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Biogenesis of Chlorophyll-Binding Proteins under Iron Stress in *Synechocystis* sp. PCC 6803

X. Liu, J. Zhao, and Q. Wu*

Department of Biological Sciences and Biotechnology, Tsinghua University, Beijing 100084, P. R. China; fax: 86-10-62781825; E-mail: qingyu@tsinghua.edu.cn

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Abstract—The biogenesis of chlorophyll-binding proteins under iron stress has been investigated *in vivo* in a *chlN* deletion mutant of *Synechocystis* sp. PCC 6803. The *chlN* gene encodes one subunit of the light-independent protochlorophyllide reductase. The mutant is unable to synthesize chlorophyll in darkness, causing chlorophyll biosynthesis to become light dependent. When the mutant was propagated in darkness, essentially no chlorophyll and photosystems were detected. Upon return of the *chlN* deletion mutant to light, 77 K fluorescence emission spectra and oxygen evolution of greening cells under iron-sufficient or -deficient conditions were measured. The gradual blue shift of the photosystem I (PS I) peak upon greening under iron stress suggested the structural alteration of newly synthesized PS I. Furthermore, the rate of biogenesis of PS II was delayed under iron stress, which might be due to the presence of IsiA.

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Key words: chlorophyll synthesis, chlorophyll-binding proteins, cyanobacterium, fluorescence emission, iron stress, IsiA

Cyanobacteria, like many other prokaryotes, are often exposed to iron-deficient environments due to the extremely low solubility of the dominating Fe^{3+} ions under oxygenic conditions. Iron deficiency results in a variety of physiological and morphological changes in cyanobacteria. The overall changes are as follows: decrease in phycocyanins (PC) and chlorophyll (Chl) content, a characteristic blue shift of the main red maximum of Chl absorption, dramatic alterations in the 77 K Chl fluorescence emission spectrum, reduction in the number of thylakoids, reduced photosystem II (PS II) and photosystem I (PS I) photochemistry, and partial replacement of ferredoxin by flavodoxin [1-3]. Most importantly, a novel Chl-binding protein, IsiA, encoded by *isiA*, is synthesized. In iron-deficient cyanobacteria up to 50% of Chl is associated with it [4]. Because of the similarity to CP43, IsiA was suggested to be associated with PS II functioning as a Chl storage protein [5] or an excitation energy dissipater [6, 7]. Recently, it was shown that 18 copies of IsiA form a ring around the trimeric PS I reaction center. This would imply a substantial increase of the PS I antenna size [8, 9]. Recent studies utilizing biochemical, physical, and genetic approaches have revealed the structural and functional rearrangement of the photosynthetic apparatus under iron stress (for a review, see [10]). In contrast, little is known about the biogenesis of these Chl-binding proteins. Understanding of the contribution of Chl molecules to functional photosynthetic complexes assembly under iron stress remains especially elusive.

Synechocystis sp. PCC 6803 contains both light-dependent and light-independent Chl biosynthesis pathways [11]. Three genes (*chlL*, *chlN*, and *chlB*) encode the subunits of the light-independent protochlorophyllide reductase. Mutants lacking any one of these genes are unable to synthesize Chl in darkness, causing Chl synthesis to become light dependent [12, 13]. When they were propagated under light activated heterotrophic growth conditions (LAHG, in darkness except for 15 min/day of weak light), the mutant cells become etiolated and contain essentially no Chl and photosystems [12, 14]. In $chlN^-$ mutant, Chl content is controled by light, and the process of "greening" (light-dependent Chl synthesis and photosystem assembly) can be monitored. Therefore, this mutant strain provides an excellent *in vivo* system to investigate biogenesis of Chl-binding proteins in relation to Chl biosynthesis.

In this work, this system was used to study the biogenesis of Chl-binding proteins under iron stress. We have performed both spectral and functional analyses under *in vivo* conditions to study the biogenesis of the photosynthetic apparatus in relation to Chl biosynthesis under iron deficiency.

Abbreviations: Chl) chlorophyll; LAHG) light activated heterotrophic growth; PC) phycocyanin; PS I) photosystem I; PS II) photosystem II.

* To whom correspondence should be addressed.

