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The roles of the chaperone-like protein CpeZ and the phycoerythrobilin lyase CpeY in phycoerythrin biogenesis

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ABSTRACT

Phycoerythrin (PE) present in the distal ends of light-harvesting phycobilisome rods in *Fremyella diplosiphon* (*Tolypothrix* sp. PCC 7601) contains five phycoerythrobilin (PEB) chromophores attached to six cysteine residues for efficient green light capture for photosynthesis. Chromophore ligation on PE subunits occurs through bilin lyase catalyzed reactions, but the characterization of the roles of all bilin lyases for phycoerythrin is not yet complete. To gain a more complete understanding about the individual functions of CpeZ and CpeY in PE biogenesis in cyanobacteria, we examined PE and phycobilisomes purified from wild type *F. diplosiphon*, *cpeZ* and *cpeY* knockout mutants. We find that the *cpeZ* and *cpeY* mutants accumulate less PE than wild type cells. We show that in the *cpeZ* mutant, chromophorylation of both PE subunits is affected, especially the Cys-80 and Cys-48/Cys-59 sites of CpeB, the beta-subunit of PE. The *cpeY* mutant showed reduced chromophorylation at Cys-82 of CpeA. We also show that, *in vitro,* CpeZ stabilizes PE subunits and assists in refolding of CpeB after denaturation. Taken together, we conclude that CpeZ acts as a chaperone-like protein, assisting in the folding/stability of PE subunits, allowing bilin lyases such as CpeY and CpeS to attach PEB to their PE subunit.

1. Introduction

Cyanobacteria are photosynthetic organisms that have existed for over 3.5 billion years and are believed to be responsible for Earth's oxidizing atmosphere as it exists today [1]. Their light-harvesting complexes, phycobilisomes (PBS)[5], are the macro-molecular protein complexes contributing to the success of these organisms by absorbing and channeling excitation energy into the chlorophyll reaction centers of photosystems I and II. PBS typically consists of two structural domains: a core, which connects to the photosynthetic thylakoid membrane, and rods, which extend out from the core [2]. The PBS core and rods consist of phycobiliproteins present in heterohexameric form $(\alpha\beta)_6$ interconnected by linker proteins to form the large antenna structure [2,3] (see Fig. S1A).

In cyanobacteria, there are four major classes of phycobiliproteins based on absorbance spectra and sequence homologies: phycocyanin (PC), allophycocyanin (AP), phycoerythrin (PE) and phycoerythrocyanin. Each phycobiliprotein is composed of α- and β-subunits, and each subunit contains between one and four isomeric bilin chromophores that are attached by thioether linkages to conserved cysteine (Cys) residues [2–4] (see Fig. S1, panels B and C): phycocyanobilin (PCB), phycoerythrobilin (PEB), phycoviolobilin and phycourobilin (PUB) [5]. These bilins absorb visible light that is not effectively captured by chlorophyll. Some PE-producing cyanobacteria are capable of

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[5] The abbreviations used are: ANOVA, analysis of variants; AP, allophycocyanin; BME, 2-mercaptoethanol; CS, citrate synthase; EICs, extracted ion chromatograms; HT-, hexahistidine-tagged; MW, molecular weight; NT-, non-tagged; Ni-NTA, nickel-nitrilotriacetic acid; PBS, phycobilisome(s); PC, phycocyanin; PCB, phycocyanobilin; PE, phycoerythrin; PEB, phycoerythrobilin; PUB, phycourobilin

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altering their PBS complexes to readily capture the most abundant wavelengths of light available in the environment through a process called chromatic acclimation. *Fremyella diplosiphon* (*Tolypothrix* sp. PCC 7601), the focus of this study, specifically performs Type III chromatic acclimation where the distal rod protein synthesis is altered under green light and red light conditions [6]. When cells are grown in red light, the rods of the PBS are composed of two different forms of PC (constitutive and red-light inducible), but if the cells are switched to green light, PE is synthesized and assembled onto the outer rods of the new PBS [7] where it takes the place of the red-light inducible PC.

Phycobiliprotein chromophorylation is catalyzed post-translationally by specific bilin lyases [8,9], a known exception being the ApcE linker which is autocatalytically chromophorylated [10]. The lyases for PE, responsible for attaching PEB to the five conserved Cys residues: α-82, α-139, β-48/59, β-80 and β-165 (based on the *F. diplosiphon* PE sequence [11]), are still not fully characterized. The first phycobiliprotein lyases characterized were the heterodimeric CpcE and CpcF proteins, which ligate PCB to the α-subunit of PC (CpcA) at Cys-84 [12–16]. All members of this "E/F" family of lyases contain HEAT-repeat motifs [17–19], and have been shown to be primarily α-helical in structure [20]. Some members have the ability to isomerize and attach a bilin [18,19,21], and others portray a dual function as a chaperone-like protein and as a lyase [16,22].

Two members of the E/F family, whose complete functions have not yet been fully elucidated, are CpeY and CpeZ [23,24]. In *F. diplosiphon* *cpeY* and *cpeZ* genes are present downstream of *cpeBA* operon (Fig. 1A) which encodes the genes for CpeB and CpeA apo-proteins. Based on sequence similarity, CpeY is a fusion of CpcE-like and CpcF-like proteins, while CpeZ is more similar to CpcE [24]. Khan et al. isolated transposon mutants in the *cpeY* and *cpeZ* genes in *F. diplosiphon.* These mutants displayed decreased levels of PE when grown in green light, suggesting CpeY/CpeZ might be a bilin lyase for either CpeA (α-subunit of PE) or CpeB, but their biochemical characterization of the mutants were incomplete [23]. Later, we showed that recombinant CpeY is a specific lyase for Cys-82 of CpeA, while recombinant CpeZ enhanced the CpeY lyase activity by 40%, but the specific function of CpeZ remained unclear [24]. In this paper, we characterize in detail the phenotypes of single deletion mutants of *cpeY* and *cpeZ* genes in *F. diplosiphon* and demonstrate that CpeY is a bilin lyase specifically for CpeA whereas CpeZ is involved in the biosynthesis of both PE subunits by acting as a chaperone-like protein.

2. Experimental procedures

2.1. Strain constructions and culture conditions

The laboratory isolate SF33 [25,26], a shortened filament mutant of *Fremyella diplosiphon* UTEX 481, was used as wild type (WT) and was cultured as previously described [27]. The *F. diplosiphon* mutants in *cpeY* or *cpeZ* were generated as clean deletions as previously described [28–30] with a few alterations. The deletion constructs of *cpeY* and *cpeZ* obtained by overlapping PCR were cloned into pJCF276 [29] between *Eco*RI and *Nco*I restriction sites. The deletion constructs were designed to include at least a 1.3 kb of flanking homology regions on each side of the gene of interest. The genes were internally truncated in-frame such that only short chimeric open reading frames were left in place. For *cpeY*, the first 18 codons at the 5′ end were fused to the last 12 codons from the 3′end, whereas for *cpeZ*, the first 8 codons at the 5′ end were fused to the last 11 codons (see Fig. 1A). All primers used are provided in Table S1. All transformed strains were obtained through triparental mating as previously described [29] with the selection of neomycin resistant colonies on 1% agar-medium D, pH 8 [31] and maintained at 25 μmol $m^{-2} s^{-1}$ of white light (GroLux - Sylvania USA F15T8-GRO). After selection on neomycin (10 μg ml^{-1}) for chromosomal integration of the deletion plasmids, the transformed *F. diplosiphon* colonies were grown in liquid BG-11 to an OD_{750} of ~0.8 to allow for the second intramolecular recombination event to occur. Afterwards, an inoculum of this culture was serially diluted on plates with 1% agar-medium D, pH 6.75 and 5% sucrose in order to select and screen for sucrose resistant isolates in which allelic replacement has occurred. Successful excision of the wild type copies of *cpeY* and *cpeZ* was verified by PCR and several independent isolates of each were obtained for further analysis. All isolates containing the correct deletions had a greenish coloration when compared to wild type.

Growth curves were generated from two independent replicates of cells grown in ~15 μmol $m^{-2} s^{-1}$ of green light monitored by absorbance at 750 nm every 24 h for 12 days.

2.2. Isolation of phycobilisomes (PBS)

PBS were isolated from *F. diplosiphon* cells as described previously [32] with a few minor alterations. All steps were performed at room temperature (~22 °C). When cultures reached an OD_{750} between 0.6 and 0.9, cells were harvested by centrifugation at 8000 ×*g* for 10 min. The cells were washed with and then resuspended in 0.65 M NaH_2PO_4/K_2HPO_4 buffer, pH 7.5before being passed through a French Pressure Cell Press at 18,000 psi three times. As previously described [32], intact PBS were extracted by ultracentrifugation on sucrose gradients and analyzed by absorbance and fluorescence emission spectroscopy (described below in Experimental procedures Section 2.8) before long term storage in the dark at −20 °C.

2.3. Isolation of phycoerythrin (PE)

PE was purified from *F. diplosiphon* cells following the protocol described by Glazer [33] with minor changes. The entire procedure was conducted in dim light and at 4 °C when possible. Cells (10 *g* wet weight) were centrifuged and resuspended in 15 mL of 1 M sodium acetate, pH 5.0 and 1 mg mL^{-1} complete mini protease inhibitor cocktail. The cells were lysed using a French Pressure Cell press three times at 18,000 psi, followed by centrifugation at 77,000 ×*g* for 1 h.

During the ammonium sulfate precipitations steps, some mutants required 10–20% more ammonium sulfate to precipitate PE in the supernatants (compared to the amount required for WT PE as referenced in [33]). Pure PE fractions were pooled and brought to 70% saturation for long-term storage at 4 °C in the dark. Samples were exhaustively dialyzed in 100 mM sodium phosphate-1 mM sodium azide, pH 7.0 before analysis.

