Localization and function of the IdiA homologue Slr1295 in the cyanobacterium *Synechocystis* sp. strain PCC 6803

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Slr1295 (and Slr0513) in the cyanobacterium *Synechocystis* sp. PCC 6803 has amino acid similarity to the bacterial FbpA protein family and also to IdiA of *Synechococcus* PCC 6301/PCC 7942. To determine whether Slr1295 is the periplasm-located component of an iron transporter, or has a function in protecting photosystem (PS) II, subcellular localization and Δ slr1295 mutant characterization studies were performed. Localization of Slr1295 provided evidence that it has an intracellular function, since virtually no Slr1295 was detected in the soluble protein fraction of the periplasm or in the cytoplasmic membrane. Characterization of a Δ slr1295 *Synechocystis* PCC 6803 mutant indicated that PS II is more susceptible to inactivation in the mutant than in the wild-type (WT). Under mild iron limitation, modification of PS I to the PS I–IsiA complex is more advanced in the Δ slr1295 mutant, indicating that iron deficiency leads more rapidly to changes in the photosynthetic apparatus in the mutant than in the WT. Biochemical fractionation procedures provide evidence that Slr1295 co-purifies with PS II. These results suggest a function of Slr1295 that is comparable to the function of IdiA in *Synechococcus* PCC 6301/PCC 7942 being a protein that protects PS II under iron limitation in an as yet unknown way.

Keywords: *Synechocystis* PCC 6803, iron deficiency, Slr1295, photosystem I and II

INTRODUCTION

In a number of previous publications we have shown that under iron-limiting growth conditions a protein of 35 kDa, called IdiA (iron deficiency induced protein A), becomes expressed in highly elevated amounts in the cyanobacteria *Synechococcus* PCC 6301 and PCC 7942 (accession numbers of *idiA* from PCC 6301 and PCC 7942 are Z48754 and AJ319672, respectively) as well as in the thermophilic *Synechococcus elongatus* (Michel *et al.*, 1996, 1998, 1999). IdiA is located almost entirely intracellularly and is mainly associated with the thylakoid membranes (Michel *et al.*, 1998). Comparative investigations about the function of IdiA in *Synechococcus* PCC 7942 wild-type (WT) and the corresponding IdiA-free mutant indicated that IdiA seems to pre-

ferentially protect the acceptor side of photosystem II (PS II) under oxidative stress – especially when oxidative stress is caused by iron deficiency (Michel *et al.*, 1996; Exss-Sonne *et al.*, 2000). However, the exact nature of the PS II protecting function of IdiA has remained unclear. Recently, the iron-responsive *idiA* promoter was characterized and IdiB, with strong similarity to helix–turn–helix transcription factors of the FNR/CAP family, was identified as a positively acting transcription factor of IdiA expression in *Synechococcus* PCC 7942 (Michel *et al.*, 2001).

The association of IdiA with the thylakoid membranes of *Synechococcus* PCC 7942/PCC 6301 and the protective function on PS II came somewhat as a surprise, because IdiA has high similarity to the FbpA (also called SfuA) protein of pathogenic bacteria, such as *Serratia marcescens* (Angerer *et al.*, 1990), *Neisseria gonorrhoeae* (Berish *et al.*, 1990a; Forng *et al.*, 1997), *Neisseria meningitidis* (Berish *et al.*, 1990b) and *Pasteurella haemolytica* (Kirby *et al.*, 1998) for example. FbpA

Abbreviations: ABC, ATP-binding cassette; Chl, chlorophyll; DCPIP, 2,6-dichlorophenol-indophenol; Dig, digoxigenin; MV, methylviologen; PBQ, phenyl-*p*-benzoquinone; PCV, packed cell volume; PS I and PS II, photosystem I and II.

belongs to the cluster 1 group of extracellular solute-binding proteins with specificity for ferric iron (Tam & Saier, 1993). The FbpA protein of pathogenic bacteria is a component of a typical ATP-binding cassette (ABC) transporter for ferric iron across the inner membrane when iron is delivered as Fe^{3+} -transferrin and/or Fe^{3+} -lactoferrin. In the Fbp transport system FbpA is the periplasm-located component, FbpB is a cytoplasmic membrane protein and FbpC is a membrane-bound protein carrying a nucleotide-binding motif (Mietzner & Morse, 1994; Adhikari *et al.*, 1996). A dendrogram was compiled for the pathogenic bacterial FbpA proteins, including the IdiA protein from *Synechococcus* PCC 6301, showing that IdiA has the highest similarity to FbpA from *Pasteurella haemolytica* (Kirby *et al.*, 1998).

