Subunit Interactions and Protein Stability in the Cyanobacterial Light-Harvesting Proteins

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Strain 4R is a phycocyanin-minus mutant of the unicellular cyanobacterium Synechocystis sp. strain 6803. Although it lacks the light-harvesting protein phycocyanin, 4R has normal levels of phycocyanin (cpc) transcripts. Sequence analysis of the cpcB gene encoding the phycocyanin β subunit shows an insertion mutation in 4R that causes early termination of translation. Other work has shown that the phycocyanin α subunit and the linker proteins encoded on the cpc transcripts are all functional in 4R, yet the defective phycocyanin β subunit results in the complete absence of the α subunit and the linkers. Phycocyanin-minus mutants were constructed in a wild-type background by interruption of cpcB and cpcA with an antibiotic resistance gene and were compared with the 4R strain. Immunoblot analysis of the mutants demonstrated that interruption of one subunit was accompanied by a complete absence of the unassembled partner subunit. Phycocyanin assembly begins with the formation of the cfp heterodimer (the monomer) and continues through higher-order trimeric and hexameric aggregates that associate with linker proteins to form the phycobilisome rods. The results in this paper indicate that monomer formation is a critical stage in the biliprotein assembly pathway and that unassembled subunits are subject to stringent controls that prevent their appearance in vivo.

Light harvesting in cyanobacteria is mediated by the phycobilisomes, which are complex protein structures located on the surface of the photosynthetic membrane (20). The major structural components of the phycobilisome are the biliproteins, which contain covalently attached bilin chromophores that constitute a resonance energy transfer pathway. Light energy in the 500- to 650-nm range can be absorbed by different classes of biliproteins and is rapidly and efficiently transferred through the phycobilisome to chlorophyll complexes in the photosynthetic membrane. The three major classes of biliproteins are distinguished by their spectral properties. The allophycocyanins (AP [λmax = 650 to 665 nm]) are located in the core of the phycobilisome, which is in direct contact with chlorophyll complexes in the membrane. Phycocyanin (PC [λmax = 617 nm]) is found in the rod substructures that are attached to the phycobilisome cores. A third major biliprotein, phycoerythrin (PE [λmax = 565 nm]), is synthesized in some cyanobacteria and is attached to PC at the periphery of the rod substructures. Each biliprotein has the same subunit organization that is based on a heterodimer (called a monomer by convention) composed of α and β subunits (11, 16, 37–39). Monomers are assembled into disc-like trimers with a central channel, which then stack to form a hexamer in the PC and PE biliproteins. Hexamers are associated with single copies of linker proteins that direct their assembly into the rod substructures. The organization of biliproteins within the phycobilisome structure establishes an energy transfer pathway, PE to PC to AP to chlorophyll, that operates at close to 100% efficiency (7, 19, 20).

Phycobilisomes are not required for phototrophic growth in white light, and assembly mutants have been useful in establishing protein function and phycobilisome architecture (1, 3, 10, 17, 28, 29). The phycobilisome can constitute 30% of the cellular dry mass and represents a major biosynthetic investment for the cyanobacterium. While they are generally not lethal, mutations that disrupt phycobilisome assembly may force a cellular response to the accumulation of unassembled proteins. In some mutants, unassembled material is stable and may remain soluble in the cell (10) or form inclusion bodies (4). Mutations that remove a biliprotein subunit from the assembly process often result in the complete loss of other phycobilisome components (7, 40), suggesting rapid degradation or downregulation of the stranded proteins. An examination of phycobilisome mutants that emphasizes the fate of defective or unassembled proteins may reveal control features that the cell uses to manage the efficient production of these light-harvesting complexes. Some mutants may also provide experimental access to the in vivo pathways that produce a complete phycobilisome.

Strain 4R is a PC-minus mutant of Synechocystis sp. strain 6803 that synthesizes intact phycobilisome cores but shows no traces of PC subunits or PC-associated proteins. We have used 4R as a transformation host for the cpc genes from Synechocystis sp. strain 6701 (2) and have demonstrated the rescue of 4R PC α subunits and PC-associated linker proteins by heterologous assembly (31). The present report establishes that the PC-minus phenotype in Synechocystis sp. strain 6803 4R is due to a mutation in the cpcB gene that removes the last 80% of CpcB by premature termination of translation. Interposon mutants interrupted in cpcA or cpcB were constructed for comparison with 4R, and the phenotypes of these strains indicate that stranded biliprotein subunits are not stable when they cannot bind to their assembly partner.

