

# Light-Induced Energy Dissipation in Iron-Starved Cyanobacteria: Roles of OCP and IsiA Proteins <sup>W</sup>

Adjélé Wilson,<sup>a</sup> Clémence Boulay,<sup>a</sup> Annegret Wilde,<sup>b</sup> Cheryl A. Kerfeld,<sup>c</sup> and Diana Kirilovsky<sup>a,1</sup>

<sup>a</sup>Unité de Recherche Associée 2096, Centre National de la Recherche Scientifique, Service de Bioénergétique, Commissariat à l’Energie Atomique Saclay, 91191 Gif sur Yvette, France

<sup>b</sup>Institute of Biology, Humboldt University, 10115 Berlin, Germany

<sup>c</sup>Molecular Biology Institute, University of California, Los Angeles, California 90095-1570

**In response to iron deficiency, cyanobacteria synthesize the iron stress-induced chlorophyll binding protein IsiA. This protein protects cyanobacterial cells against iron stress. It has been proposed that the protective role of IsiA is related to a blue light-induced nonphotochemical fluorescence quenching (NPQ) mechanism. In iron-replete cyanobacterial cell cultures, strong blue light is known to induce a mechanism that dissipates excess absorbed energy in the phycobilisome, the extramembranal antenna of cyanobacteria. In this photoprotective mechanism, the soluble Orange Carotenoid Protein (OCP) plays an essential role. Here, we demonstrate that in iron-starved cells, blue light is unable to quench fluorescence in the absence of the phycobilisomes or the OCP. By contrast, the absence of IsiA does not affect the induction of fluorescence quenching or its recovery. We conclude that in cyanobacteria grown under iron starvation conditions, the blue light-induced nonphotochemical quenching involves the phycobilisome OCP-related energy dissipation mechanism and not IsiA. IsiA, however, does seem to protect the cells from the stress generated by iron starvation, initially by increasing the size of the photosystem I antenna. Subsequently, the IsiA converts the excess energy absorbed by the phycobilisomes into heat through a mechanism different from the dynamic and reversible light-induced NPQ processes.**

## INTRODUCTION

Excess light can be lethal for photosynthetic organisms because harmful reactive oxygen species are generated in the photochemical reaction centers when energy absorption exceeds the rate of carbon fixation. To survive, photosynthetic organisms have evolved several protective processes. One such mechanism is the dissipation of the excess absorbed energy as heat in the light-collecting pigment/protein complexes, the so-called antenna. In plants, this process involves the chlorophyll-containing light-harvesting complex (LHCII) of photosystem II (PSII) and is triggered by acidification of the thylakoid lumen under saturating light conditions (reviewed in Demmig-Adams, 1990; Horton et al., 1996; Niyogi, 1999; Müller et al., 2001). A drop in the thylakoid lumen pH activates the formation of the carotenoid zeaxanthin from violaxanthin as part of the xanthophyll cycle (Yamamoto, 1979; Gilmore and Yamamoto, 1993) and induces the protonation of PsbS, a PSII subunit that belongs to the LHC superfamily (Li et al., 2000, 2004). This process also involves conformational changes in LHCII, modifying the interaction between chlorophylls and carotenoids (Ruban et al., 1992; Pascal et al., 2005). Thermal energy dissipation is accompanied

by a decrease of PSII-related fluorescence emission, known as high-energy quenching (qE), one of the nonphotochemical quenching (NPQ) processes. The qE is a dynamic, rapidly reversible process that is induced seconds after the plant is exposed to high light intensities.

Several recent studies have shown that cyanobacteria, which do not have the integral membrane chlorophyll-containing LHCII, also use a light-induced antenna-related NPQ mechanism to decrease the amount of energy funneled to the PSII reaction center (El Bissati et al., 2000; Rakhimberdieva et al., 2004; Scott et al., 2006; Wilson et al., 2006). In cyanobacteria, light is captured by a membrane extrinsic complex, the phycobilisome, which is attached to the outer surface of thylakoid membranes. These large complexes consist of phycobiliproteins with covalently bound bilin pigments and linker peptides that are required for the organization of the phycobilisomes (reviewed in MacColl, 1998; Adir, 2005). Phycobilisomes are composed of a core from which rods (usually six) radiate. The major core protein is allophycocyanin (APC), while the rods contain phycocyanin (PC) and, in some species, phycoerythrin or phycoerythrocyanin (in the distal end of the rod). The phycobilisomes are bound to the thylakoids via the core membrane linker protein Lcm, which also serves as the terminal energy acceptor. Harvested light energy is transferred from Lcm to the chlorophylls of PSII and photosystem I (PSI) (Mullineaux, 1992; Rakhimberdieva et al., 2001).

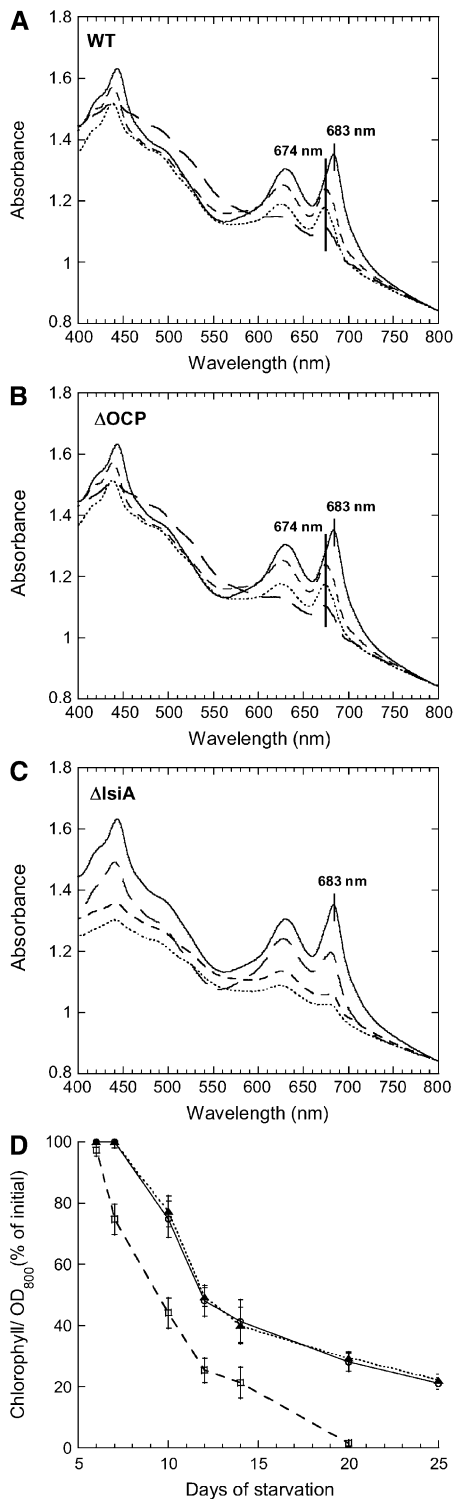
Results revealing the existence of a blue light-induced NPQ mechanism proposed to be associated with the phycobilisomes were first described in 2000 (El Bissati et al., 2000). Subsequently, spectral and kinetics data were presented suggesting that blue light-activated carotenoids induce quenching of

<sup>1</sup>To whom correspondence should be addressed. E-mail diana.kirilovsky@cea.fr; fax 33-1-69088717.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantcell.org) is: Diana Kirilovsky (diana.kirilovsky@cea.fr).

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**Figure 1.** Changes in Absorption Spectra and Chlorophyll Content Induced by Iron Starvation in Wild-Type,  $\Delta$ OCP, and  $\Delta$ IsiA Cells.

**(A)** and **(B)** Absorption spectra of wild-type **(A)** and  $\Delta$ OCP **(B)** cells grown in iron-containing medium (solid line) or in iron-depleted medium for 12 (small dashed line), 14 (dotted line), or 20 (large dashed line) d.

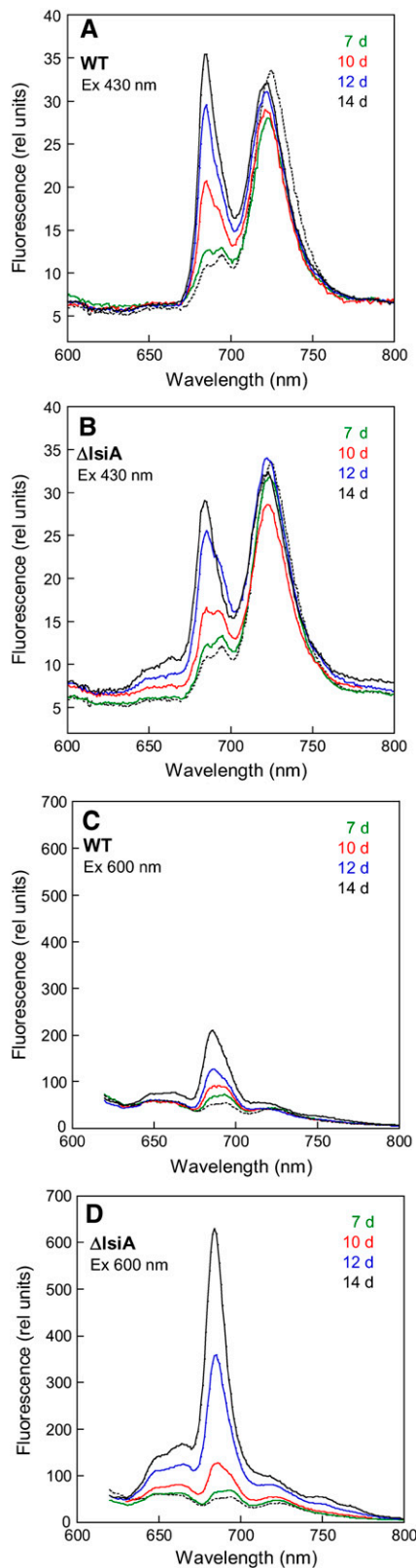
phycobilisome fluorescence emission (Rakhimberdieva et al., 2004). Wilson et al. (2006) demonstrated that a soluble carotenoid binding protein, the Orange Carotenoid Protein (OCP), is specifically involved in a phycobilisome-related NPQ that appears to be associated with a photoprotective energy dissipation mechanism. OCP, a 35-kD protein that contains a single non-covalently bound carotenoid, is encoded by the *slr1963* open reading frame in *Synechocystis* PCC 6803 (Holt and Krogmann, 1981; Wu and Krogmann, 1997; for review, see Kerfeld, 2004a, 2004b). Highly conserved homologs of OCP are found in the genomes of all cyanobacteria, with the exception of the *Prochlorococcus* strains, for which genomic data are available (Kerfeld, 2004a, 2004b).

In the absence of OCP, the NPQ induced by strong white or blue-green light in *Synechocystis* PCC 6803 cells is completely inhibited, and the cells are more sensitive to high light intensities (Wilson et al., 2006). The observation that the effective antenna size was smaller in the cells in the quenched state strongly supports the hypothesis that the OCP-related mechanism dissipates the excess absorbed energy, thereby decreasing the amount of energy arriving at the photochemical centers. The OCP phycobilisome-associated NPQ is not dependent on the presence of a transthylakoid  $\Delta$ pH, on the excitation pressure on PSII, or on changes in the redox state of the plastoquinone pool (El Bissati et al., 2000; Scott et al., 2006; Wilson et al., 2006). Instead, OCP seems to act as a photoreceptor that responds to blue-green light and induces energy dissipation (and fluorescence quenching) through interaction with the phycobilisome core.