2.4. Separation and purification of phycoerythrin (PE) α- and β-subunits

PE α- and β-subunits (GenBank Accession Numbers EKF01783.1 and EKF01782.1, respectively) were separated and purified using a combination of previously published protocols with modifications [11,34–36]. Approximately 5–10 mg of purified PE was exhaustively dialyzed against 5 mM sodium phosphate, pH 7.0 plus 5 mM 2-mercaptoethanol (BME) overnight at 4 °C. PE samples were concentrated by ultrafiltration through an Amicon Ultra centrifugal filter unit (10 kDa cutoff; Novagen/EMD Millipore Corp., Darmstadt, Germany) to a volume of 1–2 mL. The concentrated PE was denatured in 8.8% formic acid, pH 2.2 plus 10 mM BME for 1 h at room temperature in the dark. The sample was centrifuged at 20,800 ×*g* in a microcentrifuge tube for 5 min to remove any precipitates. The supernatant was gently applied to a Bio-Rex 70 sodium form column (200–400 mesh, 2.2 × 11.5 cm; Bio-Rad, Hercules, CA) pre-equilibrated with 8.8% formic acid, pH 2.2 plus 10 mM BME at room temperature. The sample was allowed to drain into the column bed before being washed with 1 column volume each of 2 M urea, pH 2.2 plus 10 mM BME and then 4 M urea, pH 2.2 plus 10 mM BME. The proteins were eluted off the resin with a stepwise gradient using 9 M urea, pH 2.2 plus 10 mM BME and dH_2O plus 10 mM BME to produce the following urea concentrations: 6 M (50 mL), 7 M (125 mL), 7.5 M (125 mL), 8 M (70 mL), 8.5 M (70 mL), and 9 M

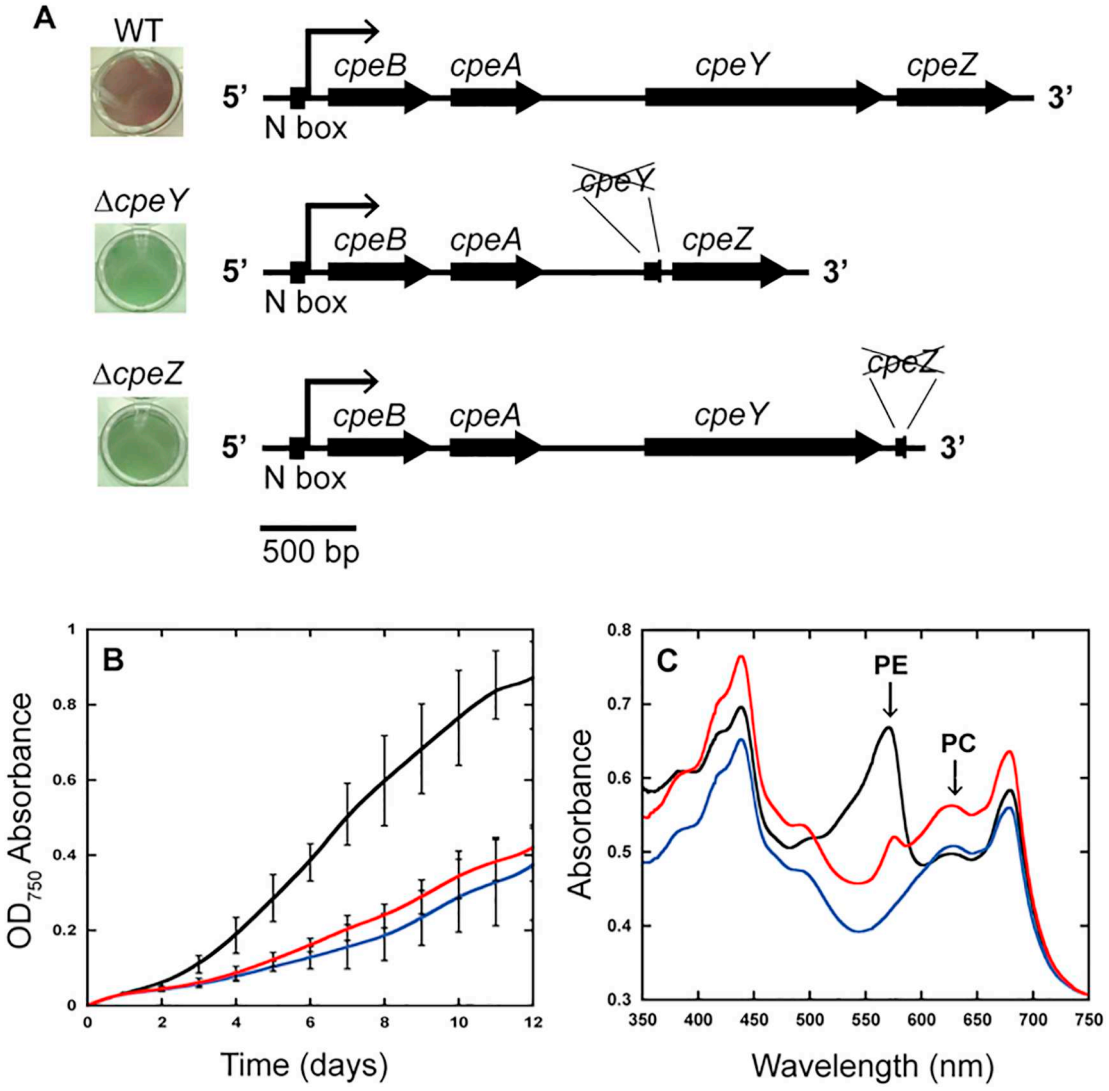

Fig. 1. Phenotypes of WT and mutants grown in ~15 μmol m^{-2} s^{-1} of green light.
(A) Diagrams of the PE operon containing the genes that encode the β- and α-subunits of PE (CpeB and CpeA, respectively), the α-Cys-82 lyase CpeY [24], and the putative chaperone-like protein CpeZ from the WT, Δ*cpeY*, and Δ*cpeZ* strains of *F. diplosiphon*. Possible transcription regulation site is labeled "N box" [7]. Liquid cell cultures of each strain grown in approximately ~15 μmol m^{-2} s^{-1} of green light are represented to the left of the operons. (B) Average growth curves of two independent replicates each for WT (black line), Δ*cpeY* (blue line), and Δ*cpeZ* (red line) cells with OD readings taken at 750 nm every 24 h for 12 days. Error bars represent the ranges. (C) Whole cell absorbance spectra of WT (black line), Δ*cpeY* (blue line), and Δ*cpeZ* (red line) cells. PE and PC peaks are indicated with arrows.

(100 mL). The fractions containing CpeA or CpeB as judged by SDS-PAGE analyses were pooled and renatured during exhaustive dialysis in 50 mM sodium phosphate, pH 7.0 plus 5 mM BME (until sample pH reached 7.0). After approximately 3 days of exchanging the dialysis buffer with fresh buffer 2 to 3 times daily, the pooled fractions were concentrated by ultrafiltration through an Amicon Ultra centrifugal filter unit (10 kDa cutoff). Pure PE subunits were stored short term in the dark at 4 °C and long term in the dark at −20 °C.

2.5. Construction of expression plasmids

Plasmids used in this study, including those that were previously described, are listed in Table S2. All expression constructs newly produced for this study were sequenced at the W. M. Keck Conservation and Molecular Genetics Laboratory (University of New Orleans). Each gene was amplified by PCR from *F. diplosiphon* chromosomal DNA or pre-existing plasmid DNA using the primers listed in Table S1. Each resulting amplicon was cloned into a Duet vector (Novagen) using restriction enzymes as designated in Table S1. The plasmid pNT-PebS was generated by restriction enzyme digestion of the construct pPebS [37] with *Nco*I and *Eco*RI, filling in overhangs with DNA Polymerase I, and vector religation which removed the coding region for the hexahistidine-tag (HT-) from the vector, thus generating the non-tagged (NT-) version of the plasmid.

2.6. In vivo heterologous expression and purification of recombinant proteins

Recombinant proteins were expressed and purified from *E. coli* BL21 (DE3) as previously described [24,38]. For CpeB solubility experiments, the induced cultures were placed at 18 °C for a total of 19 h with shaking. Cells were harvested by centrifugation at 11,000 × *g* for 10 min in 100 mL aliquots at time points 0 (time directly after induction with IPTG), 4 h, 10 h, and 19 h. The wet weight of all cell pellets (ranging from 0.22 g to 0.71 g) was measured prior to storage at −20 °C. Cell pellets were resuspended in Buffer O (20 mM Tris-HCl, pH 8.0, 50 mM NaCl, 50 mM KCl), 0.75 mg mL^{-1} complete mini protease inhibitor cocktail and 0.01 mg·mL^{-1} lysozyme in volumes based

on the relative mass of the pellets. The largest pellet was resuspended in a maximum volume of 10 mL of buffer. The masses of other cell pellets were compared to the largest and an equal ratio (volume:wet weight) was used to resuspend the smaller cell pellets. Cell lysis and centrifugation were conducted as previously described [24]. After centrifugation, the pellets containing cellular debris, inclusion bodies, and unbroken cells were diluted in 2 mL Buffer O prior to analysis.

2.7. Tryptic digestion and mass spectrometry

Tryptic digestion of proteins was conducted following the protocol described earlier [24] with a few minor alterations. Only one aliquot of trypsin (dimethylated trypsin from porcine pancreas; Sigma, St. Louis, MO) was added to 2% (w/w) from a 20 μg mL^{-1} stock to the denatured protein mixtures and incubated at 30 °C for 3 h in the dark. Digested peptides were passed through a pre-equilibrated C8 Sep-Pak cartridge (Waters Corporation, Milford, MA) before LC-MS/MS analysis using a Waters Synapt HDMS Quadrupole-TOF mass spectrometer similar to described [19] except that mass tolerances of 0.08 *m/z* were used for all extracted ion chromatograms (EICs).

2.8. Protein analysis by spectroscopy and gel-electrophoresis

Fluorescence emission and absorbance spectra were acquired on a Perkin Elmer LS55 fluorescence spectrometer (Waltham, MA) and a Lambda 35, dual-beam UV/Vis spectrometer (Perkin Elmer) as previously described [39]. The excitation wavelength was set to 490 nm to preferentially excite PEB-bound proteins and 590 nm for PCB-bound proteins. In some experiments, a Synergy MX Microplate Reader (BioTek Instruments, Inc., Winooski, VT) and a Beckman DU 640B Spectrophotometer (Beckman Coulter, Inc., Indianapolis, IN) were also used to acquire fluorescence emission and absorbance spectra, respectively.

Most polypeptide samples were resolved by polyacrylamide gel electrophoresis (PAGE, 15% w/v), in the presence of sodium dodecyl sulfate (SDS), and visualized as previously described [39]. PBS samples were precipitated from sucrose gradient fractions using 25% (w/v) TCA, and then analyzed by 10–20% Tris-HCl gradient Criterion gels (Bio-Rad).

All recombinant and native total protein concentrations were measured using the Quick Start Bradford Protein Assay kit (Bio-Rad).

