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RESEARCH PAPER

Properties of photosystem I antenna protein complexes of the diatom *Cyclotella meneghiniana*

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Abstract

Analysis of photosystem I (PSI) complexes from *Cyclotella meneghiniana* cultured under different growth conditions led to the identification of three groups of antenna proteins, having molecular weights of around 19, 18, and 17 kDa. The 19-kDa proteins have earlier been demonstrated to be more peripherally bound to PSI, and their amount in the PSI complexes was significantly reduced when the iron supply in the growth medium was lowered. This polypeptide was almost missing, and thus the total amount of fucoxanthin-chlorophyll proteins (Fcps) bound to PSI was reduced as well. When treating cells with high light in addition, no further changes in antenna polypeptide composition were detected. Xanthophyll cycle pigments were found to be bound to all Fcps of PSI. However, PSI of high light cultures had a significantly higher diatoxanthin to diadinoxanthin ratio, which is assumed to protect against a surplus of excitation energy. PSI complexes from the double-stressed cultures (high light plus reduced iron supply) were slightly more sensitive against destruction by the detergent treatment. This could be seen as a higher 674-nm emission at 77 K in comparison to the PSI complexes isolated from other growth conditions. Two major emission bands of the Fcps bound to PSI at 77 K could be identified, whereby chlorophyll a fluorescing at 697 nm was more strongly coupled to the PSI core than those fluorescing at 685 nm. Thus, the build up of the PSI antenna of several Fcp components enables variable reactions to several stress factors commonly experienced by the diatoms *in vivo*, in particular diatoxanthin enrichment under high light and reduction of antenna size under reduced iron conditions.

Key words: diatoxanthin, Fcp4, fucoxanthin-chlorophyll-proteins, high light adaptation, iron, Lhcr.

Introduction

Diatoms are eukaryotic, unicellular algae performing oxygenic photosynthesis. They are of high ecological importance due to their high abundance in oceans, accounting for up to 25% of the worldwide primary production (for review see Falciatore and Bowler, 2002). Despite a different thylakoid membrane organization in bands of three thylakoids each, the light reactions of photosynthesis in diatoms resemble those of higher plants. Diatoms possess membrane-intrinsic light-harvesting complexes (LHC), called fucoxanthin-chlorophyll proteins (Fcps), which belong to the same protein family as the light-harvesting proteins of higher plants (Green and Pichersky, 1994). However, the pigmentation is different with chlorophyll (Chl) c replacing Chl b and fucoxanthin (Fx) being the main carotenoid. The carotenoid to Chl ratio of 4:5 is much higher in Fcp complexes (FCPs) than the 4:14 ratio in plant LHCs, which also highlights the light-harvesting function of carotenoids in these antenna polypeptides (Papagiannakis *et al.*, 2005; Premvardhan *et al.*, 2008, 2009, 2010; Gildenhoff *et al.*, 2010a,b). By means of Stark spectroscopy on isolated FCPs (Premvardhan *et al.*, 2008), and electrochromic shift experiments using whole cells (Szabó *et al.*, 2010), it was proven that the binding of up to eight Fx molecules to FCP results in different bathochromic shifts. Thus, Fx molecules absorbing at slightly longer or shorter wavelengths, i.e. 'red', 'green', and 'blue' Fx could be distinguished. The overall arrangement of the Fcp polypeptides in supercomplexes seems to differ, too. Whereas trimeric complexes have been proven for several diatoms, higher oligomers have also been described. In *Cyclotella meneghiniana*, two

complexes make up the main antenna pool (Büchel, 2003), the trimeric FCPa and the oligomeric FCPb, which differ in polypeptide composition. Fcp1/2/3, belonging to the group of major Fcp polypeptides, also called Lhcf, and Fcp6/7/8, belonging to the group of Lhcx proteins, were identified in FCPa. FCPb consists of one polypeptide, most probably Fcp5, a member of the Lhcf group (Beer *et al.*, 2006). These FCPs not only function in light harvesting by each having all spectral forms of Fx ('red', 'green', and 'blue') bound, but they also act in protection, due to their ability to bind pigments of the so-called xanthophyll cycle. The main cycle in diatoms consists of diadinoxanthin (Dd) and diatoxanthin (Dt), whereby Dd is converted to Dt under high light conditions. The appearance of high amounts of Dt under high light correlates with a high non-photochemical quenching (Lavaud *et al.*, 2002), a mechanism to dissipate excess energy via heat. Recently it could be shown that Dt is reducing the fluorescence yield of one of the isolated FCPs, FCPa, thus identifying this complex as one possible quenching site (Gundermann and Büchel, 2008).

