The Highly Abundant Chlorophyll-Protein Complex of Iron-Deficient Synechococcus sp. PCC7942 (CP43') Is Encoded by the isiA Gene

Robert L. Burnap**, Tracy Troyan, and Louis A. Sherman

Department of Biological Sciences, Purdue University, West Lafayette, Indiana 47907-1392 (R.L.B., T.T., L.A.S.); and Department of Biological Sciences, University of Missouri-Columbia, Columbia, Missouri 65211 (T.T.)

A chlorophyll (Chl)-protein complex designated CPVI-4 becomes the major pigment-protein complex in Synechococcus sp. PCC7942 cells grown under conditions of iron limitation. Work by Laudenbach et al. (J Bacteriol [1988] 170: 5018-5026) has identified an iron-repressible operon, designated isiAB, containing the flavodoxin gene and a gene predicted to encode a Chl-binding protein resembling CP43 of photosystem II. To test the hypothesis that the iron-repressible operon, designated isiA/B, encodes a Chl-binding protein..., we have inactivated the isiAB operon in Synechococcus sp. PCC7942 using directed insertional mutagenesis. Mutant cells grown under conditions of iron limitation exhibit pronounced changes in their spectroscopic and photosynthetic properties relative to similarly grown wild-type cells. Notably, the strong 77 K fluorescence emission at 685 nm, which dominates the spectrum of iron-deficient wild-type cells, is dramatically reduced in the mutant. The loss of this emission appears to unmask the otherwise obscured photosystem II emissions at 685 and 695 nm. Most importantly, mildly denaturing gel electrophoresis shows that mutant cells no longer express the CPVI-4 complex, indicating that the isiA gene encodes a component of this abundant Chl-protein complex.

Cyanobacteria acquire a markedly different cellular phenotype when cells are shifted from conditions of nutrient-sufficient growth to conditions where growth is limited by iron availability (Oquist, 1971, 1974a, 1974b; Huber et al., 1977; Guikema and Sherman, 1983, 1984; Sherman and Sherman, 1983). For example, iron deprivation of the unicellular cyanobacterium Synechococcus sp. PCC7942 results in a variety of physiological and morphological changes, which include: (a) the replacement of the iron-sulfur protein Fd with the flavin-containing protein flavodoxin in the role of terminal electron carrier of the photosynthetic apparatus (Laudenbach and Straus, 1988); (b) the reduction in the number of light-harvesting phycobilisomes and a corresponding reduction in the cellular phycobilin content (Guikema and Sherman, 1983); (c) 3- to 4-fold reductions in the number of thylakoids seen in cross-section (Sherman and Sherman, 1983); and (d) changes in the spectral characteristics of Chl within the thylakoids (Guikema and Sherman, 1983, 1984; Pakrasi et al., 1985a, 1985b). Despite these changes, cells remain viable because they can be propagated indefinitely in culture under the iron-limited conditions that produce this phenotype. Furthermore, these changes are readily reversible, since readdition of iron to starved cultures rapidly initiates pigment synthesis and results in the complete restoration of the nutrient-sufficient phenotype (Sherman and Sherman, 1983; Pakrasi et al., 1985a).

The pronounced iron stress-induced changes in the spectral forms of Chl, evidenced most dramatically by changes in the low-temperature fluorescence emission spectra, indicate that Chl becomes sequestered in alternative molecular environments within the thylakoid membranes (Oquist, 1973, 1974a, 1974b; Guikema and Sherman, 1983, 1984; Pakrasi et al., 1985a). Indeed, the spectral changes induced by iron limitation are directly correlated with the loss of high molecular mass Chl-protein complexes of PSI, typical of normally grown cells, and the induction of a Chl-protein complex, designated CPVI-4 (Pakrasi et al., 1985a, 1985b). The CPVI-4 complex becomes the major Chl-binding protein in membranes of cells deprived of iron. In contrast, virtually all of the Chl found in the cyanobacterial cell during nutrient-sufficient growth is associated with either the PSI or the PSII reaction center complexes (Pakrasi et al., 1985a, 1985b). The CPVI-4 complex may be isolated from dodecyl β-D-maltoside extracts of iron-deficient thylakoids using mildly denaturing PAGE ("green gels") (Pakrasi et al., 1985a) or ion-exchange chromatography (Riethman and Sherman, 1988). The isolated complex exhibits a single 77 K fluorescence emission peak centered around 685 nm (Pakrasi et al., 1985b; Riethman et al., 1988) and appears to represent the dominant 77 K fluorescence emission source in iron-limited whole cells (Oquist, 1971; Guikema and Sherman, 1983). Denaturing PAGE of the purified CPVI-4 complex indicates that it contains...