Under iron starvation conditions, blue light causes a large reversible quenching of Fo and Fm levels (Cadoret et al., 2004; Bailey et al., 2005; Joshua et al., 2005). It was proposed that the iron stress-induced protein IsiA was essential in this NPQ process. Two mechanisms were proposed: (1) blue light converts the IsiA protein from one form that is efficient in harvesting light energy for photosynthesis into another form that converts excess energy into heat (Cadoret et al., 2004); and (2) strong light induces a change in IsiA, increasing the affinity of IsiA for the phycobilisomes and diminishing the high fluorescence of free phycobilisomes (Joshua et al., 2005).

The expression of the IsiA protein, which belongs to the core complex family of chlorophyll binding proteins, is induced by iron starvation (Laudenbach and Straus, 1988; Burnap et al., 1993) and other stress conditions (such as salt stress, oxidative stress, and high-light stress) (Jeanjean et al., 2003; Yousef et al., 2003; Havaux et al., 2005). IsiA encircles the PSI reaction center, forming complexes consisting of a trimeric PSI and 18 IsiA molecules (Bibby et al., 2001; Boekema et al., 2001). Larger

**(C)** Absorption spectra of  $\Delta$ IsiA cells grown in iron-containing medium (solid line) or in iron-free medium for 7 (large dashed line), 12 (small dashed line), or 14 (dotted line) d. The spectra were normalized at OD<sub>800</sub>. **(D)** Decrease of chlorophyll content in iron-starved wild-type (circles),  $\Delta$ OCP (triangles), and  $\Delta$ IsiA (squares) cells. The results are the average of seven independent experiments. Error bars show the maximum and minimum chlorophyll/OD<sub>800</sub> values for each point. 100% of chlorophyll/OD<sub>800</sub> = 7.5, corresponding to  $\sim$ 5.8  $\mu$ g chlorophyll/mL for a culture at OD<sub>800</sub> = 0.78.



**Figure 2.** Changes in 77K Fluorescence Emission Spectra Induced by Iron Starvation in Wild-Type and  $\Delta$ IsiA Mutant Cells.

amounts of IsiA are bound to PSI during prolonged iron starvation (Yeremenko et al., 2004). IsiA increases the absorptional cross section of PSI, acting as an additional and efficient LHC for PSI (Andrizhiyevskaya et al., 2002; Melkozernov et al., 2003). IsiA aggregates, forming empty multimeric rings (without PSI), also accumulate and are very abundant in long-term iron-depleted cells (Yeremenko et al., 2004). These IsiA aggregates, in vitro, are in a strongly quenched state, suggesting that they are responsible for thermal dissipation of absorbed energy (Ihalainen et al., 2005). In cells grown under high light conditions, IsiA is also synthesized to protect the cyanobacterial cells from photodestruction (Havaux et al., 2005).

In addition to IsiA, the high-light-inducible proteins (HLIPs; also called SCPs) seem to protect the cells by heat dissipation of the absorbed energy, and mutants lacking these proteins are more sensitive to high light conditions (He et al., 2001; Havaux et al., 2003). The HLIPs that are synthesized in cyanobacterial cells grown under high-light conditions and other stress conditions (Dolganov et al., 1995; Funk and Vermaas, 1999; He et al., 2001) are chlorophyll binding single-helix polypeptides related to the LHC proteins and to the early-light-inducible proteins (Adamska, 1997, 2001).

Our recent results demonstrating the existence of the phycobilisome OCP-related NPQ mechanism in cyanobacteria grown in the presence of iron (Wilson et al., 2006) led us to consider the possibility that this mechanism could also play a role in the (blue) light-induced fluorescence quenching observed under iron starvation conditions. To date, only IsiA had been implicated in mediating this process. To test this idea, we studied light-induced fluorescence quenching in several iron-starved *Synechocystis* PCC 6803 mutants: a mutant without IsiA ( $\Delta$ IsiA), a mutant without OCP ( $\Delta$ OCP), and a mutant without phycobilisomes (PAL). Indeed, our results demonstrate that under iron starvation the blue-green light-induced NPQ mechanism is related to the OCP-mediated quenching of the phycobilisome emission and not to modifications of IsiA. Even though the presence of the IsiA protein protects the cells from stress, this protein is not involved in the light-induced NPQ mechanism.

## RESULTS

### Pigment Changes during Iron Starvation in Wild-Type, $\Delta$ IsiA, and $\Delta$ OCP Mutant Cells

Cyanobacteria grown under iron-deficient conditions exhibit decreased chlorophyll and PC content (Öquist, 1971, 1974a;

The 77K fluorescence spectra of wild-type (**[A]** and **[C]**) and  $\Delta$ IsiA (**[B]** and **[D]**) cells grown in iron-containing medium (dotted line) or in iron-lacking medium for 7 (green), 10 (red), 12 (blue), and 14 (black) d. The excitation wavelength was 430 (**[A]** and **[B]**) or 600 nm (**[C]** and **[D]**). Each spectrum shown is the mean of four spectra. The 77K fluorescence spectra were normalized to the fluorescence emitted at 800 nm. The figure shows a representative iron starvation experiment; the experiments consistently showed similar fluorescence changes and kinetics. The cells were at 3  $\mu$ g chlorophyll/mL.

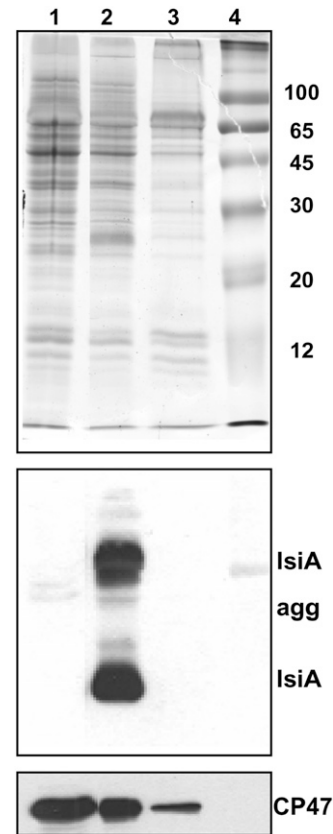
Guikema and Sherman, 1983; Sandmann, 1985). These changes are systemic; the levels of PSI, PSII (Spiller and Terry, 1980; Guikema and Sherman, 1983), and thylakoid (Sherman and Sherman, 1983) all decrease during iron starvation, with the decrease in PSI being the most marked (Guikema and Sherman, 1983; Sandmann, 1985). The presence of IsiA causes a blue shift in the room temperature chlorophyll *a* absorbance peak (680 to 673 nm), and the chlorophyll *a* fluorescence at 77K becomes dominated by a high emission at 685 nm (Öquist, 1974b; Burnap et al., 1993; Falk et al., 1995; Park et al., 1999). Odom et al. (1993) were the first to describe these changes in iron-starved *Synechocystis* PCC 6803.

Under experimental conditions of iron starvation and low-light intensities (see Methods), during the first 6 d, the content of chlorophyll per cell and the PC/chlorophyll ratio remained similar to those in unstarved wild-type,  $\Delta$ OCP, and  $\Delta$ IsiA *Synechocystis* PCC 6803 cells (Figure 1). Subsequently, the chlorophyll content of the wild-type and  $\Delta$ OCP cells decreased; the maximum of the chlorophyll absorbance peak at 683 nm down-shifted until it reached 674 nm after 10 d of starvation (Figures 1A and 1B). In  $\Delta$ IsiA cells, no shift of the chlorophyll-related peak was observed, and the chlorophyll content per cell decreased faster than in wild-type cells (Figures 1C and 1D). Even though the PC content per cell also decreased, an increase of the PC/chlorophyll ratio was observed in  $\Delta$ IsiA cells (Figure 1C), while in wild-type and  $\Delta$ OCP cells, the PC/chlorophyll ratio remained almost constant (Figures 1A and 1B). After 20 d of iron starvation, the  $\Delta$ IsiA contained almost no chlorophyll and the cells were dead (Figure 1D). By contrast, wild-type and  $\Delta$ OCP cells containing a low content of chlorophyll and a high content of carotenoids continued to survive and were still alive after 50 d of iron starvation (see Supplemental Figure 4 online).

### Fluorescence Changes during Iron Starvation in Wild-Type and $\Delta$ IsiA Cells

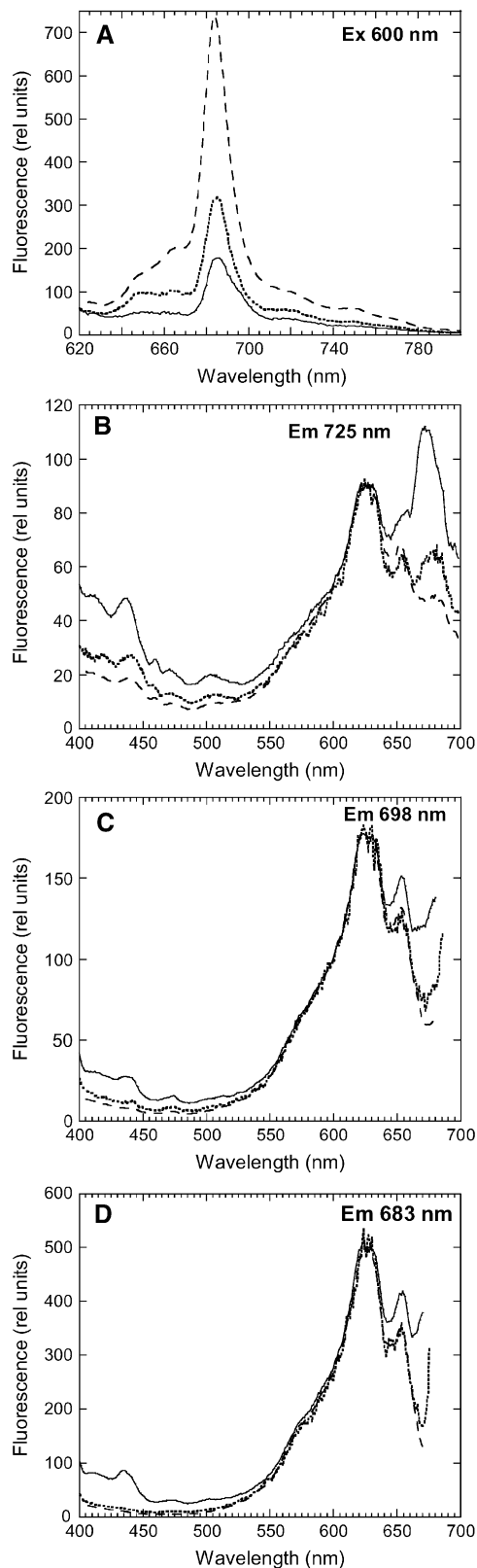
Figure 2 shows the 77K fluorescence emission spectra of wild-type and  $\Delta$ IsiA cells grown in complete medium or medium lacking iron for 7, 10, 12, and 14 d. Fluorescence excitation experiments using 430-nm light, which is preferentially absorbed by chlorophyll, and 600-nm light, which is preferentially absorbed by the phycobilisomes, were used to monitor the changes in the photosynthetic apparatus induced by iron starvation. When wild-type and  $\Delta$ IsiA cells grown in iron-containing medium were excited at 430 nm, the 77K fluorescence spectra showed bands at 685 and 695 nm (related to the CP43 and the CP47 chlorophyll antenna of PSII, respectively; Van Dorssen et al., 1987; Siefermann-Harms, 1988), and a large PSI chlorophyll-related band at 725 nm (Figures 2A and 2B). In the early phases of iron starvation, wild-type and  $\Delta$ IsiA cells showed an increase in the ratio of PSII fluorescence (685- and 695-nm peaks) to PSI fluorescence, suggesting an increased PSII/PSI ratio (Figures 2A and 2B). This is in accordance with the observation that the content of PSI, which contains three 4Fe-4S centers, decreases faster than that of PSII, which contains only three iron sulfur centers (Guikema and Sherman, 1983; Sandmann, 1985; Falk et al., 1995; Ivanov et al., 2000).