MATERIALS AND METHODS

Bacterial strains and growth conditions. All strains and plasmids used in this study are listed in Table 1. The transformable unicellular cyanobacterium Synechocystis sp. strain 6803 and its derivatives were maintained on BG-11 medium (34) supplemented with 1% Bacto-Agar (Difco) and 10 mM N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES; pH 8.0). Liquid BG-11 cultures were supplemented with 20 mM glucose–20 mM HEPES (pH 8.0) and were bubbled with 1 to 2% CO2 in air at room temperature under four warm-white fluorescent lights. The wild-type (WT) and PC-minus (4R) strains of Synechocystis sp. strain 6803 were obtained from Dzelkalns and Bogorad (Department of Biology, Temple University, and Department of Biology, University of Oklahoma) and from Life Sciences, University of Tulsa, 600 S. College Ave., Tulsa, OK 74104. Phone: (918) 631-3328. Electronic mail address: biol_lka@centum.utu.edu.
TABLE 1. Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant property(ies)</th>
<th>Reference or source</th>
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<tbody>
<tr>
<td>E. coli DH5α</td>
<td>Host strain for plasmid propagation</td>
<td>Gibco BRL</td>
</tr>
<tr>
<td>Synechocystis sp.</td>
<td>wt strain 6803</td>
<td></td>
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<tr>
<td>WT</td>
<td>Wild type strain; cpcB + cpcA +</td>
<td>V. Dzeiňalskas</td>
</tr>
<tr>
<td>4R</td>
<td>PC minus phenotype</td>
<td></td>
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<tr>
<td>R38</td>
<td>4R complemented to PC-plus phenotype by pPC338</td>
<td>This study</td>
</tr>
<tr>
<td>R38KA</td>
<td>4R cpcA::kan (by pPC38KA)</td>
<td>This study</td>
</tr>
<tr>
<td>W30KA</td>
<td>WT cpcA::kan (by pPC38KA)</td>
<td>This study</td>
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<tr>
<td>W30KB</td>
<td>WT cpcB::kan (by pPC38KB)</td>
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Plasmids

- pBluescript SK
- pPC338
- pPC396
- pBluescript SK
- pPC338

Characterization of Synechocystis sp. strain 6803

RESULTS

Characterization of Synechocystis sp. strain 6803 4R. Colonies of the 4R strain have an olive-green pigmentation that clearly distinguishes them from the blue-green Synechocystis sp. strain 6803 WT, suggesting a decreased level of PC. The absorbance spectrum of intact WT cells shows their PC content in the major peak to be near 625 nm, while the 4R cell spectrum is flat in this region (Fig. 1). The absence of PC in 4R was confirmed by SDS-PAGE comparison of phycobilisomes and cell extracts from WT and 4R (Fig. 2). The 4R phycobilisomes are core substructures that lack all rod components, including both PC subunits and the three rod-associated linker proteins (compare lanes 1 and 2), and are similar to the core structures that have been characterized in a mutant of Synechocystis sp. strain 6701 (3). The 4R strain does not appear to have any soluble PC, since cell extracts do not show the zinc-enhanced fluorescence signals of PC subunits that are visible in the WT extract (lanes 3 to 6). The membrane pellets from 4R cell extracts were also negative for PC content on the basis of zinc-enhanced fluorescence in SDS-PAGE samples (data not shown).

The apparent absence of PC subunits in 4R is not caused by a lack of cpc transcripts. A 700-bp DNA fragment that includes all of cpcB and the first 20% of cpcA was generated by amplification with Taq polymerase with pPC338 as a template. Northern (RNA) hybridization analysis with this probe against 5 µg of WT and 4R mRNA (Fig. 3, lanes 1 and 2) shows the
presence of 1.4- and 3.2-kb transcripts in both strains. The 1.4-kb mRNA represents the major transcript from cpcBA that encodes the PC β and α subunits. The less-prominent 3.2-kb transcript encodes more of the operon, cpcBAHID, as was determined with a probe specific for the linker genes cpcH and cpcI (data not shown). The level of the cpcBA transcript in 4R is comparable to that seen in WT, indicating that expression of this operon in 4R is not sufficiently reduced to account for the PC-minus phenotype.

