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THE CYANOBACTERIAL CHLOROPHYLL-BINDING-PROTEIN IsiA ACTS TO INCREASE THE IN VIVO EFFECTIVE ABSORPTION CROSS-SECTION OF PSI UNDER IRON LIMITATION[1]

Thomas J. Ryan-Keogh,[2] *Anna I. Macey*

School of Ocean and Earth Science, University of Southampton, National Oceanography Centre, Southampton, European Way, Southampton, SO14 3ZH, UK

Amanda M. Cockshutt

Department of Chemistry and Biochemistry, Mount Allison University, Sackville, NB E4L 1G7, Canada

C. Mark Moore and Thomas S. Bibby

School of Ocean and Earth Science, University of Southampton, National Oceanography Centre, Southampton, European Way, Southampton, SO14 3ZH, UK

Iron availability limits primary production in >30% of the world's oceans; hence phytoplankton have developed acclimation strategies. In particular, cyanobacteria express IsiA (iron-stress-induced) under iron stress, which can become the most abundant chl-binding protein in the cell. Within iron-limited oceanic regions with significant cyanobacterial biomass, IsiA may represent a significant fraction of the total chl. We spectroscopically measured the effective cross-section of the photosynthetic reaction center PSI (σ_{PSI}) in vivo and biochemically quantified the absolute abundance of PSI, PSII, and IsiA in the model cyanobacterium *Synechocystis* sp. PCC 6803. We demonstrate that accumulation of IsiA results in a ~60% increase in σ_{PSI}, in agreement with the theoretical increase in cross-section based on the structure of the biochemically isolated IsiA-PSI supercomplex from cyanobacteria. Deriving a chl budget, we suggest that IsiA plays a primary role as a light-harvesting antenna for PSI. On progressive iron-stress in culture, IsiA continues to accumulate without a concomitant increase in σ_{PSI}, suggesting that there may be a secondary role for IsiA. In natural populations, the potential physiological significance of the uncoupled pool of IsiA remains to be established. However, the functional role as a PSI antenna suggests that a large fraction of IsiA-bound chl is directly involved in photosynthetic electron transport.

Key index words: **chlorophyll-binding protein; cyanobacteria; iron limitation; IsiA; PSI**

Abbreviation: **σ_{PSI}, the effective absorption cross-section of PSI**

Chl in phytoplankton cells is primarily associated with the highly conserved photosynthetic reaction centers PSI and PSII, which catalyze oxygenic photosynthesis, or with peripheral, species-specific, light-harvesting protein complexes that enable niche adaptation to different light and nutrient environments (Ting et al. 2002, Chen and Bibby 2005, Varsano et al. 2006). It has been proposed, however, that the chl-binding protein IsiA, expressed in some cyanobacteria under iron stress (Burnap et al. 1993), is not directly involved in photosynthesis (Cadoret et al. 2004, Sarcina and Mullineaux 2004, Behrenfeld et al. 2006, Singh and Sherman 2007). Under iron limitation, IsiA can be the most abundant chl-binding protein in cyanobacteria cells (Burnap et al. 1993). IsiA-associated pigment may therefore significantly contribute to total chl in oceanic regions such as the Equatorial Pacific, where cyanobacteria comprise a substantial fraction of the total phytoplankton community, and the availability of the trace metal iron (Fe) has been shown to limit primary production (Martin et al. 1994, Behrenfeld et al. 1996, 2006, Behrenfeld and Kolber 1999). A recent whole-community genomic study has revealed that the cyanobacterial *isiA* gene is present specifically in this region, suggesting that it has a functional role that confers a selective advantage in situ (Bibby et al. 2009).

The IsiA protein is thought to bind 12 chl molecules in six transmembrane helices arranged in three sets of two dimers (Bricker and Frankel 2002). Its sequence and structural motif are homologous to the core antenna proteins of PSII (CP43 and CP47) (Pakrasi et al. 1985, Burnap et al. 1993) and to the light-harvesting antenna proteins (Pcbs) of the marine cyanobacterium *Prochlorococcus* (La Roche et al. 1996, Bibby et al. 2003). Both Pcbs and IsiA, however, lack the large hydrophilic loop that

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[2]Author for correspondence: e-mail t.ryan-keogh@noc.soton.ac.uk.

joins the luminal ends of helices V and VI (Burnap et al. 1993). *Prochlorococcus* can have multiple *pcb* genes, each of which has a specific function: for example, as specific light-harvesting antenna for PSII, or PSI (expressed constitutively), or as a light-harvesting antenna system for PSI expressed under iron limitation (Garczarek et al. 2000, Bibby et al. 2001d, 2003). This six-trans-membrane chl-binding motif is therefore thought to be a core building block of photosynthesis that has been retained in some cyanobacteria because it offers a photosynthetic strategy, advantageous under iron limitation (Green 2003, Chen and Bibby 2005).