In contrast to the main FCPs, much less is known about Fcp proteins that are specifically associated with one of the photosystems. For photosystem I (PSI), genetic analyses point to *Lhcr* genes, which were annotated due to their similarity with red algal genes coding for PSI antenna proteins (Oudot-Le Secq *et al.*, 2007). PSI has been demonstrated to be a monomer, like in higher plants (Veith and Büchel, 2007), allowing for Fcp proteins to be associated with it in a similar manner like LHCI. For PSI of *C. meneghiniana*, Fcp4, an Lhcr protein, could lately be identified as a specific antenna protein for PSI, whereas Fcp6/7/8 as well as Fcp1/2/3 were completely missing in the PSI-FCP (Veith *et al.*, 2009). Later *lhcr* gene products have been reported as constituents of PSI in the pennate diatom *Phaeodactylum tricornutum* (Lepetit *et al.*, 2010) and in the centric diatom *Thallassiosira pseudonana* (Grouneva *et al.*, 2011), a close relative to *C. meneghiniana*. Xanthophyll cycle pigments are located close to the photosystems as well, e.g. in a mixed photosystem fraction in *P. tricornutum*, where they were shown to be interconvertible depending on the light intensity (Lepetit *et al.*, 2007), but also in pure PSI-FCPs of *C. meneghiniana* (Veith *et al.*, 2009; Lepetit *et al.*, 2010).

In order to get more insight into the antenna composition of PSI and the characteristics of the Fcps bound, this study compared PSI complexes isolated from *C. meneghiniana* cells cultivated under different conditions. In a former publication (Veith *et al.*, 2009), the basic composition of PSI from cells grown under high light conditions was studied. Since already a slight reduction of the iron supply in the growth medium resulted in differences in chloroplast size and influenced the polypeptide composition of the FCPa complex (Beer *et al.*, 2011), this study used cells cultured with a reduced iron supply under high light (HL) or low light (LL) conditions. In addition, PSI complexes from a LL culture fully supplemented with iron were tested.

Materials and methods

Culturing of diatoms

The diatom *Cyclotella meneghiniana* (Culture Collection Göttingen, strain 1020-1a) was grown under LL (40 μmol photons m^{-2} s^{-1}) or HL (140 μmol photons m^{-2} s^{-1}) regimes in culture medium according to Provasoli *et al.* (1957), but modified so that it contained only 1 μM iron (–Fe). Usually this medium contains 12 μM iron (+) and also (+) cultures were grown under LL. Cells were kept in the different conditions by inoculating every fortnight for at least a year before experiments were started.

Preparation of PS complexes and FCP

Cells were harvested in the early light phase by centrifugation after 10–14 days of culture. Thylakoid membranes were isolated by several centrifugation steps after breaking the cells in a bead mill according to Büchel (2003). Solubilization of thylakoids was done at 0.25 mg/ml total Chl with 15 mM β-dodecyl maltoside (1:54, mol/mol) on ice. The complexes were then purified by sucrose density centrifugation according to Veith *et al.* (2009).

Biochemical characterization of PSI complexes

The purity of the complexes was checked by SDS-polyacrylamide gel electrophoresis (PAGE) as described in Schägger and von Jagow (1987), using separating gels with 15% acryl amide (w/v). Preparation of the samples, silver staining of the gels and immunodetection were carried out according to Beer *et al.*, 2006. The antibody used, α-cmFCP, was created to detect all Fcps of *C. meneghiniana*. To this end, the antenna complexes FCPa and FCPb were purified from LL(+) cells according to Beer *et al.* (2006), pooled and used for commercial antibody production (Biotrend, Germany) in rabbits. A chemically synthesized peptide, GDFRNGYIDFGWDSFD, with a sequence identical in the Lhcf1-11 sequences of *P. tricornutum* and also found in Fcp5 of *C. meneghiniana*, was used for the commercial production (Eurogentec, Belgium) of α-lhcf1-11. According to sequence comparisons this antibody should also detect Fcp1/2/3 of *C. meneghiniana* and Lhcf1-5, 8 and 9 of *T. pseudonana*, but none of the Lhcr or Lhcx proteins. The intensities of the FCP bands of the different PSI complexes after immunodecoration with α-cmFCP and using enhanced chemoluminescence for detection were analysed using the program ImageJ. In order to compare the different culture conditions, equal amounts of Chl a (5 μg) were loaded and samples were probed on the same blotting membrane.

Pigment analysis

Pigment stoichiometries of isolated PSI complexes were determined by analytical HPLC (Elite LaChrom, L-2130/L-2450, Merck). Pigments were extracted directly with 90% methanol (final concentration). Samples were centrifuged shortly at maximal speed in an Eppendorf centrifuge, and the supernatants were loaded onto the column. Pigments were separated and quantified using a RP18 column and a photo-diode array detector as described in Papagiannakis *et al.*, 2005.