The PC-minus phenotype of Synechocystis sp. strain 6803 4R can be complemented by the introduction of the cpcBA operon from Synechocystis sp. strain 6701 (31). The resulting transformants synthesize phycobilisomes with hybrid rod structures that consist of PC from the introduced cpcBA operon and linker proteins expressed from the cpcH and cpcI genes of 4R. Some PC α subunit (CpcA) from the 4R cpcA gene is also detected in these phycobilisomes. These observations lead to two conclusions: the cpe transcripts that are present in 4R can be translated and the PC α subunit and linker proteins that derive from these transcripts are functional and can be incorporated into a phycobilisome. Thus, the cpcB gene is implicated as a possible site of mutation that leads to the 4R phenotype. The DNA sequence analysis of the cpcBA from Synechocystis sp. strain 6803 WT will be reported elsewhere. Sequence information available for the cpcBA4 region has allowed a comparison between the cpcB genes of WT and 4R. The two genes show a single nucleotide difference where 4R cpcB has a C insertion at the 73rd nucleotide position (data not shown). The resulting frameshift causes premature termination of translation and would, by prediction, yield a 32-amino-acid peptide of which the first 23 residues correspond to the PC β subunit (CpcB) from WT (Fig. 4). The absence of CpcB in 4R would prohibit the formation of PC monomers and may explain the PC-minus phenotype of 4R on the basis of the simple assumption that unassembled material is degraded.

**Complementation of the PC-minus phenotype.** While the previous results suggest a viable explanation for the PC-minus phenotype in 4R, the origin of this strain as a revertant from a photosynthetic mutant background raises the possibility that mutations outside the cpc operon may contribute to the phenotype. Figure 5 shows a restriction map of the 9.6- and 3.8-kbp fragments of WT chromosomal DNA containing the cpe operon that were cloned into pBluescriptSK+, creating pPC396 and pPC338, respectively. The 4R strain was transformed with pPC396 and pPC338 by direct application of plasmid DNA to cyanobacterial cells in top agar (13) without antibiotic selection or glucose in the base BG-11 agar. Conversion to a WT phenotype occurred in the immediate radius of the site at which 5 to 10 μg of pPC396 or pPC338 was applied to the top agar. Control areas on the same plates that were treated with 5 to 10 μg of pBluescriptSK+ showed no transformants. While both plasmids produced reversion phenotypes, pPC338 transformants were much less frequent and were harvested from the 4R background with the aid of a dissecting microscope. Multiple transformants were isolated and segregated, and each clone displayed the absorbance, fluorescence energy transfer, and phycobilisome composition of Synechocystis sp. strain 6803 WT, indicating the synthesis of a complete PC β subunit and the correction of the cpcB lesion in 4R (data not shown). One of the pPC338 transformant clones, 4R CpcB

\[ MFDFVTRVSVQADARGEYLSGQLDALSATVAE... \]

\[ \alpha\text{-helices} \quad [\ldots X \ldots] \quad [\ldots Y \ldots] \]

**Fig. 4.** Consequences of the 4R mutation. The predicted amino acid sequences for CpcB from WT and 4R are presented for the first 33 residues. The X and Y α-helices from the PC structure are lined up with the amino acid sequences. The nucleotide insertion in 4R causes a reading frame shift that introduces a stop codon early in the CpcB primary structure.
R38, was analyzed by Northern hybridization and displayed PC mRNA at levels seen in both WT and 4R strains (Fig. 3, lane 3). Recombination of the WT cpeB on pPC338 with the 4R genome would correct the frameshift mutation and produce a PC β subunit that would complement the 4R mutation. Since the CpcA, CpcH, and CpcI proteins in 4R are functional when rescued by heterologous assembly (31), the complementing DNA in pPC338 must reside in the 600-bp region that includes the cpcB gene and 200 bp of noncoding DNA upstream of cpcB.

**Generation of PC-minus phenotypes by interposon mutations.** Analysis of the 4R strain shows an absolute requirement of CpcB for stable PC expression. Is there a similar requirement for CpcA? We addressed this question by constructing PC-minus mutants in the WT background using interposon mutagenesis. Plasmid pPC338 was used as the base transforming vector. An neomycin phosphotransferase gene (npt) was inserted at the npt insertion site and indicates the presence of transcripts that initiate from the cpe promoter and include cpeB. The npt insertion in W38KA occurs 160 nucleotides before the end of cpeA and introduces translational stop codons in all three reading frames within 60 bp of the cpeA-npt junction. Assuming that the major cpe transcript in W38KA is translated, the products would consist of an intact CpcB and at least 70% of CpcA. The absence of PC in W38KA implies that the truncated CpcA is not stable and is rapidly degraded, leaving CpcB without an assembly partner and subject to proteolysis.