2.9. Immunoblotting

Immunoblotting analysis was performed as described [24] using the following primary antisera generated in rabbits: Anti-CpeA (1:5000 dilution [24]), Anti-CpeB (1:20,000 dilution), Anti-CpcA (1:5000 dilution [40]) and Anti-CpcS (1:5000 [41]). Anti-CpeB antibodies were generated in rabbits against holo-CpeB purified from *F. diplosiphon* (YenZym Antibodies, San Francisco, CA). These antibodies were tested against recombinant and holo-phycobiliproteins to validate they were specific for CpeB with some minor cross-reaction with CpcB. Protein band intensities were quantified and analyzed using Quantity One 1-D Analysis Software and Image Lab Software V5.2.1 (Bio-Rad).

2.10. Pull-down assays

Pull-down assays were performed using the protocol described previously [41] with a few alterations. Proteins of interest were mixed in an approximately 1:1 ratio (determined by SDS-PAGE) in a small sample volume (≤ 1 mL), and allowed to sit on ice with gentle mixing every 25 min for 75 min in the dark. When native cyanobacterial proteins were used, 10 μg (total protein) of each protein sample were mixed. Pull-down mixtures (referred to as "mix") were purified using a small nickel-nitrilotriacetic acid (Ni-NTA) affinity column (25% (v/v) of total sample volume) pre-equilibrated with Buffer O. Samples were passed through the column three times to ensure binding (unbound proteins are referred to as the flow-through "FT"), and washed with 1 to 3.5 mL of Buffers A1, B, and A2 [38]. All samples were eluted with 250–500 μL Buffer C (referred to as eluate), and precipitated using 25% TCA.

2.11. Chaperone assays

2.11.1. Chaperone assays using native and recombinant phycobiliproteins

Chaperone assays were developed modifying the protocols described previously [16,22]. Protein concentrations, buffer conditions, temperature, and assay duration were modified for protocol optimization. Recombinant, partially chromophorylated PE subunit substrates (HT-CpeA-PEB, expressed from pCpeA, pCpeY and pNT-PebS to attach PEB to Cys-82 [24]; HT-CpeB-PEB, expressed from pCpeB2, pCpeS and pNT-PebS to attach PEB to Cys-80 [24]) were produced in liter cultures of *E. coli* and purified using Ni-NTA affinity chromatography. *E. coli* cells containing pCpeB2, pCpeS, and pNT-PebS (Table S2) were produced in larger volumes ranging from 1 to 7 L due to the low yield of soluble protein. Holo-PE subunits isolated from cyanobacteria were also used as substrates. The recombinant protein of interest (HT-CpeZ) was expressed in liter cultures of *E. coli* and purified using Ni-NTA affinity chromatography [24]. All proteins were examined by SDS-PAGE prior to the chaperone assays. Fluorescent substrates were diluted in the appropriate buffers (Buffer O for recombinant proteins, or 50 mM sodium phosphate, pH 7.0 for native PE proteins) to obtain a fluorescence emission peak within the readable range of the fluorometer. Total protein concentrations were calculated using the Bradford assay. The recombinant protein of interest (HT-CpeZ) or the BSA control was mixed with the phycobiliprotein substrate in a 1:10 ratio (phycobiliprotein:chaperone-like protein or control), and allowed to incubate on ice in the dark for 20 h. Fluorescence emission spectra were collected at time points 0, 1 h, 2 h and 20 h after gentle mixing of the sample to detect any change in fluorescence over time. Chaperone assays were performed in duplicate and represented as averages with ranges. For each replicate series, fluorescence was normalized by dividing the raw emission values by the emission value present at time 0. GraphPad Prism (v.7) was used to estimate the statistical significance of the differences observed using a repeated measures ANOVA.

2.11.2. Chaperone assays using non-native substrate citrate synthase

Citrate synthase (CS, Pig Heart), acetyl coenzyme A, oxaloacetic acid and ATP were purchased from Sigma. CS was used as a non-native substrate in chaperone assays. 75 μg of CS was unfolded in the presence of 6 M guanidine hydrochloride and 2 mM DTT as described [42], and then refolding was allowed for 1 h with aliquots removed and assayed for CS activity as described in [42] following the utilization of acetyl CoA by monitoring the thioester bond cleavage at 233 nm [42]. Activity levels (initial reaction rates) after renaturation in the presence of HT-CpeZ, BSA or Buffer O alone were compared to activity levels of non-denatured CS (and expressed as % CS activity) [42,43]. HT-CpeZ and BSA were added in a 1:10 ratio (substrate:chaperone-like protein or control) in these assays. For some samples, the renaturation step was also conducted in the presence of 2 mM ATP, 5 mM $MgCl_2$, and 10 mM KCl [42,43]. Statistical significance of obtained differences was estimated *via* a two-way ANOVA test in GraphPad Prism (v.7).

2.11.3. Chaperone assays using chemically denatured recombinant CpeB

Chaperone assays using chemically denatured recombinant CpeB were conducted using a modified version of a previously described protocol [42]. The chaperone-like HT-CpeZ (pHT-CpeZ) and the recombinant, partially chromophorylated CpeB substrate (HT-CpeB-2PEB, purified from a strain co-expressing pCpeB, pCpeSF2, pNT-CpeZ3, and pNT-PebS to attach the 2 PEBs to CpeB at Cys-80 and Cys-48/Cys-59 by the lyases CpeS [24] and CpeF [44], respectively) were produced in liter cultures of *E. coli* and purified using Ni-NTA affinity chromatography. All proteins were examined by SDS-PAGE, and total protein concentrations were determined using the Bradford assay prior to the chaperone assays.

All chaperone assays were conducted in the dark at room temperature (22 °C). The HT-CpeB-2PEB substrate was denatured in the presence of 6 M guanidine hydrochloride and 5 mM BME for 90 min. The denatured HT-CpeB-2PEB substrate was diluted 25-fold (in Buffer O) and allowed to refold for 120 min in the presence of HT-CpeZ (1:10 ratio, substrate:chaperone-like protein), BSA (1:10 ratio, substrate:control protein), or Buffer O alone. For some samples, the refolding step was also conducted in the presence of 2 mM ATP, 5 mM $MgCl_2$, and 10 mM KCl [42,43]. Refolding of HT-CpeB-2PEB was measured by monitoring the fluorescence emission (excitation at 490 nm) every 15 min for 120 min. The amount of recombinant CpeB used in the assays was based on the concentration of the protein required to have a detectable fluorescence after dilution (2 μM). All assays were performed in duplicate and represented as averages with ranges. For each replicate series, fluorescence was normalized by dividing the raw emission values by the emission value present at time 0. A two-way ANOVA test in GraphPad Prism (v.7) was used to estimate the statistical significance of the differences observed.

3. Results

3.1. Phenotypes and whole cell analyses of wild type, cpeY and cpeZ deletion strains

To better understand the functional roles of CpeY and CpeZ *in vivo*, both *cpeY* and *cpeZ* genes were each cleanly deleted from *F. diplosiphon* in a two-step procedure to minimize any potential polar effects, especially in the case of the *cpeY* deletion, which left the *cpeZ* open reading frame intact (Fig. 1A). Both mutants grew at similar rates as wild type (WT) under red light conditions (data not shown). Next, cells were grown in green light, providing an opportunity for maximal PE production. WT cells grown in green light were characteristically brick red in color due to the production of the red-colored PE (Fig. 1A). Under these conditions, both the *cpeY* and *cpeZ* deletion mutants (Δ*cpeY* and Δ*cpeZ*, respectively) displayed phenotypes that differed from WT in color and growth rates. Both Δ*cpeY* and Δ*cpeZ* cells grew at slower rates than WT when grown in green light, and both mutants were green in color (more so for Δ*cpeY* than for Δ*cpeZ)*, indicating a reduction of PE (Fig. 1, panels A and B). The absorbance spectrum for WT cells showed a large amount of PE (λ_{max} = 571 nm), whereas the spectra of the mutants indicate little PE is being produced (Fig. 1C). From these observations, we conclude that *cpeY* and *cpeZ* are two genes whose products are involved in the biosynthesis and/or stability of PE.

3.2. Characterization of purified phycobilisomes from WT and mutants

PBS from green light-grown WT and mutant cells were purified using sucrose density gradients and analyzed to determine the effects of *cpeY* or *cpeZ* deletions on PBS composition (Fig. S1). Three major fractions were collected from the samples (top, middle, and bottom) with larger and denser protein complexes migrating further within the gradient (bottom). The top fractions contained very little phycobiliproteins (data not shown), and thus were not analyzed further. The WT complete PBS (collected from the bottom fraction) were purple in color due to the presence of AP, constitutive PC and PE in the rods. The bulk of the PBS from the Δ*cpeY* mutant was collected from the middle fraction and had a blue color (Fig. S1D). PBS from the Δ*cpeZ* mutant also had a blue color, but they migrated to both the middle and bottom fractions suggesting a mixture of PBS complexes of different sizes and densities within this mutant (Fig. S1D). Thus, PBS from the mutants had reduced PE levels which appear to have resulted in smaller PBS complexes (middle fractions) when compared to the WT complete PBS.

All PBS fractions were analyzed by absorbance and fluorescence emission spectroscopy (Fig. S1E). The absorbance spectrum for WT PBS showed a large amount of PE (λ_{max} = 568 nm) present in comparison to PC (λ_{max} = 614.5 nm) and to AP (λ_{max} = 649 nm). When excited at 490 nm, which excites PE preferentially, the fluorescence emission peak at 669 nm indicates most of the energy is being transferred to AP. Both Δ*cpeY* and Δ*cpeZ* mutants had very little PE incorporated into the PBS when compared to WT, as estimated by the size of the 669 nm emission peak upon 490 nm excitation. However, some energy transfer from PE to AP was observed (Fig. S1E). We also note that PE within the PBS isolated from the Δ*cpeY* and Δ*cpeZ* mutants had a maximum absorbance of approximately 575 nm (Table S3), indicating a red shift in the absorption maxima when compared to WT.

