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Chapter 7

Accumulation of large aggregates of chlorophyll-binding protein IsiA that are not associated with photosystem I in a *psaFJ* mutant of the cyanobacterium *Synechocystis* sp. PCC 6803

**Accumulation of large aggregates of chlorophyll-binding protein
IsiA that are not associated with photosystem I in a *psaFJ*
mutant of the cyanobacterium *Synechocystis* sp. PCC 6803**

Nataliya Yeremenko*, Janne A. Ihalainen†, Roman Kouřil‡, Sandrine D'Haene†, Henk L. Dekker§, Martin Hagemann¶, Egbert J. Boekema‡, Hans C. P. Matthijs*# and Jan P. Dekker†

**Aquatic Microbiology, Institute of Biodiversity and Ecosystem Dynamics, Universiteit van Amsterdam, Nieuwe Achtergracht 127, 1018 WS Amsterdam, The Netherlands*

†*Division of Physics and Astronomy, Faculty of Sciences, Vrije Universiteit, De Boelelaan 1081, 1081 HV Amsterdam, The Netherlands*

‡*Department of Biophysical Chemistry, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Nijenborgh 4, 9747 AG Groningen, The Netherlands*

§*Swammerdam Institute for Life Sciences, Mass Spectrometry Group, University of Amsterdam, Nieuwe Achtergracht 166, 1018 WV Amsterdam, The Netherlands*

**Universität Rostock, Fachbereich Biowissenschaften, Pflanzenphysiologie, Albert-Einstein-Strasse 3a, 18051, Rostock, Germany*

Summary

In the absence of iron, a mutant of the cyanobacterium *Synechocystis* sp. PCC 6803 without the PSI subunits PsaF and PsaJ shows a much stronger decrease in its PSI content than the wild type. After prolonged growth under iron-free conditions PSI disappears nearly completely. The iron-starvation inducible protein IsiA becomes by far the most abundant chlorophyll-protein complex in the mutant. A partial purification and a characterization by mass spectrometry, spectroscopy and electron microscopy has revealed the presence of several types of IsiA aggregates, of which a 'doughnut'-like complex not associated with PSI is the most conspicuous. This ring-shaped complex is smaller than the normal ring of eighteen IsiA proteins around a wild type trimeric PSI complex, or the 17-mer homologue that was recently observed in the *psaFJ* mutant, but large enough to encircle a monomeric PSI complex. We conclude that the combination of oxidative stress and iron limitation that is exerted on PSI in the mutant results in the accumulation of IsiA in a form that does not contribute to light harvesting for PSI. The aggregates of IsiA rather provide optimal conditions for growth when the content of iron increases again.

Introduction

Cyanobacteria use several strategies to regulate the light-harvesting capacity for photosynthesis (Ting *et al.*, 2002). Normally, the major peripheral antenna complexes associated with photosystem II (PSII) are the phycobilisomes, large supramolecular assemblies of phycobiliproteins that are located at the cytoplasmic site of the thylakoid membranes. Under iron-limiting conditions, a common condition in many aquatic ecosystems, the content of the most iron-rich photosystem (photosystem I, PSI) decreases compared to that of PSII. This change in stoichiometry is usually compensated by a decreased synthesis of phycobilisomes (Sandström *et al.*, 2002) and the accumulation of the so-called iron-starvation inducible IsiA or CP43' protein, a chlorophyll-binding protein that is structurally related to the CP43 protein of PSII (Bricker and Frankel, 2002). Recent research has indicated that 18 copies of the IsiA protein can encircle a trimeric PSI core complex, both in *Synechocystis* sp. PCC 6803 (Bibby *et al.*, 2001; Nield *et al.*, 2003) and in *Synechococcus* PCC 7942 (Boekema *et al.*, 2001). All spectroscopic evidence gathered thus far suggests that within the PSI-IsiA supercomplex the IsiA antenna ring is efficiently coupled to the PSI core complex (Bibby *et al.*, 2001; Andrizhiyevskaya *et al.*, 2002; Melkozernov *et al.*, 2002) and that the coupling of a full IsiA ring increases the light-harvesting capacity of PSI from 70% (Bibby *et al.*, 2001) to 100% (Andrizhiyevskaya *et al.*, 2002). It cannot be excluded, however, that the IsiA protein also fulfills other roles, e.g., as an excitation energy dissipator (Sandström *et al.*, 2001; 2002)

or as a chlorophyll storage protein (Burnap *et al.*, 1993).