In addition to expressing IsiA under iron stress, cyanobacterial cells undergo chlorosis (loss of total chl per cell) (Guikema and Sherman 1983, Boyer et al. 1987, Strauss 1994, Erdner et al. 1999, Moseley et al. 2002), reduce the abundance of photosynthetic reaction centers (PSI and PSII) (Öquist 1974, Strauss 1994, Boekema et al. 2001, Küpper et al. 2008), and reduce their phycobilisome content (soluble light-harvesting systems that require iron-binding enzymes for synthesis) (Guikema and Sherman 1983). The biochemical isolation of an IsiA-PSI supercomplex from iron-limited cyanobacterial cells, in which 18 IsiA monomer proteins are functionally coupled to a PSI trimer (IsiA:PSI ratio 6:1) (Bibby et al. 2001a,b,c, Boekema et al. 2001), was thought to explain the role of this protein, namely to increase the functional antenna size of a restricted cellular quota of PSI reaction centers by ~70% under iron limitation, thus compensating for a reduction in PSI per cell (Andrizhiyevskaya et al. 2002, Melkozernov et al. 2003, Küpper et al. 2008). However, an increase in the cross-section of PSI (σ_{PSI}) has not been established in vivo (Ivanov et al. 2006). Instead, evidence from culture studies suggests that IsiA may have an alternative role in photoprotection or as a chl store for the cell (Guikema and Sherman 1983, Park et al. 1999, Sandström et al. 2001, 2002, Singh and Sherman 2007) and is therefore not directly involved in harvesting light for primary production (Behrenfeld et al. 2006).

The ratio of variable to maximal fluorescence (F_v/F_m) has frequently been used to establish and map phytoplankton iron stress in the field (Boyd and Abraham 2001, Behrenfeld et al. 2006, Bibby et al. 2008). However, the mechanistic basis of this response remains unclear (Suggett et al. 2009). The observed reduction in F_v/F_m within natural populations in low-iron regions (Behrenfeld et al. 2006, Nielsdóttir et al. 2009) and within cultures under iron-starvation conditions, although not necessarily steady-state iron limitation (Price 2005), probably reflects accumulation of nonphotochemically active chl. Consequently, rather than the original suggestion that lowered F_v/F_m reflects accumulation of damaged PSII reaction centers (Falkowski and Kolber 1995), it has been suggested that accumulation of uncoupled chl-binding IsiA protein, or proteins with similar iron-regulated control in eukaryotes (although a role of these other than as light-harvesting antenna also remains to be established, Varsano et al. 2006), may be primarily responsible for the observed reduction in F_v/F_m under iron stress (Behrenfeld et al. 2006). Establishing the role of IsiA and the potential for excess accumulation of this protein is thus crucial for understanding the physiological basis of active fluorescence data and potentially for interpreting large-scale satellite-based measurements of production (Behrenfeld et al. 2006, 2009). In addition, the iron-stress response of cyanobacteria may be representative of similar responses in photosynthetic eukaryotes (Varsano et al. 2006).

In this study, we measured the absolute abundance of the chl-binding proteins PSII, PSI, and IsiA, and the in vivo σ_{PSI} in cultures of the model cyanobacterium *Synechocystis* sp. PCC 6803 under conditions of increasing iron stress. We subsequently calculated a chl budget for the iron-limited cyanobacterial cell, and demonstrate that the primary function of IsiA in vivo is as a PSI antenna.

MATERIALS AND METHODS

Growth conditions. *Synechocystis* sp. PCC 6803 (Bricker et al. 1998) was grown photoheterotrophically in mineral medium in BG-11 (Williams 1988) supplemented with glucose at 30°C and with illumination of 10 μmol photons $\cdot m^{-2} \cdot s^{-1}$ on a 12:12 light:dark (L:D) cycle. Iron-stressed cultures were obtained by inoculating into BG-11 medium without added iron. All cultures were grown under batch conditions for a period of 96 h, with inoculation of new experimental cultures carried out from cells grown under iron-replete conditions. Cells were harvested every day during the midpoint of the light cycle over the course of 96 h, with samples collected for physiological and molecular analysis. Within each experiment, analyses were performed on triplicate independent cultures. We further present results from a series of three independent experiments.

Samples for chl analysis were filtered onto Whatman GF/F filters (Whatman, GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) and extracted into 90% acetone for 24 h in the dark at 5°C before analysis using a Turner Designs 10-AU fluorometer (Turner Designs, Sunnyvale, CA, USA) (Welschmeyer 1994). Cell enumeration was performed using a Becton Dickinson Facsort™ flow cytometer (Becton Dickinson Biosciences, Oxford, UK) with a reference bead stock used as an internal standard.

Biophysical measurements. The apparent PSII photosynthetic efficiency (F_v/F_m) was assessed from chl fluorescence measurements performed using a Chelsea Scientific Instruments Fasttracka™ Mk II Fast Repetition Rate fluorometer (FRRf) integrated with a FastAct™ Laboratory system (Chelsea Technologies Group Ltd., West Molesey, Surrey, UK). All samples were dark acclimated for 30 min and measurements were corrected for the blank effect (Cullen and Davis 2003). Absorbance spectroscopy was undertaken using a Varian Cary 50 Scan™ UV-Visible spectrophotometer (Agilent Technologies UK Ltd., Wokingham, Berkshire, UK), on samples that had been sonicated on a freeze-thaw cycle using a Sonics and Material VibraCell™ (Sonics and Materials Inc., Newtown, CT, USA) to extract the thylakoid membranes. Scans were performed on a medium resolution from 600 to 800 nm on triplicate cultures from both iron-stressed and iron-replete treatments over the course of 96 h.

