Biochimica et Biophysica Acta 1787 (2009) 905–912

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbabio

Identification of a specific fucoxanthin-chlorophyll protein in the light harvesting complex of photosystem I in the diatom *Cyclotella meneghiniana*

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ARTICLE INFO

Article history:
Received 5 November 2008
Received in revised form 30 March 2009
Accepted 20 April 2009
Available online 3 May 2009

Keywords:
Photosynthesis
Fluorescence
Fcp4
Light harvesting complex
Membrane protein
Diatom

ABSTRACT

Thylakoids of the diatom *Cyclotella meneghiniana* were separated by discontinuous gradient centrifugation into photosystem (PS) I, PSII, and fucoxanthin-chlorophyll protein (FCP) fractions. FCPs are homologue to light harvesting complexes of higher plants with similar function in e.g. brown algae and diatoms. Still, it is unclear if FCP complexes are specifically associated with either PSI or PSII, or if FCP complexes function as one antenna for both photosystems. However, a trimeric FCP complex, FCPa, and a higher FCP oligomer, FCPb, have been described for *C. meneghiniana*, already. In this study, biochemical and spectroscopical evidences are provided that reveal a different subset of associated Fcp polypeptides within the isolated photosystem complexes. Whereas the PSII associated Fcp antenna resembles FCPa since it contains Fcp2 and Fcp6, at least three different Fcp polypeptides are associated with PSI. By re-solubilisation and a further purification step Fcp polypeptides were partially removed from PSI and both fractions were analysed again by biochemical and spectroscopical means, as well as by HPLC. Thereby a protein related to Fcp4 and a so far undescribed 17 kDa Fcp were found to be strongly coupled to PSI, whereas presumably Fcp5, a subunit of the FCPb complex, is only loosely bound to the PSI core. Thus, an association of FCPb and PSI is assumed.

1. Introduction

Diatoms (*Bacillariophycea*) are unicellular, eukaryotic algae that carry out oxygenic photosynthesis and contribute nearly to one quarter of global primary production [1]. The most remarkable feature of diatoms is the highly patterned cell wall composed of amorphous silica [$(SiO_2)_n(H_2O)$]. Diatoms derived from a secondary endosymbiotic event. An as yet unknown eukaryotic host domesticated a phylogenetically relate of red algae. This endosymbiosis event led to the phenomenon that chloroplasts of diatoms are surrounded by four membranes. Another characteristic of diatom chloroplasts is the even arrangement of thylakoids in bands of three, instead of an organisation into stroma and grana lamellae (for reviews see [2,3]). Consequently, photosystem (PS) I and PSII are not segregated as in higher plants, which has been visualised by immuno-cytochemical experiments [4]. Despite their biological relevance our knowledge about diatom photosynthesis is relatively poor. In the last years the focus laid mainly on biochemical and spectroscopic analyses of the light harvesting complexes (LHCs) of diatoms, the fucoxanthin-chlorophyll proteins (Fcps), e.g. [5–8]. Although Fcps belong to the group of *cab* proteins (chlorophyll *a*-binding proteins), their pigmentation differs from LHCII of higher plants. Fcps bind fucoxanthin instead of lutein, chlorophyll (Chl) *c* instead of Chl *b*, and their Chl *a*: carotenoid stoichiometry is 1:1 in contrast to a 2:1 ratio in LHCII [9,10]. Furthermore, the hydrophilic loop regions connecting the transmembrane spanning helices of Fcps are shortened in comparison to higher plant LHCs.

Fcps can be placed into three groups according to sequence homologies. Group I, represented by e.g. the genes *fcp* 1–3 and 5 of *Cyclotella cryptica* and *fcp* A–F of *Phaeodactylum tricornutum*, relates with Fcps of brown algae. The gene *fcp* 4 of *C. cryptica* and their homologues in *Thalassiosira pseudonana* and *P. tricornutum*, *lhca* and *lhcr*, belong to group II and are related with a PSI associated, intrinsic light harvesting protein Lhca-R1/2 of red algae and cryptophytes. The third group shares homologies with LI818r-3 of *Chlamydomonas reinhardtii*, a member of the LHC family, and is represented by the genes *fcp* 6, 7 and 12 of *C. cryptica* [3,11–14]. Despite the knowledge about various gene sequences of Fcps, the knowledge about Fcps on protein level and their association with the photosystems remains scarce. Since Fcps possess high sequence similarities, small differences in molecular weight, and a strong hydrophobic character, it becomes a challenge to differentiate between polypeptides by immunological methods or sequencing. Still, in *Cyclotella meneghiniana* an FCP trimer

Abbreviations: Chl, chlorophyll; DD, diadinoxanthin; DDM, n-dodecyl β-D-maltoside; DT, diatoxanthin; FCP, fucoxanthin-chlorophyll protein complexes; Fcp, fucoxanthin-chlorophyll protein; HG, β-heptyl glucopyranoside; HPLC, high pressure liquid chromatography; IEX, ion exchange chromatography; LC-ESI-MS, liquid chromatography-electrospray ionisation-mass spectrometry; LHC, light harvesting complex; NG, n-nonyl β-D-glucopyranoside; OG, n-octyl β-D-glucopyranoside; PS, photosystem; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; TX, Triton X-100

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doi:10.1016/j.bbabio.2009.04.006

and a higher oligomer have been isolated and the associated Fcp polypeptides were identified [5,6]. The FCP trimer, named FCPa, is mainly composed of 18 kDa subunits encoded by the *fcp* 2 gene. In smaller amounts also 19 kDa subunits encoded by *fcp* 6 could be identified. In the higher oligomer FCPb, probably a hexa- or nonamer, only 19 kDa subunits were detected, encoded most probably by the *fcp* 5 gene. Recently, trimeric and hexameric FCP complexes were also described for *P. tricornutum* with subunits encoded by the genes *fcp* C/D and *fcp* E [8]. Thus, not only the pigmentation but also the assembly into higher oligomeric states seems to distinguish FCP complexes from e.g. higher plant LHCII trimers, although for LHCII heptamers of trimers (icosienamers) have been described as well [15].