**Abbreviations:** CP43, Chl-protein acting as proximal antenna to PSII; CPVI-4, Chl-protein complex resolved on mildly denaturing electrophoretic gels as a band designated VI-4; isiA/B, iron stress-induced operon in which co-transcribed genes A and B encode a CP43-like protein and flavodoxin, respectively; PCR, polymerase chain reaction; SmR, streptomycin resistant; SpR, spectinomycin resistant.

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2 Present address: Oklahoma State University, Department of Microbiology and Molecular Genetics, Stillwater, OK 74078.
* Corresponding author; fax 1-405-744-6790.
tains at least one polypeptide of 34 to 36 kD (Pakrasi et al., 1985b; Riethman et al., 1988).

Recent characterizations of iron stress-induced (isi) operons containing the flavodoxin gene (isiB) in both *Synechococcus* sp. PCC7942 (Laudenbach and Straus, 1988; Laudenbach et al., 1998) and *Synechococcus* sp. PCC7002 (Leonhardt and Straus, 1992) led to the putative identification of the apoprotein of the CPV4-1 complex. An iron stress-induced gene, designated *isiA*, is predicted to encode an approximately 36-kD apoprotein that has strong sequence homology to the well-studied PSII Chl-binding protein CP43, encoded by the *psbC* gene (Bricker, 1990). The *isiA* gene encoding the putative CP43 homolog is situated immediately upstream of the flavodoxin gene (isiB) and is co-transcribed with the flavodoxin gene in both species examined (Laudenbach and Straus, 1988; Laudenbach et al., 1988; Leonhardt and Straus, 1992). Transcription of the di-cistronic *isiAB* operon is derepressed in the absence of iron (Laudenbach et al., 1988; Leonhardt and Straus, 1992) according to a mechanism that appears to involve a fur-like repressor (de Lorenzo et al., 1987). Because the predicted size of the CP43-like *isiA* gene product approximates the size of the biochemically identified CPV4-1 apoprotein and because transcription of the *isiAB* operon is switched on during iron-deficient growth conditions, Laudenbach et al. (1988) proposed that the *isiA* gene encodes the apoprotein of the CPV4-1 complex. To test this hypothesis, we characterized a mutant strain of *Synechococcus* sp. PCC7942 in which the *isiAB* operon has been inactivated by insertional mutagenesis. We show here that this mutant fails to accumulate the CPV4-1 complex under iron-deficient growth, yet it is phenotypically indistinguishable from the wild type during nutrient-sufficient growth. We conclude that CPV4-1 is a CP43-like Chl-protein complex that we now refer to as CP43'.

**MATERIALS AND METHODS**

**Bacterial Strains, Plasmids, and Media**

The unicellular coccoid cyanobacterium *Synechococcus* sp. PCC7942 and its mutant derivative, *visiA* (construction described below), were grown in a modified BG-11 medium (Allen, 1968) under conditions described previously (Sherman and Sherman, 1983). For growth of *Synechococcus* under conditions of iron limitation, cells were cultured in standard media modified to contain 1/1000 the normally added Fe-citrate (1/1000 Fe medium). We found that this formulation of iron-deficient media produced a deficiency severe enough to induce the iron-deficient phenotype as judged by changes in pigment content, and yet allowed continual growth of the mutant strain in the limiting media. Large-scale cultivation under iron-sufficient or limiting conditions was performed in 15-L carboys bubbled with air (Sherman and Sherman, 1983). All glassware used for iron-deficient cell growth and media preparation was soaked in EDTA to remove residual iron. Cell numbers were determined using a Petroff-Hauser chamber. Transformation of cyanobacteria and growth with antibiotic selection on solid medium was performed as described previously (Golden, 1988).

An antibiotic resistance cassette that encodes spectinomycin/streptomycin resistances (Prentki and Kirsch, 1984) and is flanked by symmetrical polylinkers (plasmid pRL643; Elhai and Wolk, 1988) was used for insertional mutagenesis. *Escherichia coli* XL1-Blue (Stratagene Cloning Systems) was used for recombinant DNA work and was propagated in Luria broth or Luria broth supplemented with 1.5% agar for solid medium.

**DNA Isolation, PCR Amplification, and Cloning**

Chromosomal DNA was isolated from *Synechococcus* sp. PCC7942 as described previously (Burnap and Sherman, 1991). The coding sequence of the *isiA* gene was amplified from chromosomal DNA by PCR using oligonucleotides synthesized according to the gene sequence previously determined by Laudenbach and Straus (1988). The two chemically synthesized oligonucleotides used for PCR were 5'-GGAAAGCTTCCCCAGAACTCATTACGACTGG-3' and 5'-CGTCTAGACTTAGGCTTCTACAGAGCTGAG-3'. These oligonucleotides were designed to amplify a 1008-bp fragment corresponding to the entire *isiA* coding sequence minus the first six amino acids at the amino terminus of the predicted polypeptide (Laudenbach and Straus, 1988). To facilitate the subsequent cloning of the amplified fragment, the oligonucleotides were also designed to introduce *HindIII* and *XbaI* restriction enzyme sites at the 5' and 3' ends, respectively.