**Immunoblot screening of PC-minus mutants for abandoned subunits.** In light of the PC-minus phenotypes in W38KA and 4R, it appears that mutations which truncate either PC subunit may lead to the complete absence of intact partner subunits, possibly through degradation. We reasoned that trace amounts of the abandoned subunits could exist for a short time in these mutants and that more-sensitive methods might detect them in cell extracts. The nature of the abandoned proteins prior to proteolysis, whether they contained chromophore or not, might provide a window on early events in the biliprotein biosynthetic pathway. We used polyclonal antibodies raised against SDS-PAGE-purified CpcA and CpcB from Synechocystis sp. strain 6803 in immunoblot analyses of cyanobacterial cell extracts. Strain 4R was transformed with pPC338KA to create a CpcA mutant (designated R38KA) for direct comparison with 4R in the immunoblots. apo-PC subunits (synthesized in vitro) were used to indicate the positions in SDS-PAGE of CpcA and CpcB that lacked chromophores. The detection limit of anti-CpcA in immunoblots was tested under our standard conditions with a dilution series of purified PC, demonstrating a clear detection of 1 ng of CpcA (data not shown). Anti-CpcB was not assayed for a lower detection limit; however, comparative immunoblots have shown it to be less sensitive than that for anti-CpcA.

If abandoned subunits are detectable by immunoblot, extracts of 4R should contain CpcA while R38KA and W38KA are predicted to be CpcA negative because of interposon mutations. The W38KA extract may display CpcB, but 4R and R38KA should both be CpcB negative because of the reading frame shift in cpeB. Strain W38KB should be negative for both CpcA and CpcB on the basis of the level of cpe transcripts in this mutant (Fig. 3). Figure 6A shows an anti-CpcA immunoblot against cell extracts from 4R, R38KA, W38KA, and W38KB. All four extracts contain two immunoreactive proteins that migrated close to the major signal in the purified PC control lane, and none of the cell extracts indicated the presence of apo-CpcA. The zinc-enhanced fluorescence from the same gel shows that the major fluorescence signals are due to AP subunits and that anti-CpcA exceeds fluorescence in detection sensitivity for SDS-PAGE (note the weak fluorescence in the PC control lane). These results suggested that the polyclonal anti-CpcA was detecting other proteins that comigrated with CpcA in SDS-PAGE. This is a distinct possibility, since the CpcA antigen was isolated by electroporation of gel slices from high-resolution SDS-PAGE of Synechocystis sp. strain 6803 phycobilisomes. We employed SDS-PAGE in the presence of 8 M urea to alter protein mobility during electrophoresis (6) in an effort to unmask any trace amounts of CpcA in 4R (Fig. 6B). The major signals detected by anti-CpcA in all four extracts did not comigrate with CpcA in the PC control lane, and there was no detectable protein in 4R that was missing in the other three samples. Anti-CpcB was also tested against the
mRNA levels that are comparable to that of the WT strain. Inability to detect trace amounts of CpcA or CpeB as the stranded partners in each mutant suggests that nascent biliprotein subunits are rapidly and immediately degraded in the absence of their assembly partner or that unassembled subunits may initiate a process that blocks translation from the cpe transcripts.

**DISCUSSION**

**Origin of the complete PC-minus phenotype in 4R.** An explanation for the complete absence of CpcA in 4R must accommodate the high level of cpe transcripts in this strain. The rescue of 4R CpcA by heterologous assembly (31) rules out polarity effects in which the synthesis of CpcA would require translation through cpeB. The simplest explanation for the 4R phenotype is based on a proteolytic activity that degrades abandoned subunits in a rapid and efficient manner. This activity could be constitutive, or the production of unassembled biliprotein subunits may be similar to the synthesis of abnormal proteins, which is known to induce protease activities as part of a heat shock-like response in E. coli (21, 26). The degradation of proteins that lack assembly partners should be reciprocal with respect to α and β subunits and was demonstrated in comparisons of the 4R and W38KA strains. Degradation of unassembled subunits should affect other biliprotein classes as well, and this was observed for a nonsense mutation that terminates ApcA in the middle of the F-helix and causes a complete AP-minus phenotype in the UV6p strain of Synechocystis sp. strain 6803 (40).