In this work, the association of Fcp polypeptides with the photosystems, focussing on PSI, was studied by spectroscopical and immunological means. Earlier reports by e.g. Brakemann et al. [16] suggested a homogeneous distribution of Fcps despite the suggestion of PSI specific antenna proteins from sequence comparisons. Here, solubilised thylakoids of *C. meneghiniana* were separated into functional FCP, PSII, and PSI complexes. Analysis of polypeptides revealed a different subset of Fcps that are associated with the two photosystems. In this way, it could be demonstrated that a protein homologous to Fcp4 is tightly bound to PSI together with a so far non-described 17 kDa Fcp polypeptide. These data were compared to a similar approach in *P. tricornutum*, in order to demonstrate that the organisation of photosystem specific Fcp light harvesting protein complexes is species independent.

2. Materials and methods

2.1. Cell culture and preparation of pigment protein complexes

C. meneghiniana (Culture collection Göttingen (SAG), strain 1020-1a, formerly *C. cryptia* according to SAG, personal communication) was grown in ASP medium supplemented with 1 mM silica [17] under a 16 h light (140 μE m^{-2} s^{-1}) to 8 h dark cycle. Isolation of thylakoids was performed as described in [5]. In brief, thylakoids were adjusted to a concentration of 0.25 mg/ml Chl *a* + *c*, solubilised with 15 mM n-dodecyl β-D-maltoside (DDM) for 20 min on ice, and loaded onto discontinuous sucrose gradients. The gradients consisted of five sucrose layers with concentrations of 40, 30, 25, 20, and 15% in buffer B1 (25 mM Tris–HCl, 2 mM KCL, pH 7.4, 0.03% DDM). Thylakoids were centrifuged for 22 h at 28.000 rpm (rotor: Beckmann SW 28) and 4 °C. The separated pigment protein complexes were harvested and either concentrated using filtration devices (Amicon) with a 30 kDa cut-off or analysed directly. Chlorophyll concentrations of the samples were determined spectroscopically according to [18] in 90% acetone. Samples were stored at −20 °C.

Pigment protein complexes of *P. tricornutum* and pea thylakoids were purified according to [19]. Chlorophyll concentrations of the samples were determined spectroscopically according to [18,20] in 90% or 80% acetone, respectively.

2.2. Further purification of isolated PSI complexes

2.2.1. Gel-filtration

50 μl of purified and concentrated PSI complexes with a Chl concentration of approx. 0.4 mg/ml were applied to a Superdex 200 (GE Healthcare) gel-filtration column 10/300 GL (Amersham Biosciences) connected to an Äkta Purifier P-900 (Amersham Biosciences). Elution was carried out with buffer B1 at a flow rate of 0.5 ml/min and controlled spectroscopically at three different wavelengths (λ_1 = 437 nm, λ_2 = 530, λ_3 = 700 nm).

2.2.2. Discontinuous sucrose gradient centrifugation

For further analysis, PSI fractions were resolubilised at a Chl *a* concentration of 0.2 mg/ml using different detergents, and again fractionated on a sucrose gradient consisting of the same sucrose layers as described above with the exception of the 40% sucrose layer, which was omitted. Detergents used were 240 mM β-heptyl glucopyranoside (HG), 75 mM n-octyl β-D-glucopyranoside (OG), 20 mM n-nonyl β-D-glucopyranoside (NG), 3% (w/v) Triton X-100, and a combination of 10 mM *n*-nonyl β-D-glucopyranoside (NG) and 37.5 mM n-octyl β-D-glucopyranoside.

2.3. Characterisation of purified pigment protein complexes

2.3.1. Gel electrophoresis

15% Tris–Tricine Gels were cast according to [21]. Depending on their Chl concentration, samples were either precipitated in acetone before or directly denatured in Rotiload® (Roth) for 20 min at room temperature (RT). Gels were either stained with Coomassie Blue G 250 or were silver stained.

2.3.2. Western blot

Unstained gels were incubated in cathode buffer (25 mM Tris–HCl, 40 mM glycine, 10% methanol at pH 9.4) for 15 min and then blotted onto PVDF membranes (Roth) previously incubated in anode buffer II (25 mM Tris–HCl, 10% methanol, pH 10.4). Both were enclosed by a sandwich of 3MM Chr Whatman paper (Schleicher & Schüll), wetted with either cathode buffer, anode buffer II or anode buffer I (0.3 M Tris–HCl, 10% methanol, pH 10.4). Transfer was carried out for 1 h at 1.5 mA/cm^2 in a semi-dry transfer cell (BioRad Trans-blot SD). Immunodetection was performed using the ECL Plus kit (Amersham Biosciences) according to the manufacturer's instructions. Antibodies directed against higher plant reaction centre II PsbD (α-D2) and against Fcp2 (α-Fcp2), Fcp4 (α-Fcp4), Fcp6 (α-Fcp6) [22], and all Fcp polypeptides (α-ccFcp) [23] of *C. cryptica* were kind gifts from Dr. D. Godde, (University of Bochum) and Dr. E. Rhiel (University of Oldenburg), respectively. Antibodies were diluted in PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, and 1.4 mM KH_2PO_4) containing 5% (w/v) dry milk (Roth), except for α-Fcp4, which was diluted in PBS only. Dilutions used were 1:2000 (α-D2, α-ccFcp), 1:1000 (α-Fcp2, α-Fcp6), and 1:200 (α-Fcp4).

2.3.3. HPLC

Concentrated samples were extracted using 90% methanol and analysed according to [9].