After prolonged (10 to 14 d) iron starvation, both strains showed a more rapid increase in the emission at 685 nm relative to that at 695 nm (Figures 2A and 2B). This was particularly marked in wild-type cells in which the 685-nm fluorescence emission reflects the presence of PSI-less IsiA aggregates (Yeremenko et al., 2004). PSI-IsiA supercomplexes have a very small 686-nm emission that makes a negligible contribution to this band (Andrizhiyevskaya et al., 2002; Yeremenko et al., 2004). The presence of IsiA and IsiA aggregates was confirmed by protein gel blot experiments (Figure 3). IsiA was present in 10 d iron-starved wild-type cells, but it was absent in wild-type cells grown in the presence of iron and in iron-starved  $\Delta$ IsiA cells. The amount of CP47 (a measure of PSII) was increased in cells under iron-starved conditions relative to cells grown in the presence of iron (Figure 3). In iron-starved  $\Delta$ IsiA cells, the amount of CP47 was even slightly higher than in wild-type cells. These results were in accordance with the increased PSII/PSI ratio deduced



**Figure 3.** Immunodetection of IsiA and CP47 in Membranes Isolated from Nonstarved and Starved *Synechocystis* Wild-Type and Mutant Cells.

Coomassie blue-stained gel electrophoresis and immunoblot detection of IsiA and CP47 in the membrane fractions isolated from 10 d iron-starved  $\Delta$ IsiA (lane 1) and wild-type (lane 2) cells and nonstarved wild-type cells (lane 3). The IsiA antibody also reacts with IsiA aggregates (IsiA agg). Lane 4, molecular mass markers (in kilodaltons). Each sample contained 1  $\mu$ g of chlorophyll.



**Figure 4.** The 77K Excitation Fluorescence Spectra of Iron-Starved Wild-Type and  $\Delta$ IsiA Cells.

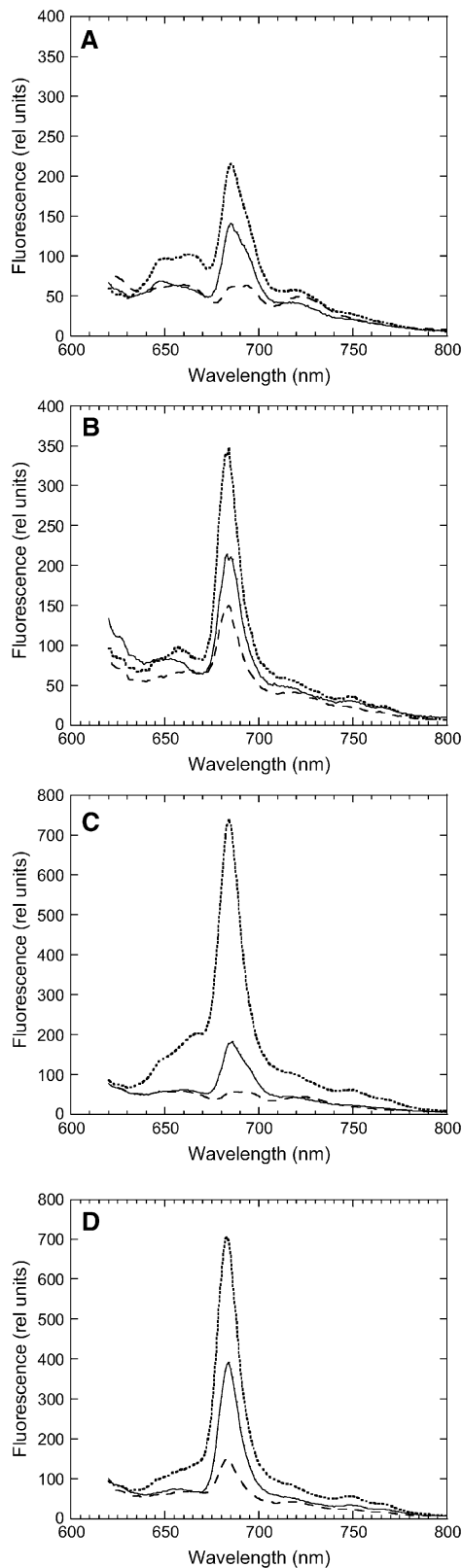
from fluorescence spectra and variable fluorescence measurements (see below). Dühring et al. (2006), using Blue-Native PAGE gels, has also shown that in  $\Delta$ IsiA iron-starved cells there is a higher concentration of PSII dimer and monomer than in wild-type iron-starved cells.

In iron-starved  $\Delta$ IsiA cells, the increase of the 685-nm emission is evidently not associated with IsiA; instead, the source of this emission may be the phycobilisome terminal emitter, the Lcm, in disconnected phycobilisomes (see Supplemental Figure 1 online). In long-term iron-starved wild-type cells, a contribution of the emission from Lcm of uncoupled phycobilisomes may also be present.

In cyanobacteria, the fluorescence spectrum generated at 77K by 600-nm excitation contains emission bands related to PC (650 nm), APC (660 nm), PSII (685 and 695 nm), and PSI (725 nm) (Figures 2C and 2D). The peak at 695 nm derives from the chlorophyll *a* emission of the PSII antenna CP47 and the reaction center. The emission at 685 nm is principally related to the phycobilisome terminal emitter, but fluorescence emission from the CP43 PSII antenna also contributes to this peak. When empty (without PSI) IsiA complexes are present, their fluorescence emission significantly contributes to the 685-nm peak. In the fluorescence spectra, the first manifestation of iron starvation in wild-type and  $\Delta$ IsiA cells was an increase in the ratio of PSII fluorescence (685- and 695-nm peaks) to PSI fluorescence (Figures 2C and 2D). Extended iron starvation resulted in a large increase of the 685-nm peak. This increase was faster and more pronounced in  $\Delta$ IsiA cells than in wild-type cells (Figures 2C and 2D). The origin of this increase was different in each strain. In  $\Delta$ IsiA cells, this increase can only be related to the phycobilisome terminal emitter, reflecting an accumulation of functionally disconnected, high fluorescent phycobilisomes. In wild-type cells, the increase could be attributed to IsiA emission (Yeremenko et al., 2004) due to energy transfer from the phycobilisomes to IsiA complexes, as suggested by Joshua et al. (2005), and to a small population of uncoupled phycobilisomes in long-term iron-starved cells.

This suggestion was confirmed by comparison of 77K excitation fluorescence spectra of the emissions at 725 (PSI emission), 698 (PSII emission), and 683 nm (IsiA aggregates and phycobilisome emission) in iron-starved wild-type and iron-starved  $\Delta$ IsiA cells. Isolated phycobilisomes also have a relatively high emission at 698 and 725 nm (see Supplemental Figure 2 online). In iron-starved wild-type cells, the spectra showed higher contributions from chlorophyll *a* (peaks at around 435 and 680 nm) and smaller contributions from components at 570, 620 (from PC), and 650 nm (from APC) than in iron-starved  $\Delta$ IsiA cells (Figure 4). Moreover, the relative contribution of PC to the spectra increased with the increase of the 685-nm emission peak in iron-starved cells. These results indicated that in  $\Delta$ IsiA cells, a large

The 77K fluorescence emission spectra (Ex 600 nm) of 11 d iron-starved wild-type cells (solid line) and 11 d (dotted line) and 15 d (dashed line) iron-starved  $\Delta$ IsiA cells (**A**). The 77K fluorescence excitation spectra for an emission at 725 (**B**), 698 (**C**), and 683 nm (**D**) of 11 d iron-starved wild-type cells (solid line) and 11 d (dotted line) and 15 d (dashed line) iron-starved  $\Delta$ IsiA cells. The cells were at  $2 \mu\text{g}$  chlorophyll/mL.



**Figure 5.** The 77K Fluorescence Emission Spectra of Iron-Starved Wild-Type and  $\Delta$ IsiA MP Fractions.

quantity of functionally disconnected phycobilisomes was present. The spectra also strongly suggest that in iron-starved wild-type cells, most of the phycobilisomes transferred the absorbed energy not only to the photosystems but also to IsiA complexes (see Supplemental Figure 2 online).

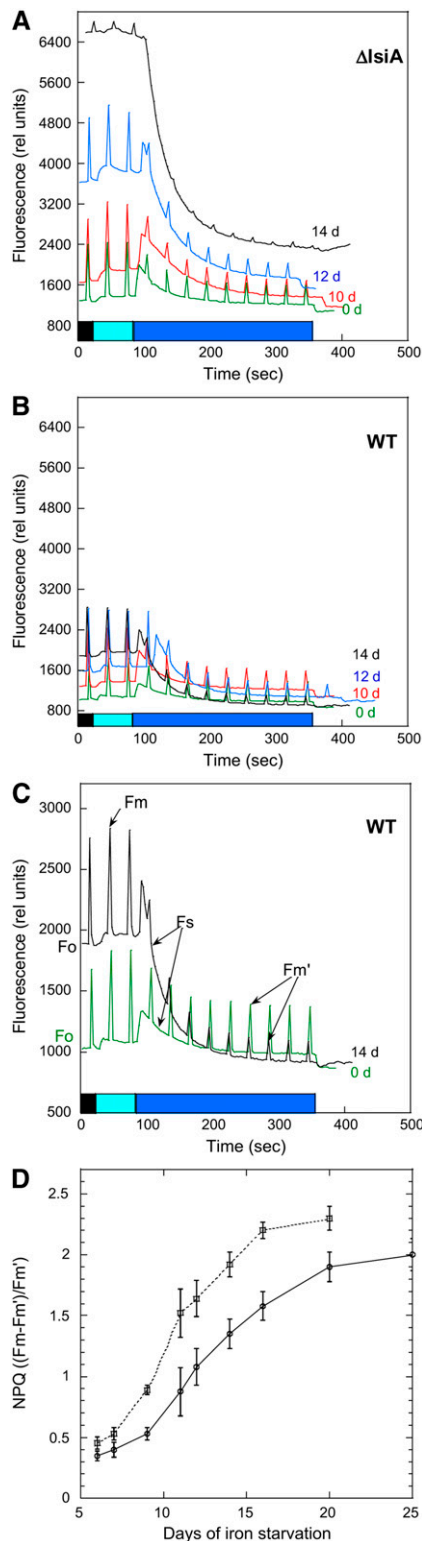
Phycobilisome-associated membrane fractions (MPs) from nonstarved and 11 or 15 d iron-starved wild-type and  $\Delta$ IsiA cells were prepared to elucidate if the functionally disconnected phycobilisomes remained associated with the thylakoids. The cells were broken in a phosphate/citrate buffer. After discarding unbroken cells, the MPs were obtained by centrifugation. In all cases, the supernatant was nearly colorless, indicating that the bulk of the phycobilisomes remained attached to the thylakoids. This was confirmed by absorption spectra (see Supplemental Figure 3 online). The PC/chlorophyll ratio was higher in  $\Delta$ IsiA MP compared with that of MP from wild-type cells. Fifteen days iron-starved  $\Delta$ IsiA cells and MPs showed high and similar fluorescence at 685 nm. The magnitude of this fluorescence suggested that already in whole cells a large quantity of phycobilisomes (perhaps nearly all) were functionally disconnected. The rest of the MP preparations produced a larger emission at 685 nm than whole cells, suggesting a compromised connection between the phycobilisomes and the thylakoids *in vitro* (Figure 5). Nevertheless, as in whole cells,  $\Delta$ IsiA MPs presented a larger 685-nm emission than wild-type MPs (Figures 5B and 5D). Thus, we conclude that in  $\Delta$ IsiA iron-starved cells, all the phycobilisomes were attached to the thylakoids as in wild-type cells, but a larger number were functionally disconnected; they did not transfer energy to any chlorophyll complex.