PCR amplification was performed according to published procedures (Sambrook et al., 1989) using *Taq* DNA polymerase (Perkin-Elmer Cetus). One hundred-microliter reactions containing 1 μg of *Synechococcus* sp. PCC7942 genomic DNA were subjected to a thermal cycle routine consisting of a 1-min denaturation step at 94°C, a 1-min annealing step at 55°C, and a polymerization step for 1.5 min at 72°C. After 30 iterations of the cycle, the reaction mixture was extracted once with an equal volume of phenol-chloroform (50:50, v/v) and once with an equal volume of chloroform and then was precipitated with ethanol.

The PCR product was redissolved and subjected to simultaneous digestion with *HindIII* and *XbaI* restriction enzymes. These restriction site cohesive ends were then used to join and ligate (with *T4* DNA ligase) the PCR-amplified fragment into the corresponding *HindIII* and *XbaI* restriction enzyme sites in the polylinker cloning region of plasmid pUC19. Following transformation into *E. coli* strain XL1-Blue, ampicillin-resistant colonies were picked at random and those harboring plasmids containing approximately 1-kb inserts were subjected to further restriction enzyme analysis. A resultant recombinant plasmid was then subjected to dideoxy sequencing to verify that the amplified and cloned PCR fragment was indeed the intended *isiA* DNA fragment. This plasmid was designated pISIAI.

**Insertional Mutagenesis of the *isiAB* Operon**

Wild-type *Synechococcus* sp. PCC7942 cells were subjected to directed insertional mutagenesis to produce a strain in which the *isiAB* operon was inactivated at its chromosomal locus. A mutagenic plasmid was constructed from pISIAI by ligating the 2-kb *Ω* antibiotic resistance cassette (Elhai and Wolk, 1988) encoding spectinomycin and streptomycin re-
sistances into the PstI restriction enzyme site located near the 5' end of the isiA coding sequence in pISIAI (Fig. 1). Wild-type Synechococcus sp. PCC7942 cells were transformed with this construct and subjected to selection for spectinomycin resistance on solid medium. Note that the transformed plasmid does not have a cyanobacterial origin of replication, and all stable transformants must arise by integration of sequences containing the antibiotic resistance cassette with the recipient cells' DNA. Homologous recombination with recipient's chromosomal DNA occurs with much higher frequency than other modes of integration, with double crossover recombinations occurring more frequently than single crossover recombinations (Williams and Szalay, 1983; Golden, 1988). Resistant colonies were passaged several times on solid BG-11 medium containing spectinomycin to produce a strain homozygous for the interrupted isiAB operon.

DNA-DNA and DNA-RNA Hybridizations

Chromosomal DNA was digested with restriction enzymes, separated by agarose gel electrophoresis, and transferred to nitrocellulose membrane filters using standard Southern blotting methods (Sambrook et al., 1989). Probes were radiolabeled with [α-32P]dCTP using the Random Prime Labeling Kit (United States Biochemical) with restriction enzyme-generated gene fragments purified from plasmid vector DNA by low melting point agarose gel electrophoresis. The digested, separated, and filter-immobilized genomic DNA was hybridized with labeled probe DNA at 65°C in 6X SSC, 10X Denhardt's solution, 1% SDS, and 500 μg of sonicated salmon sperm DNA for 18 h. Filters were washed at 65°C, first in 1X SSC and then in 0.1X SSC plus 0.1% SDS to remove nonspecifically bound probe DNA. The washed filters were air dried and used to expose x-ray film at -80°C.

Total cellular RNA was isolated according to the method of Reddy et al. (1990), except that under iron-deficient growth conditions the volume of culture harvested was increased from 500 mL to 1 L due to the reduced levels of RNA present in iron-deficient cells. Northern blot analysis was performed essentially as described by Sambrook et al. (1989). Total RNA was electrophoresed (10 μg/lane) in agarose gels containing 1.9% formaldehyde and transferred to a Nytran (Schleicher and Schuell) membrane. Blots were hybridized with radiolabeled probes (see above) in a solution consisting of 5X SSPE, 50% formamide, 10X Denhardt's solution, 1% SDS, and 500 μg of sonicated salmon sperm DNA at 42°C for 18 h. Blots were then washed and processed as described for Southern blot hybridizations.