2.3.4. Spectroscopy

Absorbance spectra were recorded between 350 nm and 750 nm at room temperature with 1 nm band pass and 1 cm optical path length using a Jasco spectrophotometer (V 550). Fluorescence spectra were measured with a Jasco fluorometer (FP-6500) at room temperature (RT) and 77 K, respectively. Band passes of 3 nm were used both on emission and excitation side. A rhodamine B spectrum served as a reference for the correction at the excitation side and the photomultiplier was corrected using a calibrated lamp spectrum. Emission spectra were taken upon excitation at λ_{ex} = 440 nm or λ_{ex} = 465 nm and measured from λ_{em} = 600 nm to λ_{em} = 800 nm. For the excitation spectra, emission was recorded at λ_{em} = 675, λ_{em} = 687 nm, or λ_{em} = 717 nm upon excitation from λ_{ex} = 400 nm to λ_{ex} = 600 nm. For direct comparison of different fractions, samples were adjusted to the same Chl *a* concentration (absorbance of 0.03 at the Q_Y band of Chl *a*) in buffer B1. In case of 77 K measurements samples were diluted in buffer B1 containing 60% glycerol.

2.3.5. Mass spectrometry

Bands of interest were cut out from silver stained gels and freeze-dried. After destaining and *in-gel* digestion with trypsin, the pellet was resuspended in 5 μL 5% (v/v) DMSO/5% (v/v) formic acid and peptides were separated by nano-HPLC and analysed by LC-ESI-MS/MS as described [24,25]. The mass spectrometer was

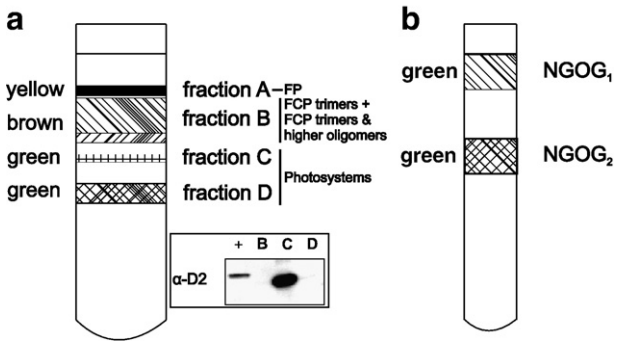

Fig. 1. Schematic representation of a sucrose gradient after fractionation of thylakoids solubilised with DDM (a) or a PSI sample of *C. meneghiniana* re-solubilised with a mixture of NG and OG (b). Pigmented bands are indicated. The inset in (a) depicts a Western blot of the sucrose gradient fractions B, C, and D and thylakoids of pea (+) as positive control. The antibody used was directed against the PSII subunit PsbD. 4 μg Chl of all samples was loaded, except for fraction B (0.5 μg Chl).

cycling between one full MS and MS/MS scans of the four most abundant ions. After each cycle, these ions were excluded from analysis for 10 s.

Data analysis was done using the Proteome Discoverer software (Version 1.0) from Thermo Electron Corp. including the SEQUEST algorithm [26]. Detection of a modification of 16 Da on Met representing its oxidized form was enabled. Peptide mass tolerance was set to 1.5 Da in MS mode. In MS^2 mode, fragment ion tolerance was set up to 1 Da. The parameters for all database searches were set to achieve a false discovery rate (FDR) of not more than 1% for each individual analysis. Thereby, the Proteome Discoverer software (Version 1.0) generates a reversed "decoy" database from the chosen database and any peptide passing the initial filtering parameter that was derived from this decoy database is defined as a false positive. Then, it automatically adjusts the minimum Xcorr filter for each individual charge state (+1, +2, +3) separately in order to optimally meet the predetermined target FDR of 1% based on the number of random false positive matches from the reversed "decoy" database. Data were searched against a combined diatom database of *C. cryptica*