All PBS samples were further analyzed by SDS-PAGE to visualize phycobiliprotein levels (Fig. S1F). Covalent bilin attachment to proteins was examined by fluorescence emission of the same gel after incubation with Zn sulfate with excitation at 532 nm (Fig. S1G) which detects both PEB and PCB or at 635 nm (Fig. S1H) to detect PCB only [39]. When compared to WT, both Δ*cpeY* and Δ*cpeZ* mutants had drastically reduced levels of CpeB as well as reduced CpeA (Fig. S1F). Zn-enhanced fluorescence (excited at 532 nm) indicated a reduced PEB fluorescence of both subunits in the Δ*cpeY* and Δ*cpeZ* mutants when compared to WT (Fig. S1G) which is consistent with the lower PE levels observed in the mutants. These results were confirmed by immunoblotting with antibodies specific for CpeA or CpeB (Fig. S1, panels I and J). CpeA was undetectable in the Δ*cpeY* PBS sample, but some CpeB was detected at reduced levels. In the Δ*cpeZ* mutant, CpeA was reduced by approximately half when compared to WT, and CpeB was detected but also was reduced in amount and partially degraded (Fig. S1J). For both mutants, the relative amount of PC also appeared to increase in the PBS when compared to WT (Fig. S1, panels G, H and K); however, while this increase was noted, we did not further investigate which version of PC (constitutive and inducible forms of PC [7]) was produced. Collectively, these analyses show that both Δ*cpeY* and Δ*cpeZ* mutants are unable to synthesize and assemble normal levels of PE into PBS.

3.3. Characterization of holo-PE from ΔcpeZ mutant

Since lower levels of PE were being incorporated into the PBS of the Δ*cpeY* and Δ*cpeZ* mutants, holo-PE was purified from the WT and mutant cells and analyzed to reveal the effects of deleting these genes on total PE content and chromophorylation. Total PE represents the entire pool of PE present in cells, including PE assembled and not assembled into PBS. First, holo-PE was purified from both WT and Δ*cpeZ* mutant cells, although only a very small amount of PE was obtained from the Δ*cpeZ* mutant. Holo-PE from WT cells had an absorbance maximum at 563 nm with a shoulder at 545 nm, and fluorescence emission maximum at 573 nm (Fig. 2A and Table S4). Fig. 2B shows that PE obtained from Δ*cpeZ* had an absorbance maximum at 572 nm, which is red shifted when compared to WT (Fig. 2A), with a shoulder at 542 nm (Table S4). To obtain the fluorescence emission spectrum (Fig. 2B), the sample was excited at 490 nm initially to observe the presence of PEB (fluorescence emission maxima at 573 nm) and at 590 nm to excite PCB (no major emission from PCB was observed). Coomassie blue staining of pure PE isolated from the Δ*cpeZ* mutant showed that CpeB levels were undetectable when compared to WT PE (Fig. 2C). Zn-enhanced fluorescence, which is more sensitive than protein staining, of PE isolated from the Δ*cpeZ* mutant showed that CpeA contained PEB and CpeB was still undetectable (Fig. 2D). Thus, deletion of *cpeZ* drastically affects the biosynthesis of the β-subunit of PE, resulting in reduced CpeB protein levels.

To identify the post-translational modifications present in the PE obtained from the Δ*cpeZ* mutant, we analyzed holo-PE samples purified from WT and the mutant that were digested with trypsin using mass spectrometry (LC-MS/MS). Sequences of expected tryptic peptides containing Cys residues with bound PEB can be found in Table S5. Peptides were separated by HPLC and ligated chromophores detected using visible absorbance in line with the mass spectrometer. Five peptides containing PEB were detected from WT; two from CpeA and three from CpeB (Fig. S2 and Table 1). The non-chromophorylated peptides containing the five Cys ligation sites were also detected but at very low amounts within the

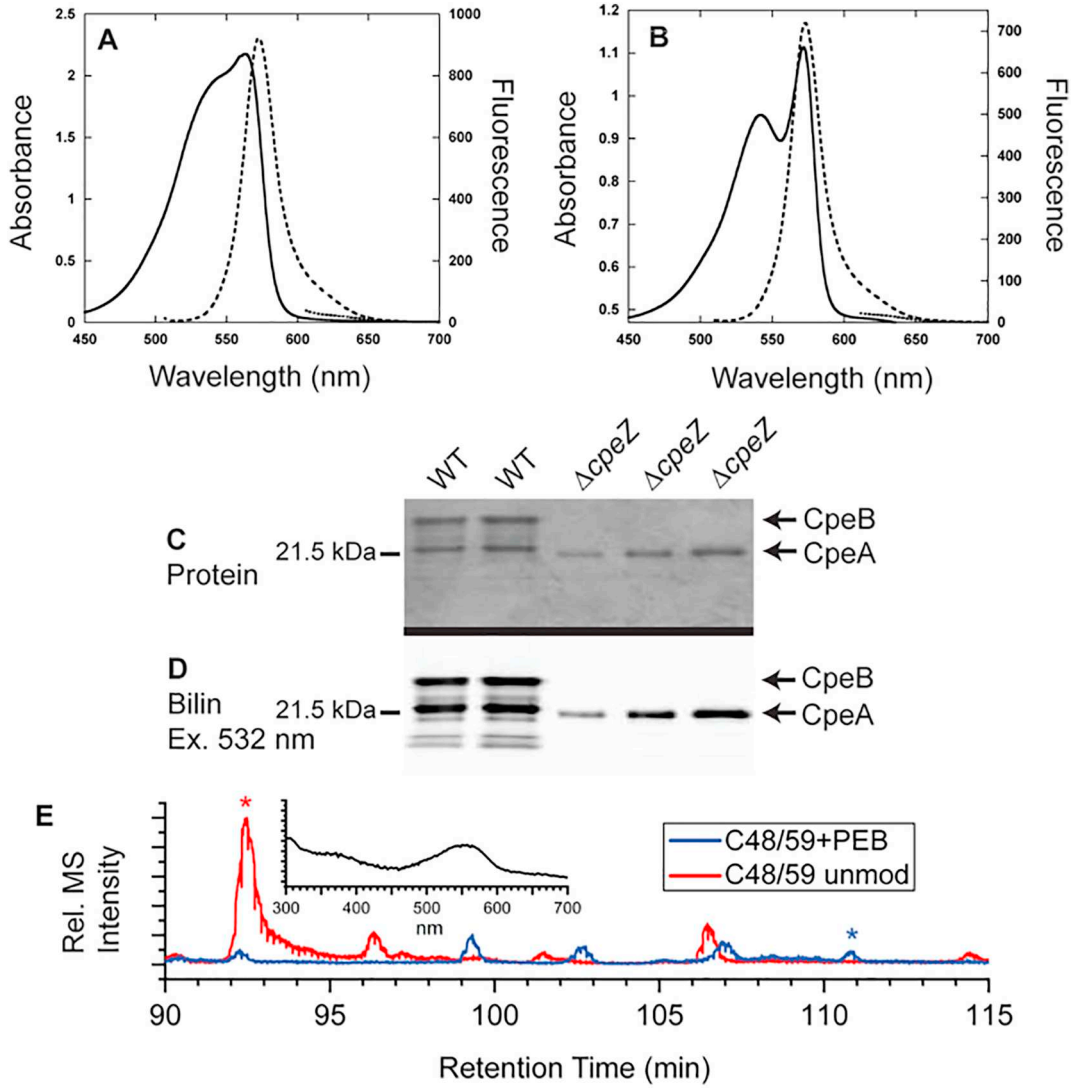

Fig. 2. Analyses of PE purified from WT and Δ*cpeZ* mutant.
Absorbance (solid line) and fluorescence emission (dashed line, excited at 490 nm; dotted line, excited at 590 nm) spectra of PE purified from (A) WT and (B) Δ*cpeZ* mutant *F. diplosiphon* cells grown in green light. (C) The Coomassie-stained SDS-polyacrylamide gel for PE purified from WT (5 and 10 μL were loaded) and Δ*cpeZ* mutant (5, 10, and 15 μL were loaded). (D) The Zn-enhanced fluorescence for the gel in panel C excited at 532 nm. (E) Combined extracted ion chromatograms for the peptides RLDAVNAIASNASC$_{48}$MVSDAVAGMIC$_{59}$ENQGLIQAGGNCYPNR at *m/z* 1200.0^{4+} (PEB modified, blue line and asterisk) and *m/z* 1404.3^{3+} (unmodified, red line and asterisk) of CpeB isolated from Δ*cpeZ* cells grown in green light. (*Inset*) UV–Vis absorption spectrum for the peak at retention time 110.8 min (blue asterisk) indicates PEB on Cys-48/Cys-59. These results are representative of two independent replicates.

Table 1
Observed LC-MS peaks of tryptic digested PE peptides.

Strain	α-82 $(m/z)^{+1}$	α-139 $(m/z)^{+1}$	β-80 $(m/z)^{+1}$	β-165 $(m/z)^{+1}$	β-48/59 $(m/z)^{+1}$
WT	936	1361	1251	2105	4797
Δ*cpeZ*	936[c]	1361	1251[c]	2105	4210[a]; 4794
Δ*cpeY*	349[b]; 936	1361	1251	2105	4797

[a] The *m/z* 4210 peptide (no PEB bound) is 10-fold more abundant than the *m/z* 4797 peptide (PEB bound) based on the areas of the relevant extracted ion chromatograms (EICs).

[b] EICs of ions from the *m/z* 349 peptide (no PEB bound) showed 2-fold more area than those from the *m/z* 936 peptide (PEB bound).

[c] Peptide mixtures contained peptides with PEB, PCB, PUB or bilin-2H bound at that specific Cys residue.

peptide mixtures (Fig. S2). Considering that these PE samples represent the entire pool of PE and not just fully assembled holo-PE, the detection of the non-chromophorylated peptides was expected.

PE purified from the Δ*cpeZ* mutant had PEB attachment to all 5 Cys residues (Table 1). Interestingly, the peptide mixtures for CpeA-Cys-82 and for CpeB-Cys-80 had PEB, PCB, or PUB covalently attached. These bilins are identical in *m/z* but vary in their UV–Vis spectra (PEB, λ_{max} = 540–565 nm; PCB, λ_{max} = 620–650 nm; PUB, λ_{max} = 490–495 nm) (Figs. S3, S4 and Table 1). In Fig. S4, panels I, J, and L show a bilin attached to CpeA-Cys-82 with an absorbance around 630 nm that is 2 Da less (labeled as "bilin-2H") than the typical bilin chromophore found in *F. diplosiphon* (PEB and PCB MW = 587 Da). The bilin's mass and visible absorbance spectrum suggest 3-*Z*-phytochromobilin [39,45] may be attached to Cys-82 of CpeA in the Δ*cpeZ* mutant. Presumably, the three bilins detected (bilin-2H, PUB and PCB) are a result of non-enzymatic addition reaction products which have been observed after *in vitro* reactions with apo-PE [34]. Another interesting result was that even though the CpeB-Cys-48/Cys-59 peptide was detected with a bilin (*m/z* 1200.0^{4+}), the peptide without PEB (*m/z* 1404.3^{3+}) was approximately 10-fold more abundant (Table 1, and Fig. 2E). These results indicate that in the absence of *cpeZ*, CpeB chromophorylation is severely affected especially at the Cys-48/Cys-59

position, but also at Cys-80. Thus, the main effect of deleting *cpeZ* leads to more promiscuous chromophorylation of CpeB. Given that recombinant CpeZ does not act as a bilin lyase for CpeA or CpeB, but it does enhance the bilin lyase activity of CpeY [24], we propose that CpeZ is functioning as a chaperone.