Recently, a protein immunologically related to IdiA from *Synechococcus* PCC 6301/PCC 7942 was detected in three marine cyanobacterial species: *Synechococcus* sp., *Crocospheera* sp. and *Trichodesmium* sp. (Webb *et al.*, 2001). In the cyanobacterium *Synechocystis* PCC 6803, whose genome has been completely sequenced (Kaneko *et al.*, 1996), there are two genes encoding proteins Slr0513 and Slr1295 which both have high similarity to IdiA from *Synechococcus* PCC 6301 and PCC 7942 (Slr1295, 53% identity and 69% similarity; Slr0513, 51% identity and 66% similarity). In addition, a third gene encoding a protein with lower similarity is present: Sll0237, with 26% identity and 42% similarity to IdiA. In a search for genes/proteins involved in iron acquisition in *Synechocystis* PCC 6803, Katoh *et al.* (2000, 2001a, b) recently investigated a number of genes encoding polypeptides with similarity to proteins of ABC-type ferric iron transporters. These genes are *slr1295*, *slr0513*, *slr0327* and *sll1878*, which were designated *futA1*, *futA2*, *futB* and *futC*, respectively. These genes are not located in an operon as is the case for the bacterial iron transporters (for a comparative listing, see Webb *et al.*, 2001). Inactivation of *futB* or *futC* or of both *futA1* and *futA2* greatly reduces the activity of ferric iron uptake and also growth. This group (Katoh *et al.*, 2001a) concludes that the Fut system in *Synechocystis* PCC 6803 is related to the Sfu/Fbp family of bacterial iron transporters (Adhikari *et al.*, 1996). In the *Synechocystis* PCC 6803 transport system, proteins FutA1 (Slr1295) and FutA2 (Slr0513) are suggested to be the periplasm-located components which are assumed to play a role in iron binding. This is supported by the recent finding that the GST-tagged recombinant FutA1 was shown to bind ferric ion with high affinity (Katoh *et al.*, 2001b). Since FutA1 and FutA2 are suggested to have redundant or overlapping substrate-binding functions, the phenotype with significantly reduced ferric iron uptake and consequently reduced growth is only obtained in the *Synechocystis* PCC 6803 double mutant lacking both proteins (Katoh *et al.*, 2001a, b). In agreement with the assumed function of Slr0513 (FutA2) are the data of Fulda *et al.* (2000) who have shown that Slr0513 is indeed present in the soluble protein fraction of the periplasm in *Synecho-*

cystis PCC 6803. On the other hand, the localization of Slr1295 (FutA1) has remained uncertain, since it was not among the group of periplasm-located soluble proteins identified by this group. To obtain more information about Slr1295, we investigated the localization of Slr1295 together with the localization of Slr0513 and have performed a detailed study of *Synechocystis* PCC 6803 WT and an Slr1295-free mutant grown under iron-sufficient and iron-deficient conditions.

METHODS

Cyanobacterial strains and growth conditions. *Synechocystis* sp. strain PCC 6803 (subsequently called *Synechocystis* PCC 6803) was obtained from the Institut Pasteur, Collection Nationale de Cultures de Microorganismes, Paris, France, and was grown as described previously (Stephan *et al.*, 2000). The bottles were illuminated with four Philips lamps (Cool Spot, $\approx 12^\circ$, 150 W) resulting in $161 \mu\text{E m}^{-2} \text{s}^{-1}$. Growth of the Slr1295-free *Synechocystis* PCC 6803 mutant was as above, except that the growth medium contained chloramphenicol (8 mg l^{-1}).

For growth of *Synechocystis* under iron limitation, BG11 medium without Fe(III) citrate was used. Prior to transfer of cells into iron-free medium, cultures were grown for several cycles of 2 days under regular conditions, then harvested by centrifugation and once washed with 20 mM sodium phosphate buffer, pH 7.0. In general, *Synechocystis* PCC 6803 [inoculation, $0.4 \mu\text{l}$ packed cell volume (PCV) ml^{-1}] was grown for 48 h under the given conditions. Growth was determined by measuring the OD_{750} of *Synechocystis* PCC 6803 cultures [OD_{750} of 0.1 (up to a total absorbance of <1) corresponds to about $0.1 \mu\text{l}$ PCV ml^{-1}] (see also Flores *et al.*, 1982).

Preparation of cell suspensions and cell-free extracts. For preparation of cell suspensions, *Synechocystis* cells were harvested by centrifugation for 30 min at 2200 g and then resuspended in MCMG buffer (20 mM MES/NaOH, pH 6.35, 5 mM CaCl_2 , 5 mM MgCl_2 , 25% glycerol) to give a cell density of $100 \mu\text{l}$ PCV ml^{-1} .

When cell-free extracts of *Synechocystis* were used, the above cell suspension ($100 \mu\text{l}$ PCV ml^{-1}) was mixed with an equal volume of glass beads (0.17–0.18 mm) and treated in a 'Bead Beater' (Biospec Products; chamber vol. 15 ml) 10 to 15 times for 25 s with cooling intervals of 4 min. After this treatment the cell extract was decanted from the beads and the beads were washed once with an equal volume of MCMG buffer. Subsequently, the two supernatants were combined. Thus, the obtained cell extract corresponded to a cell suspension of $50 \mu\text{l}$ PCV ml^{-1} . The cell extract was centrifuged for 10 min at 2000 g to remove the residual glass beads and unbroken cells.

Determination of photosynthetic activities. Photosynthetic O_2 evolution/uptake with intact cells or cell-free extracts of *Synechocystis* PCC 6803 was measured in a Clark-type electrode (Rank Brothers) at a polarization voltage of 600 mV. The reaction temperature was 30°C for cell suspensions and 20°C for cell-free extracts. Continuous red light was provided by a halogen lamp (24 V, 250 W; Spindler and Hoyer). The light was filtered through a glass cuvette containing 2% (w/v) CuSO_4 and a red plexiglass filter (RG1-610; Schott). Light intensity on the water surface corresponded to $1995 \mu\text{E m}^{-2} \text{s}^{-1}$ (400–740 nm).

O_2 evolution of whole cells was determined in a reaction mixture of 3 ml containing 54 mM HEPES/NaOH, pH 7.0, 15 mM NaHCO_3 in BG11 medium and cell suspension

corresponding to 5–20 µg chlorophyll (Chl). Alternatively, a reaction mixture containing 54 mM HEPES/NaOH, pH 7.0, 1 mM phenyl-*p*-benzoquinone (PBQ), 50 mM CaCl₂ and cell suspension was used.