Two subunits of the PSI complex, the PsaF and PsaJ proteins, are located at the outer edge of each PSI monomer (Kruip *et al.*, 1993; Jordan *et al.*, 2001; Chitnis, 2001). In contrast to its indispensable role in chloroplasts, PsaF is not absolutely needed for growth in cyanobacteria (Xu *et al.*, 2001, Hippler *et al.*, 1996). In *Synechocystis* sp. PCC 6803, PsaJ and PsaF are cotranscribed from the same operon (Chitnis *et al.*, 1991). PsaJ and PsaF also interact on the protein level, and PsaJ binds three chlorophyll molecules (Jordan *et al.*, 2001), which have been implied to participate in the energy transfer from the peripheral IsiA antenna to the PSI core complex in PSI-IsiA supercomplexes (Nield *et al.*, 2003).

The absence of PsaF and PsaJ triggers the derepression of the 'iron-stress inducible' operon *isiAB*, even when the content of iron does not limit growth (Jeanjean *et al.*, 2003). It was proposed that the accumulation of the IsiA and IsiB (flavodoxin) proteins was induced by oxidative stress, and correlated to a malfunctioning of PSI at its stromal side. The ring of IsiA around trimeric PSI centers in the *psaFJ* mutant surprisingly contained 17 IsiA monomers (Kouřil *et al.*, 2003), in contrast to the 18-mer status in the wild type (Bibby *et al.*, 2001; Nield *et al.*, 2003). Here we show that a combination of iron stress and malfunction of PSI caused by the absence of PsaF and PsaJ results in a strong decrease of the content of PSI, but not of IsiA and phycobiliproteins. In these cells, IsiA becomes by far the most abundant chlorophyll-binding protein, which predominantly occurs in linear or circular aggregates. IsiA thus becomes transcribed more than would be needed to supply the remaining PSI for the formation of PSI-IsiA supercomplexes. The results shed new light on the response of cyanobacteria to environmental stress.

Experimental procedures

Cell Culture and Isolation of Thylakoid Membranes. *Synechocystis* sp. PCC 6803 wild type and the *psaFJ* mutant (Zuther *et al.*, 1998) were grown at 30°C in liquid BG11 medium (Rippka *et al.*, 1979) 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, stationary phase cells harvested 8 to 14 days after inoculation were used. Thylakoid membranes were isolated as in Scholts *et al.* (1996). Freshly harvested cells were washed by resuspension in buffer containing 0.3 M mannitol, 10 mM MgCl_2 , 10 mM CaCl_2 , and 10 mM Hepes at pH 7.5, followed by incubation with lysozyme (5 mg/ml^{-1}) during 1 h in the dark. Cells were disrupted with Zirconium/glass beads (0.1 mm) in a Mini-bead beater (Biospec products). Unbroken cells and debris were removed by centrifugation (1,000 x g, 5 min, 4°C) and the thylakoid membranes were spun down at 15,000 x g and

washed in a buffer containing 20 mM Bis-Tris (pH 6.5), 5 mM MgCl₂, and 10 mM CaCl₂.