Preparation of Thylakoids and Electrophoresis of Chl-Protein Complexes

Synechococcus sp. PCC7942 cells were harvested, broken by three passages through a French pressure cell at 16,000 p.s.i. in HMCS buffer (50 mM Hepes, pH 7.8, 10 mM MgCl2, 5 mM CaCl2, 1 mM Suc), which was diluted 1:1 with 2 mM Tris-maleate, pH 7.0, 8 mM PMSF just prior to cell breakage. DNase I and MgCl2 were added to the lysates to a final concentration of 50 μg/mL and 6 mM, respectively, and the lysates were incubated on ice for 1 h. EDTA was added to a final concentration of 10 mM, and unbroken cells were pelleted from the lysate by centrifugation at 7000 rpm for 10 min. Thylakoid membranes were prepared by a modification (T.A. Troyan and L.A. Sherman, unpublished observations) of the Suc step gradient purification previously described (Omata and Murata, 1984). The thylakoid-enriched fraction from the gradients were collected, diluted with half with 2 mM Tris-maleate (pH 7.0), and pelleted at 150,000 g for 1 to 3 h at 4°C. Pelleted membranes were homogenized into a small volume of 2 mM Tris-maleate (pH 7.0). The Chl concentration was determined (Arnon et al., 1974) and the appropriate quantities of membranes were pelleted at 50,000 rpm in a TLA-100.3 rotor for 30 min at 4°C using a Beckman TL-100 benchtop ultracentrifuge. The membrane pellet was homogenized into a solubilization solution containing 0.45% decyl glucoside, 0.45% dodecyl-ß-o-maltoside, 0.1% lithium dodecyl sulfate, and 10% glycerol. The membranes were solubilized for 30 min on ice and cleared of insoluble material by centrifugation at 40,000 rpm for 30 min at 4°C. Prior to loading onto the gels, the density of the supernatant was increased by adding an equal volume of 80% glycerol. Mildly denaturing Chl-protein gels were prepared and run as described by Allen and Staehelin (1991). The solubilization supernatant was loaded onto a small (30 mL volume) 3-mm thick slab gel (10% acrylamide) and electrophoresed at 7 mA constant current in the dark for 6 h at 4°C.

EM

Cells were prepared for EM by chemical fixation as described previously (Sherman and Sherman, 1983). The fixation procedure used 3% acrolein, 3% glutaraldehyde in 0.1 M Na-cacodylate, pH 7.2, followed by 1% OsO4 and in-block staining in uranyl acetate. The material was then dehydrated in ethanol and embedded in Spurr's resin. Micrographs were taken on a Phillips 400 electron microscope at 80 kilo-electron volts.
Spectroscopy and \( O_2 \) Evolution

Absorption spectra were recorded with a Beckman spectrophotometer essentially as described previously. Fluorescence emission spectra of whole cells were recorded at 77 K using an SLM 8000C spectrofluorimeter equipped with a liquid \( N_2 \) temperature optical Dewar flask. Samples were concentrated by centrifugation in fresh growth medium to a known cell density. The cell suspension was frozen as a thin coat on sample tubes, placed in a liquid \( N_2 \) optical Dewar flask, and centered in the excitation beam. Particular care was given to ensure reproducible coating and orientation of the sample within the sample chamber. To approach a true measure of relative fluorescence comparing mutant and wild-type cells, samples used for coating sample tubes were adjusted to equal cell densities (approximately \( 1 \times 10^9 \) cells mL\(^{-1}\) for iron-stressed cells, \( 2 \times 10^9 \) cells mL\(^{-1}\) for cells from iron-replete cultures). \( O_2 \) evolution assays were performed under light saturation conditions in the presence of an artificial electron acceptor as described (Burnap and Sherman, 1991).

RESULTS

Insertional Inactivation of the \( isiAB \) Operon

A mutagenic plasmid designed to inactivate \( isiAB \) was constructed by ligating a 2-kb antibiotic resistance cassette encoding Sp\(^r\) and Sm\(^r\), the \( \Omega \) element (Elhai and Wolk, 1988), into a unique \( PstI \) restriction site within pISIA\(^{r} \) approximately 160 bp downstream from the ATG translational start site of the \( isiA \) gene (Laudenbach and Straus, 1988). The resultant plasmid, pISIA\(^{r} \), was used to transform the \( Synechococcus \) sp. PCC7942 wild type to spectinomycin resistance on agar plates (Fig. 1). Recombinants were isolated by selection for the Sp\(^r\) trait under conditions of iron-sufficient growth to diminish any selective advantage conferred by the possession of an intact \( isiAB \) operon. Several Sp\(^r\) transformant colonies were picked for further investigation.

These transformants were also tested for their ability to grow in the presence of ampicillin to reveal those that might have resulted from the integration of the entire plasmid by single crossover recombination. No ampicillin-resistant transformants were found, indicating that all transformants most likely arose via double-crossover/marker insertion events. The insertion was confirmed by Southern blot hybridization analysis of chromosomal DNA isolated from wild-type and transformant cells and probed with the cloned PCR-generated \( isiA \) fragment (Fig. 2). The approximately 3-kb \( BgII \) fragment containing the \( isiAB \) operon in the wild-type (Fig. 2, lane 1) was converted to an approximately 5-kb \( BgII \) fragment, indicating that the intended insertion of the 2-kb Sp\(^r\)/Sm\(^r\) \( \Omega \) element occurred in all of the transformants examined (Fig. 2, lanes 2–5). No trace of the wild-type 3-kb \( BgII \) fragment was detected in any transformants even with overexposure of the autoradiogram (data not shown), indicating that the segregation of the mutant allele is complete in all of the Sp\(^r\) isolates. One of the directed mutants, designated \( VisiA \), was selected for further analysis.