MATERIALS AND METHODS

Culture and growth. Both wild-type and mutant strains of *Synechocystis* sp. PCC 6803 were cultured at 30°C (±1) in BG 11 medium [15] containing either 30 μM ferric ammonium citrate or lacking this component completely. Cells were grown in continuous light (white light at 50 $\mu E \cdot m^{-2} \cdot sec^{-1}$) or under LAHG conditions. Under LAHG conditions, the cells were kept in complete darkness except for one 15-min light period (white light at 50 $\mu E \cdot m^{-2} \cdot sec^{-1}$) every 24 h. Aeration was provided through bubbling. The cells were harvested during the late logarithmic growth phase (around $8 \cdot 10^7$ cells/ml) by centrifugation at 6000*g* for 7 min and resuspended in fresh medium. Cell growth was determined by monitoring the optical density of the culture at 730 nm using an Ultra-Spec 2000 UV/Visible spectrophotometer (Pharmacia, Sweden). The specific growth rate was defined as an increase in OD_{730} during the time indicated.

Chlorophyll analysis. Chlorophyll was extracted with 100% methanol from cell pellets obtained from 1 ml of *Synechocystis* sp. PCC 6803 wild-type and mutant cell cultures (OD_{730} = 0.8) that had been grown in light or under LAHG conditions. The Chl content was determined according to MacKinney [16].

Measurements of PC contents, absorption spectra, and 77 K fluorescence emission spectra. Absorption spectra of intact *Synechocystis* sp. PCC 6803 cells were measured by an Ultra-Spec 2000 UV/Visible spectrophotometer and the relative PC amount was determined according to Xu et al. [17]. Low temperature (77 K) fluorescence measurements were performed on a F4500 spectrofluorimeter (Hitachi, Japan). The cells were suspended in 25 mM Hepes-NaOH, pH 7.0. Cell suspensions were adjusted to equal Chl concentration within the range of 2-10 μg Chl/ml. Cells were immediately frozen in liquid nitrogen without any further pretreatment.

Measurement of oxygen evolution. Oxygen evolution was measured with a Clark-type electrode (Hansatech, England) at 25°C. Cells were suspended in 25 mM Hepes-NaOH, pH 7.0. Light was provided by a 150-W xenon arc lamp. The incident light intensity was 1500 $\mu E \cdot m^{-2} \cdot sec^{-1}$.

RESULTS AND DISCUSSION

Growth and pigment composition. The *chlN*⁻ mutant cells exhibit similar growth rates compared to wild-type cells under iron-sufficient and -deficient conditions although the cell growth rates in darkness are lower than those in light (Table 1). These results indicate that iron stress and deletion of *chlN* do not affect the growth and viability of the cells. After growth under LAHG conditions for two weeks, the Chl content in the mutant cells was about 5-7% of that in the wild-type in both iron-sufficient and -deficient media. In addition, the Chl content decreased under iron stress in both wide-type and mutant cells compared to the control (Table 1).

Table 1. Characteristics of *Synechocystis* sp. PCC 6803 wild-type and the *chlN*⁻ mutant cells grown in iron-sufficient (+) and -deficient (–) media (cells were grown in continuous light or under LAHG conditions for two weeks; mean ± S.D., $n = 3$)

Strain	Fe	Growth conditions	Doubling time (h)	Chlorophyll (μg/ml/OD_{730})
Wild-type	+	in light	19 ± 1	3.2 ± 0.1
Wild-type	–	in light	21 ± 1	1.7 ± 0.1
chlN⁻ mutant	+	in light	19 ± 1	3.3 ± 0.1
chlN⁻ mutant	–	in light	21 ± 1	1.4 ± 0.1
Wild-type	+	under LAHG conditions	51 ± 2	3.1 ± 0.1
Wild-type	–	under LAHG conditions	53 ± 2	1.1 ± 0.1
chlN⁻ mutant	+	under LAHG conditions	54 ± 2	0.14 ± 0.02
chlN⁻ mutant	–	under LAHG conditions	56 ± 1	0.08 ± 0.01

Figure 1 shows the absorption spectra of intact cells of *chlN*⁻ mutant grown under iron-sufficient and -deficient conditions. The spectrum of cells in light has three major peaks: the peaks at 436 and 680 nm correspond to Chl [18], whereas that at 625 nm is due to PC, the major component of the phycobilisome [19]. As expected, the spectrum of *chlN*⁻ cells in darkness lacked the two Chl absorption peaks and retained the PC absorption peak. The PC absorption peak under iron stress decreased significantly both in light and darkness. Indeed, the PC level under iron deficiency was 15-20% of that under iron

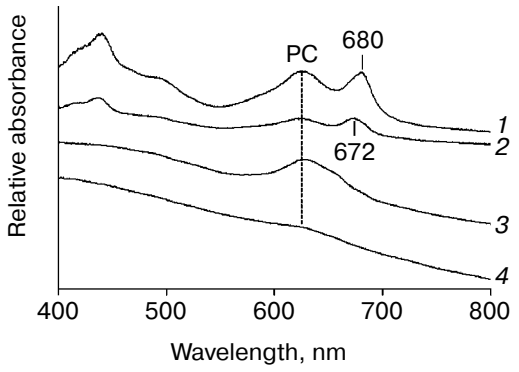

Fig. 1. Absorption spectra *of chlN*⁻ cells grown in iron-sufficient (*1*, *3*) and -deficient (*2*, *4*) media. The spectrum of the *chlN*⁻ mutant was determined after growth in continuous light (*1*, *2*) or under LAHG conditions (*3*, *4*) for two weeks.

