may be necessary for PBS degradation (10). In sequence alignments of NblA from cyanobacteria and red algae, the most conserved stretch of amino acids was found near the N terminus of the protein (supplemental Fig. S3 and Refs. 15 and 25). To examine the importance of this motif for protein function, we complemented an NblA-deficient Nostoc 7120 mutant, which is unable to degrade PBS upon nitrogen deprivation, with NblA variants (Fig. 1). Amino acid exchanges in the C-terminal helix (L51A and K53A) did not completely prevent in vitro function, although their binding to phycobiliproteins in vivo was totally inhibited (Fig. 1, B and C, spectra and lanes 8 and 9). This seems to be somewhat contradictory, but one has to take into account that the in vitro PBS binding experiments do not fully simulate in vivo conditions. For example, in vitro immobilized NblA was first incubated with cyanobacterial crude extract that was then removed by washing with buffer. Thus, in the cells but not in the pull-down experiments, an equilibrium situation between bound and free NblA is likely to exist that should strengthen the interaction of NblA and phycobiliproteins. In this context it is of note that these pull-down experiments were carried out with phycobiliprotein trimers and, possibly, higher oligomers, subcomplexes that result from dissociation of phycobilisomes. Experiments of Luque et al. (20) have demonstrated that a significant fraction of NblA copurifies with PC-rich phycobilisomes. This is indicative of a rather tight binding of NblA to phycocyanin in the native light-harvesting complexes, and furthermore, suggests that degradation of phycobilisomes may be initiated when they bind a certain amount of NblA. Hence, although phycobiliprotein binding to the L51A and K53A variants of NblA was undetectable in vitro, it might still occur to some extent in vivo.

Complementation analyses with NblA variants mutated in the conserved region of the N-terminal helix demonstrated that these amino acids, too, are essential for protein function (see Fig. 1). Based upon the crystal structure of NblA and the model of NblA binding to PBS, these residues at the N-terminal helix should be accessible for a potential interaction partner (25). This region could be the recognition site for a further factor involved in PBS degradation. It cannot be excluded that the amino acid replacements introduced into this site may affect the structure of other functionally relevant parts of the NblA
NblA Binds to ClpC

NblA, an Adaptor Protein of ClpC—Considering that expression of the *SyclpC* gene is constitutive (49) and that PBS degradation is a regulated process induced by *nblA* expression, we assume that NblA works as a subunit of the ClpC protease. When NblA expression is missing, e.g. in Δ*nblA* mutants or under nitrogen replete conditions, ClpC does not recognize the phycobiliproteins as substrates, and the PBS are not degraded. By use of a phosphate-regulated promoter, Collier and Grossmann (10) have demonstrated that PBS degradation can be induced solely by the expression of NblA even when cells grow in nitrogen-replete medium. It thus appears that NblA expression alone suffices to change the substrate binding specificity of ClpC in a way that allows for PBS degradation. The regulation of NblA expression would be an effective method to control the activity of a particular Clp protease quickly, without affecting its other activities (51).

Proteins binding to the Clp/HSP100 chaperone partner of a Clp protease to modulate substrate recognition are called adaptor proteins. In bacteria and cyanobacteria, several adaptor proteins of Clp proteases have already been characterized. Examples are ClpS (40, 52, 53), RssB (54, 55), and SspB (56–58) in *E. coli*, MecA (59–61) and YpbH (62, 63) in *B. subtilis*, as well as ClpS1 and ClpS2 in *Synechococcus* 7942 (41). Adaptor proteins are not restricted to bacteria but also occur in yeasts and other eukaryotes (64–66). No similarities in sequence and structure exist between these adaptor proteins, but they are small like NblA (51). Considering MecA, an adaptor protein of ClpC from *B. subtilis*, and ClpS, an adaptor protein of ClpA from *E. coli*, interesting similarities to NblA exist concerning the arrangement of binding sites on the surface of the proteins. Structural analysis of ClpS revealed two highly conserved patches located on opposite sites of the molecule. Whereas one patch is responsible for substrate recognition, the other is involved in the interaction with ClpA (53), the ortholog of ClpC in *E. coli* (67). MecA consists of two domains. The N-terminal domain is responsible for recognition and targeting of substrates (60, 68), and the C-terminal domain is necessary for the interaction with ClpC (61). In a similar manner, NblA binds via its C-terminal helix to the α-subunits of phycobiliproteins, which are probably substrates of ClpC, and via its N-terminal helix to ClpC (see Figs. 1 and 3). In summary, NblA resembles in many regards ClpS and MecA, well characterized adaptor proteins of Clp proteases from other organisms.