Figure 2. Southern blot hybridization of chromosomal DNA isolated from the \( Synechococcus \) sp. PCC7942 wild type (lane 1) and several Sp\(^r\) isolates (lanes 2–5) that had been transformed with the plasmid pISIA\(^{r} \) (containing the \( isiA \) coding sequence interrupted by the 2-kb \( \Omega \) element encoding Sp\(^r\) and Sm\(^r\)). Two micrograms of chromosomal DNA from each strain was digested with the restriction enzyme \( BgII \), electrophoresed on a 1% agarose gel, and blotted onto a nitrocellulose filter. The immobilized DNA was probed with the \( ^{32}P \)-labeled 1-kb fragment corresponding to the coding sequence of the \( isiA \) gene (Laudenbach et al., 1988).

Northern Blot Analysis

Previous transcriptional analysis of the \( isiAB \) operon in \( Synechococcus \) sp. PCC7942 revealed two transcripts from this operon (see Fig. 1) (Laudenbach et al., 1988; Laudenbach and Straus, 1988). The 1900-base transcript, containing both the \( isiA \) and \( isiB \) genes, was found to be much less abundant than the 1100-base transcript encoding only the \( isiA \) gene. Both transcripts were detected in wild-type cells grown in iron-deficient media, whereas neither transcript was detectable in cells grown in iron-replete media (Fig. 3). This confirms that the transcription of this operon is regulated by the availability of iron in the growth medium (Laudenbach and Straus, 1988). Transcripts originating from the \( isiAB \) operon of the \( VisiA \) mutant were not detectable in total RNA from cells grown under either iron-sufficient or iron-deficient growth conditions (Fig. 3). Probing the same RNA with a DNA fragment containing the \( isiB \) sequence also failed to produce a hybridization signal (data not shown). These results demonstrate that the insertion of the \( \Omega \) element within the \( isiA \) gene effectively prohibits the accumulation of any transcripts corresponding to the \( isiAB \) operon in mutant cells.

The strong positive signal obtained using the \( psbA1 \) gene as a probe (encoding the D1 protein of PSII) ensured that high-quality RNA was isolated from each cell type. Expression of the \( psbA1 \) gene was found to be entirely unaffected by the presence of the mutation (Fig. 3). It should be noted that the RNA yield from 1/1000 Fe cultures was roughly 5- to 10-fold less on a per cell basis compared with cells grown under iron-sufficient conditions (data not shown). This is important in relation to the larger \( psbA1 \) hybridization signals seen in the iron-deficient samples as compared with the \( psbA1 \) signals in the iron-sufficient samples; equal amounts of total cellular RNA (10 \( \mu \)g) were loaded for all samples and, consequently, 5- to 10-fold more cells are represented in the iron-deficient samples.
This is evident when the gel is illuminated with either visible green band (Pakrasi et al., 1985b), is absent in VisiA (Fig. 4). To compare Chl-protein bands from wild-type and mutant strains, we used with smaller quantities of solubilized membranes. It was shown to prevent resolution of the Chl-protein bands quite difficult. The presence of contaminating membranes increased substantially in the mutant grown under iron-deficient conditions. These characteristics rendered the content increased substantially in the mutant grown under iron-sufficient medium. In addition, the carotenoid content increased two to three-fold under these conditions compared with cells grown in iron-sufficient medium. In light to display the green bands (Fig. 4A) or UV light to display fluorescent bands (Fig. 4B). The Chl-protein band pattern from iron-starved VisiA cells is very similar to the pattern obtained from iron-sufficient wild-type cells. The pattern similarity is better revealed when larger quantities of Chl are electrophoresed for longer periods; however, these conditions usually result in a smearing of the region surrounding CP43'. The Chl-protein band pattern in VisiA mutant grown in 1/1000 iron (VisiA, LoFe) and from the wild type (wt, + Fe) or in medium containing 1/1000 the normal level of iron (wt, LoFe). Membrane samples containing 200 μg of Chl from iron-sufficient wild-type and 75 μg of Chl from the iron-limited wild-type and VisiA mutant were solubilized and electrophoresed in the dark on a 10% acrylamide gel at 7 mA constant current for 6 h at 4°C. The gel was photographed with visible (A) or UV transillumination (B). The Chl-protein band originally termed CPVI-4, now termed CP43', migrates the farthest in this gel system and was shown to be missing in the VisiA mutant. This was highlighted around CP43' (T.A. Troyan and L.A. Sherman, unpublished observations). This similarity suggests that when VisiA is grown in iron-deficient conditions Chl remains associated with the same Chl-proteins as during iron-sufficient growth. This association of Chl with PSI and PSII is masked in wild-type, iron-deficient cells by the accumulation of large quantities of CP43'. In the absence of this protein, as in the VisiA mutant, these other associations are revealed.