PS I activity was determined with cell-free extracts. The reaction mixture contained in a total volume of 3 ml: 54 mM HEPES/NaOH, pH 7.0, 0.08 mM 2,6-dichlorophenol-indophenol (DCPIP), 3.33 mM sodium ascorbate, 0.17 mM methylviologen (MV), 0.4 mM KCN, 0.01 mM 3-(3,4-dichlorophenyl)-1,1-dimethylurea and cell-free extract corresponding to 5–20 µg Chl.

PS II activity was determined in a reaction mixture of 3 ml containing 54 mM HEPES/NaOH, pH 7.0, 50 mM CaCl₂, 400 mM sucrose, 1.7 mM potassium ferricyanide and cell-free extract corresponding to 10–20 µg Chl.

Since iron-deficient growth leads to Chl reduction, photosynthetic activity values are always given on Chl basis (per mg Chl) and in addition on the basis of PCV (per 100 µl PCV). PCV was calculated from the OD₇₅₀ (see above).

Pigment content, protein content, SDS-PAGE and immunoblotting. Chl content was estimated according to Grimme & Boardman (1972), and phycocyanin and allophycocyanin content according to Tandeau de Marsac & Houmard (1988). Protein content was determined either according to Smith *et al.* (1985) or Bradford (1976).

SDS-PAGE and immunoblotting were done as described previously (Exss-Sonne *et al.*, 2000). Samples were denatured at 60 °C for 30 min. The antisera used were as follows: anti-IdiA (IdiA from *Synechococcus* PCC 6301; Michel & Pistorius, 1992), anti-Slr0513 (Slr0513 was isolated from the soluble protein fraction of the periplasm from *Synechocystis* PCC 6803; a kind gift of Dr Sabine Fulda), anti-PsbA, anti-PsbB, anti-PsbO and anti-AtpA (proteins from oat or tobacco; Exss-Sonne *et al.*, 2000), anti-PsaA/B (PsaA/B from *Synechocystis* PCC 6803) and anti-C-phycocyanin (from *Aphanotheca halophytica*; Sigma) and anti-allophycocyanin (from *Anabaena variabilis*; Boehringer).

77 K Chl *a* fluorescence measurements. 77 K Chl *a* fluorescence measurements were performed with a Perkin Elmer luminescence spectrometer LS50B. Excitation was at 435 nm and fluorescence emission was recorded between 650 and 800 nm. Cells suspensions cultivated under iron-sufficient or iron-deficient conditions were adjusted to equal Chl concentration by dilution with BG11 medium ($\pm$ iron) to give a Chl concentration within the range of 2–10 µg Chl ml⁻¹. Cells were immediately frozen in liquid nitrogen without any further pretreatment.

Isolation of *Synechocystis* PCC 6803 subcellular fractions. Preparation of the soluble protein fraction from periplasm and of the spheroplast fraction was done according to Block & Grossman (1988). The soluble protein fraction of the periplasm was concentrated (about eightfold) by dialysis against 20% carbowax (PEG 20000). The pellet containing the spheroplasts was resuspended in 5 mM Tris/HCl, pH 7.5, and broken by French pressure cell treatment at 138 MPa and 4 °C. Centrifugation for 1 h at 100 000 *g* resulted in a soluble protein fraction and a pellet, which contained cytoplasmic and thylakoid membranes and which was resuspended in 5 mM Tris/HCl, pH 7.5.

The cytoplasmic and thylakoid membranes were isolated with slight modifications according to the procedure described by Omata & Murata (1984). *Synechocystis* WT cells (2.5 l cell suspension) were harvested by centrifugation, resuspended in 5 mM HEPES/NaOH, pH 7.0, containing 600 mM sucrose and 2 mM Na₂EDTA, treated with lysozyme for 2 h at 28 °C

and broken by passage through a prechilled French pressure cell at 40 MPa. The homogenate was centrifuged at 5000 *g* for 10 min to remove unbroken cells. The supernatant was subjected to density-gradient centrifugation as described by Omata & Murata (1984).

Isolation of PS I complexes from *Synechocystis* PCC 6803 was done as described by Wenk & Kruip (2000), and isolation of PS II complexes was done as described by Burnap *et al.* (1989) with slight modifications. The chromatography of the detergent-solubilized PS II complexes was done on a MonoQ column (Pharmacia) instead of a Toyopearl DEAE-M column and the NaCl gradient was extended to 500 mM NaCl instead of 200 mM NaCl.

Ultrastructural and immunocytochemical investigations. *Synechocystis* PCC 6803 cells were grown and harvested as described above. A cell pellet obtained from 10 ml cell suspension was washed three times with EM buffer (50 mM KH₂PO₄/Na₂HPO₄, pH 7.0). The ultrastructural and immunocytochemical investigations were performed as described previously (Engels *et al.*, 1997).

Construction of the Slr1295-free *Synechocystis* PCC 6803 mutant. For construction of the Slr1295-free *Synechocystis* PCC 6803 mutant the primers P-5'-AACCTCCAGTTCCA-CTGACC-3' and P-5'-AGTCTGCCAACTGGTGACAA-3' were used to amplify a 1907 bp DNA fragment carrying the gene *slr1295*. The PCR-amplified fragment was cloned into *Sma*I-digested pK18mob (Schäfer *et al.*, 1994). The insertion of a Cm^R cassette with flanking Ω terminators preventing transcriptional read-through activities, taken from plasmid pHP45 Ω -Cm (Chang & Cohen, 1978), into the *Ava*I-site leads to insertional inactivation of the *slr1295* gene, leaving nearly equal flanking DNA regions on both sides of the fragment. Transformation of *Synechocystis* PCC 6803 with this 6987 bp construct (pK18mob1295Cm^R) was performed according to Laudenbach & Straus (1988).