Spectroscopic characterization of complexes

Absorbance of isolated PSI complexes was recorded in the Q_Y maximum of Chl a absorption and complexes were then adjusted to an absorbance of 0.03 (corresponding to roughly 0.3×10^{-3} μg/ml Chl a) with a buffer containing 60% glycerol as described in Veith *et al.* (2009). Samples prepared accordingly were used to record fluorescence spectra at 77 K with a Jasco fluorometer (FP-6500). Emission spectra were taken upon excitation at $\lambda ex = 440$ nm for preferential excitation of Chl a and measured from $\lambda em = 600$ to 800 nm in 0.1 nm steps. Excitation spectra of the same samples were recorded from $\lambda ex = 400$ to 600 nm for $\lambda em = 674$, 686, and

717 nm. Band passes of 3 nm were used both on the emission and the excitation side. A rhodamine B spectrum served as a reference for the correction of the excitation side and the photomultiplier was corrected using a calibrated lamp spectrum. Emission spectra obtained from samples of two independent preparations were fitted using Gaussian bands.

Results and Discussion

Cyclotella meneghiniana cells were grown under three different conditions: either in LL with full supplement of iron (+) or under slightly reduced iron concentrations (–), and in addition HL cells cultured under reduced iron were used as well. From the different cultures, PSI complexes were prepared using sucrose density centrifugation after a mild solubilization of the thylakoid membranes as reported in Veith *et al.* (2009). No changes in band patterns of the gradients were observed concerning the different light conditions (Fig. 1A). The lower green bands formerly attributed to PSI complexes having Fcps bound were harvested from the gradients and characterized further.

In order to determine the Fcp composition of the PSI complexes, Western blots were carried out using an antibody directed against all Fcp polypeptides of *C. meneghiniana*, and the relative intensities of the bands were quantified using two different blots from two preparations each (Fig. 1B, C). Same amounts of Chl were loaded in each case, but due to the differences in absolute intensities of the different blots, standard deviations were high. Nevertheless, this study abstained from normalizing to one band since no data existed about a constant amount of any of the proteins recognized by the Fcp antibody. As reported before for PSI from HL(+) cells (Veith *et al.*, 2009), several Fcp proteins are found in these PSI preparations. Three main bands, at 19, 18, and 17 kDa, could again be distinguished (Veith *et al.*, 2009). Since the sensitivity of the antibody used might differ between the different polypeptides, results do not represent absolute values for the distribution of different proteins inside one sample. On the other hand, a direct comparison for one certain antenna protein between different cultures is possible. Whereas three bands were detected in PSI complexes from (+) cells as already reported for HL(+) PSI, the 19-kDa band was severely reduced under (–) conditions. PSI complexes from LL(+) seemed to have the highest amounts of each of the polypeptides, but taking into account the high variations of the blots, the 18- and 17-kDa bands showed rather unchanged amounts under all conditions. 19- and 18-kDa bands are found in FCPa complexes as well and these molecular masses are thus very common to Fcps of *C. meneghiniana*. 17-kDa bands were sometimes also visible in FCPa, but only under HL conditions (Beer *et al.*, 2006), making it unlikely that they are identical to those found here under LL as well as HL conditions. Some subunits of the PSI core have similar molecular weights, so cross-reactions of the antibody cannot be ruled out, but the 17-kDa polypeptide was detected with another antibody made from Fcp proteins prepared in a different way as well (Veith *et al.*, 2009). Using a more specific antibody, which was raised against a small peptide specific for Fcps encoded by *lhcf1-11* from *P. tricornutum* and *Fcp5* as well as *Fcp1/2/3* of *C. meneghiniana*, only the 19-kDa band could be detected (Fig. 1C).

Far more genes are described for Fcp polypeptides in *C. meneghiniana* (Eppard and Rhiel, 2000) and another centric diatom, *T. pseudonana* (Armbrust *et al.*, 2004) compared to what has been identified at the protein level so far. In *C. meneghiniana*, identification, for example by mass spectrometric analysis, is further hampered by the fact that the genome is not sequenced, in contrast to the related species *T. pseudonana* and the pennate diatom *P. tricornutum* (Armbrust *et al.*, 2004; Bowler *et al.*, 2008). Thus, although altogether only five distinctive Fcp proteins (Fcp1/2/3, Fcp4, Fcp5, Fcp6/7/8, and Fcp12) have been identified in *C. meneghiniana* so far, each of the bands in PSI is most probably composed of several proteins.