Spectroscopic Characteristics

Low temperature fluorescence emission spectroscopy provides a sensitive assay for the state of organization of different Chls within the photosynthetic membrane (reviewed in Murata and Satoh, 1986). For wild-type cells grown under nutrient-sufficient conditions, preferential excitation of Chl at liquid nitrogen temperature (77 K) will yield emission that can be traced to Chl in the reaction center of PSII (signals at 685 and 695 nm) and PSI (720 nm). An example of this well-documented wild-type spectrum as seen under our experimental conditions is shown in Figure 5A, along with the corresponding fluorescence emission spectrum of VisiA, also grown under nutrient-sufficient conditions. The 77 K emission spectra of the isiA mutant and wild-type cells were virtually indistinguishable when the strains were grown in media containing sufficient iron. This analysis shows that CPVI-4, the fastest migrating green band (Pakrasi et al., 1985b), is absent in VisiA (Fig. 4). This is evident when the gel is illuminated with either visible

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Figure 3. Northern blot hybridization analysis of total cellular RNA isolated from the Synechococcus sp. PCC7942 wild type (W) and the VisiA mutant (M). Cells were grown under either iron-sufficient (+Fe) or iron-deficient (-Fe) conditions as described in “Materials and Methods.” Ten micrograms of total cellular RNA (per lane) was subjected to agarose gel electrophoresis under denaturing conditions, blotted to a nylon membrane, and hybridized with 32P-labeled fragments corresponding to the psbA1 gene encoding the PSI reaction center D1 protein of Synechococcus sp. PCC7942 (left half of figure) and the isiA gene (right half of figure).

Figure 4. Analysis of Chl-protein complexes from Synechococcus sp. PCC7942 using a non-denaturing, low ionic strength acrylamide gel system, based on that of Allen and Staehelin (1991). Membranes were obtained from wild-type cells, grown in the presence of iron (wt, + Fe) or in medium containing 1/1000 the normal level of iron (wt, LoFe), and from the VisiA mutant grown in 1/1000 iron (VisiA, LoFe). Membrane samples containing 200 μg of Chl from iron-sufficient wild-type and 75 μg of Chl from the iron-limited wild-type and VisiA mutant were solubilized and electrophoresed in the dark on a 10% acrylamide gel at 7 mA constant current for 6 h at 4°C. The gel was photographed with visible (A) or with UV transillumination (B). The Chl-protein band originally termed CPVI-4, now termed CP43', migrates the farthest in this gel system and was shown to be missing in the VisiA LoFe cells.
Previous work established that the adaptation of *Synechococcus* wild-type cells to iron-deficient growth conditions was accompanied by a dramatic change in the 77 K fluorescence emission spectrum obtained upon excitation of Chl (Oquist, 1974; GuiKema and Sherman, 1983; Pakrasi et al., 1985b). Under these iron-limited growth conditions, the fluorescence emission spectrum of wild-type cells was dominated by a peak centered around 685 nm, whereas the relative intensities of the 696- and 717-nm peaks were greatly diminished. This is precisely what we observed for wild-type cells grown under nutrient-sufficient conditions. However, mutant cells did not shift to 673 nm but was consistently found to be at 675 nm.

**Photosynthetic and Growth Characteristics**

Table I documents some of the photosynthetic and growth characteristics of *Synechococcus* sp. PCC7942 wild-type and *visiA* mutant cells from typical iron-sufficient and iron-deficient cultures (*visiA* grown in iron-sufficient media is omitted because its properties are virtually identical to those of the wild type grown under these conditions). It was observed that *visiA* grows well in a medium containing 1/1000 the normal level of iron, achieving cell densities as high or sometimes higher than comparably grown wild-type cultures. However, mutant cells do not grow well in a medium completely lacking added iron and eventually die (data not shown). Thus, some added iron is necessary to retain viability.

An important characteristic is that *visiA* grown under 1/1000 Fe conditions contain much less Chl per cell and that the Chl absorption maximum, as noted above, is shifted toward the red. As shown in Figures 5 and 6, low-iron grown *visiA* has more phycobilisome and PSII fluorescence (on a
proportional basis) than does the wild type grown under similar conditions. We believe that these properties are the result of the loss of CP43'. Light-saturated O₂ evolution, when normalized to milligrams of Chl per hour, would seem to increase 2-fold in \( \nabla isiA \) relative to the wild-type grown under the same iron-deficient conditions (Table I). However, considering that the amount of Chl per cell is decreased 2-fold in \( \nabla isiA \), it is likely that O₂ evolution per cell is rather similar in the two cases.