Southern blotting. Southern blotting was performed as described in Sambrook *et al.* (1989). Genomic DNA from *Synechocystis* PCC 6803 WT and the Slr1295-free mutant was isolated by the Sarkosyl method, purified by the phenol extraction procedure (Williams, 1988) and then digested with *Ssp*I and *Sca*I or *Xmn*I, or *Alw*NI and *Kpn*I. The digested DNA was separated in a 1% agarose gel and transferred via capillary blotting onto a positively charged nylon membrane (Hybond-N⁺; Amersham). For hybridization a digoxigenin (Dig)-labelled pK18mob1295Cm^R probe was used, according to the manufacturer's recommendation.

Northern blotting. Northern blotting was done as previously described (Michel *et al.*, 1999). The primers used to amplify the probes were 5'-AACCTCCAGTTCCACTGACC-3' and 5'-AGTCTGCCAACTGGTGACAA-3' for *slr1295*, 5'-GGC-AAGGATGGACAGCAGTA-3' and 5'-GCATTACTGCCA-GCCAACTT-3' for *slr0513*, and 5'-GGTGGACAGAGACGCTTTATT-3' and 5'-TGGCTAATGACTAGGTTTGC-3' for *isiA*.

RESULTS AND DISCUSSION

Construction of an Slr1295-free *Synechocystis* PCC 6803 mutant

Plasmid pK18mob1295Cm^R (see Methods) was used for insertional inactivation of *slr1295* in *Synechocystis* PCC 6803. The Cm^R gene in the plasmid has the same orientation as the *slr1295* gene. The mutant was verified by Southern blot analysis of the corresponding Cm^R and

Fig. 1. Northern blot analysis of *slr1295* and *slr0513* mRNA expression. *Synechocystis* PCC 6803 WT and the Slr1295-free mutant were grown for 2 days in iron-sufficient medium (A) and for 2 (B) or 4 days (C) in iron-deficient medium. Detection of *slr1295* and *slr0513* mRNA was achieved by DNA/RNA hybridization using the corresponding Dig-labelled probe.

Km^S clones. As shown below, the mutant did not contain any detectable *slr1295* mRNA, nor the Slr1295 protein (see Figs 1 and 2). This Slr1295-free *Synechocystis* PCC 6803 mutant (Δ 1295) was used in subsequent experiments.

Analysis of the expression of Slr1295 and Slr0513 at the mRNA and protein level

Expression of Slr1295 and Slr0513 was analysed in *Synechocystis* PCC 6803 cells grown under iron-sufficient and iron-deficient conditions. For these experiments, cells were cultivated for 2 days under iron-sufficient conditions (+ Fe: 2d) and for 2 days (– Fe: 2d) or 4 days (– Fe: 4d) under iron-deficient conditions (medium without Fe³⁺ citrate). In the experiments with cells grown for 4 days under iron deficiency (– Fe: 4d), the cells were grown for 2 days under iron deficiency and

then diluted into fresh iron-deficient medium and grown for another 2 days. The results of the Northern blot analysis are given in Fig. 1. Under the chosen conditions, only a low level of mRNA for *slr1295* and *slr0513* was present in WT cells grown under iron-sufficient conditions. The transcript level substantially increased under iron limitation. This increase was already seen in 2-day iron-depleted cells. As expected, the Slr1295-free mutant did not contain any detectable *slr1295* mRNA, confirming that the mutant was fully segregated. In the Slr1295-free mutant, as well as in the WT, the *slr0513* mRNA level was low under iron-sufficient conditions and increased under iron-deficient conditions.

Expression of Slr1295 and Slr0513 at the protein level was analysed with an anti-IdiA antiserum raised against IdiA isolated from *Synechococcus* PCC 6301 (Michel & Pistorius, 1992), and with an antiserum raised against the Slr0513 protein which was isolated from the soluble protein fraction of the periplasm from *Synechocystis* PCC 6803. The results of the immunoblot analysis are given in Fig. 2. Two very weak protein bands were detectable after immunostaining with the anti-IdiA antiserum in WT cells cultivated under iron-sufficient conditions. The expression of both proteins was highly increased under iron limitation. As expected, in the Slr1295-free mutant only one protein with an increased expression under iron limitation was detected, corresponding to the lower band in WT cells. Thus, the upper band in WT cells represents Slr1295, which is missing in the mutant, and the lower band represents Slr0513, which is present in the mutant. The results in Fig. 2 also show that the anti-Slr0513 antiserum only recognizes the lower band in WT cells and thus only cross-reacts with Slr0513, the protein against which the antiserum was raised. This antiserum is slightly more sensitive in detecting small amounts of Slr0513 than anti-IdiA antiserum. Thus, a 10% SDS-polyacrylamide gel (Tris-glycine buffer system) can resolve the two proteins under investigation: Slr1295 migrates slightly slower (upper band, apparent molecular mass 35 kDa)

Fig. 2. Immunoblot analysis of Slr1295 and Slr0513 protein expression. *Synechocystis* PCC 6803 WT and Slr1295-free mutant cells were grown for 2 days in iron-sufficient medium (A) and for 2 (B) or 4 days (C) in iron-deficient medium. Detection of the Slr1295 and Slr0513 proteins was achieved with the anti-IdiA antiserum (dilution 1:500), which recognizes both proteins, and the anti-Slr0513 antiserum (dilution 1:1000) which only cross-reacts with Slr0513, but is slightly more sensitive. Total cell-free extracts corresponding to 25 μ g protein were applied in each lane.

Table 1. Growth and pigment content of *Synechocystis* PCC 6803 WT and the Slr1295-free mutant

After growth for 2 days under iron-sufficient conditions (+Fe: 2d) or for 2 days (–Fe: 2d) or 4 days (–Fe: 4d) under iron-deficient conditions, cells were harvested and resuspended to give a cell density of 100 µl PCV ml⁻¹. Chl was determined in the cell suspensions; phycocyanin and allophycocyanin were determined in broken cell extracts. Details are given in Methods.