Cell Fractionation. Freshly isolated thylakoid membranes (0.15 mg chlorophyll a ml⁻¹) were solubilized with 0.5% (w/v) n-dodecyl-β-D-maltoside and centrifuged at 9,000 x g for 3 min. The supernatant was filtrated on a Millipore nitrocellulose membrane (0.45 μm) and analyzed by size exclusion chromatography as described by (Boekema *et al.*, 1999), using a Superdex 200 HR 10/30 column (Pharmacia), a running buffer consisting of 20 mM Bis-Tris (pH 6.5), 5 mM MgCl₂ and 0.03% n-dodecyl-β-D-maltoside, and an on line diode array detector (Shimadzu SPD-M10A).

SDS-PAGE and Mass Spectrometry. FPLC-fractions were concentrated on Microcon YM-10 (Millipore), the protein content was estimated by the assay according to Bradford (Bio-Rad). The protein composition was analyzed by Tris-Tricine SDS-PAGE according to (Schägger *et al.*, 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) as described by (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 μm 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 x 10⁻⁵ bar measured on the quadruple pressure gauge. The acquired peptide MSMS spectra were used to search the SwissProt database.

Spectroscopy. For low-temperature spectroscopic measurements, the samples were diluted in a buffer containing 20 mM Bis-Tris (pH 6.5), 10 mM MgCl₂, 10 mM CaCl₂, 0.02% β-DM and 66% (w/w) glycerol. The OD of the samples used for fluorescence and absorbance measurements was about 0.1 and 0.6 cm⁻¹, respectively, at the Q_y absorption maximum. Low-temperature absorption was measured on a home-built spectrophotometer. Fluorescence emission spectra at 5 K were recorded on a home-built fluorimeter as described in (Ihalainen *et al.*, 2000), using an excitation wavelength of 420 nm.

Electron Microscopy. EM was performed as described in Germano *et al.* (2002). 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 CM10 electron microscope at 52,000 x magnification. Images were scanned with a Nikon Coolscan 8000 ED at 20 μm, equivalent to 3.85 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 using mutual correlation functions (Van Heel *et al.*, 1992). The 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.

Results

Characterization of the growth properties of wild type and *psaFJ* mutant cells.

In iron-replete medium the growth rates of wild type (WT) and *psaFJ* mutant cells (hereafter referred to as mutant) are similar. Differences between both strains are visible in the absorption spectra (Fig. 1A). The mutant features a high phycobilisome (628 nm peak) content relative to the assembly of peaks in the 670-690 nm area that represent the chlorophyll proteins of PSI, PSII and, if present, also IsiA. In iron-depleted conditions the WT shows a broader 670-690 nm domain, largely due to added absorption at 675 nm. The mutant also features this addition, but shifted to the blue due to a decrease of the original peak in iron-replete spectrum at 680 nm (Fig. 1B).

