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## **Chapter 8**

### **Flexibility of light-harvesting in iron-stressed cyanobacteria**

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## Flexibility of light-harvesting in iron-stressed cyanobacteria

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## Summary

A significant part of global primary productivity is provided by cyanobacteria, which are abundant in most marine and fresh-water habitats (Ting *et al.*, 2002). In many oceanographic regions, however, the concentration of iron may be so low that it will limit growth (Behrenfeld *et al.*, 1996; De Baar *et al.*, 1995; Martin *et al.*, 1994). Cyanobacteria respond to this condition by expressing a number of iron-stress-inducible genes (Singh *et al.*, 2004), of which the *isiA* gene encodes a chlorophyll-binding protein known as IsiA or CP43'. It was recently shown that 18 IsiA proteins encircle trimeric photosystem I (PSI) under iron-deficient growth conditions (Bibby *et al.*, 2001; Boekema *et al.*, 2001), increasing the light harvesting ability of PSI by a factor of about two (Bibby *et al.*, 2001; Boekema *et al.*, 2001; Andrizhiyevskaya *et al.*, 2002; Melkozernov *et al.*, 2003). We report here that upon prolonged iron stress the number of bound IsiA proteins can be up to 35 units in a double ring around a PSI monomer, increasing the light-harvesting ability of PSI by a variable factor of up to seven, and that a significant part of IsiA builds supercomplexes without PSI, possibly to provide photoprotection. These results highlight the capacity of the IsiA protein to form flexible supramolecular structures of variable size to optimally respond to the degree of iron stress.

The IsiA protein belongs to the so-called core complex antenna family of chlorophyll-binding proteins, a family of proteins that is more widespread and important than was realized until only a few years ago (Green, 2003). Its most well-known members are the CP47 and CP43 core antenna proteins of photosystem II (PSII), which are structurally resolved up to 3.5 Å resolution (Ferreira *et al.*, 2004). CP47 and CP43 are located at fixed positions in the PSII supercomplex and mediate the transfer of excitation energy from the peripheral antenna to the photochemical reaction center of PSII (Van Amerongen and Dekker, 2003). The IsiA protein shows sequence homology with CP43 (Burnap *et al.*, 1993) and is therefore often referred to as CP43'. Other members of the core complex antenna family are the prochlorophyte chlorophyll *a/b* (Pcb) proteins, which also show sequence homology with CP43 (Green, 2003). It was shown that Pcb proteins from *Prochloron didemni* (Bibby *et al.*, 2003) and a moderate-light adapted *Prochlorococcus* strain (Bibby *et al.*, 2003) can bind to dimeric PSII, whereas other Pcb proteins from the latter species and from an extremely low-light adapted *Prochlorococcus* species form a ring of 18 units around trimeric PSI (Bibby *et al.*, 2003; Bibby *et al.*, 2001), giving rise to a supercomplex with very similar overall structure as the (PSI)<sub>3</sub>(IsiA)<sub>18</sub> supercomplex of cyanobacteria.

We recently showed that a mutant of *Synechocystis* sp. PCC 6803 without the PsaF

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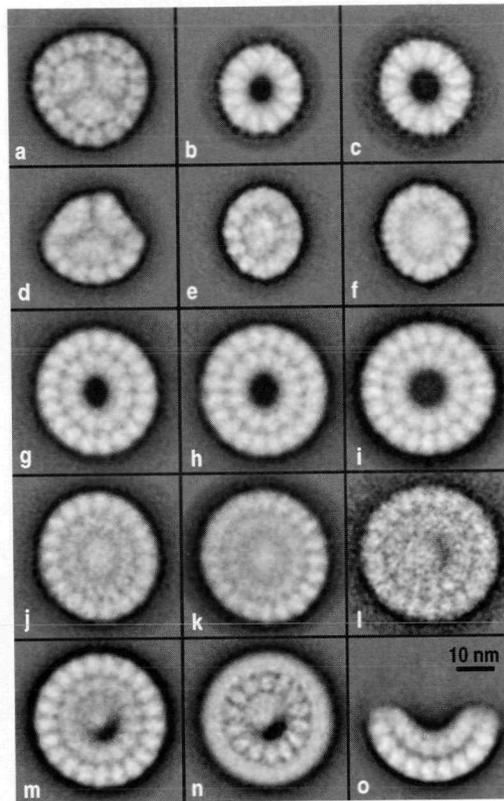
and PsaJ subunits (Jeanjean *et al.*, 2003) forms an IsiA ring of 17 units around trimeric PSI (Kouřil *et al.*, 2003). The smaller number of bound IsiA proteins was explained by the smaller circumference of the PSI trimer of the mutant (the PsaF and PsaJ subunits are located at the outer rim of the PSI trimer (Jordan *et al.*, 2001)) and it was concluded that the size of the PSI complex determines the number of units in the IsiA ring (Kouřil *et al.*, 2003).