<i>Synechocystis</i> PCC 6803	Growth conditions	Growth (OD ₇₅₀)	Chl (mg per 100 µl PCV)	Phycocyanin/allophycocyanin (mg per 100 µl PCV)	
WT	+Fe: 2d	0.4→3.1±0.3	0.560±0.11	2.89±0.51	1.10±0.33
	–Fe: 2d	0.4→2.9±0.5	0.432±0.03	2.91±0.54	1.08±0.32
	–Fe: 4d*	0.4→1.7±0.6	0.323±0.02	0.96±0.10	0.37±0.04
Mutant Δ1295	+Fe: 2d	0.4→2.9±0.3	0.577±0.09	2.57±0.43	1.16±0.47
	–Fe: 2d	0.4→2.5±0.3	0.295±0.08	1.85±0.45	0.70±0.25
	–Fe: 4d*	0.4→1.1±0.4	0.165±0.01	0.52±0.10	0.21±0.03

* In the experiments with cells grown for 4 days under iron deficiency (–Fe: 4d), the cells were grown for 2 days under iron deficiency and then diluted into fresh iron-deficient medium and were grown for another 2 days. The values of the second cycle are given.

Table 2. Photosynthetic activities of *Synechocystis* PCC 6803 WT and the Slr1295-free mutant

Cells were cultivated as described in the legend to Table 1. The activity measurements were performed with cell suspensions (H₂O→NaHCO₃) or with broken extracts (PS II and PS I activity) as described in Methods. Data from two representative experiments are given.

<i>Synechocystis</i> PCC 6803	Growth conditions	H ₂ O→NaHCO ₃		H ₂ O→K ₃ [Fe(CN) ₆]		DCPIP/Asc.→MV	
		μmol O ₂ evolved or taken up h ⁻¹ per:					
		100 μl PCV	mg Chl	100 μl PCV	mg Chl	100 μl PCV	mg Chl
WT	+ Fe: 2d	135.7	201.7	137.0	203.6	125.3	186.2
		94.1	180.9	149.8	287.9	99.1	190.5
	116.6	286.9	69.2	170.4	70.5	173.5	
	33.0	97.3	56.8	167.8	31.6	93.2	
	MutantΔ1295	+ Fe: 2d	167.1	250.5	143.6	215.3	127.5
106.0			197.4	101.3	188.6	107.7	200.4
65.3		270.9	36.8	152.8	41.5	172.0	
2.4		13.8	27.7	156.9	25.0	141.4	

* In the experiments with cells grown for 4 days under iron deficiency (–Fe: 4d), the cells were grown for 2 days under iron deficiency and then diluted into fresh iron-deficient medium and were grown for another 2 days. The values for the second cycle are given.

than Slr0513 (lower band, apparent molecular mass 32 kDa). Both proteins are detected by anti-IdiA antiserum, while the anti-Slr0513 antiserum only cross-reacts with Slr0513.

Comparative analysis of growth, pigment content and photosynthetic activities

Growth, pigment content and photosynthetic activities of *Synechocystis* PCC 6803 WT and the Slr1295-free mutant were analysed under iron-sufficient and iron-deficient growth conditions. The results (Table 1) show that growth measured as OD₇₅₀ was reduced after 4 days of iron-deficient conditions (–Fe: 4d) from 3.1 to 1.7

and from 2.9 to 1.1 for the WT and mutant, respectively, showing that the reduction was higher in the mutant than in the WT (45 vs 62 %). A higher decrease was also observed in pigment content. The reduction in Chl, phycocyanin and allophycocyanin content in the WT corresponded to 42, 67 and 66 %, respectively; the corresponding values in the Slr1295-free mutant were 71, 80 and 82 %, respectively.

The O₂-evolving activity with bicarbonate was determined in intact cells, and partial activities were measured in cell-free extracts or intact cells. Since the Chl content per cell decreased under iron depletion, values shown in Table 2 (data for two representative

Table 3. O₂-evolving activity with PBQ as electron acceptor

Activity was determined in cell suspensions of *Synechocystis* PCC 6803 WT and the Slr1295-free mutant grown for 2 days under iron-sufficient conditions. Data for two representative experiments are given.

<i>Synechocystis</i> PCC 6803	Chl (mg per 100 µl PCV)	H ₂ O→PBQ µmol O ₂ evolved h ⁻¹ per:	
		100 µl PCV	mg Chl
WT	0.48	165.6	345.5
	0.42	115.5	274.2
Δ1295	0.53	72.4	135.6
	0.40	77.1	191.4

experiments are shown in Table 2 and mean values are given in the text) were always calculated on the basis of 100 µl PCV and on the basis of Chl. On the PCV basis, the overall photosynthetic activity dropped after 4 days under iron depletion by 71 % in the WT and by 94 % in the mutant (the corresponding values on the Chl basis are 46 and 79 %). On the PCV basis, PS II as well as PS I activities were reduced by about 65 % in the WT and by about 80 % in the mutant. Measurements with intact cells and with PBQ as electron acceptor gave evidence that in mutant cells the PS II activity was already lower than in the WT when cells were grown under iron-sufficient conditions (Table 3). A higher susceptibility of PS II for inactivation in the mutant was also obvious when cells were cultivated for 3 h in the presence of 30 mM H₂O₂. The PS II activity measured with PBQ as electron acceptor dropped by about 10 and 40 % in the WT and mutant, respectively (not shown).