For the 19-kDa band, only three proteins identified in *C. meneghiniana* (Fcp5, Fcp6/7/8, and Fcp12) come into question. Two of them (Fcp6/7/8 and Fcp12) belong to the Lhcx family, whereas Fcp5 is a Lhcf protein. Grouneva

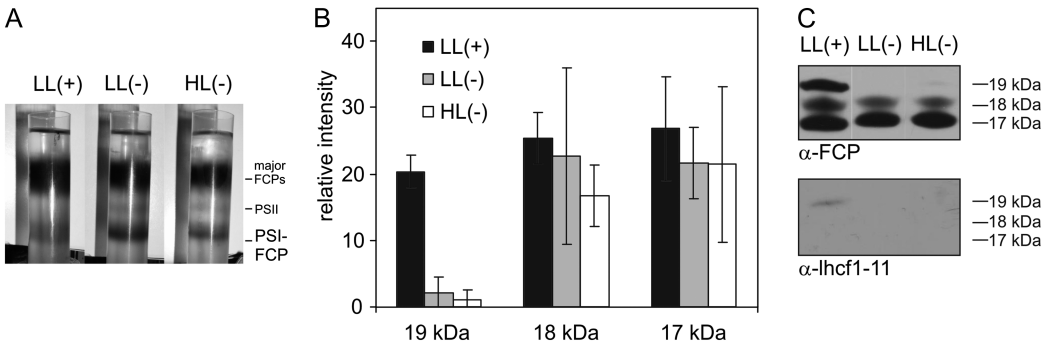

Fig. 1. Protein composition of photosystem I complexes. (A) Results of separating complexes from the differently grown cultures using sucrose density centrifugation [low light plus 12 μmol iron (LL(+)), LL plus 1 μmol iron (LL(–)), and high light plus 1 μmol iron (HL(–))]: for attribution of bands see Veith *et al*. (2009). (B) Signal intensities from immunoblots of complexes from cells grown under LL(+) (black), LL(–) (grey), and HL(–) (white), developed using α-cmFCP: identical amounts of chlorophyll a (5 μg each) were loaded and preparations from all three cultures were run on two blots each. (C) One example of the original blots and the results using the α-lhcf1-11 antibody.

et al. (2011) identified mainly Lhcr proteins, but also several Lhcx and Lhcf proteins when analysing PSI complexes isolated by blue native (BN) PAGE using mass spectrometry. Fcp6/7/8 can be ruled out, since a specific antibody did not react with the 19-kDa band (Veith *et al.*, 2009). Surprisingly, the homologous protein, Lhcx1, was found in PSI of *T. pseudonana* (Grouneva *et al.*, 2011). Fcp12 might be present in PSI as well, although its homologue, Lhcx5, was not found in PSI of *T. pseudonana* (Grouneva *et al.*, 2011). Other members of the Lhcx family have been detected as well, but no identical sequences are known from *C. meneghiniana* so far. The 19-kDa proteins were earlier shown to be more loosely bound to PSI than the 18- or 17-kDa polypeptides (Veith *et al.*, 2009), which might explain an absence after BN-PAGE. Since the Lhcf antibody reacted with the 19-kDa band, Fcp5 might be one of the constituents, although none of the *T. pseudonana* proteins which should be detected by this antibody were found in PSI, including the Fcp5 homologue Lhcf8 (Grouneva *et al.*, 2011). The most obvious feature is the reduction of the 19-kDa band under iron reduction. Allen *et al.* (2008) tested the expression levels of some of the Fcp genes under iron limitation in *P. tricornutum* and also reported on *T. pseudonana* genes in their supplementary material. One of the latter, annotated *lhcr6* (jgi: 3815), was downregulated under iron limitation, but again not found in PSI by Grouneva *et al.* (2011). On the other hand *lhcr2* was downregulated in *P. tricornutum* and the most similar sequence in *T. pseudonana* belongs to Lhcr7, a protein of higher molecular mass and a constituent of PSI according to Grouneva *et al.* (2011). None of these Lhcr proteins have been identified so far in *C. meneghiniana*. In summary, the Lhcf protein Fcp5, the Lhcx protein Fcp12, and/or as-yet unknown Lhcx proteins of *C. meneghiniana* and/or homologues of Lhcr6 and Lhcr7 of *T. pseudonana* may be constituents of the 19-kDa band. The severe decrease in the amount of this protein seen here under iron reduction and the reduced expression of Lhcr2 in *P. tricornutum* under iron limitation make a similar protein the most likely candidate.

Concerning the 18-kDa proteins, it is only known from mass spectrometric analyses that Fcp4 is one of the constituents of this band (Veith et al 2009). Fcp4 is most closely related to Lhcr3 of *T. pseudonana* and *P. tricornutum*. This protein was also detected in PSI by Grouneva *et al.* (2011) in *T. pseudonana*, and in *P. tricornutum* this gene is downregulated during HL (Nymark *et al.*, 2009), fitting to the biochemical analysis shown here. The current study could not identify polypeptides in higher abundance in HL, although the genes for Lhcr6, 7, 8, and 10 were reported to be significantly upregulated in cells of *P. tricornutum* under HL conditions (Nymark *et al.*, 2009). Moreover, the proteins were detected exclusively under HL conditions by Lepetit *et al.* (2010), but only in the FCP pool. It has to be emphasized that none of these *P. tricornutum* proteins has close homologues in *T. pseudonana*. Most probably, some of the other Lhcr polypeptides of similar size as Fcp4 contribute to the 18-kDa band in *C. meneghiniana*.