In order to investigate the relation between the number of bound IsiA units in PSI-IsiA supercomplexes, the monomeric or trimeric aggregation state of PSI (Karapetyan *et al.*, 1999) and the degree of iron starvation, we analyzed these complexes at various times after the start of culture in an iron-free medium. We used both the wild type and a *psaFJ* mutant (Jeanjean *et al.*, 2003) of *Synechocystis* sp. PCC 6803, and investigated detergent-solubilized supercomplexes by electron microscopy and image analysis of a very large set of single particle projections.

Fig. 1 shows that besides the 'standard'  $(\text{PSI})_3(\text{IsiA})_{18}$  complex (of wild type cells, Fig. 1a) also various other ring-shaped complexes can be discerned, both with smaller and larger contents of IsiA, in particular under conditions of prolonged iron limitation. Relatively well-resolved are ring-like structures without central complex. The largest complex is an almost circularly shaped double ring structure (Fig. 1i) that consists of inner and outer rings of 14 and 21 units, respectively. Slightly smaller and oval-shaped complexes consisting of 13 and 20 (Fig. 1h) and of 12 and 19 units (Fig. 1g) were also detected in significant numbers. Single rings of 12 and 13 units with a size similar to those of the inner rings of the double rings (Fig. 1bc), as well as partial double and single rings of variable sizes (see Fig. 1o for an example) were observed as well.

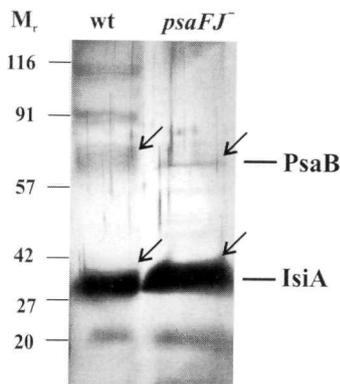
Many other complexes, however, do contain a central complex resembling monomeric or trimeric PSI. After a few days of culture in an iron-free medium a  $(\text{PSI})_3(\text{IsiA})_7$  complex is abundant (Fig. 1d), whereas after about 20 days of culture single and double closed rings of IsiA around a particle with the size and shape of monomeric PSI can be detected in significant numbers (Fig. 1efjkl). The central mass of the best-resolved complex (Fig. 1e) reveals the typical features of PSI. In the largest complex (Fig. 1l) stain accumulation in the center of the complex suggests that the inner ring of 14 units (Fig. 1n) is too large for the central complex, which is expected if a ring of 12 units is just large enough to encircle monomeric PSI (Fig. 1e). The shapes and sizes of the rings with a central complex appear to be very similar to those without.

In order to verify that the supercomplexes depicted in Fig. 1 consist of IsiA and PSI we analyzed the supercomplex-containing fractions from cells grown for about 20



**Figure 1. Processed top views of PSI-IsiA and IsiA supercomplexes obtained by electron microscopy.** **a-c,** Results of statistical analysis and classification of 59,000 particles from wild-type *Synechocystis* sp. PCC 6803. A total of 47,000 projections could be assigned to specific complexes. Closed rings of IsiA consist of 12, 13 or 18 copies in a single ring (b, c, a, respectively) plus 19, 20 and 21 copies in a second ring surrounding 12, 13 and 14 copies in an inner ring (g, h, i). The center of the complexes can be occupied by either a PSI trimer (a) or a monomer (e, f, j, k, l). The relative abundance of these complexes was about 19% (a), 8% (b), 16% (c), 9% (e), 19% (f), 4% (g), 5% (h), 1% (i), 4% (j), 6% (k), 5% (l) and 4% (o). Note that a relatively low amount of detail is resolved in the projection maps j-l. The fuzziness is attributed to rotational flexibilities of a few degrees between the monomer and both rings. For complexes with 14 + 21 IsiA copies the monomer has the expected shape, but only in classes with small numbers of projections (l). By increasing the number of summed projections the features of the outer ring become more apparent (m), whereas the features of the inner ring could be improved by masking the outer ring during additional alignment (n). **d,** Results of analysis of 6,000 particles from a *Synechocystis* *psaFJ* (Kouřil *et al.*, 2003). A specific complex of trimeric PSI plus 7 IsiA proteins attached (d) was observed in this set. In addition, this set yielded PSI trimers with a closed ring of 17 units (Kouřil *et al.*, 2003) and rings and partial rings similar to b-c and o, respectively.