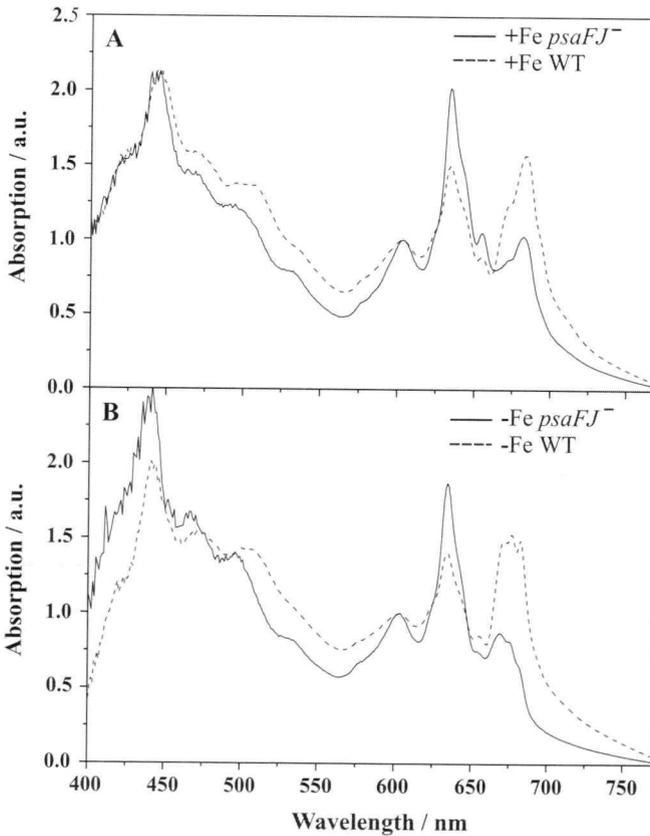


Figure 1. Absorption spectra monitored at 5 K of WT and *psaFJ* mutant cells grown in iron-replete (Frame A) and iron-depleted media (Frame B).

The 5 K fluorescence emission spectra from iron-replete grown cultures are depicted in Fig. 2A. These spectra are dominated by a broad band peaking at about 724 nm, which can be attributed to PSI (van Grondelle *et al.*, 1994). The region between 685 and 695 nm usually represents emission from PSII. The mutant shows a strong increase in emission around 685 nm. The fluorescence spectrum from the mutant cells grown in the absence of iron is very pronouncedly dominated by a band peaking at 687 nm (Fig. 2B) (the shift from 685 to 687 nm will be referred in the Discussion). The emission between 650 and 680 nm shows that there is still some phycobiliprotein emission in these cells, but emission originating from PSI is reduced in time of culture in iron-depleted medium as seen from the strong decrease between 3 and 8 days. The fine structure observed between 710 and 770 nm can be almost

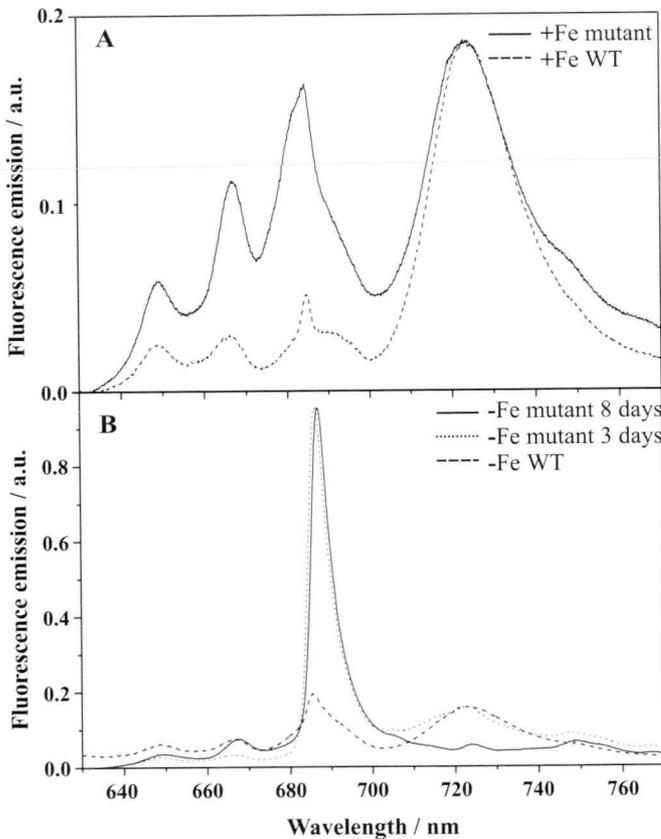


Figure 2. Low temperature fluorescence emission spectra from WT and the *psaFJ* mutant cells grown in iron-replete (Frame A) and iron-depleted media (Frame B). The excitation wavelength was 420 nm.

fully attributed to vibrational transitions of the main 687 nm band (Kwa *et al.*, 1994). It is most likely that the 687 nm fluorescence arises from chlorophyll-protein complexes that are not associated with a photosystem. A possible candidate for such a complex is IsiA, though the peak wavelength is slightly longer than has been observed before for this complex (Bibby *et al.*, 2001; Andrizhiyevskaya *et al.*, 2002). Though present, the amplitude of emission at 687 is much less pronounced in the WT than in the mutant (Fig. 2A, 2B). In the WT PSI remains present, even after prolonged culturing in iron-free medium.