When predicting proteins from gene sequences, another difficulty for these nuclear-encoded plastidic proteins is the lack of data about the precise start of the mature protein. Diatoms possess secondary plastids, and nuclear-encoded proteins like Fcps have thus to be transferred across four envelope membranes. The signal peptide responsible for the first part of this transfer is known, but for the transit peptide, needed for further transport, no typical sequence has been identified so far, making it difficult to predict the molecular mass of the mature protein. In many cases, the calculated molecular mass still including the target signal is about 20 kDa or below, and thus the proteins with an apparent mass of 17 kDa most probably belong to the group of proteins discussed above.

All the PSI complexes contained pigments specific for Fcp polypeptides. The ratio of these typical pigments, Chl c/Fx was constant (Fig. 2), and the amount of xanthophyll cycle pigments, Dd plus Dt, was only slightly decreased per Fx in PSI complexes from LL(–) cells compared to the two other growth conditions. These results point to a similar pigmentation of the various Fcp polypeptides despite the different composition of Fcps associated with PSI seen in the Western blots. In contrast, the de-epoxidation ratio expressed as Dt/(Dd + Dt) differed. Here the Fcps in PSI complexes followed the same trends as reported for the major FCPs before (Beer *et al.*, 2006, 2011). PSI from HL cells had a high de-epoxidation ratio whereas reduced iron led to a decreased de-epoxidation ratio. There are several reports in the literature also indicating a light-dependent increase in xanthophyll cycle pigments and de-epoxidation of Dd to Dt in PSI complexes (Lepetit *et al.*, 2007; Veith *et al.*, 2009). Lepetit *et al.* (2010) also demonstrated that this Dt is protein bound and not associated with the lipids

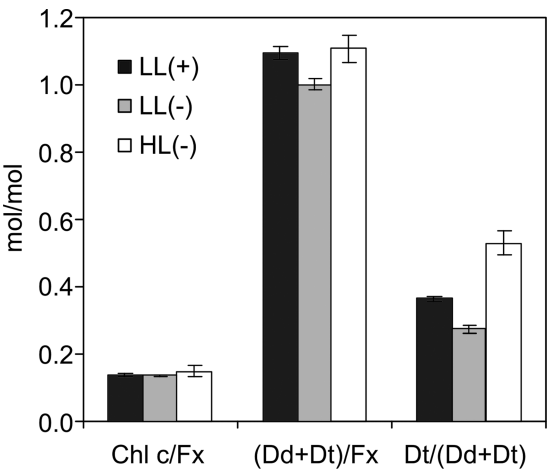

Fig. 2. Pigment composition of the photosystem I complexes. Ratios of the pigments specific for fucoxanthin-chlorophyll protein polypeptides [chlorophyll (Chl) c, fucoxanthin (Fx), diadinoxanthin and diatoxanthin (Dd + Dt)] are shown for complexes isolated from cells grown under low light plus 12 μmol iron [LL(+), black], LL plus 1 μmol iron [LL(–), grey], and high light plus 1 μmol iron [HL(–), white]. Values are means ± standard deviations from two independent preparations each.