days in an iron-deficient medium by SDS-PAGE (Fig. 2), and subjected the main bands to a mass spectrometry analysis. The lower main bands revealed peptide fragmentation spectra consistent with IsiA, while the upper band revealed peptide fragmentation spectra consistent with PsaB, a core subunit of PSI. These data confirm that IsiA and PSI are the main constituents of supercomplexes from iron-depleted wild-type cells.

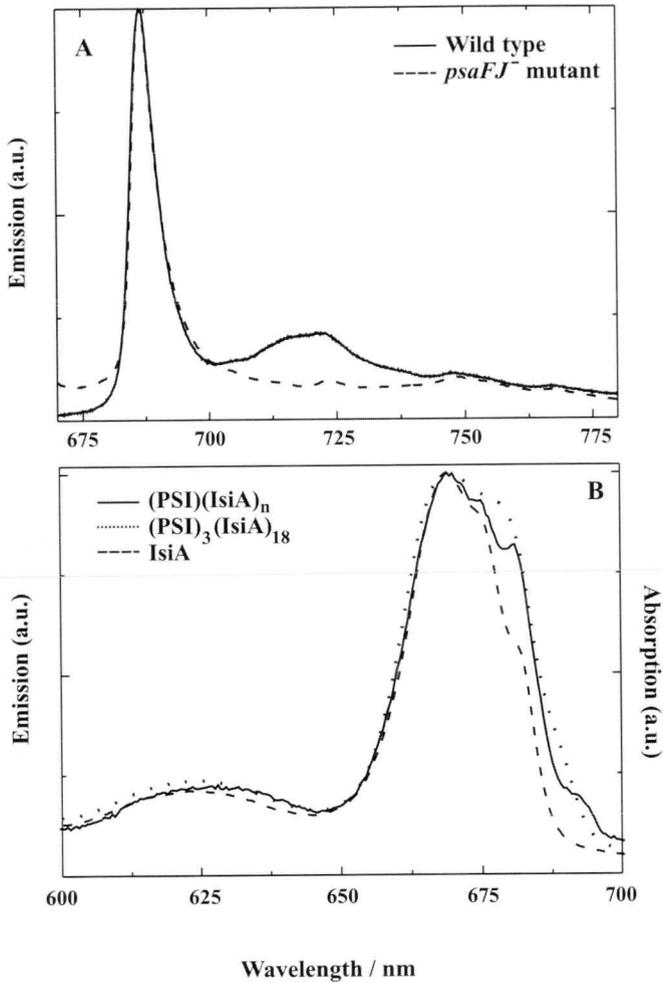


**Figure 2. Silver stained SDS-PAGE of proteins of fractions from WT and *psaFJ* mutant cells grown in iron-deficient media.** Arrows show the bands analyzed by mass spectrometry.

has a full width at half maximum (fwhm) of 14 nm (E. G. Andrizhiyevskaya, unpublished observations). The fluorescence spectrum from iron-starved mutant cells is dominated by a band peaking at 686-687 nm but with a fwhm of only 6 nm, which therefore cannot arise from PSII and in fact must arise from IsiA aggregates. IsiA is abundant in the iron-starved mutant cells (Fig. 2) and IsiA monomers show a blue-shifted 5 K emission maximum at 682 nm (Andrizhiyevskaya *et al.*, 2002). The 686 nm emission in wild-type cells must for a large part arise from PSI-less IsiA aggregates, because in isolated PSI-IsiA supercomplexes only a very small 686-687 nm emission band is observed (Andrizhiyevskaya *et al.*, 2002).