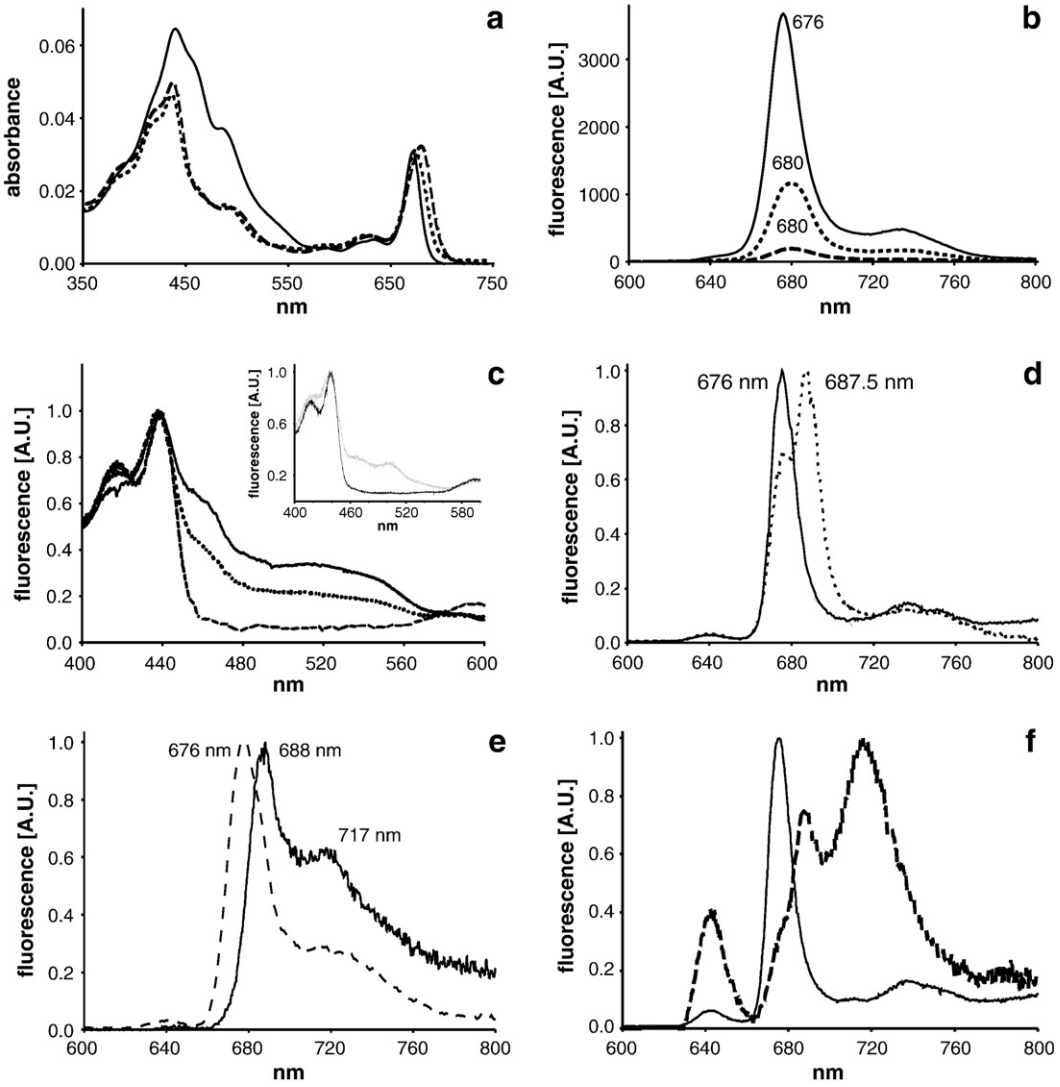

Fig. 2. Absorbance and fluorescence spectra of purified pigment protein fractions B (solid line), C (dotted line), and D (dashed line). In (a) the absorbance spectra recorded between 350 nm and 750 nm of fractions are shown. Samples were adjusted to about the same Chl *a* Q_y absorbance. Room temperature fluorescence emission spectra are shown in (b). Actinic light was set to 440 nm. 77 K fluorescence excitation spectra measured at 675 nm are represented in (c). For comparison the 77 K fluorescence excitation spectra of fraction D measured at 675 nm (black, solid line) and 717 nm (grey, dotted line) are shown in the inset. 77 K fluorescence emission spectra of fractions B and C excited at 440 nm can be seen in (d). The same excitation was used for the spectra shown in (e) of fraction D and D*gf1* (obtained after gel-filtrating fraction D, solid line). 77 K fluorescence emission of fractions B and D excited at 465 nm can be seen in (f). Fluorescence emission and excitation spectra shown in (c–f) were normalised to 1 at their Chl *a* Q_y or Soret bands, respectively. In (d) and (e) the wavelengths of the emission maxima are indicated.

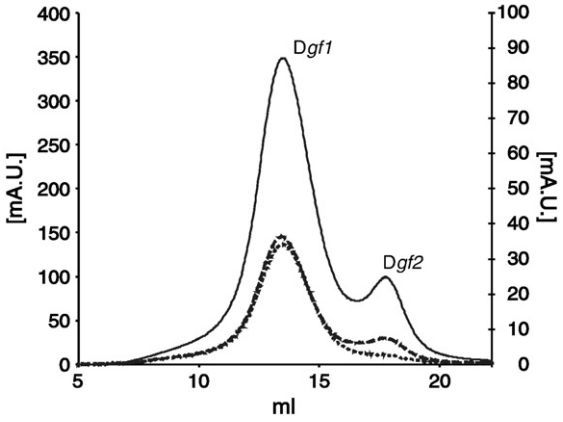

Fig. 3. Elution profile of an analytical gel-filtration chromatography of the PSI fraction D. Elution was controlled by measuring absorbance at three different wavelengths: 437 nm (solid line), 530 nm (dashed line), and 700 nm (dotted line). For better visualisation the absorbance at 437 nm is depicted on the left *y*-axis, whereas the two other wavelengths were plotted according to the right *y*-axis. Eluted peaks were named D*gf1* and D*gf2*.

(http://www.uniprot.org/), *T. pseudonana* (http://genome.jgi-psf.org/Thaps3/Thaps3.home.html) and *P. tricornutum* (http://genome.jgi-psf.org/Phatr2/Phatr2.home.html).

3. Results

3.1. Isolation of pigment protein complexes and spectroscopic analysis

To analyse the association of Fcp polypeptides with the photosystems, thylakoids of *C. meneghiniana* solubilised with 15 mM DDM were separated by discontinuous sucrose gradient centrifugation. A scheme of the resulting separation of pigment protein complexes is shown in Fig. 1a and resembles the results published by [27]. Fraction A consisted mainly of free pigment and was not considered further. Fraction B represented the free FCP pool. No differentiation was made concerning the upper phase consisting of FCPa trimers and the lower phase consisting of FCPa trimers and FCPb higher oligomers [6]. Fractions C and D were green fractions and thus seemed to consist of photosystems. Western blot analysis revealed a strong reaction of fraction C with an antibody directed against the PSII core subunit PsbD (see inset Fig. 1), whereas no reaction was observed in the other fractions B and D. Thus, PSII is solely located in fraction C and fraction D is completely devoid of PSII. All three fractions were analysed spectroscopically (Fig. 2). For comparison the three samples were adjusted to the same Chl *a* concentration. The absorbance spectrum of fraction B represented a typical FCP with Chl *a* peaks at 442 and 671 nm, a broad carotenoid shoulder including fucoxanthin extending the absorbance up to 580 nm, and Chl *c* peaks at 465 nm and 645 nm [5–8]. Fraction C showed a similar Chl *a* absorbance, with a Q_y peak slightly shifted towards 673 nm. Absorbance of Chl *c* and fucoxanthin were hardly visible in the spectrum. Fraction D gave a similar spectrum as fraction C but showed a broadened Q_y band of Chl *a* with a maximum at 679.5 nm, thus resembling a PSI spectrum. Fluorescence emission spectra recorded at the same Chl *a* concentration with excitation at 440 nm measured between 600 and 800 nm showed great differences between the three samples (Fig. 2b). Whereas fraction B fluoresced strongly at 676 nm, fluorescence of fraction C was shifted towards 680 nm and reduced by 68% in comparison to fraction B. Fraction D however, showed a decrease of fluorescence of about 95% and a shift towards 680 nm. As expected, the 77 K fluorescence excitation spectrum of fraction B measured at 675 nm showed transfer from the pigments Chl *a*, Chl *c*, and fucoxanthin towards Chl *a* (Fig. 2c). Although the absorbance spectrum of fraction C showed little fucoxanthin, energy transfer from this pigment to Chl *a* was still visible, whereas this was almost not the case for fraction D.