Gel Filtration Chromatography

In order to obtain more information on the properties of the pigment-protein complexes in the thylakoid membranes of the cells, we solubilized the membranes by the mild detergent n-dodecyl- β -D-maltoside and separated the complexes by gel filtration chromatography. Fig. 3 (solid line) shows that in the iron-depleted cells from the mutant most complexes elute at about 18 min. We designate in the following the fractions eluting at around 17.5 and 18.5 min as Fraction A and Fraction B, respectively. The complexes in these fractions must be larger in size than trimeric PSI complexes (which are abundant in iron-replete WT and elute at about 19.5 min, Fig. 3, dotted line), and have about similar sizes as the PSI-IsiA supercomplexes (which are abundant in the iron-depleted WT, Fig. 3, dashed line). Fraction A was studied separately from the notion that it should contain smallest amount of trimeric PSI complexes and may represent largest particles. Its separate identity is also apparent from the slight

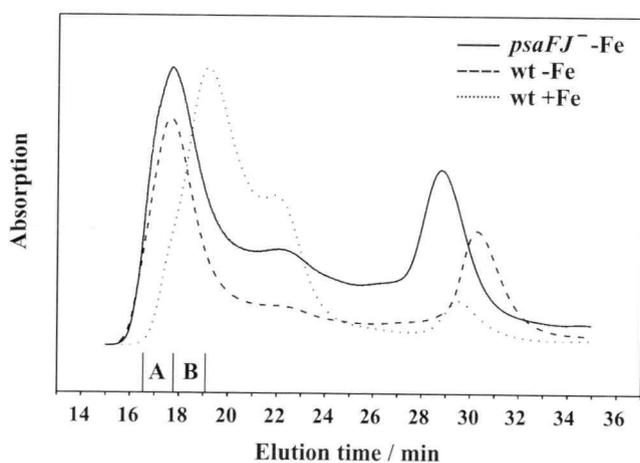


Figure 3. FPLC size-exclusion chromatography of β -DM solubilized thylakoids from the mutant cells grown in iron-deficient medium and from WT cells grown in iron-sufficient and iron-deficient (dashed line) media. The major peak at 18 min. has been collected as two separate fractions, A, 16.85 to 17.9 min and B, 18-19.1 min. The chromatograms shown were recorded at 400 nm.

shoulder on the left of the 17.5 to 18.5 min elution in the mutant.

The 5 K absorption spectrum of fraction A displays between 660 and 690 nm a fine structure with peaks at 682, 676 and 669 nm (Fig. 4A, dotted line). This structure is very similar to that observed for the iron-depleted mutant cells (Fig. 4A, full line), suggesting that the absorption of the chlorophylls in Fraction A largely represents the absorption of the chlorophylls in the cells. We note that the iron-replete cells show a very different absorption