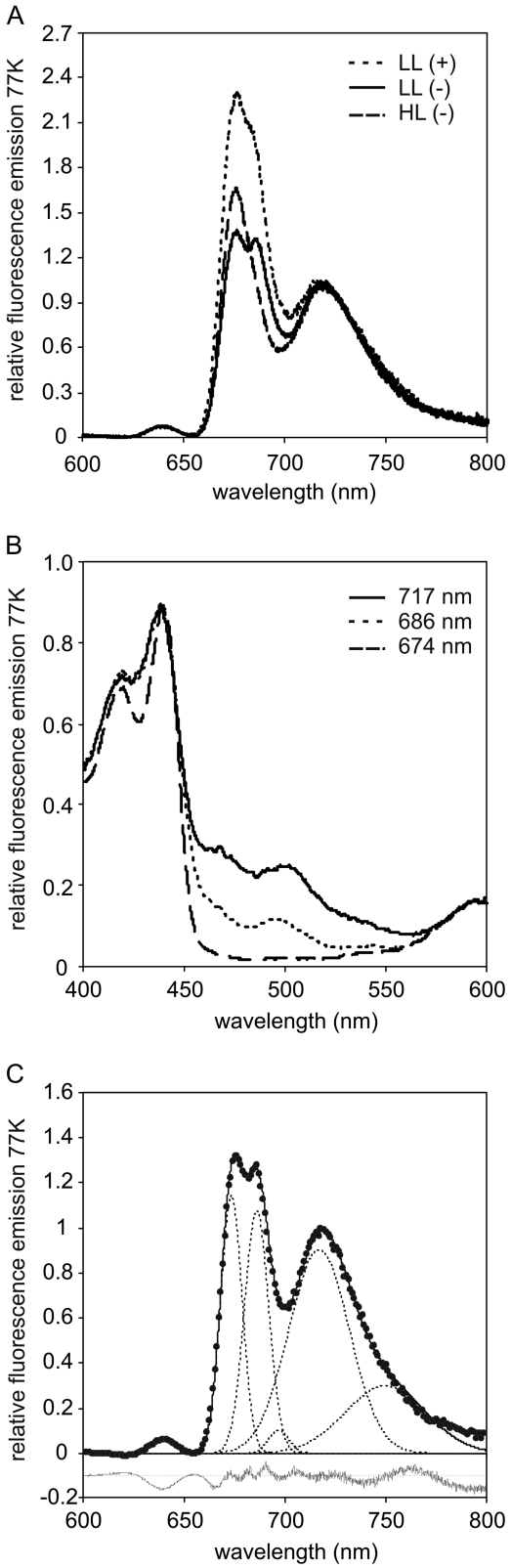

Fig. 3. (A) Fluorescence emission spectra at 77 K of photosystem I complexes from low light plus 12 μmol iron [(LL(+), dotted line], LL plus 1 μmol iron [LL(–), solid line], and high light plus 1 μmol iron [HL(–), dashed line] cells excited at 440 nm and normalized to identical 717-nm emission. (B) Excitation spectra at 77 K of complexes from LL(–) cells, recorded at 674 nm (dashed line), 686 nm (dotted line), and 717 nm (solid line). (C) A fit of the emission spectra with five Gaussians is shown exemplarily for complexes from LL(–): the original spectrum is shown with circles, the fit is demonstrated as a solid line, and the single Gaussians as dotted lines. Residuals (lower panel) were plotted on the same axis but shifted by –0.1.

present in the preparations as well. The presence of Dt in fair amounts inside the PSI complexes has already been discussed with regard to protection, especially as a scavenger of ^{3}Chl a and $^1O_2$ (Lepetit *et al.*, 2010), although this is a general feature of carotenoids and not restricted to Dt. Here it is important to note that neither the ratio of xanthophyll cycle pigments to Fx nor the Chl c/Fx ratio depend on the amount of the 19-kDa polypeptide, the only polypeptide for which the amount changed significantly. Since further solubilization led to removal of the 19-kDa proteins with similar pigment stoichiometries as reported here (Veith *et al.*, 2009), the current results point to all Fcp polypeptides of PSI having similar pigmentation. Thus, the binding of xanthophyll pigments and the de-epoxidation are located in the 19-kDa as well as in the other Fcp polypeptides.

Fig. 3 demonstrates the low-temperature fluorescence emission spectra obtained from the different PSI preparations. The spectra were recorded at very low Chl concentrations (0.3 ng/ml) to avoid reabsorption artefacts as much as possible. As reported several times before, the core of PSI of diatoms is characterized by a long wavelength emission at around 710–717 nm at 77 K (Berkaloff *et al.*, 1990; Veith and Büchel, 2007; Ikeda *et al.*, 2008; Yamagishi *et al.*, 2010). In addition, two additional peaks became obvious, especially in PSI from LL(–) grown cultures, with maxima around 674 and 685 nm as reported before for PSI from HL(+) cells (Veith *et al.*, 2009). However, at 77 K, theoretically all energy should end up on the Chls of the core emitting at long wavelength, in case a full coupling is achieved. Thus, both short wavelength emissions have to be attributed to Chls decoupled from the core to various extents. In principle this could be due to complete uncoupling, i.e. broken FCPs, or functional FCPs having partly lost the functional connection to the core. To examine whether the Fcps themselves are intact, i.e. whether Chl c and Fx are transferring energy to the different emitters, low-temperature excitation spectra were recorded at 674, 686, and 717 nm, respectively (Fig. 3B), for all different PSI complexes. Since these spectra were almost identical (data not shown), this study only reports on PSI from LL(–) here. From the excitation spectrum at 674 nm, it is pretty obvious that those emitting Chls can be identified as fully decoupled, since no other pigments are transferring energy to them. The 685-nm spectrum shows coupling of Fx (around 530 nm) and Chl c (at 465 nm), albeit to a lesser extent than in the spectrum recorded at 717 nm. A higher energy transfer from, for example, Fx to the final 717-nm emitter than to the 685-nm emitters can only be explained if Fx molecules other than those connected to the 685-nm emitter transfer their energy to the 717-nm Chls as well.

These could either be Fx molecules bound directly to the core, or Fx molecules coupled to another emitter inside the Fcps, which is transferring most of the energy to the core and is thus not visible in the emission spectra. The cores containing Fx molecules are unlikely since the polypeptides show high similarities to the core proteins of higher plants and cyanobacteria, which do not bind accessory pigments. Thus, a better coupling of some of the Fx to an emitter inside the Fcps other than the 685-nm Chl a is highly likely. The same reasoning holds for Chl c.