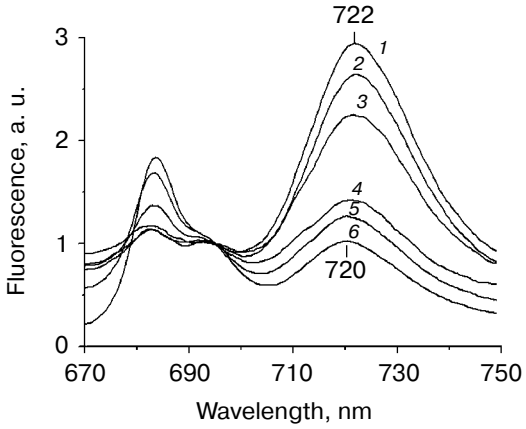

Fig. 2. Time course of 77 K Chl fluorescence emission spectra of wild-type cells after a shift from iron-sufficient to -deficient conditions. Spectra were taken after cells had been transferred from iron-sufficient to -deficient conditions for 0 (*1*), 6 (*2*), 12 (*3*), 24 (*4*), 48 (*5*), and 72 h (*6*). The excitation wavelength was 435 nm. The spectra are normalized to 1.0 at 695 nm.

sufficiency, regardless of the Chl content (data not shown).

77 K Chl fluorescence emission under iron stress. The Chl fluorescence emission spectra of iron-sufficient wild-type cells at 77 K exhibit characteristic peaks centered at 684 nm (Chl antenna of PS II), 695 nm (CP47 of PS II), and 722 nm (PS I Chl–protein complex) [20]. Transferring the iron-sufficient cells to an iron-deficient medium caused a gradual increase in the 684 nm peak and a decrease in the PS I associated peak at 722 nm. Concomitant with this, a gradual shift in the PS I related peak from 722 to 720 nm was registered. These gradual changes completed after 72 h, resulting in a dramatic change in the emission spectrum (Fig. 2). The specific increase in the 685 nm peak and a typical blue shift in the red Chl absorption peak (Fig. 1) are characteristic of the induction of IsiA expression [8]. The blue shift in the PS I characteristic peak was also observed in *Synechococcus* 7942, which is suggested to be a structural alteration in the PS I centers during acclimation to iron deficiency [3]. This shift is not a simple consequence of the IsiA–PS I supercomplex, because it was not observed in a mutant overproducing IsiA [9].

No differences in 77 K Chl fluorescence emission spectra were observed between the wild-type and *chlN*$^-$ strains grown in light. However, in *chlN*$^-$ mutant grown under LAHG conditions, no significant fluorescence emission that correlated with PS II or PS I could be detected under iron-sufficient and -deficient conditions (data not shown).

Biogenesis of photosynthetic complexes in relation to Chl biosynthesis under iron stress. By deletion of *chlN*, a system in which Chl synthesis can be triggered by light was set up. By means of this system, it is possible to investigate the biogenesis of the Chl-binding proteins in relation to Chl biosynthesis under iron stress. To address this question, both diagnostic (fluorescence emission spectra) and functional (oxygen evolution) analyses were performed.

First of all, 77 K fluorescence emission spectra were measured using the *chlN*$^-$ cells under iron-sufficient and -deficient conditions at their different stages of greening (Fig. 3). Under the iron-sufficient condition, a 684 nm fluorescence emission peak became apparent during the early stages of greening and was maximal after about 12 h of greening. The 722 nm emission could be detected after 6 h of greening and increased until about 48 h after the