3.4. Characterization of holo-PE from ΔcpeY mutant

Small amounts of holo-PE were also purified from the Δ*cpeY* mutant and compared to WT PE. The PE obtained from the Δ*cpeY* mutant had its major absorbance peak at 566 nm with two shoulders at 599 nm and 616 nm (which might be due to the presence of some PCB; Fig. S5A and Table S4). The normal 545 nm shoulder seen in the WT PE absorbance spectrum (see Fig. 2A) was not present in the Δ*cpeY* mutant PE sample, possibly due to a missing bilin. To obtain the fluorescence emission spectra, the sample was excited at 490 nm to observe the presence of PEB (fluorescence emission maximum at 573 nm) and then at 590 nm for PCB (fluorescence emission maxima at 635 nm; Fig. S5A and Table S4). We observed that the Δ*cpeY* mutant sample had a prominent emission peak at 635 nm that is not present in the WT sample (see Fig. 2A). This PCB presence in the PE sample from the Δ*cpeY* mutant is likely due to contamination by PC and/or AP subunits. Zn-enhanced bilin fluorescence confirmed the presence of PC and AP subunits as evidenced by the migration of these proteins below CpeB and CpeA in the SDS polyacrylamide gel (Fig. S5, panels B and C). Since the amount of PE purified from the Δ*cpeY* mutant was very small, residual PC and AP contamination during the purification process is unsurprising.

LC-MS/MS on holo-PE from the Δ*cpeY* mutant revealed that only PEB was covalently attached to the subunits (Table 1). CpeA had PEB attached at both Cys-82 and Cys-139; however, the amount of the Cys-82 peptide without PEB (m/z 349^{1+}) was approximately 2-fold more abundant than the amount of the peptide with PEB (m/z 468^{2+}) (Fig. S5D and Table 1). PEB attachment was detected at Cys-80, Cys-165, and Cys-48/Cys-59 of CpeB in the Δ*cpeY* mutant PE sample. Overall, these results are consistent with the previously published work that recombinant CpeY is the bilin lyase responsible for attaching PEB to CpeA at Cys-82 [24]. In the absence of *cpeY,* chromophorylation at CpeA-Cys-82 is reduced, affecting the accumulation/stability of PE in cells [24,46].

3.5. Investigating the role of recombinant CpeZ in solubilizing CpeB

Recombinant CpeZ was shown to increase the amount of chromophorylation of CpeB by CpeY, although CpeZ alone did not demonstrate lyase activity on either subunit of PE. This suggested a chaperone-like function for CpeZ [24]. Previous attempts to express recombinant CpeB using a heterologous *in vivo* plasmid expression system in *E. coli* resulted in extremely low yields of soluble protein [24]. To better characterize CpeB biogenesis using a recombinant expression system, we attempted to increase the production and accumulation of soluble CpeB in *E. coli*. Since deleting *cpeZ* negatively affects CpeB accumulation in cyanobacteria, recombinant CpeZ was investigated for its putative involvement in the folding and solubility of recombinant CpeB. For this, we examined the expression of hexahistidine-tagged CpeB (HT-CpeB) over time in different conditions in both the soluble (sup) and pellet (pel) fractions obtained from induced *E. coli* cultures (Fig. 3A). We find that the amount of soluble HT-CpeB (MW = 20.9 kDa), as estimated by immunoblotting, is very low but increases in abundance early on during induction (time 4 and 10 h) and tapers off after 19 h (Fig. 3A). This loss of soluble HT-CpeB may be due to increased aggregation in inclusion bodies or proteolytic degradation of misfolded proteins.

In order to test if CpeZ is able to increase the solubility of recombinant CpeB, CpeZ was coproduced with HT-CpeB in *E. coli* using a similar expression experiment. When CpeZ was coexpressed with HT-CpeB, the amount of soluble HT-CpeB increased early on but tapered off at a later time point (Fig. 3B); however, the ratios of soluble to insoluble HT-CpeB appeared slightly higher than without CpeZ (compare Fig. 3, panels A and B). Overall, recombinant CpeZ may be slightly increasing the solubility of recombinant CpeB over time by helping CpeB fold properly or preventing its denaturation or aggregation, but the effect in *E. coli* was not large.

As previously shown, the solubility of recombinant CpeB increased when CpeB was partially chromophorylated at the central Cys-80 position by the lyase CpeS [24,34]. It is possible that CpeZ acts after this initial chromophorylation and increases CpeB stability or stimulates the ligation of additional chromophores during the biosynthesis of holo-CpeB in cyanobacteria. In order to test if CpeZ is able to further increase the solubility of recombinant CpeB, we produced a partially chromophorylated substrate (HT-CpeB-PEB) by expressing HT-CpeB and CpeZ in the presence of the CpeS lyase (which chromophorylates CpeB-Cys-80 [24]) and the PEB-producing enzymes (PebS/HoI [37]). As controls, HT-CpeB was also expressed with CpeS and PebS/HoI but without CpeZ. Cells were processed as described, and the supernatant fractions of HT-CpeB-PEB expressed with and without CpeZ at time points 10 h and 19 h were subjected to Western blot analysis using Anti-CpeB antibodies (Fig. 3C). At 10 h of induction, the solubility of HT-CpeB-PEB in the presence of CpeZ was increased by approximately 2-fold (Fig. 3C, lane labeled “B + S + Z” at 10 h) when compared to the sample not expressed with CpeZ (Fig. 3C, lane labeled “B + S” at 10 h). After 19 h of protein production, the solubility of HT-CpeB-PEB was increased 5-fold in the presence of CpeZ (Fig. 3C, compare lane labeled “B + S + Z” at 19 h to lane “B + S” at 19 h). These results indicate that recombinant CpeZ is able to increase the solubility of partially chromophorylated recombinant CpeB, either by preventing CpeB from degradation/aggregation (thus allowing CpeS to chromophorylate CpeB) or by serving as an adaptor protein facilitating interaction of CpeB with CpeS.

3.6. Analysis of CpeZ interactions with putative lyases and PE substrates

As shown in Fig. 3C, CpeZ increased the solubility/chromophorylation of CpeB in the presence of CpeS. We wondered if CpeZ directly interacts with the lyase CpeS and/or other lyases or with CpeB itself. To answer this question, recombinant HT-CpeZ was used in pull-down assays in the presence of CpeS as well as the lyase CpeY and the CpeB lyase CpeF [44]. HT-CpeZ was purified from the protein mixtures (Fig. S6A) using Ni-NTA affinity chromatography and the concentrated eluates were analyzed by SDS-PAGE (Fig. S6B) to determine if a stable interaction was occurring. HT-CpcU and CpcS (heterodimeric lyase for β-PC at Cys-82) from *Synechococcus* sp. PCC 7002 were previously shown to interact in pull-down assays [41] and were used as a positive control. As previously shown, CpeY and CpeZ from *F. diplosiphon* do not form a stable interaction [24] and were, therefore, used as a negative control for HT-CpeZ interactions. The putative lyase CpeF did not appear to interact with HT-CpeZ as there was no visible protein of the expected size of 32.4 kDa co-purifying with HT-CpeZ (Fig. S6B). The lyase CpeS did appear to co-purify with HT-CpeZ, albeit weakly (Fig. S6B, indicated with arrow). To further test whether the protein interacting with HT-CpeZ was CpeS, a Western blot was conducted using Anti-CpcS antibodies (generated against CpcS-I from *Synechococcus* sp. PCC 7002) as shown in Fig. S6C. Due to the similarity of these two proteins, the anti-CpcS antibodies cross-react with CpeS. The 8-fold concentrated eluate sample labeled “elu (8×)” showed very little CpeS co-purifying with HT-CpeZ. Most of the CpeS protein was found in the “FT” flow-through fraction that consisted of proteins that did not bind the Ni-NTA slurry during the purification process prior to the washing and elution steps. Experiments reversing the His-tagged bait protein (CpeY, CpeS, or CpeF) and the non-tagged prey protein (CpeZ) was conducted to further confirm CpeZ interactions or lack-there-of; however, non-tagged CpeZ alone was retained on the Ni-NTA slurry (data not shown) and, thus, could not be used for confirmation. However, based on these assays, CpeZ did not interact stably with other *E. coli*-produced lyases such as CpeY and CpeF, and only formed a weak