Despite the phenotypic differences between wild-type and \( \nabla isiA \) cells grown in 1/1000 Fe media, their growth characteristics in the same medium were virtually identical. On the other hand, mutant cells appeared to exhibit a longer lag period in growth upon return to iron-replete medium than did wild-type cells. To investigate this phenomenon, we transferred equal numbers of iron-deficient cells of each strain (during exponential growth) to separate flasks of iron-sufficient growth medium to assess their growth rates upon reexposure to iron-sufficient conditions. As shown in Figure 7, \( \nabla isiA \) cells exhibited a lag period before exponential growth that was approximately 40 h longer than that observed for the wild-type cells. Whether or not this observed increase in the lag period is due to a higher cell mortality in the mutant or reflects retardation in a cellular process due to the inactivation of the isiAB operon will require further investigation.

Ultrastructure

We carefully analyzed the ultrastructure of Synechococcus sp. PCC7942 grown under iron-sufficient and iron-deficient conditions (Sherman and Sherman, 1983). The electron micrographs indicate that iron-deficient cells produce many fewer thylakoids. Indeed, low iron-grown wild-type cells often have no more than one complete membrane per cell seen in cross-section, whereas iron-sufficient cells have three to four membranes per cell in cross-section. As shown in Figure 8, similar results have been obtained in the present comparison of the mutant grown in iron-sufficient (Fig. 8A) and iron-deficient media (Fig. 8, B-D). The decline in thylakoids induced by iron starvation is roughly paralleled by the 3-fold decline in the amount of Chl per cell, suggesting that cellular Chl content may be roughly proportional to thylakoid surface area. In the case of iron-deficient \( \nabla isiA \) cells, virtually all micrographs revealed only one complete membrane per cell (Fig. 8B). In addition, many of these membranes appeared "broken" in that there were discontinuities in the photosynthetic lamellae (Fig. 8, B-D). Many iron-deficient \( \nabla isiA \) cells also had membrane fragments, as seen in Figure 8. Such breaks or fragments are rarely detected in wild-type cells.

DISCUSSION

The main objective of this work was to gain a better understanding of the CPVI-4 complex, which is an iron stress-induced Chl-protein in Synechococcus sp. PCC7942. Work done independently by Laudenbach and Straus (1988) with the same cyanobacterial strain revealed the existence of an iron-regulated gene with striking sequence similarity to the psbc gene of cyanobacteria and higher plants. This gene, designated isiA, was found upstream from the co-transcribed isiB gene encoding flavodoxin. In the context of these observations, we wished to evaluate the hypothesis that the predicted isiA gene product is the apoprotein of CPVI-4. We constructed and isolated a strain of Synechococcus sp. PCC7942 with an insertion mutation in the isiA gene (\( \nabla isiA \)) that lacked any detectable transcripts from the now interrupted isiAB operon. Importantly, the disruption of the isiAB operon results in the loss of the CPVI-4 complex. We believe that the most likely explanation for this result is that isiA encodes the apoprotein for the CPVI-4 complex (CP43'), and that CP43' is not synthesized in the \( \nabla isiA \) mutant. However, this conclusion is rendered tentative since the entire isiAB operon was inactivated and we still cannot exclude the possibility that the loss of CPVI-4 is due to a pleiotropic effect resulting from the absence of flavodoxin.

One of the phenotypic characteristics of the \( \nabla isiA \) mutant grown under iron-deficient conditions is the reduction in the
level of Chl per cell. The mutant has approximately one-half of the cellular level of Chl found in the wild-type cells when both strains were cultured in 1/1000 Fe media (Table I). The lower cellular levels of Chl in the mutant account for its altered spectroscopic properties, including the reduced Chl fluorescence emission and absorption. The deletion of CP43' in VisiA created an overall reduction in 77 K Chl fluorescence emission per cell. This results in what appears to be an unmasking of the PSII reaction center 77 K fluorescence (685 and 695 nm peaks), which are otherwise almost completely obscured by the strong 685-nm emission from CP43' in the wild type. The inactivation of the isiAB operon does not appear to affect the per cell levels of PSII, as judged by O2 evolution activity measurements, nor does the absence of CP43' affect the level of PSI, as judged by the intensity of the 77 K fluorescence.

The function of CP43' remains to be determined. We present three possibilities, none of which are mutually exclusive. First, CP43' can function as an alternative light-harvesting complex, perhaps replacing phycobilisomes during iron-deficient growth. Second, CP43' may act as a direct replacement for CP43 during conditions of iron deprivation. Finally, CP43' may provide a pool of Chl that is mobilized for the production of Chl-protein complexes during recovery from iron stress.