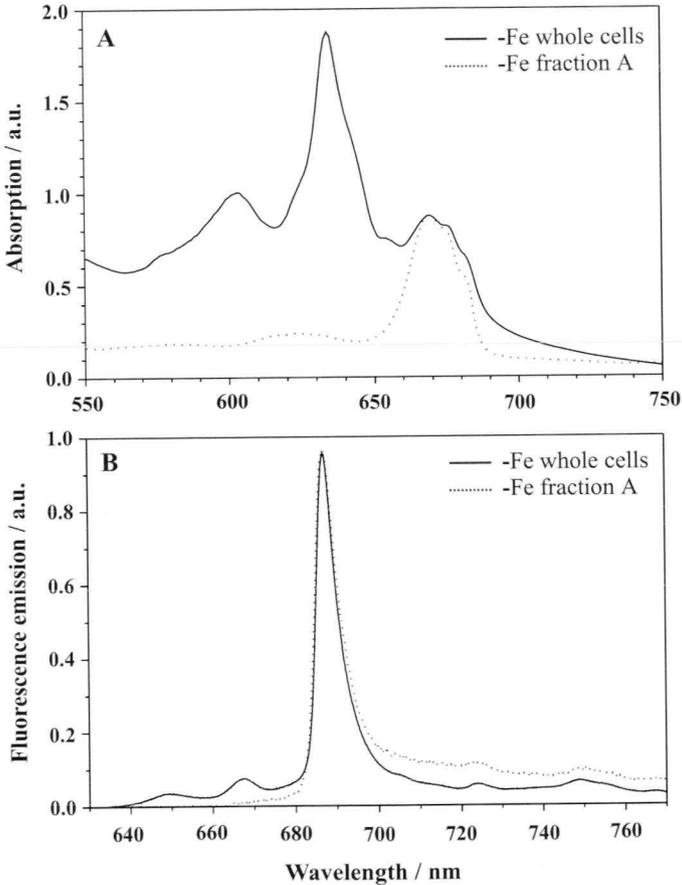


Figure 4. Spectroscopic characterization of the Fraction A. The 5 K absorption spectrum of cells of the *psaFJ* mutant of *Synechocystis* sp. PCC 6803 grown under iron-deficient conditions and the 77 K absorption spectrum of the FPLC resolved fraction A from iron-deficient *psaFJ* mutant cells. Solid line cells, dotted line fraction A (Frame A). Fluorescence emission spectra at 5 K of cells of the *psaFJ* mutant of *Synechocystis* sp. PCC 6803 grown under iron-deficient conditions and the 5 K emission spectrum of the FPLC resolved fraction A from iron-deficient *psaFJ* mutant cells. Solid line cells, dotted line fraction A. The excitation wavelength was 420 nm (Frame B).

spectrum (Fig. 1A, dashed line), where the increased absorption around 680 nm can be attributed to PSI (Kwa *et al.*, 1994). The absence of this absorption in the spectrum from the iron-depleted mutant cells confirms the almost complete absence of PSI in these cells (see Fig. 1 for a comparison to the WT). The low-temperature fluorescence spectrum of Fraction A (Fig. 4B, dotted line) is also very similar to that of the iron-depleted mutant cells (Fig. 4B, full line), except for the absence of phycobiliprotein emission in Fraction A and the presence of a very small PSI contribution.

Biochemical Characterization

Fig. 5 shows the protein composition of Fractions A and B from the iron-depleted WT and mutant. Selected protein bands from gels subjected to MSMS analysis revealed peptide fragmentation spectra that permitted identification of IsiA and PsaB in the gel (see arrows). These data indicate that IsiA is the most abundant protein in Fractions A and B from the iron-depleted mutant cells, and that Fractions A and B from the iron-depleted WT cells contain significantly more PSI core complexes (in which PsaB is one of the major constituents) than the corresponding fractions from the iron-depleted mutant cells.

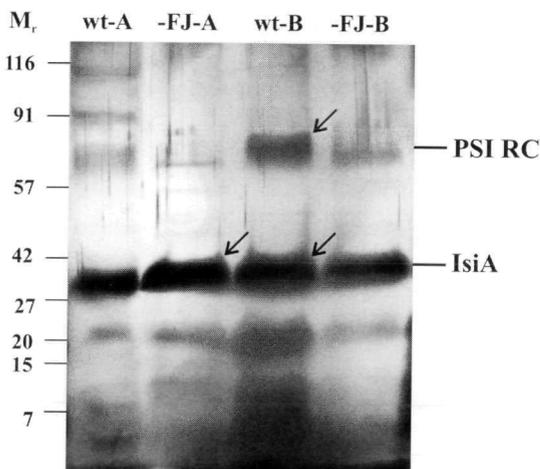


Figure 5. Silver stained SDS-PAGE of proteins of Fractions A and B from WT and *psaFJ* mutant cells grown in iron-deficient media. Lane 1, Fraction A from WT; lane 2, Fraction A from the *psaFJ* mutant, lane 3, Fraction B from WT; lane 4, Fraction B from the *psaFJ* mutant. Arrows show the bands analyzed by mass spectrometry.