Immunoblot analysis of a selected number of thylakoid proteins (Fig. 3) gave evidence that the PsbA content decreased greatly under iron deficiency in *Synechocystis* PCC 6803 WT and the Slr1295-free mutant. However, in the mutant the amount of PsbA was less than in the WT even under iron-sufficient conditions. On the contrary, the amount of PsaA/B increased under iron deficiency in the WT as well as in the mutant, although the PS I activities were lower under iron deficiency. This inconsistency might be due to the observed modification of PS I under iron deficiency (see below).

77 K Chl *a* fluorescence and isolation of PS I complexes

One major difference between *Synechocystis* PCC 6803 WT and Slr1295-free mutant cells grown under iron deficiency was the height of the 77 K Chl *a* fluorescence peak at 685 nm (Fig. 4). As previously shown, the increase of 77 K Chl *a* fluorescence at 685 nm is due to the expression of IsiA, a CP43 homologue, under iron limitation (Burnap *et al.*, 1993; Falk *et al.*, 1995; Park *et al.*, 1999; and see reviews Ferreira & Straus 1994; Straus 1994). Moreover, evidence was recently presented that IsiA forms a new PS I antenna under iron deficiency in *Synechocystis* PCC 6803 (Bibby *et al.*, 2001a, b) as well

Fig. 3. Immunoblot analysis of some selected thylakoid membrane proteins. *Synechocystis* PCC 6803 WT and the Slr1295-free mutant were grown for 2 days in iron-sufficient medium (A) and for 2 (B) or 4 days (C) in iron-deficient medium. Detection was with antisera raised against PsbA (dilution 1:500), PsbB (1:50), PsbO (1:250), PsaA/B (1:1000), AtpA (1:1000), phycocyanin (PC, 1:1000) and allophycocyanin (APC, 1:1000). Total cell-free extracts corresponding to 25 µg protein (except for PsaA/B, 50 µg protein) were applied to each lane.

as in *Synechococcus* PCC 7942 (Boekema *et al.*, 2001). As can be clearly seen in Fig. 4, the fluorescence emission peak at 685 nm was significantly higher in the mutant than in the WT when cells were grown for 2 days under iron deficiency.

Because of these differences PS I complexes were isolated

Fig. 4. 77 K Chl *a* fluorescence of *Synechocystis* PCC 6803 WT and Slr1295-free mutant cells grown under iron-sufficient and iron-deficient conditions. *Synechocystis* PCC 6803 WT and the Slr1295-free mutant were grown for 2 days under iron-sufficient conditions and for 2 days under iron-deficient conditions. Excitation was at 435 nm, fluorescence emission was recorded between 650 and 800 nm and emission spectra were normalized to the PS I peak. Cells were adjusted to equal Chl values by dilution ($3 \mu\text{g Chl ml}^{-1}$) and then immediately frozen in liquid nitrogen. The insert shows the expression of the *isiA* mRNA in WT and mutant cells cultivated for 2 days either under iron-sufficient (+ Fe) or iron-deficient (- Fe) conditions.

from *Synechocystis* PCC 6803 WT and the Slr1295-free mutant grown under iron-sufficient and iron-deficient conditions (Fig. 5). As the elution profiles from an anion

exchange column show, after growth for 2 days under iron deficiency in the WT as well as in the mutant the PS I-IsiA complex was formed, which is not present under iron-sufficient growth conditions. The major difference between the WT and the Slr1295-free mutant was the presence of a substantial amount of regular PS I trimers besides the PS I-IsiA complexes in the WT, while in the mutant only a minor amount of PS I trimers was present. This also indicates that in the Slr1295-free mutant iron limitation is more severe than in the WT, and therefore the modification of regular PS I trimers to PS I-IsiA supercomplexes is more advanced in the mutant than in the WT.

Biochemical investigations about the localization of Slr1295 and Slr0513

The localization of Slr1295 and Slr0513 was investigated by two different experimental approaches. The soluble protein fraction of the periplasm and the spheroplast fraction were isolated from *Synechocystis* PCC 6803, according to the procedure of Block & Grossman (1988). Subsequently, the spheroplast fraction was further treated by passing it through a prechilled French press. The extract was separated by centrifugation into a soluble protein fraction and a membrane fraction containing cytoplasmic and thylakoid membranes. The presence of Slr1295 and Slr0513 in these cell fractions was analysed with the anti-IdiA antiserum which recognizes both proteins, and with the anti-Slr0513

Fig. 5. Elution profiles of detergent solubilized PS I complexes from *Synechocystis* PCC 6803 WT and the Slr1295-free mutant. PS I complexes were solubilized from thylakoid membranes of *Synechocystis* PCC 6803 WT and the Slr1295-free mutant grown for 2 days under either iron-sufficient or iron-deficient conditions. The detergent-solubilized PS I complexes were submitted to an anion-exchange chromatography column (Poros 50 HQ) and the corresponding elution profiles are given. A, PS I monomers; B, PS I trimers; C, PS I-IsiA complexes.

Fig. 6. Detection of Slr1295 and Slr0513 in various subcellular fractions of *Synechocystis* PCC 6803 WT grown under iron-deficient conditions. All subcellular fractions were obtained from *Synechocystis* PCC 6803 WT grown for 2 days under iron limitation. Immunoblot analysis was performed with the anti-IdiA antiserum (dilution 1:500) and the anti-Slr0513 antiserum (1:1000). A, Total cell-free extract; B, soluble protein fraction of the periplasm; C, total cell-free extract of spheroplast fraction after French press treatment; D, soluble protein fraction of spheroplast preparation; E, total membrane fraction of spheroplast preparation; F, thylakoid membranes; G, cytoplasmic membranes. Twenty-five micrograms protein (except for periplasm fraction, 15 µg protein) was applied in each lane.