Since the fluorescence yield of PSI complexes at RT is known to be very low [28] we measured the fluorescence emission of all fractions at 77 K. Upon excitation at 440 nm, fraction B showed a strong Chl *a* peak at 676 nm. The same was true for fraction C with the exception that the Chl *a* peak was split into a 676 nm and a stronger 687.5 nm signal resembling a typical PSII spectrum at 77 K (Fig. 2d) [29]. Fraction D showed a maximum at 676 nm, a shoulder at 688 nm and a second peak at 717 nm (Fig. 2e). It is noteworthy to mention that all three fractions exhibited also a slight Chl *c* emission, indicating a partial decoupling of Chl *c* from Chl *a* inside the co-purified Fcp antenna. Since fraction D showed a subset of different Chl *a* emitters, the participating pigments inducing the longer wavelength components had to be identified. Therefore the fluorescence excitation of fraction D was repeated with emission measured at 688 nm and 717 nm. In contrast to the measurements before, in both cases transfer from Chl *c* and a carotenoid, probably fucoxanthin, was detected (a comparison between fraction D measured at 675 nm and 717 nm is shown in the inset of panel c). These pigments clearly belong to an Fcp antenna. To be sure that no free Fcp not properly bound to the PSI complex in fraction D contaminated the spectra, and in order to find out which of the detected emission peaks were caused by Fcps, an analytical gel-filtration chromatography was performed (see Fig. 3). Elution resulted in the separation of two peaks, named D*gf1* and D*gf2*. The main peak D*gf1* was eluted first and showed absorption at all detection wavelengths, 437 nm, 530 nm, and 700 nm. D*gf2* showed absorption only at 437 nm and 530 nm. It was suggested that the first peak represents PSI, whereas the second peak represents polypeptides, which were washed off, probably Fcps. When analysed spectroscopically, the absorbance spectrum of D*gf1* resembled that of fraction D, whereas the concentration of D*gf2* was too low to analyse it properly (data not shown). A Chl *a* Q_y absorbance at approximately 670 nm could be estimated, but no further analysis of this sample was performed. 77 K fluorescence measurements of D*gf1* revealed Chl *a* maxima at 688 nm and 717 nm when excited at 440 nm (Fig. 2e), and no Chl *c* emission was observed. The 676 nm peak detected in fraction D was not visible, and therefore this signal was interpreted as fluorescence that originated from partially uncoupled Fcp polypeptides, which were removed by gel-filtration. The presence of uncoupled and most probably unfunctional Fcp polypeptides became also visible when exciting fraction D at 465 nm, i.e. into the Chl *c* absorption band, causing a very obvious Chl *c* emission. However, Fcps properly bound were also present in fraction D, since

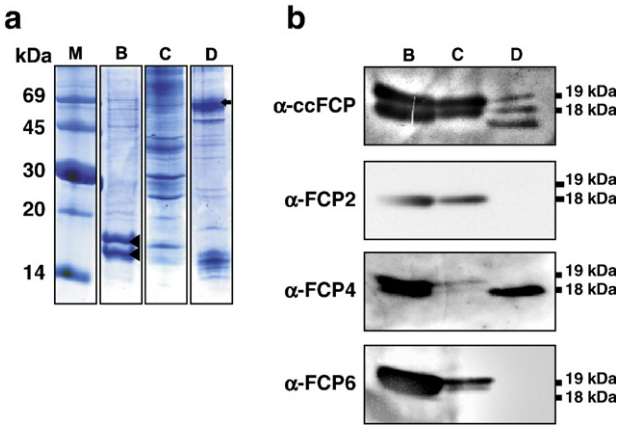

Fig. 4. SDS-PAGE (panel a) and Western blots (panel b) of isolated pigment protein fractions B, C, and D. Marker proteins (M), 18 kDa and 19 kDa Fcp polypeptides (arrowheads), and PSI core subunits PsaA/B (arrow) are indicated in panel a. In panel b the antibodies used (α-ccFcp, α-Fcp2, α-Fcp4, and α-Fcp6) are indicated on the left side. The samples contained 4 μg Chl each, besides fraction B (0.5 μg Chl).

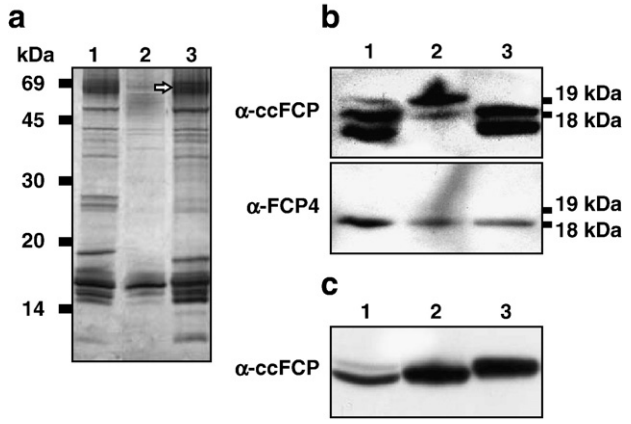

Fig. 5. SDS-PAGE (panel a) and Western blots (panel b) of purified fractions derived from resolubilisation of fraction D and further separation by sucrose density centrifugation. In panel a, silver stained fractions $NGOG_1$ and $NGOG_2$ (lanes 2 and 3) are presented. As a control fraction D was used (lane 1). All samples contained 4 μg Chl. Marker proteins are indicated (M) and PsaA/B proteins are marked with an arrow. In panel b the respective Western blots are displayed. Antibodies used were α-ccFcp, and α-Fcp4. A Western blot using α-ccFcp against pigment protein complexes of *P. tricornutum* isolated by ion exchange chromatography is depicted in panel c showing the Fcp polypeptide distribution in a PSI-FCP complex (lane 1), free FCP fraction (lane 2), and a PSII containing fraction (lane 3). 1 μg of Chl was loaded for each sample, except for the FCP complex (0.5 μg).