#### Fluorescence Quenching in Iron-Starved Wild-Type and $\Delta$ IsiA Cells

The induction of the blue light-induced fluorescence quenching was monitored using a pulse-amplitude modulated fluorimeter (PAM). In cyanobacteria, the fluorescence detected by a PAM fluorimeter is emitted from chlorophyll and phycobiliproteins (Campbell et al., 1998). In the PAM fluorimeter, the measuring light has a maximum of excitation at 650 nm, and the fluorescence is detected at wavelengths beyond 700 nm. In cyanobacteria, which lack chlorophyll *b*, most of the measuring light is absorbed by the phycobilisomes. Thus,  $F_0$ , the minimal fluorescence level in dark-adapted cells, varies according to the cellular phycobiliprotein concentration (e.g., very low in mutants without phycobiliproteins) (Campbell et al., 1998; El Bissati and Kirilovsky, 2001). The  $F_0$  level also depends on the coupling of phycobilisomes: energetically coupled phycobilisomes show

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The 77K fluorescence spectra of nonstarved (dashed line), 12 d iron-starved wild-type (solid line; **A**), and iron-starved  $\Delta$ IsiA (dotted line; **A**) cells, 15 d iron-starved wild-type cells (solid line; **C**), and iron-starved  $\Delta$ IsiA (dotted line; **C**) cells and of the corresponding MP fractions (**B**), wild-type; **D**),  $\Delta$ IsiA). The 77K fluorescence emission spectra were normalized to the fluorescence emitted at 800 nm. The cells were at 2  $\mu$ g chlorophyll/mL.



**Figure 6.** Blue-Green Light-Induced Fluorescence Quenching in Iron-Starved Wild-Type and  $\Delta$ IsiA Cells.

**(A)** to **(C)** The 0 d (green), 7 d (data not shown; similar to 0 d), 10 d (red), 12 d (blue), and 14 d (black) iron-starved  $\Delta$ IsiA **(A)** and wild-type **(B)** and

low-yield fluorescence emission, while uncoupled phycobilisomes show high-yield fluorescence emission. Figures 6A to 6C compare the room temperature fluorescence traces measured with a PAM fluorometer after 0, 7, 10, 12, and 14 d of iron starvation from wild-type and  $\Delta$ IsiA cells. Dark-adapted cells were successively illuminated by dim and strong blue-green light. The cells under dim blue-green light, which preferentially excites PSI, showed a high level of fluorescence characteristic of State 1, which is induced by oxidation of the plastoquinone pool upon illumination of dark-adapted cells. Subsequently, exposure of cells to strong blue-green light induced the quenching of all levels of fluorescence ( $F_m'$ ,  $F_s$ , and  $F_o$ ) in both wild-type and  $\Delta$ IsiA cells. The fluorescence quenching increased as iron starvation was prolonged. Figure 6D shows the increase of NPQ [( $F_m - F_m'$ )/ $F_m'$ ] values during iron starvation in wild-type and  $\Delta$ IsiA cells. This increase was faster and more marked in  $\Delta$ IsiA cells.

We also observed that after 6 to 7 d of iron starvation, differences in  $F_o$  values and  $F_v/F_o$  or  $F_v/F_m$  ratios appeared. First, in both strains at equimolar chlorophyll concentrations,  $F_o$  and  $F_v$  increased in parallel, and the ratios  $F_v/F_o$  and  $F_v/F_m$  remained high (even slightly higher than in nonstarved cells) (Figure 6, Table 1), suggesting that the increase of  $F_o$  and  $F_v$  was due to an increase in the PSII/PSI ratio, in agreement with the 77K fluorescence spectra and the observation that the per cell PSI content decreased faster than PSII content. With prolonged iron starvation,  $F_v$  began to decrease while  $F_o$  continued to increase, indicating a loss of active PSII (Figure 6, Table 1). In  $\Delta$ IsiA cells, this process was faster. After 14 d of Fe starvation,  $F_v$  was very small, while it was still relatively large in wild-type cells (Figure 6, Table 1). Wild-type cells were still alive after 48 d of iron starvation and had partially recovered the lost  $F_v$ , while  $\Delta$ IsiA cells died after 18 d of iron starvation (see Supplemental Figure 4 online).

In  $\Delta$ IsiA cells, the large increase of  $F_o$  can be explained by a rapid increase of a population of energetically uncoupled phycobilisomes detected in 77K fluorescence spectra. In wild-type cells, the increase of  $F_o$  could be related not only to an increase of fluorescence emitted by the uncoupled phycobilisome population but also to fluorescence emitted by IsiA complexes. In wild-type cells, the presence of IsiA protects the cell (previously shown in Park et al., 1999; Sandström et al., 2001; Havaux et al., 2005) from the stress generated by iron starvation (Havaux et al.,

**(C)** cells (at 3  $\mu$ g chlorophyll/mL) were dark-adapted and then illuminated successively with low-intensity blue-green light (400 to 550 nm, 80  $\mu$ mol photons  $m^{-2} s^{-1}$ ) and high-intensity blue-green light (740  $\mu$ mol photons  $m^{-2} s^{-1}$ ). Saturating pulses were applied to measure maximal fluorescence levels.  $F_m$ , maximal fluorescence under low intensities of blue light;  $F_m'$ , maximal fluorescence under high intensities of blue light;  $F_s$ , steady state fluorescence;  $F_o$ , minimal fluorescence (see **(C)**). In **(C)**, the changes of fluorescence traces in 7 and 14 d iron-starved wild-type cells are shown with a different scale than **(A)** and **(B)** to clarify the differences in fluorescence quenching.

**(D)** Increase of NPQ [( $F_m - F_m'$ )/ $F_m'$ ] during iron starvation of wild-type (circles and solid line) and  $\Delta$ IsiA (squares and dotted line) cells. The graph is the average of four independent experiments. Error bars show the maximum and minimum NPQ values for each point.

**Table 1.** Changes in Fluorescence Levels during Iron Starvation in Wild-Type and  $\Delta$ IsiA Cells

	0 d Wild- Type/ $\Delta$ IsiA	7 d Wild- Type/ $\Delta$ IsiA	10 d Wild- Type/ $\Delta$ IsiA	12 d Wild- Type/ $\Delta$ IsiA	14 d Wild- Type/ $\Delta$ IsiA	19 d Wild- Type/ $\Delta$ IsiA
Fo (% $\pm$ 10%)	100/100	100/105	125/160	154/350	180/642	260/–
Fv (% $\pm$ 10%)	100/100	100/147	140/187	148/183	120/27	30/–
Fv/Fo ( $\pm$ 0.05)	0.78/0.78	0.76/1.1	0.80/0.95	0.74/0.4	0.5/0.035	0.09/–
Fv/Fm ( $\pm$ 0.05)	0.44/0.44	0.43/0.50	0.47/0.47	0.43/0.3	0.33/0.03	0.085/–

Each result is the average of four independent iron starvation experiments.

2005; Latifi et al., 2005). Alone, in  $\Delta$ IsiA cells, the blue light-induced NPQ mechanism seems to be insufficient protection from this specific stress.

When the iron-starved, quenched, wild-type, and  $\Delta$ IsiA cells were exposed to dim blue-green light in the presence of chloramphenicol, an inhibitor of protein synthesis, they recovered their maximal level of Fm' (Figure 7). These results confirmed that the blue-green light-induced fluorescence quenching (in both iron-starved strains) was not related to photoinhibition/D1 damage. Similar effects were seen in comparable experiments in wild-type cells grown in the presence of iron (El Bissati et al., 2000; Wilson et al., 2006).

#### Phycobilisome or Chlorophyll Emission Quenching?

As stated above, a decrease in the fluorescence levels observed in a PAM fluorometer could be a result of (1) a diminution of the phycobilisome emission, (2) a decrease in the chlorophyll antenna emission, or (3) a decrease of energy transfer from the phycobilisomes to PSII. We have already demonstrated that in *Synechocystis* PCC 6803 grown in iron-containing medium, the blue-green light-induced fluorescence decrease observed in the PAM fluorometer is due to the quenching of the phycobilisome fluorescence emission and a concomitant decrease in the energy transfer from the phycobilisomes to the photosystems (Wilson et al., 2006).

Room temperature fluorescence spectra were used to elucidate the origin of the fluorescence quenching in iron-starved cells. When cells iron-starved for 12 d were excited at 600 nm (light principally absorbed by phycobilisomes), the peak at 660 nm (phycobilisome-related) was more pronounced in iron-starved  $\Delta$ IsiA cells than in iron-starved wild-type cells (Figures 8A and 8B). The large 660-nm fluorescence in  $\Delta$ IsiA cells is most probably the result of disconnected phycobilisomes (see also Figure 2D). In iron-starved wild-type cells, an increase of the shoulder at 680 nm (chlorophyll related) compared with non-starved cells was observed (see Supplemental Figure 5 online). This was attributed to the presence of IsiA and to energy transfer from the phycobilisomes to IsiA. In iron-starved wild-type and  $\Delta$ IsiA cells that were illuminated with strong blue-green light for 5 min (quenched cells), a very large decrease of the 660-nm band was observed (Figures 8A and 8B). Figure 8C shows that in wild-type cells iron-starved for an extended period (48 d), the 660-nm fluorescence emission increased and the fluorescence quenching was larger.

When iron-starved wild-type cells were excited at 430 nm (light principally absorbed by chlorophyll), a large band at 680 nm was

observed, corresponding to the accumulation of IsiA complexes. This chlorophyll-related band was not decreased after illumination of iron-starved wild-type cells with strong blue light (Figures 8D, 12 d, and 8F, 48 d). In iron-starved  $\Delta$ IsiA cells, even when the cells were excited at 430 nm, a relatively large emission at 660 nm (a phycobilisome related band) was observed (Figure 8E; see Supplemental Figure 5 online). A decrease of this emission was induced by strong blue-green light illumination. In conclusion, room temperature fluorescence spectra of unquenched and quenched iron-starved wild-type and  $\Delta$ IsiA cells strongly suggested that under iron starvation conditions the blue-green-induced fluorescence quenching was attributable to a decrease of phycobilisome fluorescence emission and a concomitant decrease in the energy transfer from the phycobilisome to the photosystems and to IsiA (in the wild type).