**Subunit interactions and biliprotein stability.** Formation of the monomer by the PC β and α subunits is accompanied by the burial of 1,040 Å² (1 Å = 0.1 nm) of surface area and is mediated predominantly by conserved hydrophobic residues (5, 38). The monomer is quite stable and requires high concentrations of urea (19) or 63 mM formic acid (30) to separate the subunits. Trimers and hexamers exist in an equilibrium with the monomers that can be affected by moderate changes in protein concentration, salt concentration, and pH (18). The complete absence of CpcA in 4R is in strong contrast with the PC content of another PC assembly mutant, strain UV16 of Synechocystis sp. strain 6701 (2–4). A mutation in the CpcA of UV16 blocks the formation of PC hexamers, yet this strain contains soluble trimers and monomers at 30% of the wild-type PC level. This difference between 4R and UV16 suggests the existence of a rapid and efficient salvage mechanism that primarily recognizes unassembled subunits over the higher-order biliprotein complexes. Since the α and β subunits of PC do not show significant structural changes upon monomer formation (30), the degradation process must be initiated by exposure of hydrophobic residues in the subunit interface domain that are buried upon monomer formation.

If subunit interactions can protect the biliproteins from degradation, why does the W38KA strain have a PC-minus phenotype? CpcA in W38KA would be missing 28% of the C terminus because of the interposon mutation; however, inspection of the structure (12) shows that this part of the protein has no direct involvement in the subunit interface and that all contact residues for a monomer would be intact in the truncated CpcA. The instability of CpcA in W38KA must derive exclusively from the absence of the C terminus, which could expose new sites for proteolysis, or it might affect protein folding if the biliproteins utilize a folding pathway that has been proposed for the structurally related myoglobin (8, 24, 25). Either case would yield a stranded CpeB protein that is also subject to rapid degradation. The importance of the CpcA

**FIG. 6.** Immunoblot analysis of PC contents in WT and mutant strains. (A) Standard SDS-PAGE of cell extracts; the upper panel is an immunoblot with anti-CpcA, and the lower panel is a zinc stain of the same tracks prior to transfer to nitrocellulose. Lanes: 1, isolated PC; 2, in vitro transcription-translation extract with pPC02 as a template; 3, 4R; 4, R38KA; 5, W38KA; 6, W38KB. Only lane 3 is expected to show CpcA if it is there. Double caret marks the positions of CpcA. (B) SDS-PAGE in 8 M urea of cell extracts; upper and lower panels are as in panel A. Lanes: 1, isolated PC; 2, 4R; 3, R38KA; 4, W38KA; 5, W38KB. Double caret marks the positions of CpcA. (C) Standard SDS-PAGE of cell extracts and immunoblot with anti-CpeB. Lanes are identical to those in panel A, but only lane 5 is expected to show CpeB if it is there. Double caret marks the position of CpeB. Gel images were obtained with a UVP Imagestore 7500 and were composed as figures with Aldus Photostyler software on PC-compatible hardware.

four cell extracts, purified PC, and apo-CpeB (Fig. 6C) and showed no CpeB signals in any of the extracts. The immunoblot results establish that these cell extracts do not contain abandoned PC subunits. If CpeB is present in 4R prior to degradation, the yield recoverable by our methods must be less than 1 μg of protein per liter of cell culture on the basis of the detection capability of anti-CpeA. It is possible that this simple extraction procedure does not preserve the proteins of interest. We have used multiple protease inhibitors in addition to phenylmethylsulfonyl fluoride in the cell extraction procedure, including EDTA, benzamidine, ε-aminocaproic acid, and leupeptin. We have also achieved a more-rapid, less-efficient extraction by grinding cells in liquid nitrogen with a mortar and pestle. No combination of these modified protocols produced data that supported the presence of CpeA or CpeB in any of the cell extracts.

In summary, strains 4R and W38KA have mutations that truncate the CpeB and CpeA proteins, respectively, and both mutants have a complete PC-minus phenotype in spite of PC production datathat supported the presenceof CpeA or CpeB in any of the cell extracts.
terminus for subunit stability can also be seen in the UV6p mutant (40), in which truncation of ApcA in the middle of the F-helix causes complete loss of both ApcA and ApcB.

**Conclusion.** Mutations that disrupt the structure of one biliprotein subunit can result in the phenotypic absence of both subunits. The major difference between a stranded subunit and a monomer-bound subunit is the exposure of the subunit-binding domain to the environment, and stable expression of a biliprotein requires protection of this domain by formation of the monomer. Since degradation of stranded subunits appears to be quite rapid, the early events of biliprotein biosynthesis, which include folding, covalent modifications (14, 15, 41), and subunit interactions, must occur with a speed and precision that prevent unwanted proteolysis of functional proteins.

**ACKNOWLEDGMENTS**

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**REFERENCES**