The Interaction between NblA and ClpC Depends on ADP/ATP Binding—Using in vitro pull-down experiments we could show that NblA forms a complex with ClpC only in the presence of ATP, ATPγS, or ADP (Fig. 3, A and B). No interaction was detected in binding experiments with ClpC and NblA in the absence of nucleotides and in the presence of AMPPNP or GTP (Fig. 3, A and B). Our results clearly demonstrate that the interaction between these two proteins depends on ATP/ADP binding or is strongly stabilized by the presence of either nucleotide. This result is consistent with reports that ATP binding but not hydrolysis is required for the stable formation of the hexameric ring of the Clp/HSP100 chaperones and for their association with adaptor proteins and substrates, respectively. For example, Kirstein *et al.* (61) reported that the adaptor protein MecA from *B. subtilis* forms a larger oligomeric complex with *BaClpC* in the presence of ATP, ATPγS, or ADP. *ClpC*<sub>Alr2999</sub> behaves similarly. According to size exclusion chromatography (data not shown), it oligomerizes in the presence of ADP and ATPγS. In the presence of ATP, we observed oligomerization of *ClpC*<sub>Alr2999</sub> only when the oligomer was stabilized by the chemical cross-linker *ortho*-phthalaldehyde.

Of the two non-hydrolysable ATP analogs used, ATPγS promoted binding but AMPPNP did not (Fig. 3A). Similar findings were described for *BaClpC* and MecA (59), as well as HslU (ClpY), an ATPase with chaperone function like ClpC (69). In accordance with this, Lee and co-workers (70) found that either ATP or ATPγS is essential for substrate binding to the chaperone ClpB from *Thermus thermophilus*, and that these nucleotides cannot be replaced by AMPPNP. By cryo-EM reconstruction of the ClpB hexamer in different nucleotide-bound states, they showed that AMPPNP binding, unlike ATPγS binding, does not produce the conformational change required for stable substrate binding. In the present study, no attempts were made to determine the effects of AMPPNP on oligomerization and conformational changes of *ClpC*<sub>Alr2999</sub>.
Our results (Fig. 4B) suggest that three NblA dimers bind to one ClpC\textsubscript{A}\textsubscript{1}2999 hexamer. This binding stoichiometry would be consistent with the fact that there are two potential ClpC binding sites on the NblA dimer. Other adaptor proteins (MecA and ClpS) have been reported to bind to their respective chaperones with a 1:1 stoichiometry when calculated as a monomer to monomer ratio (61, 71), i.e. the stoichiometries are essentially identical.

On the Function of NblA in PBS Degradation—When cyanobacteria are starved for combined nitrogen, PBS degradation is induced by NblA. Although it has been shown that NblA binds to phycobiliproteins (22, 25), the exact function of the NblA protein has not yet been elucidated. Here we provided evidence for an interaction between NblA and ClpC, suggesting that PBS degradation occurs via a Clp protease. According to these results, NblA binds via a conserved motif near its N terminus to ClpC and via another motif near its C terminus to phycobiliproteins (Fig. 5). Neither the NblA variants affected in phycobiliprotein binding nor the NblA variants affected in ClpC binding were functional in vivo (Fig. 1B). In the in vitro pull-down assays, no direct interaction between the phycobiliproteins and ClpC was observed unless NblA was added (Fig. 4), indicating that NblA binding to phycobiliproteins mediates its recognition by ClpC. Thus, a ternary complex between NblA, the phycobiliproteins, and ClpC appears to be necessary for PBS degradation, probably by a thylakoid-associated ClpP complex like the one tentatively identified by Stanne et al. (41). However, because ClpC itself can function as a molecular chaperone even in the absence of a Clp protease (72), we cannot exclude the possibility that it helps unfold phycobiliproteins, which are then degraded by other proteases.

In a recent publication, Dines et al. (23) proposed a model of the action of NblA in PBS degradation that is very different from the model presented here. By comparing the crystal structures of NblA molecules from three cyanobacterial species, it was found that their three-dimensional structures are much more conserved than their amino acid sequences. They, furthermore, found that not only amino acid exchanges in the conserved regions near the N and C termini, but also at other positions of the NblA molecule affect its function in PBS degradation, and that the NblA protein from \textit{Nostoc} 7120 complements a \textit{NblA} mutant of \textit{Synechococcus} 7942 despite a rather low conservation in amino acid sequence (cf. supplemental Fig. S2). Dines et al. (23) propose that NblA may penetrate into a gap between two phycobiliprotein hexamers of the PBS rods, thereby loosening or disrupting the rod structure and making them susceptible to proteolytic degradation. Further biochemical and mutational analyses will be required to distinguish between this model and the more specific one put forward here.

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