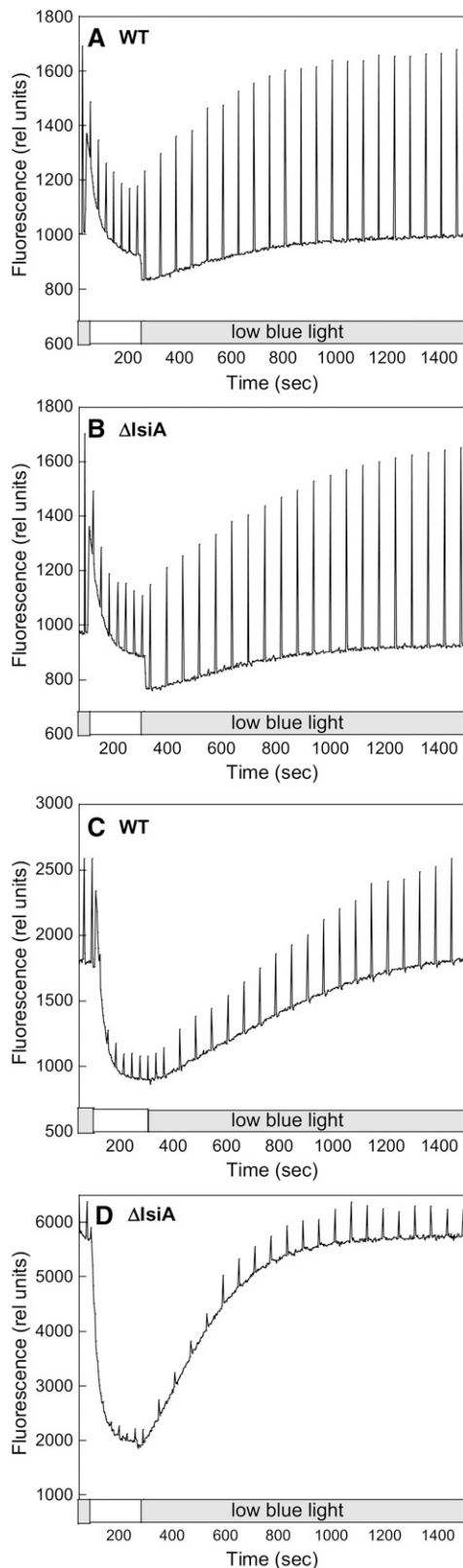
To elucidate if there was a relationship between the larger NPQ in iron-starved cells relative to nonstarved cells and to the quantity of OCP present, protein gel blot analyses were undertaken. Total cellular proteins or MP proteins were separated by SDS-PAGE, and the OCP was detected by an anti-OCP antibody (Figure 9). The comparison was done on a per chlorophyll basis because all the fluorescence experiments were performed at the same chlorophyll concentration. The antibody reacts with a 35-kD polypeptide absent in the  $\Delta$ OCP mutant. The immunoreaction was more pronounced in iron-starved cells and their MP fractions than in non-iron-starved cells and their MP fractions (per chlorophyll). Moreover, there was more OCP in  $\Delta$ IsiA iron-starved cells and MP fractions (per chlorophyll) than in wild-type iron-starved cells and MP fractions (Figure 9).

#### Mutant Strains Lacking Phycobilisomes or OCP

To further characterize the light-induced NPQ generated under iron starvation conditions, two additional mutants were studied: one lacking phycobilisomes (PAL mutant,  $\Delta$ apcAB,  $\Delta$ apcE, and PC<sup>-</sup>) (Ajani and Vernotte, 1998) and a second lacking the OCP, the protein essential for the induction of the phycobilisome-related NPQ under normal growth conditions ( $\Delta$ OCP mutant) (Wilson et al., 2006).

In the PAL mutant, the PSII/PSI ratio is higher than in wild-type cells (Ajani and Vernotte, 1998). This is reflected in the 77K fluorescence emission spectra obtained by 430-nm excitation: the 685- and 695-nm fluorescence emission bands are larger in PAL than in wild-type cells (cf. Figures 10A and 2A). After only 7 d of starvation, an increase of the 685-nm emission was observed; after 10 d of iron starvation, a sharp peak at 685 nm, characteristic of IsiA, dominates the 77K fluorescence spectra (Figure 10A).





**Figure 7.** Blue Light-Induced Quenching in Iron-Starved Wild-Type and  $\Delta$ IsiA Cells Is Reversible without Protein Synthesis.

In PAL cells grown in iron-containing medium, high intensities of blue-green light were unable to induce any fluorescence quenching (Wilson et al., 2006; Figure 10B). Figure 10B shows that this is also the case with PAL cells grown under iron starvation conditions. Since OCP is present in the PAL mutant (Figure 9), the lack of fluorescence quenching is due to the lack of phycobilisomes.

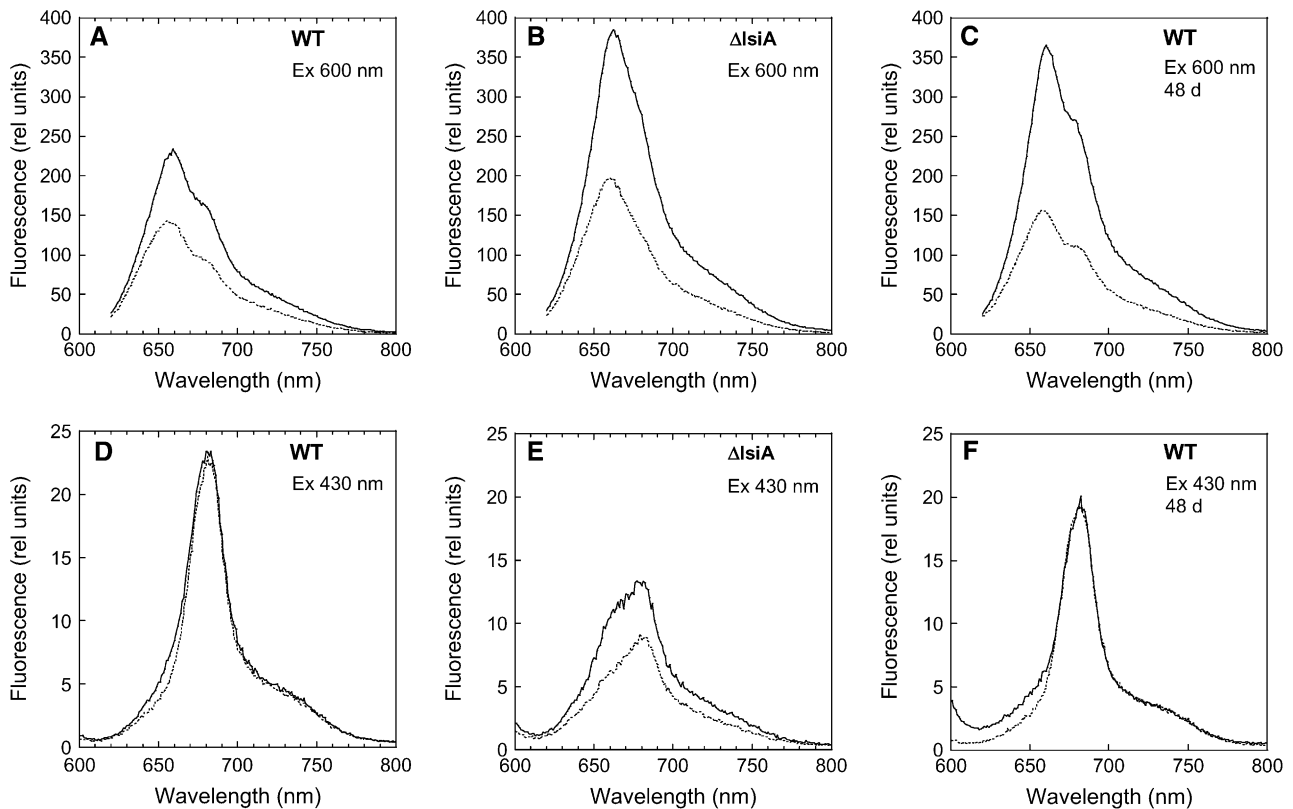
As with iron-starved wild-type cells, PAL cells showed changes in chlorophyll content and  $F_o$  and  $F_v$  values during iron starvation. During the first days of iron starvation, the chlorophyll content decreased to 60% of that of nonstarved cells and then it remained constant. During this time,  $F_v$  increased 1.5 times compared with nonstarved PAL cells, suggesting a larger number of active PSII centers on a chlorophyll basis (Figures 10B and 10C). The increase of  $F_o$  was higher (2.2 times), suggesting that part of the  $F_o$  increase was due to fluorescence emitted by IsiA complexes. As the duration of iron starvation increased,  $F_o$  remained almost constant and  $F_v$  slowly decreased to a slightly slower value than that in nonstarved cells (Figure 10C). The cells were still alive after 1 month of iron starvation.

The chlorophyll content and the PC/chlorophyll ratio of  $\Delta$ OCP cells under iron starvation conditions were similar to that of wild-type cells (Figures 1A and 1B). Likewise,  $F_o$  and  $F_v$  values were similar in wild-type and  $\Delta$ OCP cells grown in iron-containing medium. However, in general, the  $F_o$  level was higher in the wild-type than in  $\Delta$ OCP iron-starved cells, while the  $F_v$  value was larger in  $\Delta$ OCP than in wild-type cells (Figures 11A and 11B; see Supplemental Figure 4 online).

When the cells were excited at 600 nm, the emission peak at 685 nm was consistently smaller in  $\Delta$ OCP relative to wild-type cells, suggesting a smaller quantity of uncoupled phycobilisomes in the  $\Delta$ OCP iron-starved cells (Figure 11E). This was confirmed by comparison of 77K excitation fluorescence spectra of the emission at 685 nm in iron-starved wild-type and iron-starved  $\Delta$ OCP cells. In iron-starved  $\Delta$ OCP cells, the spectrum showed higher contributions from chlorophyll *a* (peaks at  $\sim$ 435 nm) and smaller contributions from components at 570, 620 (from PC), and 650 nm (from APC) than in iron-starved wild-type cells (Figure 11F). In iron-starved PAL cells, as expected, the major contribution to the excitation spectrum came from chlorophyll *a* (435 nm) (Figure 11F).

In general, fluorescence emission spectra at 77K using 430-nm excitation showed a larger 685-nm peak, suggesting a larger population of IsiA complexes in  $\Delta$ OCP than in wild-type cells (Figure 12A). This was confirmed by protein gel blot experiments (Figure 12B). The iron-starved  $\Delta$ OCP cells contained  $\sim$ 1.4 to 1.6 times more IsiA than iron-starved wild-type cells.

Measurements of fluorescence yield by a PAM fluorometer in iron-starved wild-type ([A] and [C]) and  $\Delta$ IsiA ([B] and [D]) cells for 7 ([A] and [B]) and 14 d ([C] and [D]) at  $3 \mu\text{g}$  chlorophyll/mL illuminated successively with low-intensity blue-green light ( $400$  to  $550 \text{ nm}$ ;  $80 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ ) and high-intensity blue-green light ( $740 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ ) and then again with dim blue-green light. Chloramphenicol was present during all experiments. The figure shows a representative experiment.