We recorded 5 K excitation spectra of the fraction analyzed in Fig. 3A to find out whether the IsiA proteins in these IsiA-rich complexes transfer excitation energy to PSI. Fig. 3B shows that there is a larger contribution of 670 nm states belonging to IsiA (Andrizhiyevskaya *et al.*, 2002). in these spectra than in the  $(PSI)_3(IsiA)_{18}$  complexes, which suggests that the bound IsiA proteins in the PSI-IsiA supercomplexes contribute to the light-harvesting for PSI. We calculated an average number of 10 functionally bound

We recorded 5 K fluorescence emission of the fraction from wild-type cells analyzed in Fig. 2 to further confirm the nature of the pigment-protein complexes. The broad band peaking at about 720 nm shown in Fig. 3A can be attributed to PSI (Gobets *et al.*, 2001). The fact that this peak is almost absent in iron-stressed mutant cells is consistent with the SDS-PAGE (Fig. 2). The region between 685 and 695 nm usually represents emission from PSII (Van Amerongen and Dekker, 2003), which in the PSII core complex of *Synechocystis* sp. PCC 6803 consists at 5 K of a broad band peaking at 688 nm and



**Figure 3. 5 K fluorescence emission and excitation spectra.** **a**, 5 K fluorescence emission spectra of a fraction from WT cells (solid line) and from *psaFJ* mutant cells (dashed line) grown in iron-deficient media. **b**, 5 K fluorescence excitation spectrum (solid line) of fluorescence detected at 722 nm of a fraction from WT cells grown in iron-deficient medium. The free IsiA contribution (20% at 722 nm, see a) has been subtracted from the excitation spectrum. The excitation spectrum of free IsiA was obtained by taking the difference of spectra detected at 742 and 722 nm. The 77 K absorption spectra of the IsiA-fraction from the *psaFJ* mutant grown in iron-deficient medium (dashed line) and the 5 K absorption spectrum from isolated (PSI)<sub>3</sub>(IsiA)<sub>18</sub> particles (dotted line) are shown for comparison.

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IsiA complexes per PSI monomer, which is significantly more than the number of 6 for the  $(\text{PSI})_3(\text{IsiA})_{18}$  complex.

The current data show that cyanobacteria accumulate IsiA in excess of what is needed for functional light harvesting for PSI. The increase of narrow-banded 77 K (Burnap *et al.*, 1993; Sandström *et al.*, 2002) or 4 K (this work) fluorescence emission at 685-687 nm almost immediately after a shift from iron-sufficient to iron-deficient conditions suggests that PSI-free IsiA aggregates also occur in early stages of iron deficiency. Whether this excess synthesis of IsiA serves to protect PSII, which is very vulnerable to photooxidation in the absence of PSI (Sandström *et al.*, 2002), and/or constitutes a stock of chlorophyll to rapidly resume growth after relief from the iron limitation stress (Burnap *et al.*, 1993), is not clear yet. The remaining PSI complexes show increasingly larger amounts of bound IsiA proteins with increasing iron starvation. The largest particles with a double IsiA ring around monomeric PSI (Fig. 11) would contain about  $35 \times 16 = 560$  chlorophylls, which together with 96 chlorophylls of the PSI core complex (Jordan *et al.*, 2001) would give a total number of about 656 chlorophylls. PSI particles with such large antenna sizes have not been observed before. The addition of this number of chlorophylls would give an almost seven-fold enlargement of the light-harvesting capacity of PSI. The increased antenna size will thus provide compensation for the light need of the lower number of PSI centers compared to PSII under iron stress.

The image analysis indicates that the two rings that encircle monomeric PSI have rotational disorder. For the efficiency of excitation transfer between the rings and from the rings to PSI a precise match of the IsiA proteins is probably not necessary because of very fast and multiple excitation transfer routes from IsiA to PSI (Melkozernov *et al.*, 2003; Andrizhiyevskaya *et al.*, 2004).

It thus appears that the IsiA protein has evolved optimally from the common ancestor of the core complex antenna family of chlorophyll-binding proteins. It not only lacks the large extrinsic loop that CP43 in PSII needs to assist water oxidation (Green, 2003; Burnap *et al.*, 1993), it also very likely contains a few additional 'linker' chlorophylls (Andrizhiyevskaya *et al.*, 2002) that could assist in fast and efficient excitation energy transfer from the outer to the inner IsiA ring and from the inner ring to PSI. This situation resembles that in green plant PSI, in which several linker chlorophylls have been detected between the peripheral antenna of LHCI proteins from the unrelated Cab-gene family and the PSI complex (Scholts *et al.*, 1996). But most important of all is the ability of the IsiA protein to form large and flexible supramolecular structures, both with itself and with PSI. In this sense, IsiA can be viewed

as the cyanobacterial counterpart of LHCII, the main light-harvesting complex for PSII in green plants (Liu *et al.*, 2004), which forms supramolecular structures with and without PSII (Ruban *et al.*, 2003) and thus provides the photosystem with a large functional antenna and a means to regulate the light need for photosynthesis.