Electron microscopy

Electron micrographs from Fractions A and B from the iron-depleted mutant cells revealed several types of complexes. Fig. 6 shows two typical aggregates, which according to the biochemical characterization of this fraction should entirely consist of IsiA. One has the shape of a rather irregular rod with a variable length and a maximal width of 10 nm (Fig.

6A). The packing of the IsiA molecules in these rods is not clear. The other has the shape of a doughnut with a diameter of 24.5 nm and an empty centre with a diameter of 10.5 nm (Fig. 6B). Image analysis showed that the doughnuts have a well-defined shape (Fig. 6C), but the present data permit no statistically reliable estimation of the number of IsiA units within the doughnut. The distance of 7 nm across the protein areas in the doughnuts is similar to the distance of 6.4 nm observed for IsiA in PSI-IsiA supercomplexes. We note that other types of aggregates have been observed as well (not shown), including half doughnuts (open, C-shaped complexes with sizes comparable to those of the doughnuts shown in Fig. 6B), smaller aggregates and PSI-IsiA supercomplexes. The latter complexes occurred in very small numbers, but were abundant after a limited time of culture of the mutant cells in iron-free medium and were shown to consist of PSI trimers encircled by rings of 17 IsiA proteins (Kouřil *et al.*, 2003)

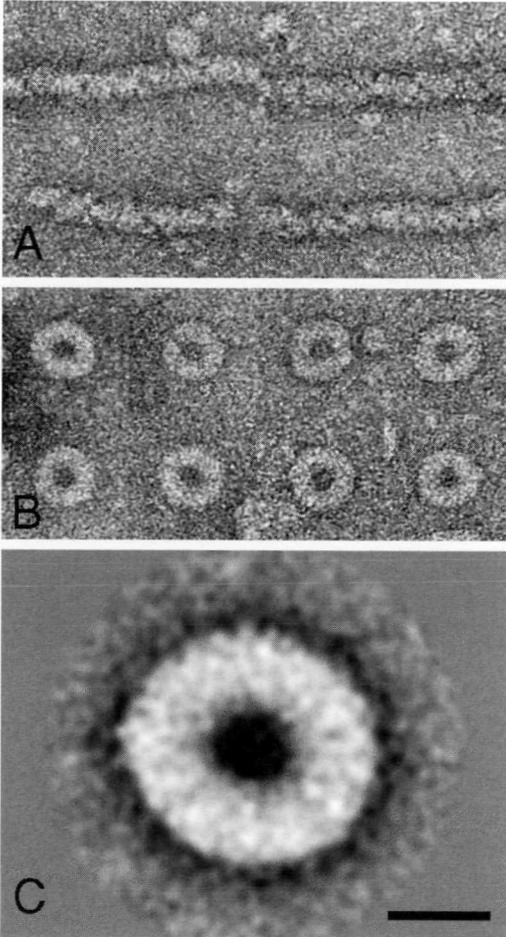


Figure 6. (A) Parts of electron micrographs showing rod-like projections of IsiA aggregates negatively stained with 2% uranyl acetate. (B) A gallery of doughnut-like top view projections from IsiA aggregates. (C) Averaged images of 128 top view projections from IsiA doughnuts. The scale bar for (C) is 10 nm.