Redox kinetics of P700, the PSI primary donor, were measured by following absorption changes at 830 nm relative to 870 nm using a Walz Dual-PAM 100^{TM} (Heinz Walz GmbH, Effeltrich, Germany). A custom protocol was developed to quantitatively estimate σ_{PSI} by measuring the oxidation of P700 in response to 3 ms duration 635 nm LED light pulses at intensities from 5 to a saturating 20 mmol photons $\cdot$ m^{-2} $\cdot$ s^{-1}. Harvested whole cells were gently concentrated to increase signal to noise ratios and allow resolution of kinetic measurements of P700 oxidation at 7.5 μs time resolution over 50 ms timescales. Accurate estimates of absolute σ_{PSI} on cultures dense enough for ΔA_{830} measurements require correction for absorption of the actinic light within the culture (Zipfel and Owens 1991). Indeed, even relative changes in σ_{PSI} between cultures would be susceptible to artifacts resulting from the need to make such measurements in optically dense situations. Culture absorption was thus measured at the wavelength of the saturating pulse using a Varian Cary 50 Scan^{TM} UV-Visible spectrophotometer. Raw ΔA_{830} measurements were analyzed using custom software in a MATLAB^{TM} (Mathworks, Cambridge, UK) computing environment following Zipfel and Owens (1991). Briefly, the effective photochemical rate constant for P700 oxidation (K_{eff}) (ms^{-1}) measured under monochromatic saturating light (E[635]) (photons $\cdot$ m^{-2} $\cdot$ s^{-1}) will be related to the total absorption of the sample at that wavelength (A[635]) (m^{-1}) and the functional absorption cross-section (Zipfel and Owens 1991):

$$K_{\text{eff}} = \sigma_{\text{PSI}} E \frac{(1 - e^{-A})}{A} \quad (1)$$

Values for K_{eff} were derived from nonlinear least squares fitting of ΔA_{830} saturation kinetics as a function of time (t) to:

$$\Delta A_{830} = \Delta A_{830\max}(1 - e^{K_{\text{eff}} t}) \quad (2)$$

where $\Delta A_{830\ max}$ is the maximum absorption change measured in a given sample under saturating light. Correction for rereduction of P700^{+} during the saturation flash (Zipfel and Owens 1991) was not performed, as kinetic measurements indicated that the effective reduction rate constant was an order of magnitude lower than K_{eff} (see below).

Formation of the IsiA-PSI supercomplex is expected to be capable of increasing σ_{PSI} by ~70% (Bibby et al. 2001b). For further comparison with measured values of σ_{PSI}, absolute theoretical cross-sections for an isolated PSI (trimer) and the IsiA-PSI supercomplex (Bibby et al. 2001b) were calculated using the in vivo absorption of a single chl molecule (Bidigare et al. 1990, Johnsen et al. 1994), and assuming 100 chl molecules in a single PSI protein complex (Jordan et al. 2001) and that every IsiA protein binds at least 12 chl molecules (Ferreira et al. 2004, Murray et al. 2006).

Protein immmunodetection and quantification. Protein samples were extracted using a liquid nitrogen freeze-thaw cycle combined with sonication to rupture the cell membrane. Protein concentrations were determined using a Lowry protein assay (Lowry et al. 1951, Peterson 1979). Quantitative Western blotting was performed using the Tricine-SDS-PAGE method as described in Schägger (2006). The SDS-PAGE and membrane transfer were run using an Invitrogen Life Technologies PowerEase 500^{TM} (Invitrogen, Paisely, UK) (see Results). Custom Agrisera^{TM} (Agrisera AB, Vännäs, Sweden) antibodies and protein standards were used for protein immunodetection according to the manufacturer's instructions (Brown et al. 2008). The membranes were imaged using a BioRad VersaDoc^{TM} (Bio-Rad Laboratories Ltd., Hemel Hempstead, Hertfordshire, UK) imaging system, and the images were processed using QuantityOne^{TM} software to quantify protein samples and standards. Quantification was performed within the unsaturated part of the calibration curve.

RESULTS

Physiological response. Iron-stress was induced by growing *Synechocystis* cells in BG-11 media lacking iron. When compared with control *Synechocystis* cultures (BG-11 containing iron), a marked difference in total chl concentration and cell density (Fig. 1a) and a marked decline in F_v/F_m (the photosynthetic