The Fx molecules bound to the complexes and actively transferring energy to Chl a molecules of the cores showed an extended absorption in the long wavelength range up to 550 nm (Fig. 3B). Thus, Fcps bound to PSI most probably also contain 'red' Fx molecules like found in both major FCPs of *C. meneghiniana*. Since the spectra of PSI complexes from different cultures were indistinguishable, these red forms occur in comparable amounts. Szabó *et al.* (2010) reported a slight preferential excitation of PSI by 'green' Fx molecules *in vivo*. This preferential excitation energy transfer occurred to a similar extent under HL and LL conditions, albeit different amounts of 'green' Fx in the cells. However, the current spectra do not allow a quantification concerning the ratio of 'blue', 'green', or 'red' Fx molecules bound in the vicinity of PSI.

In order to identify the different Chl a emitters and to get a better insight into the various contributions seen in the different samples, the spectra were fitted using Gaussians. Five Gaussians, with maxima at 674, 685, 697, 717, and 750 nm, were needed to optimize the fits as shown exemplarily for the LL(–) spectrum measured upon excitation at 440 nm in Fig. 1B. Using more Gaussians did nothing to improve the fit (data not shown). The parameters used for calculations are reported in Table 1. It has to be emphasized that all spectra could be fitted using the same parameters, including spectra recorded using other excitation wavelengths (data not shown). As to be expected from the excitation spectra, one additional emitter with a tight coupling was identified, emitting at 697 nm. Whereas the 750-nm band is certainly a mixed band containing the vibrational bands of all others, the emission around 717 nm was earlier attributed to the PSI core as stated above (Berkaloff *et al.*, 1990; Veith and Büchel, 2007; Ikeda *et al.*, 2008; Yamagishi *et al.*, 2010). These Chl a species probably represent the 'red Chls' with energy levels below P700 found in all PSI complexes, like those from higher plants or cyanobacteria (Mullet *et al.*, 1980; Gobets *et al.*, 2001; Mangels *et al.*, 2002). In order to compare the complexes from algae of different growth regimes, the current study assumed that the number of long-wavelength-emitting Chl a per core is fixed for a species, as shown for different cyanobacteria (e.g. Shubin *et al.*, 1991; Wittmershaus *et al.*, 1992; Mangels *et al.*, 2002). In Fig. 4A, the summarized areas of the fluorescence bands at 674, 685, and 697 nm normalized to the 717-nm emissions are compared. The values were obtained using two independent preparations each and thus demonstrate the reproducibility of the complexes obtained. As already seen from the emission spectra, PSI from LL(+) was characterized by high emission at short wavelengths, whereas complexes from LL(–) and HL(–) cells showed a lower, but similar amount. The higher emission can be either due to a stronger decoupling or to Fcps equally decoupled but more abundant. In Fig. 4B the relative contributions of the 674-, 685-, and 697-nm bands to the short wavelength emission are depicted. The figure reveals that the contribution is similar for LL(+) and LL(–) PSI complexes, but that HL(–) complexes showed significantly higher emission at 674 nm. Taking the excitation spectra into account, the emission at 674 nm is due to stronger uncoupling of Chl a: that is, PSI from HL(–) cells showed a higher sensitivity against the isolation procedure. This is in line with the observation that an additional gel filtration step did remove this emission from the PSI preparations from HL(+) cells (Veith *et al.*, 2009). The other two bands, at 685 and 697 nm, changed in intensity in parallel in all PSI complexes. This became more obvious when setting the sum of both emissions to one and then comparing the relative contributions (Fig. 4C). No difference between the PSI complexes from the various conditions could be detected. The very obvious enrichment in 19-kDa polypeptides as seen in LL(+) PSI has thus no counterpart in the fluorescence yield of one single band. Furthermore, since the 685- and 697-nm emissions changed in parallel, no attribution of certain fluorescence maxima to special polypeptides is possible.

In summary, the 674-nm emission is due to decoupled Chl a, and the 685- and 697-nm emissions are due to Chl a molecules that have different pools of Fx and Chl c molecules bound, which are active in energy transfer. Thereby the Chl a emitting at 697 nm are far better coupled to the cores than the 685-nm Chls.

The parallel change of the fluorescence yield of the two emitters poses the question whether these emitters work in parallel or in series. In the latter case the excitation harvested by Fx and Chl c would first be transferred into the 685-nm emitter, where most of it would get lost in the preparations, and then into the 697-nm emitting species before reaching the core. This is in contradiction to the excitation spectra, since in this case no better coupling of Fx and Chl c to the core (717-nm emission) than to the 685-nm emitter would be measurable. The excitation spectra also rule out the possibility that the 697-nm emission is an

Table 1. Wavelengths at peak maximum and half width at half peak height of the Gaussians used to fit the 77 K fluorescence emission spectra of photosystem I complexes

Data are means ± standard deviations of two spectra from three culture conditions each.