similar maxima at 718 nm and 688 nm were exhibited (Fig. 2f), and the 676 nm signal became a shoulder which was hardly visible. Thus, by exciting the bound Fcp antenna in fraction D, energy is transferred towards PSI and subsequently emitted at 688 nm or 717 nm. The latter emission maxima cannot be due to Fcps having the same properties as the main FCP complexes, since fraction B showed a different emission maximum.

3.2. Polypeptide analysis

Fractions B, C, and D were separated by SDS-PAGE (Fig. 4a). Fraction B showed the typical pattern of Fcp polypeptides for this fraction, two dominant bands at 18 and 19 kDa. One band at approximately the same size was found in fraction C together with several bands between 40 and 25 kDa which probably resemble the core subunits of PSII. Fraction D seemed to contain a PSI complex showing a double band around 65 kDa (PsaA/B) and several bands below 20 kDa which could be Fcps as well as smaller PSI subunits (for comparison see: [19,30]). The same samples were analysed by Western blot with an antibody against all Fcp polypeptides of *C. cryptica* (α-ccFcp) (Fig. 4b). Signals at 18 kDa and 19 kDa confirmed the presence of two differently sized polypeptides in fraction B [5]. The same was true for fraction C. In fraction D the antibody reacted with three different Fcp polypeptides. 19 kDa and 18 kDa signals were detected, resembling the signals in fractions B and C. The third band was located below the 18 kDa band. To elucidate which Fcp polypeptides belong to the three different fractions, the experiment was repeated with antibodies against Fcp2, Fcp4, and Fcp6 of *C. cryptica*. Fcp2 belongs to class I Fcps with a predicted molecular weight of 18.4 kDa [3]. In fractions B and C the antibody detected a single 18 kDa band, whereas fraction D showed no signal at all. Completely different results were obtained when using the α-Fcp4 antibody, which should detect a protein of 18.1 kDa. Whereas strong signals were detected in fractions B and D, fraction C only showed a faint band of the wrong molecular weight. Thus, the latter is most probably due to a cross reaction of the antibody. In contrast, the α-Fcp6 antibody reacted mainly with the 19 kDa polypeptides of the fractions B and C (predicted MW of Fcp6: 19.2 kDa). Again minor unspecific signals at 18 kDa were detected in fractions B and C, but no reaction at all was observed in fraction D. None of the antibodies used, besides the α-ccFcp antibody, detected the 17 kDa band in fraction D. In summary, fraction B contained all polypeptides probed, whereas fraction C showed strongest signals for Fcp2 and Fcp6. In fraction D, only Fcp4 could be identified. The presence of the latter was also checked by tandem MS. LC-ESI-MS/MS analysis of tryptic digested peptides from this band along with a combined diatom database yielded the peptide FSDFVPIDFLR (charge: 2; Xcorr: 3,26; FDR ≤ 1%). Its MS/MS spectrum is shown in Figure S1 (supplemental material). The peptide identified the polypeptide as Lhca4 from *T. pseudonana* (JGI protein ID: 33606), a member of the Lhca clade of the LHC superfamily, i.e. a PSI associated LHC homologous but not identical to *fcp* 4 of *Cyclotella*. Unfortunately, only the sequence of one of the four copies of similar Fcp4 genes identified [11] in *Cyclotella* is available in the data base. The known Fcp4 sequence is more similar to Lhcr3, which was also annotated in *T. pseudonana* (JGI protein ID: 18077). However, again sequences of the Lhca clade yield the strongest homology towards Lhcr3. Figure S2 of the supplementary material shows a sequence comparison of the Lhca4 and Lhcr3 proteins from *T. pseudonana* with the known sequence of Fcp4, and the fragment that was used for antibody production [22]. From this, we have to conclude that an Fcp4-like protein exists in *Cyclotella*, which is according to the data obtained by LC-ESI-MS/MS more similar to Lhca4 than Lhcr3 but still detected by the α-Fcp4 antibody. This protein is found in PSI, but not in PSII.

3.3. Further purification of PSI

To elucidate how the Fcps of fraction D are structurally organised and how strongly they are bound to PSI, a method was developed to sequentially remove the Fcp polypeptides from the PSI complex. Therefore, fraction D was resolubilised with different detergents e.g. HG, OG, NG, and TX 100 and then again ultracentrifuged on a discontinuous sucrose gradient. Independent of the detergents, the ultracentrifugation always resulted in the splitting of the sample into two green bands (schematically drawn in Fig. 1b). Fractions were named after the detergent used and "$_1$" for the upper band and "$_2$" for the lower band. None of the chosen treatments allowed for the isolation of a PSI complex devoid of Fcps (detected immunologically, data not shown). Exemplarily, results for resolubilisation with a mixture of NG and OG are presented. After harvesting these fractions and separation by SDS-PAGE one could observe the loss of the PsaA/B subunits (~65 kDa) in the upper fraction $NGOG_1$ (Fig. 5a, lane 2), but several bands below 20 kDa were preserved. In contrast, the lower fraction $NGOG_2$ (lane 3) nearly reflected the band pattern of fraction D

Table 1
Pigment ratios of pigment protein complexes purified by discontinuous sucrose gradient centrifugation measured by reverse phase HPLC.