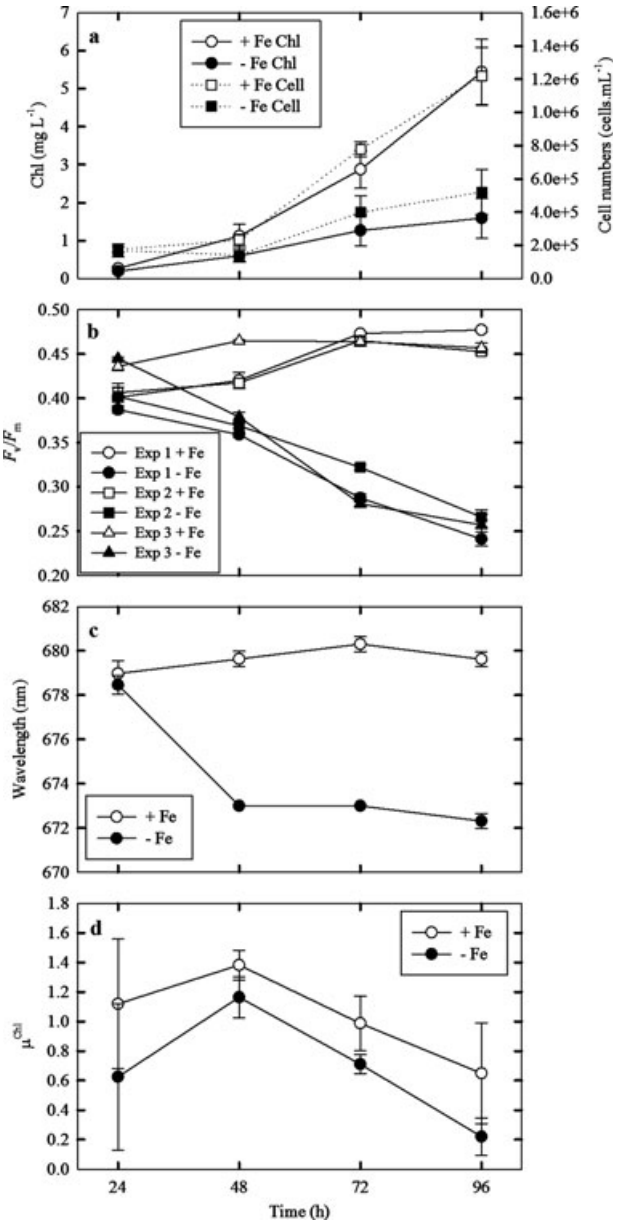

FIG. 1. Physiological measurements of *Synechocystis* PCC 6803 under iron-replete (+Fe) and iron-deplete conditions (−Fe). (a) Average chl concentrations (mg $\cdot$ L^{-1}) and average cell numbers (cells $\cdot$ mL^{-1}) enumerated using flow cytometry from three independent experiments. (b) Individual average F_v/F_m from the three independent experiments. (c) Wavelength shift of maximum absorption value between 670 and 685 nm measured at room temperature. (d) Growth rates (μ^{chl}) calculated from chl concentrations. Displayed are results averaged from triplicates from three independent experiments with ±standard errors (in a, c, d).

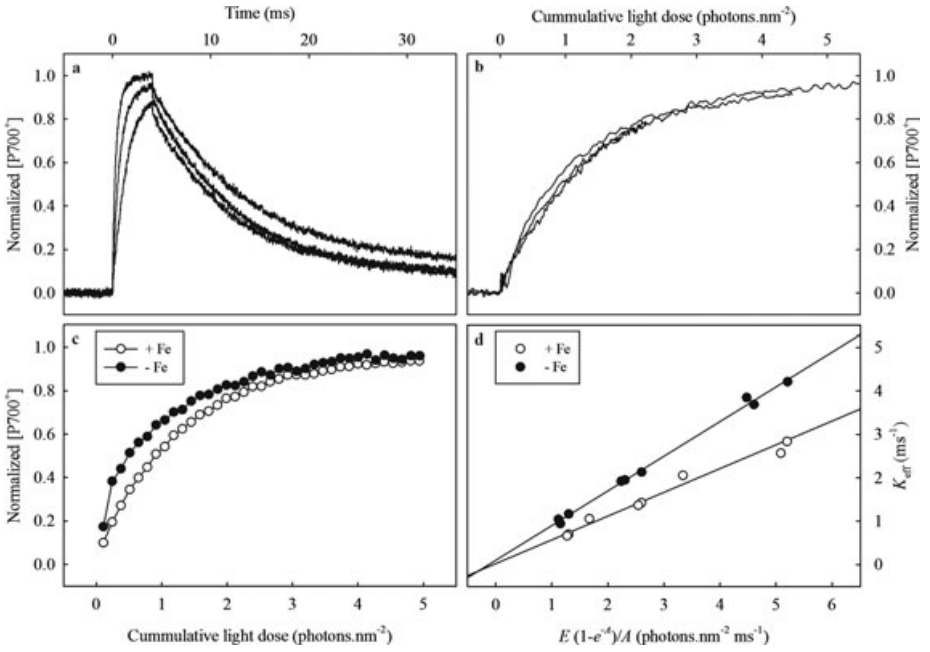

FIG. 2. Measurements of ΔA_{830} kinetics over a range of different light intensities on the same sample (a). (b) The rate of ΔA_{830} saturation normalized to cumulative photon dose. (c) Example measurements of ΔA_{830} normalized to cumulative photon dose of iron-replete and iron-deplete cultures. (d) K_{eff} (ms^{-1}) as a function of mean light intensity within the measurement cuvette for an iron-replete and iron-deplete culture measured at multiple excitation intensities and cell densities.