**Figure 8.** Room Temperature Fluorescence Spectra of Iron-Starved Unquenched and Quenched Cells.

Room temperature fluorescence spectra of dark-adapted (solid line) 12 d iron-starved wild-type cells (**A**) and (**D**), 48 d iron-starved wild-type cells (**C**) and (**F**), and 12 d iron-starved  $\Delta$ IsiA cells (**B**) and (**E**) and after 5 min of high-intensity blue-green light illumination ( $740 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) (dotted line) at  $3 \mu\text{g}$  chlorophyll/mL. Excitation was performed at 600 nm (**A**) to (**C**) and at 430 nm (**D**) to (**F**).

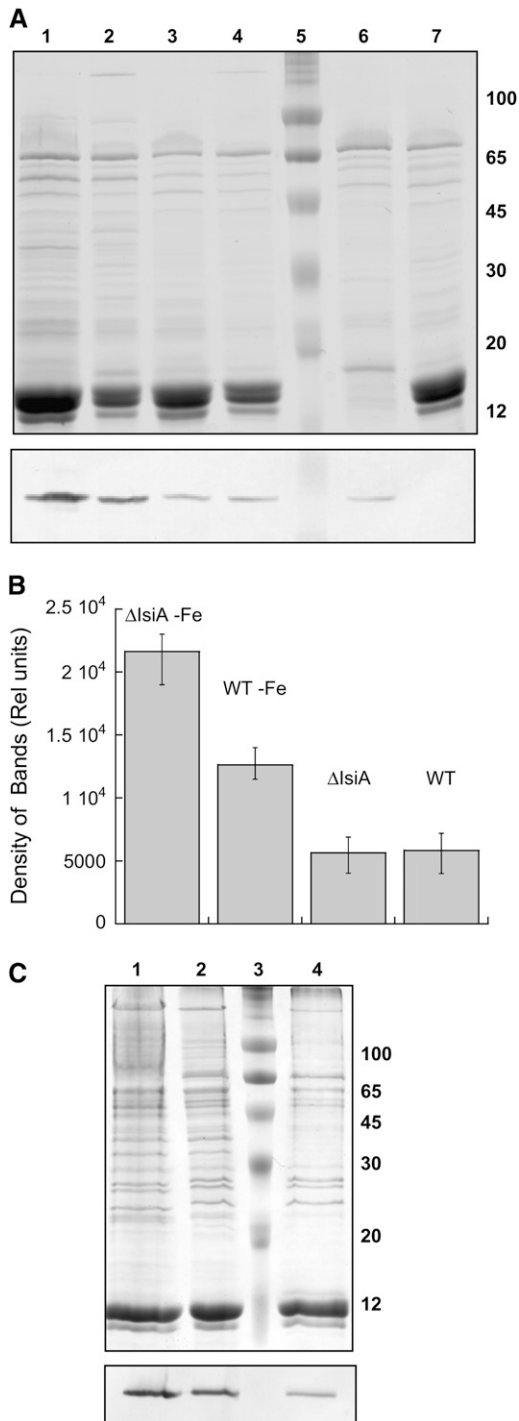
Figures 11A and 11B show the fluorescence traces in 14 d iron-starved wild-type and  $\Delta$ OCP cells. In the absence of the OCP, blue-green light did not induce any fluorescence quenching (Figure 11B). Room temperature fluorescence spectra confirmed the absence of blue-green light-induced fluorescence quenching (Figures 11C and 11D). Figure 11D clearly demonstrates that blue light did not induce any quenching of chlorophyll fluorescence, which in the iron-starved  $\Delta$ OCP cells is principally related to IsiA aggregates. In conclusion, under low light growth conditions, the presence of IsiA was sufficient to protect the  $\Delta$ OCP cells, despite the absence of the blue light-induced NPQ mechanism.

## DISCUSSION

When cyanobacteria grow under iron-limited conditions, the expression of IsiA, encoded by the *isiA* gene (Laudenbach and Straus, 1988), is synthesized in abundance. IsiA, which is a chlorophyll binding protein closely related to CP43, acts as an LHC of PSI under iron-deficient conditions (Bibby et al., 2001; Boekema et al., 2001). On the basis of results with a  $\Delta$ IsiA of *Synechococcus elongatus* 7942 and a mutant of *Synechococcus elongatus* 7942 overproducing IsiA, a protective function of IsiA

against photoinduced damage was suggested with IsiA acting as a dissipator of energy (Park et al., 1999; Sandström et al., 2001). Subsequent experiments using isolated IsiA complexes showed that these complexes are indeed efficient energy dissipators (Ihalainen et al., 2005).

It was proposed that this protection was associated with a strong blue (or white) light-induced NPQ mechanism: strong light could induce conformational changes of IsiA that transform IsiA complexes into energy dissipators (Cadoret et al., 2004) or modify the affinity of IsiA for phycobilisomes (Joshua et al., 2005). Our results clearly demonstrate that IsiA is not involved in a light-induced NPQ mechanism. In iron-starved cells (just as in iron-containing cells), the blue light-induced fluorescence quenching is associated with the phycobilisomes and with the OCP and not with IsiA. In the  $\Delta$ IsiA mutant, a large reversible fluorescence quenching was always induced by blue light. By contrast, in mutants lacking phycobilisomes (PAL mutant) or OCP ( $\Delta$ OCP mutant), blue light was unable to induce fluorescence quenching, even after very long periods of iron starvation. In addition, in iron-starved wild-type and  $\Delta$ IsiA cells, fluorescence emission spectra showed that no chlorophyll-related fluorescence quenching was induced, while a large decrease of the fluorescence emitted by phycobilisomes and of the energy transfer from phycobilisomes to chlorophyll complexes was detected.



**Figure 9.** OCP Detection in Whole Cells and MP Fractions from Nonstarved and Starved Wild Type and Mutants.

**(A)** Coomassie blue–stained gel electrophoresis and immunoblot detection (bottom panel) of OCP in 12 d iron-starved  $\Delta$ IsiA (lane 1) and wild-type (lane 2) cells and nonstarved  $\Delta$ IsiA (lane 3), wild-type (lane 4), PAL (lane 6), and  $\Delta$ OCP (lane 7) cells. Lane 5 shows molecular mass markers. Each lane contained 1.5  $\mu$ g of chlorophyll.

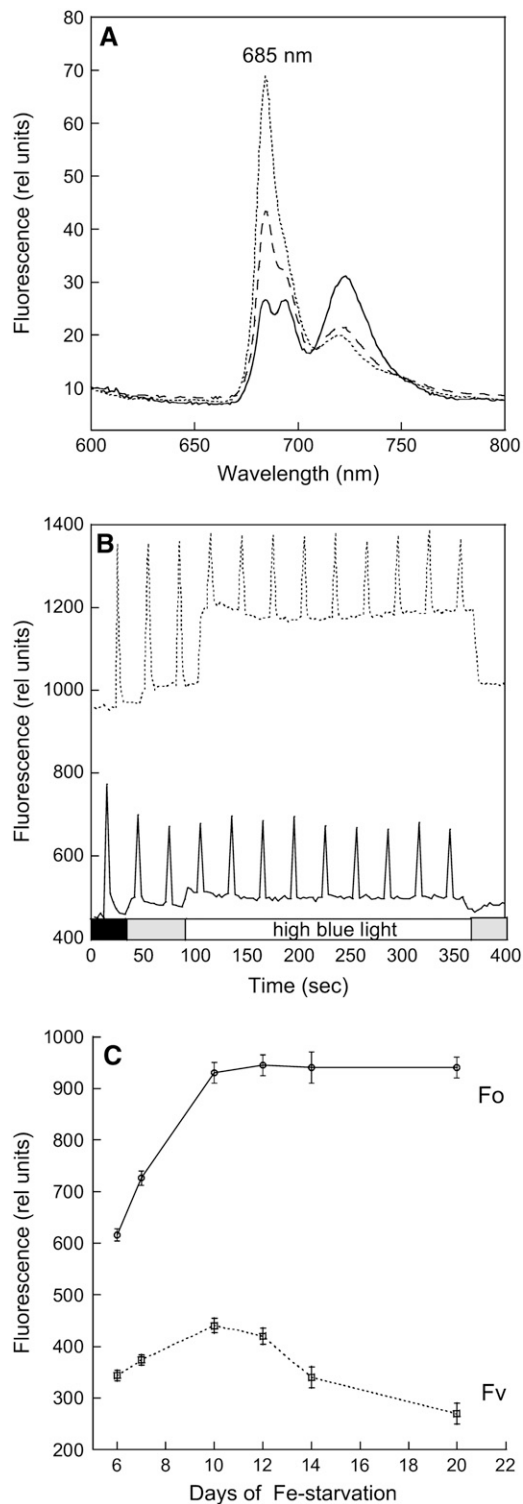
**(B)** Comparative densitometry of OCP bands in nonstarved and iron-

Blue light–induced quenching was observed in wild-type and  $\Delta$ IsiA iron-containing cells (El Bissati et al., 2000; Wilson et al., 2006) but was more marked in iron-starved cells (Cadoret et al., 2004; this article). Moreover, during iron starvation, the increase in fluorescence quenching was faster in  $\Delta$ IsiA cells than in wild-type cells. In iron-starved  $\Delta$ IsiA cells, a rapid increase of the 685-nm peak in 77K fluorescence emission spectra, of the 660-nm peak in room temperature fluorescence spectra, and of the F<sub>o</sub> level indicated a very fast increase of a population of functionally disconnected highly fluorescent phycobilisomes. In wild-type cells, the number of disconnected phycobilisomes also increased, but more slowly than in  $\Delta$ IsiA cells. Thus, our results clearly reveal a relationship between the increase of fluorescence quenching and the number of functionally disconnected phycobilisomes. Cells containing a higher number of disconnected phycobilisomes showed a higher level of thermal energy dissipation (NPQ), thereby protecting themselves by diminishing the energy arriving at the photosystems and the thylakoids. The increased NPQ is associated with a higher concentration of the OCP: a higher concentration of the OCP was observed in iron-starved cells compared with nonstarved cells. The abundance of OCP is even greater in iron-starved  $\Delta$ IsiA cells. The transcription of the *slr1963* gene is known to be increased under other stress conditions: high white light illumination (Hihara et al., 2001), UV-B light (I. Vass, personal communication), and saline stress (Fulda et al., 2006). It is possible that other stresses also upregulate the expression of this gene. It may be that the OCP-related NPQ mechanism plays a significant protective role under a range of stress conditions.

In  $\Delta$ OCP cells under prolonged iron starvation, blue light was unable to induce any fluorescence quenching even in the presence of functionally disconnected phycobilisomes and empty IsiA complexes. Thus, the OCP is essential for the NPQ occurring under iron starvation conditions. Interestingly, in iron-starved  $\Delta$ OCP cells, the increase in the amount of uncoupled phycobilisomes and the decrease of F<sub>v</sub> were slower than in wild-type cells. This appears to be the result of a higher concentration of IsiA. Increased IsiA concentration could be explained by the fact that  $\Delta$ OCP cells, being more sensitive to light, are permanently under a greater oxidative stress than wild-type cells. It has already been demonstrated that oxidative stress induces *isiA* transcript accumulation (Jeanjean et al., 2003; Yousef et al., 2003). Singh et al. (2005) have shown that  $\Delta$ IsiA cells were slightly more resistant to the presence of H<sub>2</sub>O<sub>2</sub> than wild-type cells. They proposed that this higher resistance is related to the greater transcription induction of a gene cluster, including a gene encoding a peroxiredoxin that is involved in the detoxification of

starved wild-type and  $\Delta$ IsiA whole cells. The results represent the average of four independent experiments. Error bars show the maximum and minimum density of band values for each point.