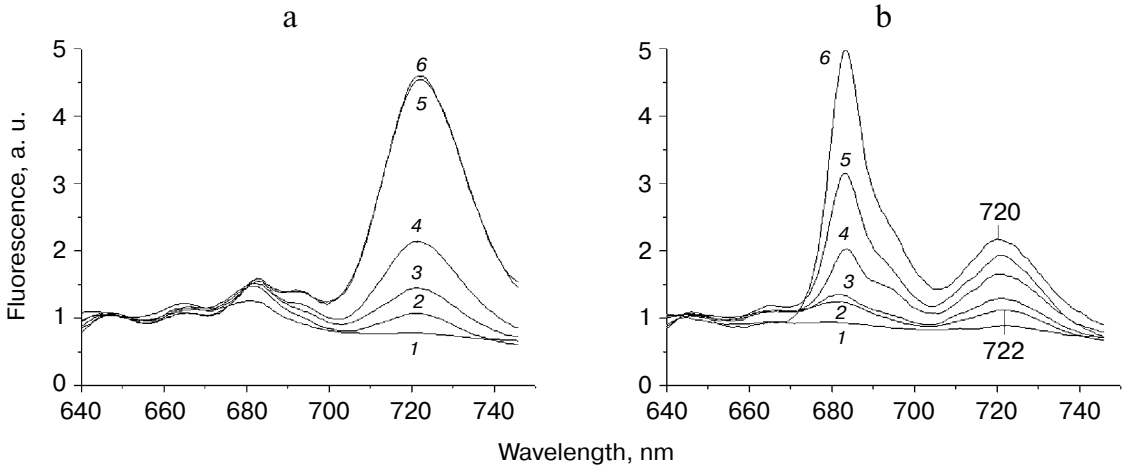

Fig. 3. Chlorophyll fluorescence emission spectra of *chlN*$^-$ cells measured at 77 K at different stages of greening under iron-sufficient (a) and -deficient (b) conditions. The time between the exposure of the *chlN* mutant grown under LAHG conditions to continuous light and measuring the spectra was 0 (*1*), 6 (*2*), 12 (*3*), 24 (*4*), 48 (*5*), and 72 h (*6*). The excitation wavelength was 435 nm. The spectra are normalized to 1.0 at 650 nm.

Table 2. Rates of oxygen evolution in greening cells grown in iron-sufficient (+) and -deficient (–) media (all cells were propagated under LAHG conditions for two weeks and then transferred to light; mean ± S.D., $n = 3$)

Greening time, h		0	6	12	24	48	72
	Fe	Oxygen evolution, μmol O_2 $(OD_{730}\cdot L\cdot h)^{-1}$					
chlN$^-$ mutant	+	0	30	38	52	186	211
chlN$^-$ mutant	–	0	34	39	41	61	198

initiation of Chl synthesis. The 695 nm emission band appeared only after 24 h of illumination. Finally, a normal pattern of the fluorescence emission spectrum similar to that of the wild-type grown in light was established at 48 h of greening (Fig. 3a). The results in Fig. 3b show the biogenesis of photosystems under iron-deficient conditions. After 6 and 12 h of greening, no difference was observed between the iron-deficient and -sufficient cells. The 695 nm peak was also observed only after 24 h of greening as in iron-sufficient cells. However, the 684 nm peak increased sharply after 24 h of illumination until about 72 h after the initiation of Chl synthesis. In addition, the 722 nm emission was blue shifted to 720 nm gradually until about 72 h of greening.

At the time cells were transferred to the light, both the *chlN*$^-$ cells under iron-sufficient and -deficient conditions exhibited no oxygen evolution. Correlating with fluorescence measurements, the oxygen evolution rates increased to about the maximal rates after 48 h of greening under iron-sufficient conditions and after 72 h of greening under iron-deficient condition (Table 2). This indicates that the rate of biogenesis of functional PS II is delayed under iron stress.

An interesting observation was the gradual blue shift of PS I peak upon greening under iron stress. This change in PS I fluorescence emission is even similar to that during the time course of wild-type transferred to iron-deficient conditions. Our results suggest that newly synthesized PS I under iron stress might have normal structure as that of iron-sufficient cells and hence alter substantially.

Under iron stress, the 684 nm peak increased sharply when the 695 nm peak appeared. This suggests that the biogenesis of IsiA and CP47 upon greening began at almost the same time but was subsequent to that of PS I. The greening time lasts for 72 h under iron deficiency, and lasts for about 48 h under iron sufficiency. As each IsiA subunit binds about 16-17 Chl molecules [21], abundance of IsiA may compete with PS II in binding newly synthesized Chl. Therefore, the delay in biogenesis of PS II under iron stress might be due to the presence of a large amount of IsiA.

In conclusion, the experimental system described here has opened up an opportunity to study the biogenesis of Chl-binding proteins *in vivo* under iron stress. The gradual blue shift of PS I peak upon greening under iron stress suggests the structural alteration of newly synthesized PS I. Furthermore, the delayed biogenesis of PS II might result from the large amount of IsiA under iron stress.

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