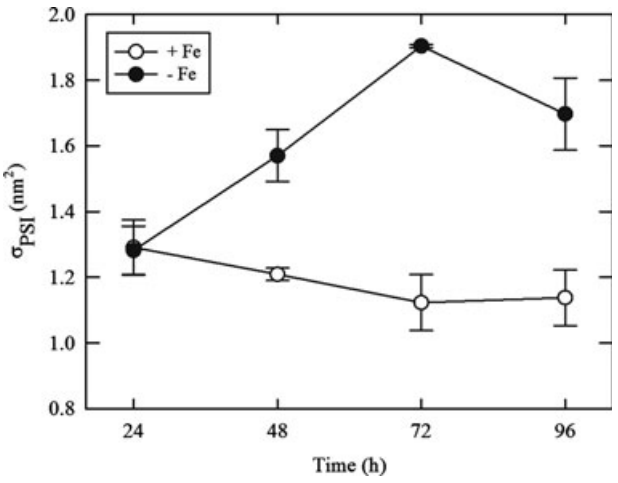

FIG. 3. The in vivo effective absorption cross-section of PSI (σ_{PSI}) measured on *Synechocystis* PCC 6803 under iron-replete (+Fe) and iron-deplete (−Fe) conditions. Displayed are results averaged from triplicates from three independent experiments with ±standard errors.

energy conversion efficiency) (Fig. 1b) became apparent 48 h after inoculation into low-iron media, indicating the point when the photophysiology switches to the iron-limited phenotype. This change was accompanied by a blue-shift of ~8 nm in the red-absorption peak of chl (indicative of expression of the *isiA* gene and accumulation of the IsiA protein) (Fig. 1c) and a decline in growth rate to ~84% of iron-replete cultures (Fig. 1d). Prolonged growth in iron-deplete media (>48 h) resulted in a continued reduction in F_v/F_m to 0.26, with significant differences from 48 h onward (*t*-test, $P < 0.05$) and further declines in growth rates to ~34% of iron-replete cultures, which had a μ^{max} of 1.38 ± 0.1. Alongside measurements of F_v/F_m, we calculated the absolute changes in F_o and F_m normalized to chl (data not shown). F_o and F_m remained relatively constant within iron-replete cultures, but increased from 48 h onward in the iron-deplete culture.

Measurements of ΔA_{830} during high-intensity light pulses displayed first-order saturation kinetics (Fig. 2a), with K_{eff} ranging from ~0.7 to 5 ms^{-1} (Fig. 2d). In contrast, first-order rate constants estimated from post-light-pulse ΔA_{830} relaxation kinetics ranged from 0.07 to 0.1 ms^{-1} (Fig. 2a). The rate of ΔA_{830} saturation normalized to cumulative photon dose at different saturation pulse intensities confirmed measurement of a photochemical absorption cross-section (Fig. 2b), and further confirmed the relative insensitivity of saturation kinetics to $P700^+$ re-reduction. When corrected for sample absorption, K_{eff} thus conformed to the expected linear function of excitation intensity (Fig. 2d), with a slope that will be equal to σ_{PSI} (eq. 1).

Clear differences in saturation kinetics were observed between iron-replete and iron-deplete cultures (Fig. 2, c and d). Measured values of σ_{PSI} indicated a significant shift between the two cultures from 48 h onward (Fig. 3), with the iron-replete culture remaining relatively constant at ~1.2 ±

0.1 nm^2, higher than, but still in reasonable agreement with, the theoretical cross-section for a PSI complex receiving absorption from only 100 chl molecules (Jordan et al. 2001, Ferreira et al. 2004). In contrast, σ_{PSI} increased from 48 h onward in the iron-starved culture to a maximum value of 1.90 ± 0.01 nm^2 on 72 h, ~60% larger than the iron-replete value at the same time point. Differences between treatments were significant from 48 h onward (*t*-test, $P < 0.05$).

Protein abundance. The absolute concentrations of the key photosynthetic proteins PSI, PSII, and IsiA, representing the vast majority of the chl-binding complexes in the cyanobacterial cell, were measured by quantitative Western blotting (Fig. 4) (Brown et al. 2008). In agreement with physiological measurements (Fig. 1c), protein abundances normalized to total protein concentration, indicated that the IsiA protein was only detectable from 48 h onward (Fig. 5a), increasing to a maximum of 0.53 ± 0.15 pmol IsiA · μg^{-1} total protein by 96 h. PSI decreased throughout to final value of 0.028 ± 0.012 pmol PSI · μg^{-1} total protein. PSII decreased between 48 and 72 h, remaining relatively constant until 96 h with a value of 0.035 ± 0.018 pmol PSII · μg^{-1} total protein.

When normalized to total cellular chl, IsiA:chl increased from 0.017 ± 0.002 to a maximum of 0.094 ± 0.041 mol · mol^{-1} on 96 h (Fig. 5b). This increase in IsiA:chl occurs alongside a decrease in PSI:chl while PSII:chl remained relatively constant, with final values of 0.0035 ± 0.0012 mol · mol^{-1} and 0.0015 ± 0.0006 mol · mol^{-1}, respectively (Fig. 5b). These measured values of protein abundance relative to total cellular chl were in good agreement with theoretical values (not shown) (Brown et al. 2008). These changes led to marked shifts in relative protein ratios, with the IsiA:PSI ratio increasing past the predicted ratio of 6:1 for an IsiA-PSI supercomplex after 48 h growth to a maximum of 27:1 at the end of the experiment, while PSII:PSI increased slightly from 0.4:1 to a final value of 0.8:1, in agreement with previous findings (Küpper et al. 2008).

The relative contribution of each protein complex (PSI, PSII, and IsiA) to the total cellular chl content was calculated using the protein ratios combined with numbers of chl molecules per complex (100, 36, and 12 per PSI, PSII, and IsiA, respectively) taken from structural studies that used the CP43 protein of PSII as a homolog for IsiA (Ferreira et al. 2004, Murray et al. 2006). On the basis of relative protein abundance and predicted chl budget, we further inferred how much of the IsiA protein could be structurally coupled to PSI based upon a theoretical IsiA:PSI ratio of 6:1 for the IsiA-PSI supercomplex combined with the observed ~60% increase in the functional PSI cross-section (from 72 h under Fe limitation), which suggested conversion of >80% of the total PSI pool to this state (Fig. 6a).