**(C)** Coomassie blue–stained gel electrophoresis and immunoblot detection (bottom panel) of the OCP in MP fractions isolated from 12 d iron-starved  $\Delta$ IsiA (lane 1) and wild-type (lane 2) cells and nonstarved wild-type cells (lane 4). Lane 3 shows molecular mass markers. Each lane contained 1  $\mu$ g of chlorophyll.



**Figure 10.** Fluorescence Changes in Iron-Starved PAL Cells.

**(A)** The 77K fluorescence spectra of iron-containing (solid line) and iron-starved PAL cells for 7 (dashed line) and 10 d (dotted line). The excitation wavelength was 430 nm.

**(B)** Dark-adapted iron-containing (solid line) and 12 d iron-starved PAL

peroxide. Thus, under stress conditions,  $\Delta$ OCP cells can synthesize not only IsiA but also other proteins (e.g., HLIPs, catalases, and peroxidases) to try to compensate for the lack of the phycobilisome-related NPQ mechanism. This could explain why  $\Delta$ OCP cells were slightly more resistant to iron starvation than wild-type cells.

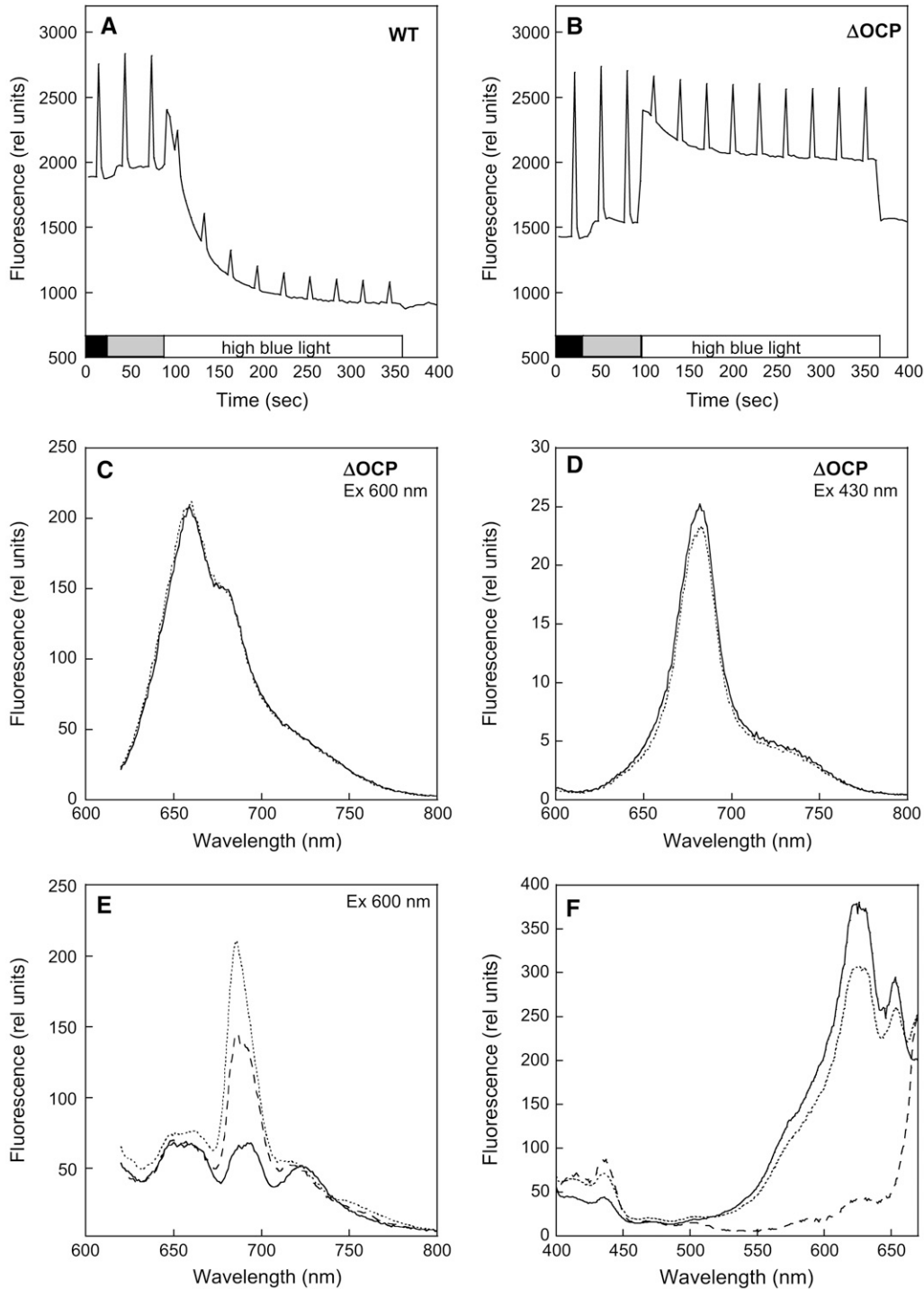
By comparing the effect of iron starvation in  $\Delta$ IsiA cells to that in  $\Delta$ OCP cells, we conclude that even though the phycobilisome-related NPQ mechanism is an important photoprotective process under high light conditions, under iron starvation (under low light conditions), the IsiA-related mechanism provides more effective protection of the cells. Under low light growth conditions, the phycobilisome-related NPQ mechanism is not induced. We have already shown that this mechanism is only induced by high intensities of blue or white light (Wilson et al., 2006). Thus, even if the capacity of induction of the phycobilisome-related NPQ mechanism is increased during iron starvation, this will not protect the cells from the iron starvation stress. However, in the iron-starved cells that are more sensitive to photoinhibition, the synthesis of OCP and the capacity of quenching are increased, providing greater photoprotective capacity under high light.

The blue light-induced fluorescence quenching is clearly not associated with the binding of free phycobilisomes to IsiA or to conformational changes in the IsiA protein that might cause the protein to become a more efficient thermal energy dissipator. Since the absence of IsiA renders *Synechocystis* cells more sensitive to the stress generated by iron starvation and high light (Park et al., 1999; Havaux et al., 2005; Latifi et al., 2005; this article) but does not inhibit the NPQ process, IsiA protects the cells via other mechanism(s). Combining our observations with results presented in the literature, we posit that during the first days of iron starvation, IsiA protects the cells by acting as the LHC of PSI, increasing the cross section of the PSI antenna (Andrizhiyevskaya et al., 2002; Melkozernov et al., 2003). In this way, a higher activity of PSI is maintained and a balance between PSII and PSI activities, decreasing the excitation pressure on PSII and, hence, the oxidative stress. During prolonged iron starvation, empty IsiA complexes may receive the energy coming from phycobilisomes and convert it into heat, thereby decreasing the energy arriving at the PSII reaction centers and at the thylakoids. Isolated IsiA aggregates, which accumulate upon chronic iron starvation, show very short fluorescence lifetimes, indicating that they are good energy dissipators (Ihalainen et al., 2005).

We observed that the increase of the population of highly fluorescent phycobilisomes was inversely proportional to the concentration of empty IsiA complexes (very high in  $\Delta$ IsiA cells and

(dotted line) cells were illuminated successively with low-intensity blue-green light (400 to 550 nm;  $80 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) and high-intensity blue-green light ( $740 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ ).

**(C)** Changes in  $F_o$  and  $F_v$  in PAL cells during iron starvation. Fluorescence yield changes were detected in a PAM fluorometer. The cells were at  $2 \mu\text{g chlorophyll/mL}$ . The results represent the average of three independent experiments. Error bars show the maximum and minimum chlorophyll/OD<sub>800</sub> values for each point.



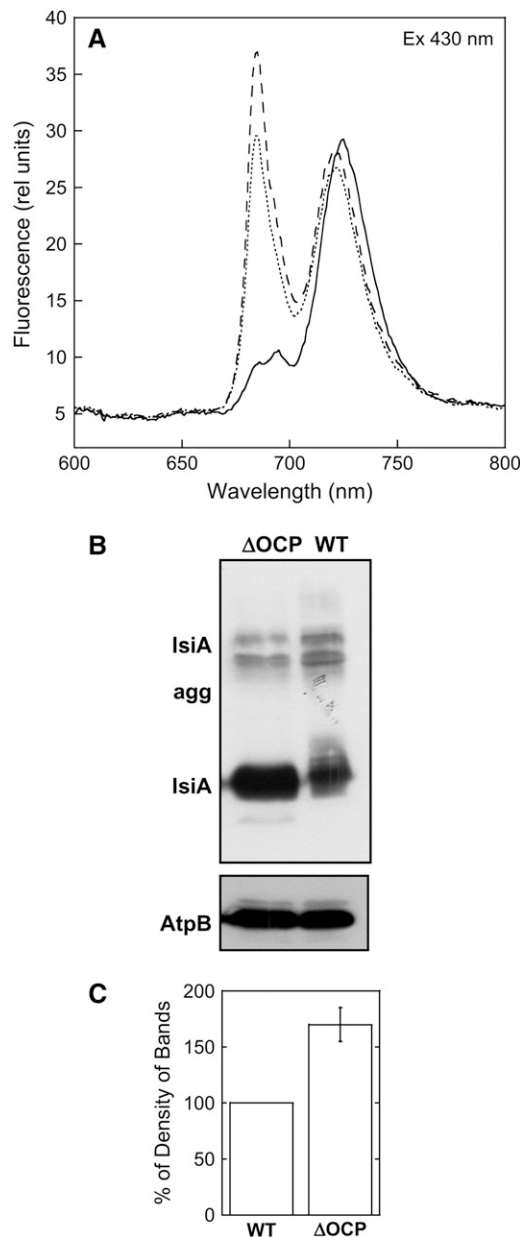
**Figure 11.** No Blue Light-Induced NPQ in Iron-Stressed  $\Delta$ OCP Cells.

**(A)** and **(B)** Fluorescence changes in iron-starved  $\Delta$ OCP cells. Dark-adapted 14 d iron-starved wild-type **(A)** and  $\Delta$ OCP **(B)** cells were illuminated successively with low-intensity blue-green light (400 to 550 nm;  $80 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) and high-intensity blue-green light ( $740 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ ). Fluorescence yield changes were detected in a PAM fluorometer. The cells were at  $3 \mu\text{g chlorophyll/mL}$ .

**(C)** and **(D)** Room temperature fluorescence spectra of  $\Delta$ OCP cells adapted to low light intensities of blue-green light (solid line) and after 5 min of high intensities of blue-green light illumination (dotted line). Excitation was done at 600 nm **(C)** and at 430 nm **(D)**.

**(E)** The 77K fluorescence emission spectra of iron-containing (solid line), 14 d iron-starved (dotted line) wild-type cells, and 14 d iron-starved  $\Delta$ OCP cells (dashed line). Excitation was done at 600 nm.

**(F)** The 77K fluorescence excitation spectra of 14 d iron-starved wild-type (solid line) and  $\Delta$ OCP (dotted line) and PAL cells (dashed line). Emission was monitored at 685 nm. The cells were at  $3 \mu\text{g chlorophyll/mL}$ .



**Figure 12.** IsiA Presence in Iron-Starved  $\Delta$ OCP Cells.

**(A)** The 77K fluorescence emission spectra of iron-containing (solid line) and 14 d iron-starved (dotted line) wild-type cells and of 14 d iron-starved  $\Delta$ OCP cells (dashed line). Excitation was done at 430 nm. The cells were at 3  $\mu$ g chlorophyll/mL.