	Chl *c*	Diadinoxanthin (DD)	Diatoxanthin (DT)	Fucoxanthin	β-carotene	DT/(DD + DT)
B	0.181 ± 0.019	0.188 ± 0.013	0.237 ± 0.023	0.935 ± 0.081	0.012 ± 0.008	0.56 ± 0.03
D	0.015 ± 0.001	0.068 ± 0.008	0.096 ± 0.019	0.137 ± 0.019	0.122 ± 0.010	0.58 ± 0.06
$NGOG_1$	0.008 ± 0.002	0.043 ± 0.004	0.035 ± 0.008	0.068 ± 0.006	0.006 ± 0.001	0.44 ± 0.06
$NGOG_2$	0.005 ± 0.000	0.034 ± 0.002	0.033 ± 0.001	0.067 ± 0.003	0.060 ± 0.003	0.49 ± 0.00

Values represent mol pigment/mol Chl *a*. Ratios are given as mean ± standard deviation of 6 measurements on 2 independent preparations (B), 10 measurements on 3 independent preparations (D) and of 3 measurements on 1 preparation ($NGOG_{1/2}$), respectively.

(lane 1), albeit with different strengths of the various bands. To characterise these sucrose gradient fractions further, they were compared to fractions B and D by HPLC analysis. The resulting pigment stoichiometries are presented in Table 1. The data for fraction B resembled the results published in [5,9], although with a slightly reduced amount of Chl *c*, which might be due to the fact that the 10% sucrose layer was omitted and thus only a mixture of FCPa and FCPb was obtained. Significantly lower amounts of Chl *c*, fucoxanthin, diadinoxanthin and diatoxanthin with respect to Chl *a* were found in fraction D. Fucoxanthin was decreased by a factor of ~7, whereas diadinoxanthin (DD) and diatoxanthin (DT) decreased only by a factor of ~2.5. Still, the de-epoxidation rate in both samples was nearly the same (~0.6). As expected for a PSI fraction, the β-carotene ratio in D was increased by a factor of ten. In the resolubilised fractions $NGOG_1$ and $NGOG_2$, all the pigments were reduced further. In both fractions the Chl *c*:Chl *a* ratio was below 1% and fucoxanthin was reduced to approximately 7%. However, fraction $NGOG_2$ still contained ten times more β-carotene than $NGOG_1$. Absorbance and fluorescence spectra of the resolubilised fractions led to comparable characteristics of $NGOG_2$ if compared to fraction D. $NGOG_1$ rather resembled a spectrum of a damaged FCP with a blue-shifted absorbance of Chl *a* at 672 nm, a reduced amount of Chl *c* as well as a noticeable Chl *c* fluorescence emission around 645 nm (data not shown). This effect held true for all upper bands collected in the resolubilisation experiments, independent from the detergents used. Taken together with the results from SDS-PAGE it was concluded that $NGOG_2$ represents a further purified PSI complex, although not completely devoid of Fcps, whereas $NGOG_1$ contains the Fcp polypeptides, which were removed but also lost their abilities of excitation energy transfer.

Again the samples were checked by Western blot (Fig. 5b). Incubation with the α-ccFcp antibody showed that after resolubilisation with NGOG the typical Fcp 18/19 kDa signal remained in fraction $NGOG_1$ with a stronger reaction at 19 kDa. Interestingly, a strong 18 kDa signal can be seen in fraction $NGOG_2$, together with the signal at 17 kDa. Since neither α-Fcp2 nor α-Fcp6 reacted with fraction D, we only used α-Fcp4 here. If incubated with the α-Fcp4 antibody, the 18 kDa signal was detected in both NGOG fractions. Still, the reaction in $NGOG_2$ was more pronounced compared to $NGOG_1$.

Since Fcp6 was only found in fraction C, i.e. the only PSII containing fraction, and a reaction with α-Fcp4 was only strongly visible in fraction D, i.e. PSI, we had to conclude that the distribution of these polypeptides differs between the photosystems. To check whether this is species independent we repeated parts of the experiments with *P. tricornutum*. Recently, the separation of thylakoids by ion exchange chromatography has been published [19]. An Fcp-binding PSI-complex, an FCP fraction, and a PSII containing fraction were isolated and the authors could prove a different composition of Fcp polypeptides in the PSI-FCP complex in comparison to the FCP fraction. Both fractions were taken as controls in a Western blot experiment together with the PSII containing fraction. As the antibodies α-Fcp2, 4, and 6 are species specific, only the α-ccFcp antibody could be used (Fig. 4c). As expected, in the PSI-FCP fraction two signals around 18 kDa were detected, whereas only one signal was detected in the FCP fraction. In contrast, the PSII containing fraction inhabited a single Fcp band with higher molecular weight than the two other samples.

4. Discussion

Discontinuous sucrose density centrifugation of solubilised thylakoids of *C. meneghiniana* led to the separation of five pigmented fractions. In earlier publications the first three bands, a fraction consisting mainly of free pigment (fraction A) and two FCP fractions (herein considered as fraction B, consisting of trimers and higher oligomers) have been analysed thoroughly [6]. In this study, the photosystems and their associated Fcps laid in the focus of interest. First of all, it had to be clarified if the latter two green bands (fractions C and D) were mixtures of PSII and PSI or if the photosystems were separated, as had been reported before [16]. A Western blot experiment proved that the PSII core subunit PsbD was entirely located in fraction C. Absorbance spectra confirmed this result. Both fractions showed a decreased content of fucoxanthin in comparison to the FCPs and a red shift of the Chl Q_y band. This red shift was most prominent in fraction D (band 4) and the whole peak was broadened in comparison to all other fractions. Furthermore, fraction D exhibited a strong quench in fluorescence, a unique feature of PSI.