The possibility that CP43' functions as an alternative light-harvesting antenna is consistent with its relatively high abundance in proportion to the number of reaction centers it might theoretically serve. Our measurements suggest that CP43' may bind up to 50% of the Chl in iron-starved cells. This is based upon per cell Chl measurements and the conclusion that the VisiA mutant lacks only CP43', whereas the abundance of the other Chl-protein complexes remains relatively unchanged by the mutation. Room temperature fluorescence induction measurements indicate that CP43' functions very inefficiently as an antenna for the photosynthetic reaction centers and, instead, reemits absorbed light as prompt fluorescence. We conclude that, although CP43' may function as an alternative light-harvesting antenna during periods when there are few phycobilisomes, the complex cannot be considered as a functionally equivalent replacement for the phycobilisome, a conclusion supported by the O2 evolution data. Energy utilization efficiency, however, may not be the most important parameter for cells under nutrient stress.

The homology between CP43 and CP43' suggests another possibility, namely that CP43' functions as a replacement for CP43 during periods of iron deprivation. A structural model for CP43', based upon similar models of CP43, is presented in Figure 9. CP43' is predicted to be very similar to CP43 in overall structure. The major difference is the lack of approx-

Figure 8. Electron micrographs of Synechococcus sp. PCC7942 VisiA cells grown in iron-sufficient (A) or iron-deficient media (B-D). The iron-sufficient cells were indistinguishable from wild-type cells, whereas iron-deficient VisiA cells often contained broken membranes or membrane fragments (arrows). Such fragmentation was rarely detected in wild-type cells.
Asp. Symbols enclosed by a circle indicate that the residue is present in CP43', but not in CP43. Symbols enclosed by a box indicate that the residue is present in CP43', but not in CP43. Symbols enclosed by a circle indicate that the residue is present in CP43, but not in CP43'.

Figure 9. Predicted membrane topology and distribution of His and charged residues for the CP43' and CP43 proteins based upon the model described by Bricker (1990). +, Arg or Lys; −, Glu or Asp. Symbols enclosed by a circle indicate that the residue is present in CP43, but not in CP43'. Symbols enclosed by a box indicate that the residue is present in CP43', but not in CP43.

imimately 120 amino acids in the lumenal loop, labeled E'. From all other perspectives, the protein is very similar and could conceivably replace CP43 in the formation of PSII reaction center complexes. We have found that CP43 is still produced in iron-deficient cells, although at lower levels. In our attempts to isolate PSII particles from iron-deficient cells, we have never been able to show a close association of CP43' with PSII. However, CP43' is far more labile than CP43 and the loss of CP43' may be a biochemical artifact.

We have indirect evidence that supports the contention that CP43' is of great importance to cells during the recovery from iron stress. Addition of iron to iron-deficient wild-type cultures results in the resynthesis of thylakoids and the restoration of normal levels of photosynthetic reaction centers. This process results in the reappearance of normal photosynthetic capacity and is largely completed within 24 to 36 h, depending upon the degree and duration of iron deficiency that the reconstituted cells have experienced. Previous work suggests that Chls associated with CP43' may be used for the biosynthesis of new reaction centers following the addition of iron to iron-starved cultures (Troyan et al., 1989). These results were obtained using the compound gabaclidine, which is known to inhibit Chl biosynthesis in cyanobacteria (Guikema et al., 1986). When gabaclidine was added along with iron to iron-deficient cultures, normal PSII reaction center Chl-protein complexes, CP47 and CP43, were shown to accumulate, although at reduced levels. Therefore, the Chls associated with CP43' can be reorganized into CP47 and CP43 upon iron addition and despite the absence of new Chl biosynthesis. Therefore, it was concluded that at least one function of CP43' is to act as a reservoir for Chls under conditions of severe iron stress and that this Chl can later be reorganized into normal complexes (Riethman et al., 1988).

It is proposed that this mechanism promotes a more rapid reacquisition of photosynthetic capacity, thereby allowing cells to grow and multiply more rapidly following periods of slow growth imposed by limiting nutrient levels. The absence of the capacity to reorganize preexisting Chl may therefore account for the observation that VisiA exhibits a longer lag before the resumption of exponential growth as iron-starved cells are returned to fresh iron-sufficient media (Fig. 7). However, further experiments are required to distinguish this possibility from the alternative that the increased lag is due to higher cell mortality.

In summary, we conclude that CP43' is a Chl-binding protein that complexes a great many Chls throughout the membranes of iron-deficient cells. This Chl-protein complex can be used as a weak antenna for PSII (and possibly PSI) and represents a pool of Chl for reorganization into new complexes when iron availability improves. It may be that the loss of the luminal loop E' permits the protein to migrate more readily throughout the thylakoid membrane and, thus, makes it a more flexible donor for new Chl-protein assembly. Now that we have verified that isiA is the gene that codes for this Chl-protein apoprotein, we can perform further molecular biological manipulations to test this hypothesis.

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