**(B)** Immunoblot detection of IsiA of the membrane fraction isolated from 13 d iron-starved  $\Delta$ OCP (lane 1) and wild-type (lane 2) cells. An antibody against the subunit AtpB of the ATP synthase was used as internal standard (bottom panel). Each sample contained 2  $\mu$ g of chlorophyll.

**(C)** Comparative densitometry of IsiA levels in iron-starved wild-type and  $\Delta$ OCP thylakoids. The results represent the average of three independent experiments. Each error bar shows the maximum and minimum density of band values for each point.

lower in wild-type cells), suggesting that, indeed, the phycobilisomes transferred the absorbed energy to the IsiA complexes. This was supported by the fluorescence emission and excitation spectra of wild-type cells. The peak at 685 nm observed in fluorescence emission spectra (77K) with excitation at 600 nm was largely related to IsiA emission, indicating energy transfer from phycobilisomes to IsiA. Park et al. (1999) and Sandström et al. (2001) have already reported that the PSII antenna size was inversely proportional to the concentration of IsiA: the more IsiA present, the smaller the antenna size. Thus, IsiA complexes could protect PSII by competing for the energy coming from the phycobilisomes, diminishing the effective antenna size.

We suggest that the energy absorbed by the phycobilisomes that is not used for photochemistry could also be responsible for oxidative damage (e.g., by peroxidation of lipids). For example, Havaux et al. (2005) have already demonstrated that in  $\Delta$ IsiA mutants, there is more lipid peroxidation and higher concentrations of reactive oxygen species are produced than in wild-type cells. This can be caused by a higher production of oxygen radicals in the chlorophyll antenna but also by production of carbon-centered radicals in other proteins of the membrane that by reacting with oxygen will make peroxy radicals. Our results demonstrate that a mutant lacking phycobilisomes (PAL) seemed to be less affected by iron starvation conditions. In PAL cells, IsiA was accumulated and the PSII/PSI ratio increased, indicating a faster degradation of PSI complexes relative to PSII. However, a high concentration of active PSII complexes remained after a long period of iron starvation. These results support the idea that the presence of functionally disconnected phycobilisomes is deleterious to the cell.

## Conclusions

The recently described light-induced photoprotective phycobilisome OCP-mediated NPQ mechanism also occurs under iron starvation conditions, where it is solely responsible for the light-induced fluorescence quenching observed in iron-starved cyanobacteria. In our working model for this process, blue-green light absorbed by the carotenoid of the OCP induces changes in the carotenoid and/or the protein that facilitates the interaction between the OCP and the phycobilisome core and renders the OCP capable of absorbing the energy arriving from the phycobilisomes and dissipates it as heat. Under iron starvation conditions, conditions in which the phycobilisomes become disconnected from the photosystems, energy dissipation in phycobilisomes increases (increased NPQ) to better protect the cells by diminishing the energy arriving to the thylakoids.

The empty IsiA complexes that accumulate during iron starvation could be responsible for a permanent form of thermal energy dissipation. However, this IsiA-based mechanism is not modulated by light. Ihalainen et al. (2005) have shown that the quenched state of the IsiA complexes was independent of the quality or intensity of light, at least in vitro. Under our experimental conditions, cells lacking IsiA were more sensitive to iron starvation than cells unable to activate blue light-induced NPQ. Thus, under iron starvation and low light conditions, IsiA complexes provide better protection than the phycobilisome OCP-related NPQ mechanism. The absence of IsiA and HLIPs, like the

absence of the OCP, renders *Synechocystis* PCC 6803 cells more sensitive to high light intensities. In wild-type cells, IsiA and HLIPs are present only under prolonged high light conditions, whereas the OCP is always present, suggesting that the IsiA- and HLIP-related mechanisms appear when the OCP-related NPQ mechanism is insufficient to protect the cell. A comparative study of the role and effectiveness in photoprotection of these proteins under high light conditions will increase our understanding of the different NPQ mechanisms existing in cyanobacteria.

## METHODS

### Culture Conditions

Wild-type and mutant *Synechocystis* PCC 6803 cells were grown photoautotrophically in a modified BG11 medium described by Herdman et al. (1973) containing twice the concentration of sodium nitrate. Cells were shaken in a rotary shaker (120 rpm) at 30°C and illuminated by fluorescent white lamps giving a total intensity of ~30 to 40  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  under a CO<sub>2</sub>-enriched atmosphere. The  $\Delta\text{OCP}$ ,  $\Delta\text{IsiA}$ , and PAL mutants were grown in the presence of 25  $\mu\text{g/mL}$  spectinomycin and 10  $\mu\text{g/mL}$  streptomycin. The cells were maintained in the logarithmic phase of growth and were collected at OD<sub>800</sub> = 0.6 to 0.8. The construction of the  $\Delta\text{OCP}$  and  $\Delta\text{IsiA}$  mutants in which the *slr1963* gene and the *isiA* gene are interrupted by a spectinomycin/streptomycin resistance cassette was described by Wilson et al. (2006). Construction of the PAL mutant, lacking PC, APC, and the phycobilisome linker protein Lcm, was described by Ajlani and Vernotte (1998).

### Iron Starvation

Wild-type and mutant *Synechocystis* PCC 6803 cells were collected at the logarithmic phase of growth, precipitated, resuspended (OD<sub>800</sub> = 0.6) in modified BG11 medium (Herdman et al., 1973) lacking Fe, and grown under low light (30 to 40  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ). During the first week, the cells were diluted each day to maintain the same cell concentration (OD<sub>800</sub> ranging from 0.6 in the morning to 1.2 to 0.9 the next morning). When the chlorophyll concentration per cell decreased, the cells were diluted once every 2 d, then once every 3 d, and finally undiluted. The PAL mutant was diluted only every 3 d consistently. When the cell concentration was maintained at less than OD<sub>800</sub> = 0.5, the phenotype of protractedly iron-starved cells was reached faster (in 6 to 7 d) (data not shown). This facilitated study of the different stages of iron starvation.

### Fluorescence Measurements

The yield of chlorophyll fluorescence was monitored in a modulated fluorometer (PAM; Walz) adapted to a Hansatech oxygen electrode as previously described (El Bissati et al., 2000). All NPQ induction and recovery experiments were performed in a stirred cuvette of 1 cm diameter (32°C) at a chlorophyll concentration of 3  $\mu\text{g}$  chlorophyll/mL (with the exception of the PAL mutant, 2  $\mu\text{g}$  chlorophyll/mL) and in the presence of chloramphenicol (30  $\mu\text{g/mL}$ ) to inhibit protein synthesis. Recovery was realized under 50 to 80  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  of blue-green light. Fluorescence quenching was induced by a blue-green light (400 to 550 nm) at 740  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  of light intensity. Nomenclature used is as follows: Fo, minimal fluorescence level, the fluorescence emitted by open reaction centers in dark-adapted cells; Fm, the maximal fluorescence level in samples adapted to dim blue-green light; Fm', maximum fluorescence under strong blue-green illumination, corresponding to the fluorescence emitted by the maximum concentration of closed reaction centers; and Fs, the steady state fluorescence level. The Fo level was determined by illuminating dark-adapted cells with a low

intensity of red-modulated light (pulses of 1  $\mu\text{s}$ , 1.6 kHz, and 0.024  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ). For PAL measurements, the measuring light was 2.5 times more intense. Saturating pulses (2000  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ , 1 s) were applied to measure Fm and Fm' levels. Application of such pulses that transiently close all the PSII centers serves to distinguish between photochemical quenching (qp) and NPQ.

Fluorescence emission and excitation spectra at room temperature and at 77K were done in a CARY Eclipse fluorescence spectrophotometer fluorometer (Varian). All samples were at a concentration of 2 to 3  $\mu\text{g}$  chlorophyll/mL. For 77K spectra, samples in nuclear magnetic resonance tubes (5-mm ratio) were quickly frozen by immersion in a mixture of ice, CO<sub>2</sub>, and ethanol and then in liquid nitrogen.

### MP, MP-Free, and Phycobilisome Preparations

Cells (at a 1 mg chlorophyll/mL concentration) were resuspended in a 0.7 M K-phosphate/0.3 M Na-citrate, pH 6.8, buffer to obtain MP or in a 20 mM MES, pH 6.8, buffer to obtain MP-free (M) and broken in a mini-bead-beater in the presence of glass beads. The M and MP fractions were collected by centrifugation and frozen at -80°C until used for gel electrophoresis. For fluorescence spectra, the MP fraction was immediately used without freezing. The PC/chlorophyll absorption ratio was similar in MP fractions and in whole cells.

Entire phycobilisomes were isolated as described by Ajlani et al. (1995). Cells were broken in a buffer containing 0.75 M K-phosphate, pH 7.5, and treated with Triton 2% during 1 h. The nonsolubilized material was separated by centrifugation, and the supernatant was applied to a sucrose gradient and centrifuged. The blue band (in the interface between 1.5 M sucrose and 0.75 M sucrose) contained intact phycobilisomes used in fluorescence spectra.

### Gel Electrophoresis and Protein Gel Blot Analysis

Total cell protein and MP and M fractions were analyzed by SDS-PAGE on a 12% polyacrylamide/2 M urea (Figures 9A and 12B) or on a 12% polyacrylamide/6M urea (Figures 3 and 9C) in a TRIS/MES system (Kashino et al., 2001). The IsiA protein was detected by a polyclonal antibody against the IsiA protein (Dürring et al., 2006), and the OCP protein was detected by a polyclonal antibody against OCP. Anti-OCP polyclonal rabbit antisera was made (CoVance) using recombinant *Synechocystis* PCC6803 OCP (1.4 mg/mL in 20 mM Tris, pH 8.0, and 30% sucrose). Anti-OCP was purified from the sera using Affi-Gel 15 (Bio-Rad) following the manufacturer's instructions. Binding of OCP antibody was monitored by an alkaline phosphatase colorimetric reaction. The blots were scanned and the density of bands was measured using the Image MasterTotal Lab v1.11 software (Amersham Pharmacia). Binding of IsiA, CP47, and ATPase antibodies was visualized with a goat anti-rabbit IgG-peroxidase conjugate and SuperSignal West Pico as chemiluminescent substrate (Pierce).

### Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers NP\_441508 (*slr1963*) and NP\_441268 (*isiA*; *slr0247*).

### Supplemental Data

The following materials are available in the online version of this article.

**Supplemental Figure 1.** The 77K Emission Fluorescence Spectra of Iron-Starved  $\Delta\text{IsiA}$  Cells and Isolated Phycobilisomes.

**Supplemental Figure 2.** The 77K Emission and Excitation Fluorescence Spectra of Nonstarved Wild-Type Cells, the MP Fraction, and Isolated Phycobilisomes.

**Supplemental Figure 3.** Absorption Spectra of Wild-Type and  $\Delta$ IsiA Cells and MP Fractions Corresponding to the Samples Used in Figure 5.

**Supplemental Figure 4.** Blue-Green Light-Induced Fluorescence Quenching in Long-Term, Iron-Starved, Wild-Type,  $\Delta$ OCP, and  $\Delta$ IsiA Cells.

**Supplemental Figure 5.** Comparison of Room Temperature Fluorescence Spectra of Nonstarved and Iron-Starved Wild-Type and  $\Delta$ IsiA Cells.

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