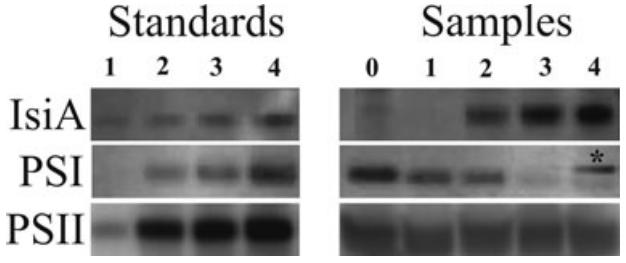

FIG. 4. Example of quantitative Western-blot detection of the chl-binding proteins (IsiA, PSI, and PSII). The left panel shows specific peptide standards at increasing concentrations (1–4). The right panel shows detection of specific peptide targets from total protein extracted from *Synechocystis* PCC 6803. The ''*'' indicates a band present under severe iron limitation that cross-reacts with PSI-specific global antibody not considered in quantification of this target.

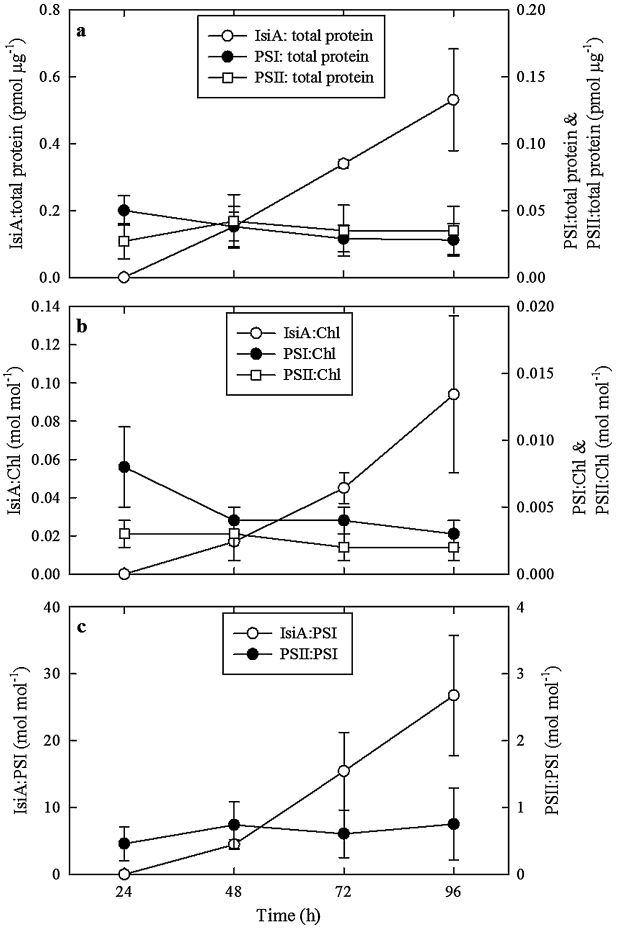

FIG. 5. Results from the protein quantification experiment. (a) Protein abundances determined by immunodetection on an iron-deplete culture. Measurements are normalized to total protein concentration in pmol · μg^{-1}. (b) Protein abundances normalized to chl in mol · mol^{-1}. (c) Protein ratios of an iron-deplete culture calculated from protein abundances normalized to chl, ratios in mol · mol^{-1}. Displayed are results averaged from three independent experiments with ±standard errors.