### Methods

**Cell culture.** *Synechocystis* sp. PCC 6803 wild-type and the *psaFJ* mutant (Jeanjean *et al.*, 2003) were grown at 30°C in liquid BG11 medium at a light intensity of 50  $\mu\text{mol photons m}^{-2}\text{s}^{-1}$  in ambient air. Iron deficiency was achieved by omitting all iron sources from the medium. Inoculation for iron-depleted culture was by 20 to 30 fold dilution of 3 times washed cells that were pregrown in normal medium. For the present study, cells harvested 2 to 23 days after inoculation were used.

**Cell fractionation.** Cells were broken and thylakoid membranes were isolated as described before<sup>24</sup>. Freshly isolated thylakoid membranes (0.15 mg chlorophyll *a* ml<sup>-1</sup>) were solubilized with 0.5% (w/v) *n*-dodecyl- $\beta$ ,D-maltoside and centrifuged at 9,000  $\times$  g for 3 min. The supernatant was filtrated on a Titan PVDF syringe filter (0.45  $\mu\text{m}$ ) and subjected to size exclusion chromatography as before (Kouřil *et al.*, 2003), using a Superdex 200 HR 10/30 column (Pharmacia), a running buffer consisting of 20 mM Bis-Tris (pH 6.5), 5 mM MgCl<sub>2</sub> and 0.03% *n*-dodecyl- $\beta$ ,D-maltoside, and an on line diode array detector (Shimadzu SPD-M10A). Fractions containing particles with sizes of supercomplexes were used for further analysis.

**SDS-PAGE and mass spectrometry.** FPLC-fractions were concentrated on Microcon YM-10 (Millipore), and the protein content was estimated by the assay according to Bradford (Bio-Rad). The protein composition was analyzed by Tris-Tricine SDS-PAGE (Schägger and von Jagow, 1987). Protein bands were cut out off the gel, reduced with DTT, S-alkylated with iodoacetamide, and digested with trypsin (sequence grade, Roche, Basel, Switzerland) (Shevchenko *et al.*, 1996). The digested peptide mixture was loaded onto the precolumn of an Ultimate nano-HPLC system (LC Packings, Amsterdam, the Netherlands) and separated on a PepMap C18 nano reversed phase column (75  $\mu\text{m}$  I.D.). Elution was performed using a gradient of 5 to 40% acetonitrile with 0.1% formic acid. The flow was infused directly into an ESI-QTOF mass spectrometer (Micromass) via a modified nano-electrospray device (New Objective, Woburn, MA). MSMS (tandem mass spectrometry) experiments were conducted with Argon as collision gas at a pressure of  $4 \times 10^{-5}$  bar measured on the quadruple pressure gauge. The acquired peptide MSMS spectra were used in a search of the SwissProt database.

**Spectroscopy.** For 5 K fluorescence measurements, the samples were diluted in a buffer containing 20 mM Bis-Tris (pH 6.5), 10 mM MgCl<sub>2</sub>, 10 mM CaCl<sub>2</sub>, 0.02%  $\beta$ -DM and 66% (w/w) glycerol to an optical density of about 0.1 at the Q<sub>y</sub> absorption maximum of the chlorophylls. Fluorescence emission spectra were recorded on a home-built spectrograph-CCD fluorimeter, using an excitation wavelength of 420 nm. Fluorescence excitation spectra were recorded on a home-built spectrometer with emission wavelengths at 722 nm and 742 nm (fwhm 5 nm).

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**Electron microscopy.** EM was performed as described in Ref. 15. Briefly, EM specimens were prepared on glow-discharged carbon-coated grids, using 2% uranyl acetate as a negative stain. EM was performed on a Philips FEG20 electron microscope. Semi-automated data acquisition was used to record a total of 7,500 2048 x 2048 pixel images at 66,850x magnification with a Gatan 4000 SP 4K slow-scan CCD camera. The step size (after binning the images) was 30 nm, corresponding to a pixel size of 4.5 Å at the specimen level and projections were selected for single particle averaging (Harauz *et al.*, 1988) with Groningen Image Processing (GRIP) software. Projections were aligned by multi-reference alignment and aligned images were subjected to multivariate statistical analysis (MSA). After MSA, particles were classified and summed and class sums were used in a next cycle of multi-reference alignment, MSA and classification. Final sums within homogeneous classes were obtained by reference-free alignment procedures (Penczek *et al.*, 1992).

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