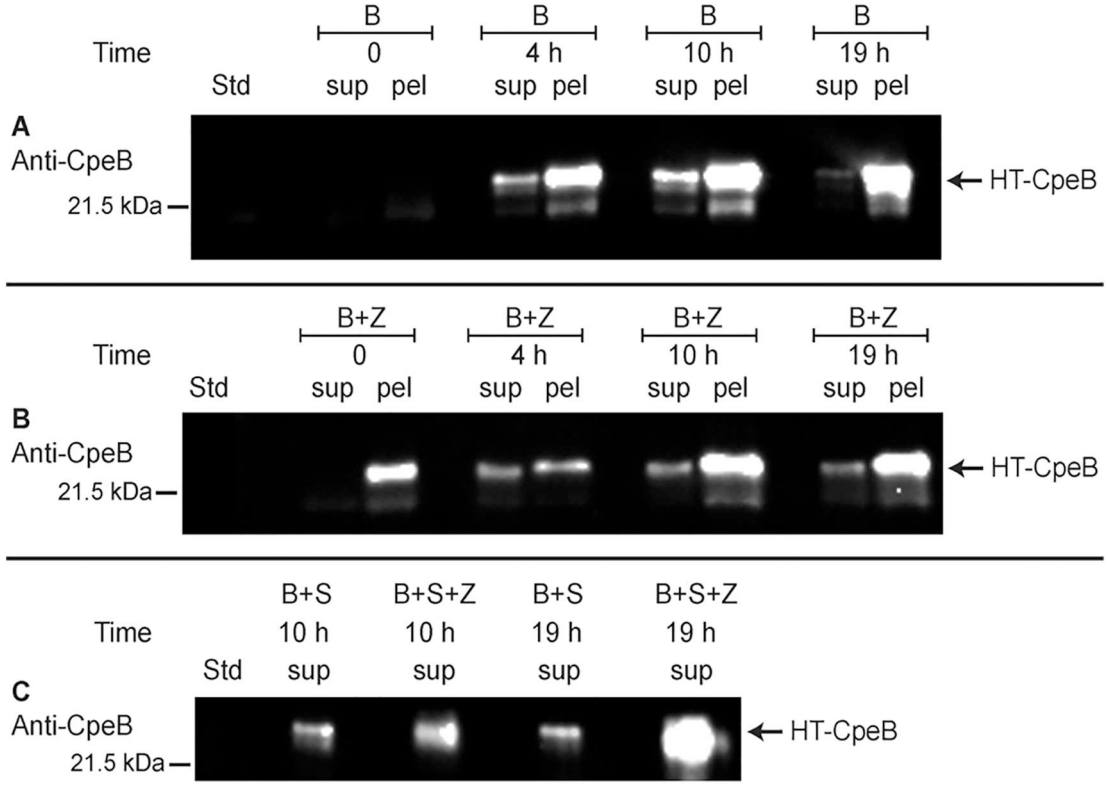

Fig. 3. Recombinant CpeB solubility analyses.
For all panels, whole cell supernatant and cell pellet fractions are labeled "sup" and "pel," respectively. (A) Western blot analysis of HT-CpeB from cells expressing pCpeB2 at time points 0, 4 h, 10 h, and 19 h post induction with IPTG using Anti-CpeB antibodies. The gel was loaded with 0.5 μg of total protein for sup samples and 0.1 μg of total protein for pel samples. Lane "Std" indicates the molecular weight standard. (B) Western blot analysis of HT-CpeB from cells expressing pCpeB2 and pNT-CpeZ2 at time points 0, 4 h, 10 h, and 19 h post induction with IPTG using Anti-CpeB antibodies. The gel was loaded with 1 μg total protein per sample. Lane "Std" indicates the molecular weight standard. (C) Western blot analysis of recombinant HT-CpeB-PEB using Anti-CpeB antibodies. "B + S" indicates samples from cells expressing pCpeB2, pCpeS, and pNT-PebS. "B + S + Z" indicates samples from cells expressing pCpeB2, pCpeS, pNT-CpeZ2, and pNT-PebS. The gel was loaded with 0.5 μg total protein per sample. These results are representative of two independent replicates.

interaction with CpeS (Fig. S6).

As CpeZ was shown to increase the solubility of recombinant CpeB with and without PEB attached by CpeS (Fig. 3) and to weakly interact with CpeS (Fig. S6), it is possible that the function of CpeZ could be to interact with the CpeB substrate itself at some point during PE biogenesis. Unfortunately, apo-CpeB and apo-CpeA are not very soluble in *E. coli*. Therefore, individual holo-PE subunits were used in pull-down assays with recombinant HT-CpeZ (Fig. S7, panels A–C). Additionally, a control assay replacing HT-CpeZ with buffer (to test interactions with the Ni-NTA slurry) was conducted (Fig. S7, panels D and E). The pre-pull-down mixtures "mix" and the 4-fold concentrated eluates "elu (4×)" were analyzed by SDS-PAGE as well as immunoblotting. HT-CpeZ did not form a stable interaction with CpeA (Fig. S7B); however, some CpeB co-purified with HT-CpeZ (Fig. S7C; lane "elu (4x), CpeB +Z"). A small amount of CpeB contamination (from the CpeA subunit separation/purification process) was detected in the pre-pull-down mixture with HT-CpeZ and CpeA (Fig. S7C, lane "mix, CpeA + Z"). The controls confirm that the interaction between holo-CpeB and HT-CpeZ was genuine since neither of the native PE subunits interacted with the Ni-NTA slurry itself (Fig. S7, panels D and E). Together these data show that CpeZ interacts with holo-CpeB.

3.7. Characterizing the chaperone-like function of recombinant CpeZ

Given the CpeZ-induced enhancement in solubility and chromophorylation of CpeB, it is possible that CpeZ is functioning as a chaperone protein which facilitates the folding and stability of PE subunits in cyanobacteria. To test this premise, we modified the fluorescence stability protocols previously developed for CpcE and PecE [16,22]. This modified protocol used CpeZ, recombinant and native PE subunits for the chaperone assays. Recombinant and partially chromophorylated CpeA (HT-CpeA-PEB) and CpeB (HT-CpeB-PEB) were individually incubated with CpeZ and the changes in fluorescence signal (a proxy of their folding and appropriate bilin interaction) over time were monitored as shown in Fig. 4. When the isolated PE subunits were kept in buffer alone on ice, fluorescence decreased dramatically over time (data not shown) indicating a lack of stability/folding in solution, especially when the total protein content was low. Therefore, BSA was used as a control rather than just using buffer alone (at a 10:1 ratio of BSA:substrate to mirror CpeZ's concentration in the test assays). The fluorescence of HT-CpeA-PEB was significantly altered in the presence of CpeZ when compared to the control at each time point (Fig. 4A; $p = 0.0203$ at 1 h, $p = 0.0286$ at 2 h, and $p = 0.0267$ at 20 h). However, when HT-CpeB-PEB was mixed with CpeZ, the initial fluorescence remained relatively unchanged over time after 2 h (Fig. 4B). The fluorescence after 20 h with CpeZ was significantly different than the result observed with the BSA control (Fig. 4B; $p < 0.0001$). These results indicate that the presence of CpeZ prevented the aggregation and/or unfolding of HT-CpeB-PEB in solution, stabilizing its fluorescence during the assay. Repeated measures ANOVA was used to analyze the collected data with time and added protein (CpeZ *vs* BSA) as the variables (Table S6). For HT-CpeB-PEB, the interaction of time and added protein (CpeZ or BSA) was significant (Table S6; $p < 0.0001$). The effect of the added protein was such that the fluorescence decreased over time when BSA was added but significantly increased over time when CpeZ was added (see Fig. 4B; $p < 0.0001$). For HT-CpeA-PEB, the effect of added protein

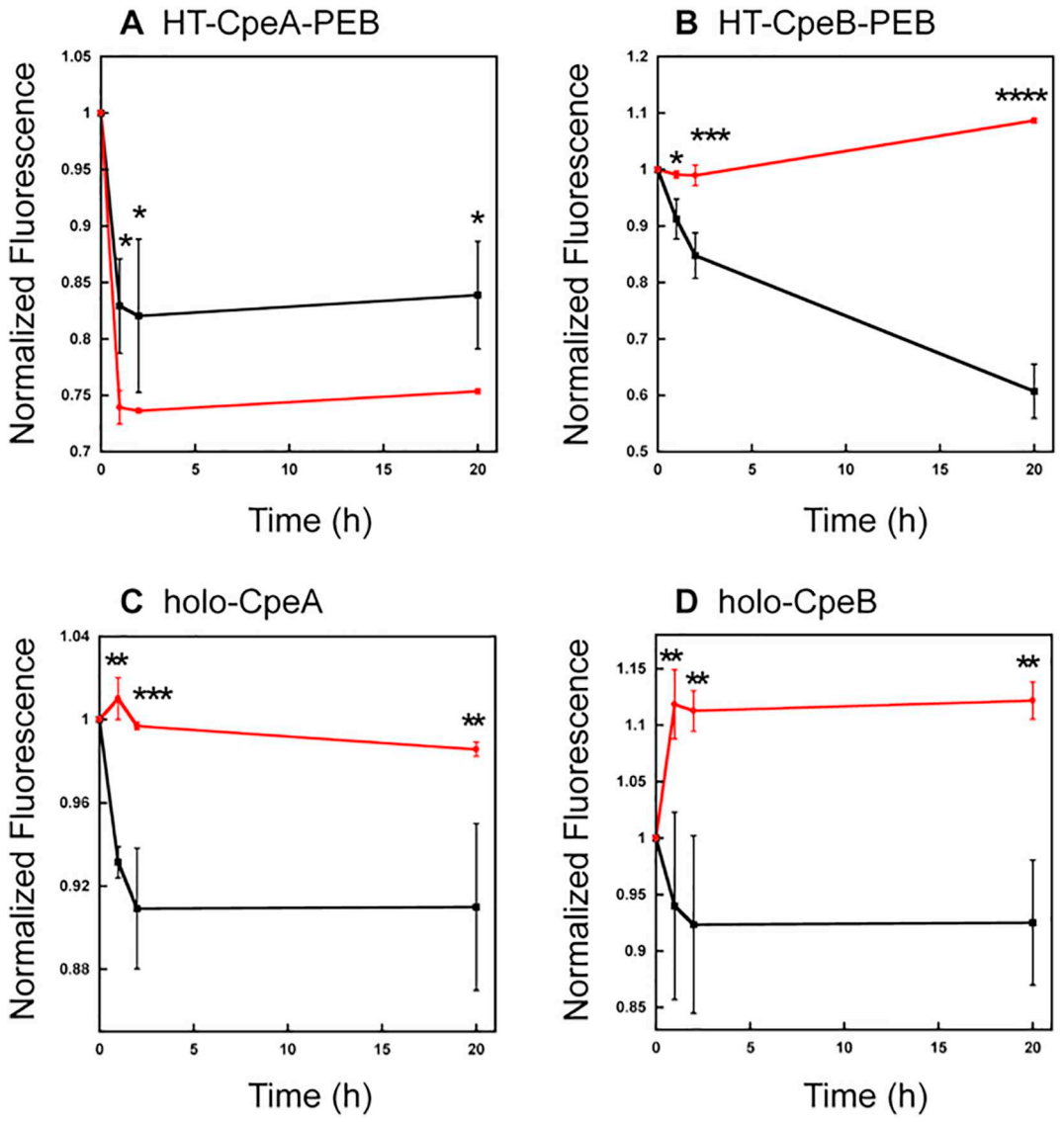

Fig. 4. HT-CpeZ chaperone assays with recombinant and native PE subunits.
All chaperone assays were conducted in the dark on ice for 20 h. (A) Graph representing the change in normalized fluorescence (excitation at 490 nm) over time of partially chromophorylated CpeA (0.75 μM; HT-CpeA-PEB; expressed in *E. coli* using pCpeA, pCpeY, and pNT-PebS) incubated with either HT-CpeZ (red) or BSA (control; black). (B) Graph representing the change in normalized fluorescence over time of partially chromophorylated CpeB (25.1 μM; HT-CpeB-PEB; expressed in *E. coli* using pCpeB2, pCpeS, and pNT-PebS) incubated with either HT-CpeZ (red) or BSA (control; black). (C) Graph representing the change in normalized fluorescence over time of holo-CpeA (0.16 μM) incubated with either HT-CpeZ (red) or BSA (control; black). (D) Graph representing the change in normalized fluorescence over time of holo-CpeB (0.095 μM) incubated with either HT-CpeZ (red) or BSA (control; black). For each replicate series, fluorescence was normalized by dividing the raw emission values by the emission value present at time 0. For all panels, error bars represent the ranges. Asterisks indicate statistical significance levels of HT-CpeZ to control protein at each time point using a repeated measures ANOVA: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$. These results are representative of two independent replicates.

was not significant (Table S6).