Fig. 7. Detection of PsbO in the soluble protein fraction of the periplasm. The subcellular fractions were obtained from *Synechocystis* PCC 6803 WT grown for 2 days under iron limitation. Immunoblot analysis was done with the anti-PsbO antiserum (dilution 1:250) and the anti-PsbA antiserum (1:500) (as a control for an intrinsic PS II polypeptide). A, Total extract of spheroplast fraction after French press treatment; B, soluble protein fraction of the periplasm. Twenty micrograms protein was applied in each lane.

antiserum which recognizes only Slr0513. As can be seen in Fig. 6, Slr0513 is present in substantial amounts in the soluble protein fraction of the periplasm. The same is true for PsbO (Fig. 7), which was used as a reference protein. PsbO is associated with the donor side of PS II, but is also found in the periplasm (see e.g. Zak *et al.*, 2001). Besides being present in the periplasm, Slr0513 was also found in the spheroplast fraction, being about equally distributed between the soluble and membrane protein fractions. In contrast, Slr1295 was not detectable in the periplasm fraction (although the periplasmic fraction was concentrated about eightfold). Slr1295 was only present in the spheroplast fraction where it was about equally distributed between soluble and membrane protein fractions.

Fig. 8. Detection of Slr1295 in detergent-solubilized PS II-enriched fractions of thylakoid membranes from *Synechocystis* PCC 6803 WT grown for 2 days under iron limitation. PS II complexes of *Synechocystis* PCC 6803 WT grown for 2 days under iron limitation were isolated as described in Methods. Detection of Slr1295 and Slr0513 in immunoblots was achieved with the anti-IdiA antiserum (dilution 1:500). As a reference for PS II enrichment the detection of PsbA was used (detected by the anti-PsbA antiserum, 1:500). The applied samples were: WT(+), total cell-free extract of WT grown under iron-sufficient conditions; WT(-), total cell-free extract of WT grown under iron limitation; WT(-) solubilized protein fraction 1 (SPF1), containing proteins only loosely attached to the thylakoid membrane, as is the case for Slr0513; WT (-) solubilized protein fraction 2 (SPF 2), the PS II-enriched fraction containing Slr1295.

To answer the question, whether the membrane-associated part of Slr0513 and Slr1295 was attached to the cytoplasmic and/or thylakoid membrane, these membranes were isolated separately, according to the procedure described by Omata & Murata (1984). As shown in Fig. 6, Slr1295 was not detectable in the cytoplasmic membrane fraction, while minor amounts

Fig. 9. Co-purification of Slr1295 with PS II complexes. The detergent-solubilized PS II complexes were submitted to chromatography on a MonoQ column. The fractions (8–12) containing PS II complexes (identified by the anti-PsbA antiserum, dilution 1:500) also contain Slr1295 (identified by the anti-IdiA antiserum, 1:500).

of Slr0513 were found (lane G). In the thylakoid membrane fraction a substantial amount of Slr1295 was detected, while in contrast only minor amounts of Slr0513 were present (lane F).

Since a substantial amount of Slr1295 was associated with the thylakoid membranes, the question was asked how tightly this protein is associated and whether it interacts preferentially with PS I or PS II. Neither Slr1295 nor Slr0513 was detectable in isolated PS I complexes (not shown). For PS II isolation, the membrane fraction obtained after breaking the cells was washed first with a low concentration of dodecyl maltoside to remove phycobili proteins and only loosely associated proteins. In the wash supernatant a small amount of Slr0513 but no Slr1295 was detectable, implying that Slr1295 was relatively tightly associated with the thylakoid membranes, while Slr0513 was only loosely bound (Fig. 8, SPF 1). Subsequently, PS II complexes were solubilized with a combination of dodecyl maltoside and octyl glucopyranoside. In the PS II-containing fraction an enrichment of Slr1295 was clearly seen in parallel to the enrichment of the PsbA protein (Fig. 8, SPF 2). The PS II complexes were further purified on an FPLC-MonoQ column. Subsequent immunoblot analysis gave evidence that the Slr1295 protein co-purified with the reaction centre of PS II (Fig. 9). Only a very slight shift of Slr1295 relative to the D1 polypeptide was seen, most likely due to different subpopulations of PS II.

Immunocytochemical detection of Slr1295 and Slr0513

For immunocytochemical investigations *Synechocystis* PCC 6803 WT and Slr1295-free mutant cells were grown under iron-sufficient or iron-deficient conditions for

2 days. Subsequently, the cells were prepared as previously described (Engels *et al.*, 1997) and then treated with anti-IdiA (Fig. 10) or anti-Slr0513 (Fig. 11) antiserum followed by gold-conjugated anti-rabbit IgG. When electron micrographs of WT cells grown under iron-sufficient conditions were compared to those cells cultivated for 2 days under iron-deficient conditions, it became apparent that there was a substantial increase in gold labels (compare panels a and b in Figs 10 and 11). The same was true for the Slr1295-free mutant (not shown). However, unexpectedly, the majority of labels for Slr1295 as well as Slr0513 was found intracellularly – mainly associated with the thylakoid membrane. Only some minor labels were present in the periplasm.