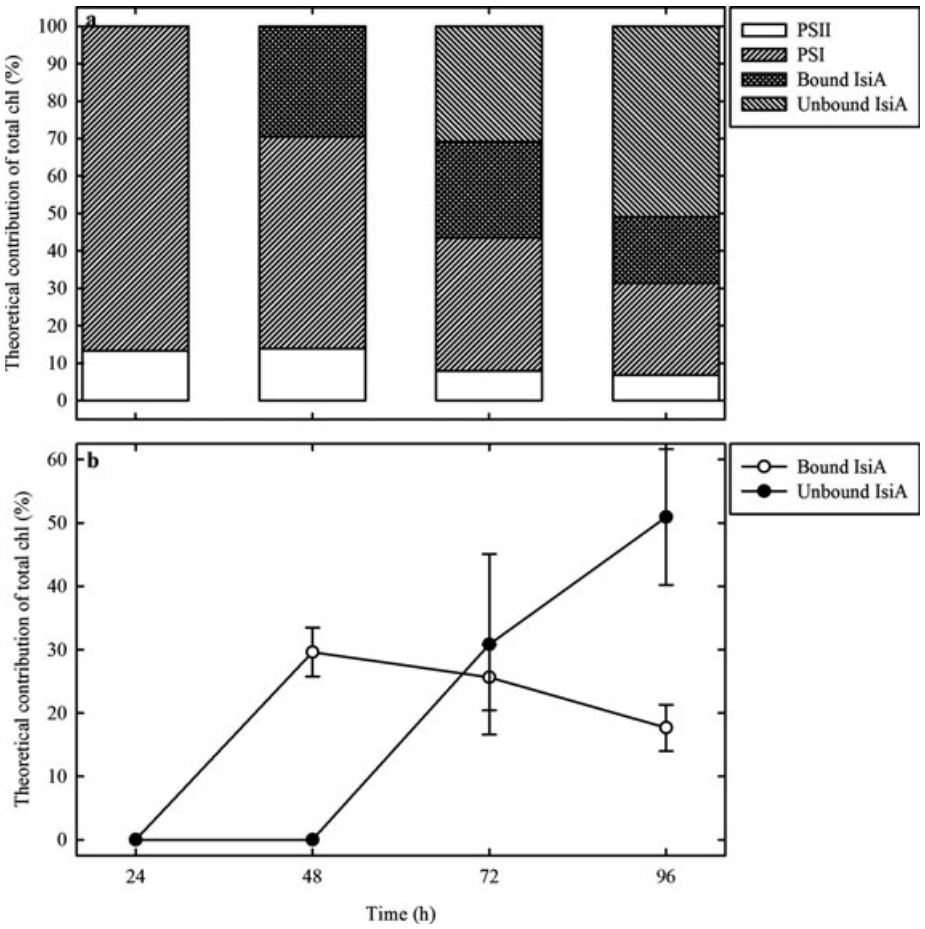

FIG. 6. The contribution to total chl content (%) by PSI, PSII, and IsiA proteins in an iron-deplete culture (a) and the contribution to total chl content (%) of bound and unbound IsiA plotted against time (b). Displayed are results averaged from three independent experiments with ±standard errors.

Using these calculations, it is clear that the IsiA protein is a substantial fraction of the total cellular chl content from 48 h onward. However, unbound IsiA was only inferred to represent a substantial fraction of total chl on 72 and 96 h. The potential contribution of bound and unbound IsiA thus shows differing trends (Fig. 6b). In particular, bound IsiA, as indicated by increased σ_{PSI}, becomes significant at the onset of iron stress. In contrast, the proportion of unbound IsiA only becomes significant after prolonged growth under iron stress.

DISCUSSION

In this paper, we describe the photosynthetic physiology of the cyanobacterium *Synechocystis* PCC 6803 grown under conditions of increasing iron stress at both biophysical and molecular levels. Biophysical measurements confirmed the development of increased iron-stress physiology over time. When compared with Fe-replete cultures, iron-depleted *Synechocystis* PCC 6803 showed a blue shift in the red chl absorption peak (Fig. 1c), indicative of accumulation of the iron-stress-induced protein IsiA (Burnap et al. 1993), and reductions in F_v/F_m (the photosynthetic energy conversion efficiency) (Fig. 1b) and growth rate (Fig. 1d). These are well-characterized responses of iron limitation and reflect an acclimation to growth under iron-limited conditions; the resulting different photosynthetic strategy enables photosynthesis to continue with a lower demand for iron (Behrenfeld et al. 1996, Bibby et al. 2001a).

Alongside clear evidence of iron stress, we provide the first demonstration of an increase of ~60% in σ_{PSI} in vivo (Fig. 3). This increase is consistent with the notion that the IsiA protein forms an antenna ring around PSI trimers, forming the IsiA-PSI supercomplex that has been biochemically isolated and shown to be energetically coupled (Melkozernov et al. 2003, Andrizhiyevskaya et al. 2004). Formation of this IsiA-PSI supercomplex has previously been proposed to represent a strategy to minimize the number of iron-containing PSI reaction centers required under iron-stress conditions (Bibby et al. 2001b,c, Küpper et al. 2008). The

increased effective cross-section was temporally correlated with both the ~8 nm blue shift in the red peak of the chl absorption and a reduction in F_v/F_m (Fig. 1, b and c).

In parallel with this physiological measurement, we have quantified the abundance of the photosynthetic reactions centers PSII and PSI and the iron-stress chl-binding protein IsiA, together representing the major pools of chl in the cell. Coincident with the initial increase in σ_{PSI}, the IsiA:PSI ratio increased from undetectable levels (no IsiA present) past the 6:1 ratio after 48 h (Fig. 5c). This is the ratio of PSI reaction centers to IsiA antenna proteins revealed in the 10 Å structure of the IsiA-PSI supercomplex (Bibby et al. 2003). At this point in progressive iron-stress development (48 h), the culture shows many signs of an iron-limited photophysiology, including a shift in the red-adsorption peak of chl (indicative of the accumulation of IsiA), and reductions in phycobilisomes (not shown), F_v/F_m and growth rate. At least 80% of the chl associated with expressed IsiA appears to be associated with PSI (Fig. 6a) if we are to account for the measured increase in σ_{PSI} (Fig. 3).

Under more severe iron stress (>48 h into iron limitation), while no further increase in σ_{PSI} or blue-shift in the red-absorption peak of chl is observed, IsiA continues to accumulate in the cell and F_v/F_m becomes severely reduced, alongside further reductions in growth rate. Cellular IsiA concentrations and IsiA:PSI ratios continued to increase to a maximum of 27:1 without a parallel increase in σ_{PSI}. The increase in IsiA:PSI ratios beyond 6:1 thus appears to represent the accumulation of IsiA that was functionally uncoupled from PSI in vivo. This pool of IsiA is therefore likely to be inactive in photosynthesis and may have an alternative role in the cell, such as a chl store or in photoprotection. It has been shown that IsiA can form a double ring around PSI (Chauhan et al. 2011). Theoretically, a double ring would have a cross-section of >2 nm^2 at 635 nm based upon an approximated 6-IsiA inner ring and an 8-IsiA outer ring. This value is higher than the maximal observed values of σ_{PSI} measured in this study (Fig. 3). The lack of a further increase in σ_{PSI} beyond day 48–72 suggests that the proportion of double ring complexes remains small in vivo.