The chaperone-like function of CpeZ was further investigated using purified holo-PE subunits in similar chaperone assays (Fig. 4, panels C and D). When holo-CpeA was incubated with CpeZ, the fluorescence of holo-CpeA was stabilized by CpeZ compared to the BSA control at each time point (Fig. 4C; $p = 0.0011$ at 1 h, $p = 0.0005$ at 2 h, and $p = 0.0014$ at 20 h). When native, holo-CpeB was incubated with CpeZ, the fluorescence was stabilized at each time point which was significantly different than the fluorescence observed with the BSA control (Fig. 4D; $p = 0.0027$ at 1 h, $p = 0.0018$ at 2 h, and $p = 0.0014$ at 20 h). The repeated measures ANOVA analyses showed that the effect of the added protein for holo-CpeA and holo-CpeB were both significant (Table S6). CpeZ appears to stabilize and prevent the aggregation of native PE subunits, consistent with its proposed role as a chaperone-like protein in the biosynthesis of PE.

CpeZ aggregates when the temperature is increased to 38 °C (data not shown), so typical chaperone assays using heat-denatured citrate synthase (CS from pig heart [42,43]) monitoring increased, heat-induced scattering were not possible. However, refolding assays of chemically denatured CS in the presence of CpeZ (with and without ATP) were performed and compared to assays in which BSA or buffer alone was used (with/without ATP; Fig. S8). BSA on its own has been previously shown to enhance the refolding of CS [47]. However, CpeZ (no ATP) showed significantly lower chaperone-like activity on CS during refolding assays than BSA (no ATP) in a two-way ANOVA ($p = 0.0154$). There was no significant effect of ATP addition for CpeZ. Given that CpeZ-dependent enhancement of CS activity is very modest and does not need ATP, we interpret that CpeZ must be a PE-specific chaperone-

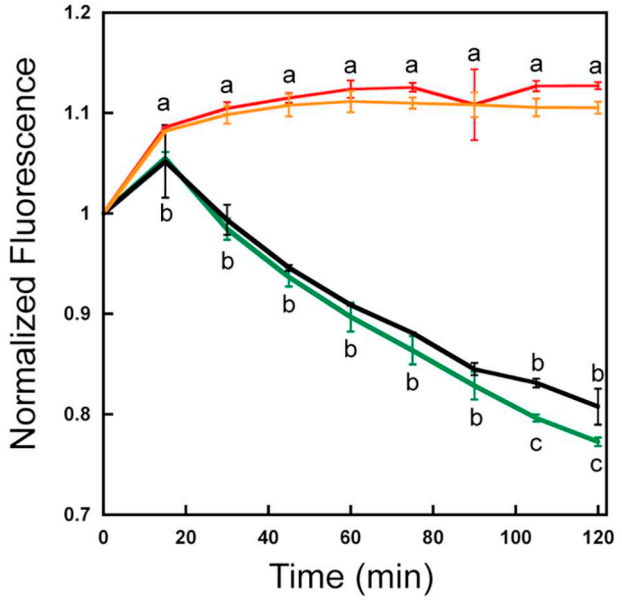

Fig. 5. Refolding of chemically denatured recombinant CpeB.
Recombinant, partially chromophorylated CpeB (HT-CpeB-2PEB; 50 μM; expressed in *E. coli* using pCpeB, pCpeSF2, pNT-CpeZ3, and pNT-PebS to attach 2 PEBs to CpeB at Cys-80 and Cys-48/Cys-59 by the lyases CpeS [24] and CpeF [44], respectively) was chemically denatured for 90 min prior to a 25-fold dilution with either Buffer O (control; green), BSA (control; black), HT-CpeZ (red), or HT-CpeZ + ATP (orange). HT-CpeZ and BSA were added in a 1:10 ratio (substrate:chaperone-like protein or control). Refolding of HT-CpeB-2PEB was measured by monitoring the fluorescence emission (excited at 490 nm) every 15 min for 120 min. All assays were performed in duplicate and represented as averages with ranges. For each replicate series, fluorescence was normalized by dividing the raw emission values by the emission value present at time 0. There was no statistical difference between CpeZ with/without ATP. Letters a and b indicate a statistically significant difference at each time point indicated ($p < 0.0001$), and letters b and c indicate statistically significant difference at each time point indicated ($p = 0.0007$) using two-way ANOVA analyses (Tukey's). These results are representative of two independent replicates.

like protein.

Next, we decided to test if CpeZ can assist partially-chromophorylated CpeB in refolding by measuring fluorescence over time. We chemically denatured recombinant HT-CpeB-2PEB in the presence of CpeZ *versus* BSA or buffer alone (see Experimental procedures Section 2.11.3) and monitored CpeB's signal over time (Fig. 5). Both buffer and BSA controls showed a loss of CpeB fluorescence over time, presumably due to aggregation of CpeB. However, CpeZ significantly enhanced the fluorescence of HT-CpeB-2PEB over time ($p = 0.0001$). There was no significant effect of ATP on CpeZ's activity, which is consistent with its lack of an obvious ATP binding site. Thus, CpeZ is able to prevent the loss of CpeB fluorescence over time, presumably by assisting CpeB's refolding process.

4. Discussion and conclusions

Our mutant analyses showed that CpeY and CpeZ are involved in PE biosynthesis in *Fremyella diplosiphon*. CpeZ functions as a chaperone-like protein, likely important during early PE biosynthesis, especially for CpeB, and CpeY functions as a CpcE/CpcF-type lyase for CpeA by attaching PEB to Cys-82 [24]. The role of CpeZ on CpeB biogenesis was not evident from previous work with recombinant proteins.

We observed a reduced PE phenotype in our Δ*cpeY* and Δ*cpeZ* mutant cells when grown in green light. Further, the PE in the purified PBS samples from both Δ*cpeY* and Δ*cpeZ* mutants showed a slight red-shifted absorbance peak. One potential explanation for this red-shift is the presence of PCB on CpeA or CpeB. Although our MS data for Δ*cpeY* PE shows CpeA and CpeB only had PEB on the Cys attachment sites, the PE from the Δ*cpeZ* PE sample showed that some peptides contained PCB attached to CpeA-Cys-82 and CpeB-Cye-80. It is possible that endogenous lyases in cyanobacteria, such as CpcE/CpcF, are capable of ligating PCB to these Cys positions in the absence of other PEB lyases. Jung et al. observed similar results from insertion mutants in the genes encoding the PecE/PecF lyase/isomerase in *Nostoc* (*Anabaena*) sp. PCC 7120 [48]. When both *pecE* and *pecF* were mutated, phycoerythrocyanin levels were severely reduced and PCB was attached to the Cys-84 site in PecA, presumably by the endogenous CpcE/CpcF lyase, instead of phycoviolobilin which is naturally found at that site [48]. Indeed, we demonstrated that CpcE/CpcF lyase can attach PCB to CpeA using our heterologous expression system (data not shown; [49]). CpcS was also shown to be capable of attaching PEB to recombinant CpeA as well [50].

Another possibility for the red-shifted absorbance spectrum of the PE in the purified PBS from the mutants is the presence of a non-covalently attached PEB chromophore in the bilin-binding pocket of one of the PE subunits, as observed in other lyase mutants [13,14,40,51]. Non-covalently bound bilins would have an extra double bond within the bilin which would cause the red shift. Consistent with this, our MS analysis of purified PE from the mutants revealed that in the absence of *cpeZ*, chromophorylation at CpeB-Cys-48/Cys-59 was drastically reduced. In the absence of *cpeY*, chromophorylation at CpeA-Cys-82 was also reduced but to a lesser extent than was observed in the Δ*cpeZ* mutant at this same position. Since bilins naturally have a high affinity for the bilin binding pockets in phycobiliproteins [34], the low amounts of PEB chromophores found at the CpeA-Cys-82 site in the Δ*cpeY* mutant or to the CpeB-Cys-48/Cys-59 site in the Δ*cpeZ* may be attaching non-enzymatically after a slow ligation process. MS analyses of PE from the Δ*cpeZ* mutant also revealed that CpeB-Cys-80 and CpeA-Cys-82 peptides had small amounts of PEB, PCB, PUB, or bilin-2H covalently attached. Given that CpeZ alone does not have lyase activity [24], these results suggests that CpeZ plays a critical role in preparing PE for chromophore ligation at CpeB-Cys-80 and CpeA-Cys-82 sites. The small amount of PE that is produced in the Δ*cpeZ* mutant implies that the subunits may not be in the right conformation for recognition by bilin lyases or they are not stable and aggregate without CpeZ. This may allow some bilins in the mutant to attach non-enzymatically, thus, resulting in the slow ligation of small amounts of bilin to CpeB-Cys-80 and CpeA-Cys-82 peptides that were detected by MS. Such non-enzymatic addition of bilins to the apo-protein is consistent with the results of Fairchild and Glazer who demonstrated that PEB addition to recombinant CpeA *in vitro* from *F. diplosiphon* occurs with very slow reaction rates and in some cases, the resultant products contained oxidized or isomerized bilin adducts [34].

Here we show that CpeZ functions as a chaperone-like protein which aids in the folding and stability of CpeB, allowing more time for interaction with bilin lyases. It may also assist in the enhancement of lyase/substrate interactions during chromophorylation events. For example, when CpeB was chromophorylated by CpeS, CpeZ increased the solubility of CpeB; however, CpeZ only weakly interacted with CpeS in pull-down assays. Furthermore, CpeZ was also unable to form a stable interaction with PE lyases CpeY and CpeF [44] which again suggests that CpeZ functions *in vivo* by directly interacting with CpeB. Indeed, this was shown to be the case when native, holo-CpeB co-purified with HT-CpeZ in pull-down assays. The presence of five HEAT-repeat motifs (involved in protein-protein interactions [52]) and one ARM repeat motif (polypeptide binding site [52]) in the predicted structure of CpeZ also supports the chaperone-like role of CpeZ.