The results shown here on the localization of Slr1295 give evidence that Slr1295 mainly has an intracellular function, since virtually no Slr1295 was detected in the soluble protein fraction of the periplasm or in the cytoplasmic membrane of WT cells when grown under mild iron limitation. These results are consistent with the immunocytochemical localization showing that the majority of the gold labels was found intracellularly. The same was true for Slr0513. Only minor labels were present in the periplasm. However, in the case of Slr0513, in contrast to Slr1295, the biochemical investigations showed that substantial amounts of Slr0513 were present in the soluble protein fraction of the periplasm, a result in agreement with the results of Fulda *et al.* (2000). A possible explanation of this discrepancy could be that thylakoid membranes in part are connected with the cytoplasmic membrane (Nierzwicki-Bauer *et al.*, 1983; see also Zak *et al.*, 2001). Therefore, it might be possible that some of the Slr0513 which was found in the soluble protein fraction of the periplasm originated from the intrathylakoid space (lumen). This might also explain the presence of PsbO in this fraction. Thus for

Fig. 10. Electron micrographs of *Synechocystis* PCC 6803 WT cells grown under iron-sufficient and iron-deficient conditions: immunocytochemical detection of Slr1295 and Slr0513 by the anti-IdiA antiserum. (a) *Synechocystis* PCC 6803 cells were cultivated for 2 days under iron-sufficient conditions and treated with the anti-IdiA antiserum (dilution 1:100) in combination with gold-labelled anti-rabbit IgG (1:30). (b) Cells were grown for 2 days under iron-deficient conditions and treated as described for (a). Bars, 0.5 μ m. Electron micrographs of cells grown for 2 days under iron-deficient conditions and treated with the corresponding pre-immune serum (1:100) in combination with gold-labelled anti-rabbit IgG showed hardly any gold labelling (not shown).

Fig. 11. Electron micrographs of *Synechocystis* PCC 6803 WT grown under iron-sufficient and iron-deficient conditions: immunocytochemical detection of Slr0513 by the anti-Slr0513 antiserum. (a) *Synechocystis* PCC 6803 cells cultivated for 2 days under iron-sufficient conditions and treated with the anti-Slr0513 antiserum (dilution 1:100) in combination with gold-labelled anti-rabbit IgG (1:30). (b) Cells were grown for 2 days under iron-deficient conditions and treated as described for (a). Bars, 0.5 μ m. Electron micrographs of cells grown for 2 days under iron-deficient conditions and treated with the corresponding pre-immune serum (1:100) in combination with gold-labelled anti-rabbit IgG showed hardly any gold labelling (not shown).

Slr0513, transport into the periplasm and the lumen is indicated.

A comparison of the presumed signal peptide sequence of Slr1295 and Slr0513 of *Synechocystis* PCC 6803 (not shown) provided evidence that both proteins have a characteristic sequence motif in the N-terminal region before the hydrophobic region [core sequence of (S/T)-R-R-x-F] that includes two consecutive and invariant arginine residues characteristic for the bacterial Tat (twin-arginine translocation) system (Berks 1996; Berks *et al.*, 2000). Bacterial Tat signal peptides are characterized by a high occurrence of a proline residue at position -6 to the signal peptidase cleavage site and of basic amino acids in the c-region. When analysing the N-terminal amino acid region in the two proteins, it became apparent that Slr0513 meets all these criteria, while in Slr1295 the corresponding proline is missing and there is an acidic amino acid in the c-region before

the signal peptidase cleavage site ('PSORT' prediction; von Heijne, 1986). Moreover, there is a possible additional cleavage site recognized by the program SignalP (Nielsen *et al.*, 1999). This might be the reason why only significant amounts of Slr0513, but not of Slr1295, are found in the soluble protein fraction isolated from the periplasm. It has been shown that the 'ΔpH-dependent' protein import pathway of thylakoid membranes in plant chloroplasts and the bacterial Tat system are very closely related (Klöggen 1997; Brink *et al.*, 1998; Wexler *et al.*, 1998; Halbig *et al.*, 1999). This might explain the high amounts of gold labels located intracellularly and suggests that Slr0513 is transported into the periplasm and also into the intrathylakoid space due to some misdirection. In contrast, Slr1295 does not seem to be exported in significant amounts into the periplasm (or lumen) – at least not under mild iron limitation and when Slr0513 is present.

Concluding remarks

The intracellular localization and the co-purification of Slr1295 with PS II in *Synechocystis* PCC 6803 suggest a function for Slr1295 in protecting PS II under iron limitation. This is in agreement with the higher susceptibility of PS II for inactivation in the Slr1295-free mutant as compared to the WT. Thus, the function of Slr1295 in *Synechocystis* PCC 6803 is comparable to the suggested function of IdiA in *Synechococcus* PCC 6301/PCC 7942 (Michel *et al.*, 1996; Exss-Sonne *et al.*, 2000) and to some extent also to the function of a plant PS II-associated 22 Ku heat-shock protein (Downs *et al.*, 1999a, b). The cyanobacterial Slr1295 and IdiA, as well as the plant 22 Ku protein, represent proteins that are not integral proteins of PS II, but function in protecting PS II under specific stress conditions. In the case of Slr1295 (and also IdiA), we suggest that PS II becomes protected or more effectively supplied with iron on the D1/D2 reaction centre heterodimer (Q_A -Fe- Q_B). We think that it is highly likely that specific proteins exist to optimize PS II function under iron limitation since iron limitation is a commonly occurring nutrient limitation in natural habitats (see e.g. Geider & La Roche, 1994; Behrenfeld & Kolber, 1999). PS II has been proven to be the most labile reaction of the photosynthetic process, and the D1 (and also D2) polypeptide has an extraordinary high turnover under various environmental stresses (Aro *et al.*, 1993; Ke, 2001), thus having an elevated iron requirement due to the PS II repair cycle. Based on our results, Slr1295 seems to be a good candidate for such an optimizing and protective function, since due to its assumed original function as a component of an iron transporter it is expressed in elevated amounts under iron limitation and can interact with iron. From an evolutionary point of view, Slr1295 seems to be a protein acquiring a different and/or additional function during evolution (for review see Murzin, 1993), but retaining significant homology to the protein family of its original function.

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