Peak wavelength (nm)	Half width (nm)
673.8 ± 0.6	5.4 ± 0.1
685.3 ± 0.5	6.0 ± 0.0
697.0 ± 0.0	5.0 ± 0.0
716.5 ± 0.6	15.0 ± 0.0
750.0 ± 0.0	20.0 ± 0.0

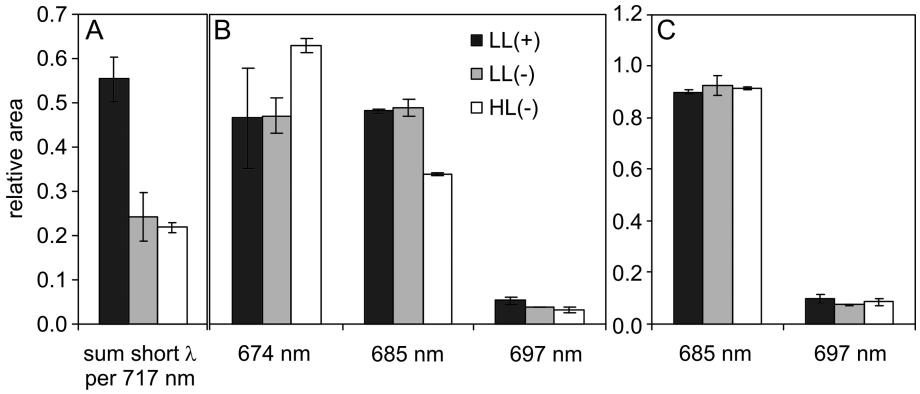

Fig. 4. Areas under the single Gaussians, calculated using the fits shown in Fig. 3 and the parameters given in Table 1, depicted for photosystem I complexes from low light plus 12 μmol iron [LL(+), black], LL plus 1 μmol iron [LL(–), grey], and high light plus 1 μmol iron [HL(–), white] cultures. (A) Sum of the areas of the 674-, 685-, and 697-nm bands normalized to the area of the 717-nm band. (B) Relative contribution of the three emitters to the short wavelength emission. (C) Relative contribution of the 685- and 697-nm bands: sum of the areas set to one. Values are means ± standard deviations from two independent preparations each.

artefact of the fitting (see above). On the other hand, if the two emitters work independently, then each of them will have Fx and Chl c bound, which is active in excitation energy transfer, explaining the excitation spectra. However, since the 685- and 697-nm emissions were always changing in parallel, both seem to be equally sensitive against the isolation procedure. For another diatom, *Chaetoceros gracilis*, Ikeda *et al.* (2008) concluded from time-resolved spectra that two emitters are located in the core. Here, LL(+) PSI has the highest absolute value of short wavelength emission per core, in accordance with the highest amount of Fcp protein found. In addition, from sequence analyses, no hints exist for a binding of accessory pigments to the core proteins as stated above. Since both emitters are served by accessory pigments independently, it is much more likely that both emitters are located in Fcps.

The comparative analysis of PSI complexes from differently grown alga cultures identified two groups of inner antenna proteins, having molecular weights of 18 and 17 kDa. Only for the 18-kDa protein group, one constituent, Fcp4, has been identified so far. The 19-kDa proteins have been demonstrated to be peripherally bound earlier and are not found in PSI from algae grown with lower iron. Thus, in addition to reducing the total amount of PSI (Allen *et al.*, 2008), iron stress seems to lead to smaller PSI complexes. Under iron reduction, the HL antenna composition is similar to the one under LL, at least within the resolution of the current blots. The xanthophyll cycle pigments are found in all Fcps, with PSI of HL(–) cultures having significantly more Dt per Dd. The PSI complexes from the double-stressed cultures (HL(–)) were in addition slightly more sensitive against destruction by the detergent treatment as judged from a higher 674-nm emission at 77 K. Two major emission bands of Fcps could be identified, whereby those Chl a fluorescing at 697 nm were more strongly coupled to the core than those fluorescing at 685 nm.

As already known for long, a higher Dt/Dd ratio helps to protect cells from a surplus of light, thus explaining the higher levels of Dt found in PSI-FCPs from HL cells. When comparing the sucrose gradients, displayed in Fig. 1A, it also seems that LL cells did contain more PSI-FCP compared to HL, in line with a higher reduction of the plastoquinone pool found in the latter (Beer *et al.*, 2011). Under lowered iron supply, not only the amount of PSI is reduced (Allen *et al.*, 2008), but also the antenna size is decreased, helping in avoiding over-excitation of the existing complexes. Thus, under HL, protection is increased by converting Dd to Dt and the overall amount of PSI-FCPs is decreased. In contrast, iron reduction induces a decrease in antenna size per PSI. The different components of the PSI antenna enable, therefore, variable reactions to several stress factors commonly experienced by the cells *in vivo*.

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