Discussion

The present data show that under selected conditions of growth or through specific mutation the 'iron-stress-inducible' chlorophyll-binding protein IsiA may accumulate in cells and associates itself into large linear or circular aggregates. Image analysis of the circular aggregates revealed a surprisingly homogeneous size distribution, suggesting that the number of units within these rings is constant. The diameter of these 'doughnuts' is 24.5 nm, which is significantly smaller than the diameter of about 32.5 nm of the 18-unit IsiA ring around wild-type PSI trimers (Bibby *et al.*, 2001; Nield *et al.*, 2003; Boekema *et al.*, 2001) and is also much smaller than the diameter of 29.5 nm of the 17-unit IsiA ring around PSI trimers without the PsaF and PsaJ subunits (Kouřil *et al.*, 2003).

The present data do not permit a precise estimation of the number of IsiA units within the doughnut. Based on the diameter of the doughnuts, numbers of 12-14 are possible. The inner diameter of the doughnut is about 10.5 nm or about 12.5 nm if the detergent contribution to this diameter is taken into account. This diameter is just sufficient to encircle a monomeric PSI complex, which has outer dimensions of 14 x 10 nm (Jordan *et al.*, 2001). This suggests that the IsiA protein has evolved in such a way that its association into a ring of a size that potentially can encircle a monomeric PSI complex is thermodynamically more stable than one that can encircle a trimeric PSI complex. However, the structure seems to be quite flexible allowing a modular built up of complexes able to associate with PSI structures of different sizes, i.e. trimeric WT complexes as well as slightly smaller complexes in the *psaFJ* mutant.

The current data show that under certain growth conditions, the cyanobacterium *Synechocystis* sp. PCC 6803 accumulates IsiA in clear excess of what is needed for functional light harvesting for PSI. 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; Asada *et al.*, 1998), or constitutes a stock of chlorophyll to rapidly resume growth after relieve from the iron limitation stress (Burnap *et al.*, 1993), is topic of current investigation. The latter option is perhaps the most likely, also because there is no sign of degradation of phycobilisomes under these conditions. The fluorescence spectra of the iron-depleted mutant cells show phycobilisome emission but clearly lack the typical PSII emission at 695 nm (Fig. 2B), which suggests that also the phycobiliproteins are synthesized in excess over their main target for light harvesting. Alternatively, the remaining phycobilisomes are almost completely decoupled from PSII. The quantum yield of the 695 nm emission of PSII is quite high at 5 K (Van Amerongen and Dekker, 2003), so differences in quantum yield between IsiA and PSII

cannot explain the absence of PSII emission from the emission spectrum of the cells. It thus appears that IsiA can serve more than one role. Under mild PSI stress conditions, it serves as an additional light-harvesting complex for PSI. The observation that in the 5 K fluorescence emission spectrum the 685 nm emission as present in the WT shifts to 687 nm in the mutant may reflect that light harvesting by PSII is shielded by the abundantly present IsiA protein (Sandström *et al.*, 2001). Third option is that under more severe stress conditions it provides a stock of protein-bound chlorophyll that can immediately be used for light harvesting when the stress is relieved again. Those more severe conditions are the combination of iron stress and photo-oxidative stress present in the *psaFJ* mutant already in iron-replete conditions (Jeanjean *et al.*, 2003).

Effects of iron stress have also been studied in various eukaryotic algae (red, brown and green algae). In contrast to cyanobacteria, these eukaryotic algae can not produce proteins related to the IsiA protein, but they do contain one or more chlorophyll *a/b* or *a/c* binding proteins encoded by the Lhc super-gene family (Jansson, 1999) that serve as peripheral light-harvesting complex for PSI (LHCI). It has recently been shown that iron stress in the red algae *Rhodella violacea* leads to a relative enrichment of both phycobilisomes and LHCI compared to PSII and PSI, and that the LHCI that is free in the membrane after prolonged iron depletion is used as a pre-assembled antenna for newly synthesized PSI when the content of iron increases again (Doan *et al.*, 2003). Our results suggest a similar mechanism with pre-existing IsiA aggregates for cyanobacteria and thus point to a common mechanism by which cyanobacteria and eukaryotic algae respond to iron stress and other stress exerted on PSI.

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