In 1994, Fairchild and Glazer first demonstrated a chaperone-like function of the E/F-type lyase CpcE/CpcF from *Synechococcus* sp. PCC 7002 [16]. Böhm et al. also provided evidence for a chaperone-like function of the PecE lyase subunit which was shown to improve the spectral properties of PecA-PCB in the absence of ATP or GTP by inducing

a conformational change in the phycobiliprotein for a more stable and fluorescent product [22]. In this study, CpeZ, which is 32% similar to PecE from *Anabaena* sp. PCC 7120, was shown to stabilize the protein/bilin complex and the fluorescence of HT-CpeB-PEB as well as native, holo-CpeB and native holo-CpeA during chaperone assays. It is likely that CpeZ is functioning in a similar manner as the other chaperone lyases by interacting with and stabilizing the structure of the its target, or assisting in protein refolding to produce a more fluorescent product [11,34].

Partially or inefficiently chromophorylated phycobiliproteins do not possess the same 3D structure as holo-phycobiliproteins [22,34,53,54] which may result in protein degradation or aggregation. Toole et al. generated site-directed mutants in *Synechocystis* sp. PCC 6701 by changing the bilin-binding residues from Cys to Ala in both subunits of PC [46] which resulted in a dramatic loss of PC production as observed in other lyase mutants [12,38,40,48,55,56]. Attachment of the central bilin (Cys-82 equivalent) facilitates stability of the subunits, thus allowing for heterodimer formation as well as hexamer formation and assembly into the PBS [46]. Biswas et al. also demonstrated this concept using an *in vivo* heterologous expression system showing that chromophorylation at this central position (Cys-82 of CpeA in *F. diplosiphon*) increased recombinant CpeA solubility and stability in *E. coli* [24]. We propose that the role of CpeZ is to assist in folding and to stabilize CpeB (*e.g.* prevent aggregation) as each of the three PEB molecules is attached by its lyase. It also increases the efficiency of PEB ligation on CpeA at Cys-82 by CpeY, but the effect on CpeA was not as dramatic as the effect on CpeB in the Δ*cpeZ* mutant. This may be because apo-CpeA is more soluble and aggregates less than apo-CpeB in cyanobacteria, analogous to what we observed in *E. coli* [24].

When recombinant HT-CpeB-PEB was incubated with buffer (data not shown) or with control protein (BSA) and left on ice overnight, a reduction of the fluorescence was observed which is a measure of the protein-bilin interaction and conformation. This process was significantly slowed down in the presence of CpeZ. Thus, CpeZ is stabilizing the partially chromophorylated HT-CpeB-PEB utilized during chaperone assays. This function of preventing CpeB from aggregating may be useful in providing bilin lyases enough time to attach PEB to the three Cys residues during the biosynthetic process of CpeB. A protection from degradation function was demonstrated for other molecular chaperonins, such as GroEL. GroEL is a chaperone that binds unfolded proteins unless the interaction is disrupted by ATP [57]. This unfolded state is maintained in the absence of ATP to promote protein refolding and preventing protein aggregation or degradation [58]. CpeZ was capable of performing this function without ATP and has no predicted ATP binding site. Because of this lack of a requirement for ATP for function, we term CpeZ a chaperone-like protein for the binding and stabilization of CpeB and CpeA during biosynthesis. Indeed, there are many examples of proteins which are not conventional chaperones but play important roles in the assembly of protein complexes especially those involved in photosynthesis [59–62]. CpeZ's role as a chaperone seems to be specific for CpeB (and CpeA) as it played no significant role in the renaturation assays for the non-native substrate protein CS [42,43].

The analyses presented in this and a companion study [44] were used to generate a model for the proposed biosynthetic pathway of PE in cyanobacteria which is represented in Fig. 6 (modified from [7,63,64]). The process begins with gene transcription and protein translation of apo-CpeA and apo-CpeB with the assistance of

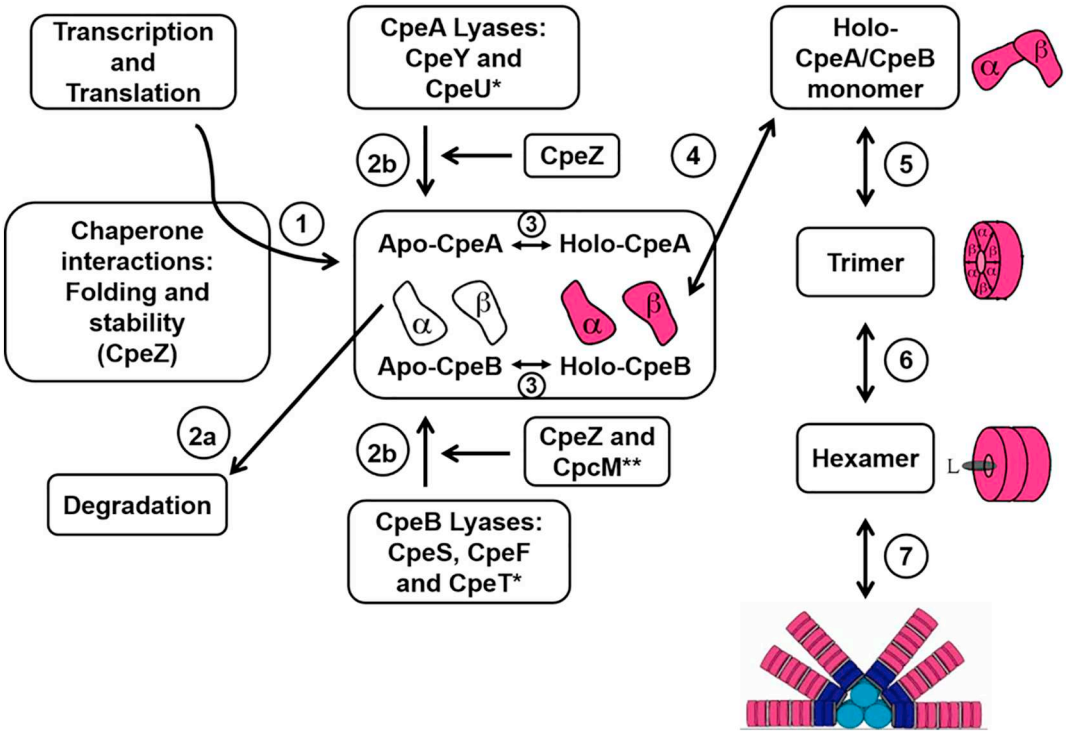

Fig. 6. A model of PE biosynthesis in cyanobacteria.
Steps in the proposed biosynthetic process of PE are labeled with numbered-circles. Arrows indicate the direction of the pathway. Double-sided arrows do not represent equilibria but represent the possible pathway *in vivo*. (1) PE biosynthesis begins with gene transcription and protein translation of apo-CpeA and apo-CpeB with the help of chaperones, such as the chaperone-like protein CpeZ which may assist with folding and stability. (2a) Improperly folded proteins are degraded. (2b) Folded apo-CpeA and apo-CpeB are stabilized by CpeZ long enough for CpeA lyases (CpeY and possibly CpeU [24,40,65]) and CpeB lyases (CpeS, CpeF, and possibly CpeT [24,38,44,65–67]) to chromophorylate their designated Cys residues. The putative lyases CpeT and CpeU are marked with asterisks. The CpcM methyltransferase (methylates CpeB-Asn-70) is marked with a double asterisk [68,69]. (3) Apo-CpeA and apo-CpeB (uncolored) become holo-CpeA and holo-CpeB (pink), respectively, when steps 1 and 2b are completed. (4) Holo-CpeA and holo-CpeB form monomers (pink; αβ). (5) Three monomers form a trimer (pink; $(\alpha\beta)_3$). (6) Two trimers are connected by linker proteins "L" to form hexamers (pink; $(\alpha\beta)_6$). (7) Hexamers are stacked together with the help of linker proteins to form the PE rods that are assembled into the PBS complex. This model was modified from [7,63,64].

chaperones, such as the chaperone-like protein CpeZ, which functions in protein folding and stability/aggregation prevention (step 1). As seen in step 2a, when apo-subunits are denatured or not properly folded, they are degraded by cellular processes. However, if the apo-subunits are folded and stabilized by CpeZ, CpeA lyases (CpeY and possibly CpeU [24,40,65]) and CpeB lyases (CpeS, CpeF, and possibly CpeT [24,38,44,65–67]) are able to chromophorylate the specific Cys residues (step 2b). Other post-translational modifications occur as well, including the methylation of CpeB-Asn-70 by the methyltransferase CpcM [68,69]. When the apo-subunits are fully chromophorylated and become holo-CpeA and holo-CpeB (step 3), they bind each other and form a monomer (αβ; step 4). Three monomers combine to form a trimer ($(\alpha\beta)_3$; step 5), and two trimers are bound together by linker proteins (abbreviated "L") to form hexamers ($(\alpha\beta)_6$; step 6). PE hexamers are further stacked upon each other by linker proteins and are assembled into the PBS complexes (step 7). As represented in this model and demonstrated in the research presented in this study, CpeZ is especially crucial in the early PE biosynthetic process (steps 1 and 2b). Our evidence suggests that CpeZ plays a role in stabilizing the conformation of both subunits, but its role is most important for the CpeB subunit. Once stabilized, we suggest this allows higher levels of activity of the other lyases. It is also possible that CpeZ acts as an adaptor protein facilitating the binding of lyases to these subunits as well. This model ignores the spatial constraints of PBS assembly imposed by the thylakoid arrangement and stacking in the cell. It would be worthwhile to understand how PE synthesis and PBS biogenesis as a whole is integrated with the biogenesis of the photosystem complexes which are ordered in time and space and occur at the membrane [70].

In conclusion, both CpeY and CpeZ play critical roles in the biosynthesis and stability of PE in cyanobacteria. In the absence of the *cpeY* lyase gene, chromophorylation on CpeA-Cys-82 is reduced, consequently affecting the accumulation and stability of PE in cells. Both subunits of PE were affected in the Δ*cpeZ* mutant, causing drastic effects to multiple chromophorylation sites, thus leading to protein instability and degradation. CpeZ behaves as a chaperone-like protein and is critical to the stabilization of PE subunits during the biosynthetic process.

Transparency document

The Transparency document associated with this article can be found, in online version.

Declaration of Competing Interest

The authors declare that they have no conflicts of interest with the contents of this article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbabio.2019.06.001.

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