Our estimated levels of uncoupled IsiA under progressive iron-stress conditions provide further evidence that this protein may not act solely as a peripheral antenna for PSI. A number of alternative roles have previously been suggested, including protecting PSII from excess light (Park et al. 1999) or acting as an alternate antenna for PSII (Pakrasi et al. 1985). The most likely alternative role would be as a chl storage protein (Sarcina and Mullineaux 2004, Singh and Sherman 2007); with ~50% of the total chl content in unbound IsiA, there is at least the possibility that this pool could act to rapidly increase photosynthesis under conditions of iron resupply (Behrenfeld et al. 2006). It has also been shown that an IsiA ring can form without a PSI trimer (Aspinwall et al. 2004), although whether such structures act as chl storage proteins or as a mechanism for protecting PSII via nonphotochemical quenching (Horton et al. 1996, Cadoret et al. 2004) also remains to be fully determined (Wilson et al. 2007, 2008). Irrespective of the functional role, increased levels of uncoupled IsiA observed under high iron-stress conditions should act to lower F_v/F_m by increasing F_o, as confirmed by the biophysical measurements.

The potential accumulation of significant amounts of unbound, and hence photochemically inactive, IsiA within natural iron-stressed phytoplankton populations where cyanobacteria dominate would have major implications for the interpretation of both in situ and remotely sensed data (Behrenfeld et al. 2006, 2009). As discussed above, unbound IsiA will likely have a high and nonvariable fluorescence yield, which will depress F_v/F_m as a result of increased F_o (Behrenfeld et al. 2006), thereby invalidating the strict interpretation of F_v/F_m as a measure of the photochemical quantum efficiency of PSII (Suggett et al. 2009). In addition, highly fluorescent unbound chl might contribute to the inferred high quantum yields of chl fluorescence in low-iron oceanic regions (Behrenfeld et al. 2009). The recent description of a eukaryotic chl-binding protein expressed under iron-stress conditions, Tidi (thylakoid iron-deficiency-induced) (Varsano et al. 2003, 2006), suggests that eukaryotic phytoplankton in oceanic regions could also potentially have a depressed F_v/F_m due to accumulation of nonphotosynthetically active chl. However, analogous to the IsiA-PSI supercomplex, Tidi has also only been shown to be an antenna system for PSI to date (Varsano et al. 2003, 2006). It is clear that a more detailed understanding of the role of iron-stress-induced chl-binding proteins in a range of marine phytoplankton may be required to interpret satellite-derived production estimates based on chl levels in large oceanic regions (Behrenfeld et al. 2006).

Establishing the contribution of unbound IsiA in natural populations will require an increased understanding of the levels of iron stress experienced by natural populations and how these relate to culture conditions. The batch culture experiments employed here and elsewhere (Bibby et al. 2001b,c, Yeremenko et al. 2004, Ivanov et al. 2006, Wang et al. 2010) will drive *Synechocystis* PCC 6803 into increasing levels of iron stress/starvation. Under these conditions, it appears that the primary role of IsiA is as a functioning PSI antenna (Fig. 3). However, subsequent excess accumulation may result from continued IsiA expression combined with down-regulation of the photosynthetic complexes (Fig. 5, a–c). Within natural populations, iron-limited

growth is more likely to approach a steady-state balance between iron uptake and resupply by regeneration owing to tight coupling between growth and loss terms (Cullen 1991, Morel et al. 1991). The relative contributions of bound and unbound IsiA to total chl (Fig. 6, a and b) suggests that unbound IsiA only increases under more severe cases of iron stress. Although we clearly cannot directly extrapolate from our laboratory measurements on a monoculture of a model organism, grown photoheterotrophically, to natural populations, we can propose that the time required under iron-starvation to accumulate large cellular pools of unbound IsiA may have limited ecological relevance. Although in situ growth rates of autotrophic prokaryotes clearly vary greatly in iron-limited systems (Mann and Chisholm 2000), given the high mortality rates reported (Landry et al. 1997), organisms with very low iron-limited growth rates would probably be outcompeted by other species.

Given the apparent role of IsiA as a functioning PSI antenna in combination with evidence for subsequent expression of uncoupled IsiA only under longer term iron-stress conditions, it may thus be premature to ascribe large-scale biophysical/biogeochemical patterns to the in situ expression of this protein and potential eukaryotic proteins with similar function (Behrenfeld et al. 2006, 2009). The role of chl-binding proteins, such as Tidi, which apparently acts as a functioning PSI antenna in eukaryotic algae, also needs to be studied further in culture and their presence verified in iron-limited oceanic regions (Varsano et al. 2006). In addition to model culture work to determine the functional role and identify the environmental conditions under which bound and unbound IsiA dominate, it is clearly necessary to obtain absolute quantification of IsiA and functionally similar proteins from iron-limited regions before it will be possible to determine whether a significant fraction of in situ chl is dominated by unbound pigment-protein complexes having no direct role in photosynthesis.

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