If analysed at 77 K fraction D also showed an increased far red fluorescence at 717 nm when excited at 440 nm. Such a fluorescence maximum was attributed to PSI in the spectra of whole diatom cells and isolated PSI complexes [28]. This effect was only observed in fraction D, since fractions B and C exhibited only Chl *a* maxima at wavelengths shorter than 700 nm. In addition, peaks at 676 nm and 688 nm were observed, as already reported for *C. cryptica* by Brakemann and et al. [16]. When Chl *c* was excited, the 688 nm peak became even stronger, together with the 717 nm peak, whereas the 676 nm emission was strongly reduced. After removing Fcps that were only loosely bound by gel-filtration, the latter signal got lost completely. This led to the interpretation that the 676 nm emission in fraction D is due to those Fcp polypeptides. However, since Chl *c* excitation led to an enhancement of both the 688 nm and the 717 nm emission bands, some Fcps were properly bound to PSI in fraction D.

None of our further attempts of purification led to PSI cores completely devoid of Fcp polypeptides, and Fcps isolated by these methods from PSI complexes were all impaired in excitation energy transfer. Thus, we cannot directly identify the origin of the long wavelength emissions at 688 nm and 717 nm, which could arise from the Fcps specifically bound to PSI or from the core itself. However, in a recent publication decay-associated spectra (DAS) of a PSI-FCP complex of the diatom *Chaetoceros gracilis* led to the conclusion that only the longer wavelength components are located within the PSI core [31]. Only the DAS component with the shortest wavelength at 685 nm could be attributed to Fcps by comparison to DAS of isolated FCP complexes. If both the 688 nm and the 717 nm emission maximum would belong to core chlorophylls, we would have to assume a 100% energy transfer from the Fcps into the cores at 77 K, because no other emission maxima are found. Since this is unlikely, our data support the idea that the 688 nm emission is due to Fcp polypeptides closely bound to the PSI cores.

The 77 K spectra of fraction C resembled those described for higher plant PSII, where the 687.5 nm emission is attributed to the core complex, thus pointing to the similarity of PSII among all organisms [32].

Analysis by HPLC revealed that the known pigment values of FCPs have been preserved in fraction B. Smaller changes were certainly due to changes in the isolation procedures, i.e. fraction B was a pooled mixture of FCPa trimers and FCPb higher oligomers since a 10% sucrose layer was omitted [5,9]. Concerning the data of the PSI fraction D, quite large differences can be seen in comparison to other PSI preparations of diatoms. If one interprets the fucoxanthin/Chl *a* ratio as a measure of the Fcp antenna size of PSI complexes, the antenna in fraction D (~14%) is relatively small. Ratios of 50% and 31% fucoxanthin were reported for *P. tricornutum* and 24% for *C. gracilis* [19,31,33]. This discrepancy is even increased if the Chl *c*/Chl *a* ratios are considered. Whereas for *P. tricornutum* and *C. gracilis* ratios of 8%, 5.8%, and 9% are reported, in fraction D only a ratio of 1.5% was determined. In contrast, the added ratios of the diatom specific xanthophyll cycle pigments, diadinoxanthin (DD) and diatoxanthin (DT), were approximately 17% in fraction D, whereas 13% where reported for *C. gracilis* and 2.4% [19] and 6.5% for *P. tricornutum* [33]. Besides species related differences, the different pigment stoichiometries are certainly caused by the stronger light regime used during the cell culturing of *C. meneghiniana* (140 μE m^{-2} s^{-1}) in comparison to *P. tricornutum* (40 μE m^{-2} s^{-1}) and *C. gracilis* (13 μE m^{-2} s^{-1}).

Therefore, a rearrangement in size and pigmentation of the light harvesting antennae of PSI has to be expected. This assumption is hardened by the fact that the de-epoxidation rates (DT / DD + DT, a measurement that correlates with non-photochemical quenching) in fraction D as well as in fraction B were 3 to 4 times higher than those reported for FCPs isolated under low light regimes, but comparable to high light FCPs [5,27].

There has been an ongoing discussion whether the two photosystems of diatoms share one antenna system, or if two separate light harvesting systems exist, as in higher plants and green algae [6,11,34]. The first theory might be supported by the fact that there is no segregation of thylakoids into grana and stroma regions, and therefore no separation of the two photosystems. On the other hand, sequence homologies relate Fcp4 of *C. cryptica* to a PSI associated Lhc protein of red algae [11]. Therefore, one could interpret Fcp4 to be part of a PSI specific light harvesting antenna. In several publications, mainly concerning the light harvesting properties of diatoms, the isolation of PSI or PSII enriched fractions was reported [7,8,28]. However, specific analyses of Fcp polypeptides in photosystem fractions were not performed. Also the recent isolation of PSI and PSII complexes from *C. gracilis* only proved a coupling of Fcp polypeptides to both photosystems, but did not provide any information about differences or similarities of these Fcps [31,35]. Solely, Brakemann et al. [16] studied the association of Fcp polypeptides towards PSI and PSII isolated by sucrose density centrifugation and Deriphate-PAGE in *C. cryptica*. In their work, two Fcp polypeptides were identified in both photosystems and in an FCP fraction using an antibody directed against all Fcp polypeptides of *C. cryptica* with apparent molecular weights of 18 kDa and 22 kDa. Furthermore, Fcp2 and Fcp4 at 18 kDa were identified in both photosystems via specific antibodies. Thus, it was suggested that indeed both photosystems share the same antenna system.

This is in contrast to the results presented herein. Fcp2 and Fcp6 were solely found in FCP and PSII. On the other hand none of these polypeptides was found in PSI, where the α-Fcp4 antibody reacted strongly with an 18 kDa polypeptide. The differences in the results compared to Brakemann et al. [16] are most probably due to several reasons: First, light conditions during growth were different, since we used high light conditions, known to change the amount of the different antenna proteins [5]. Second, we chose milder solubilisation conditions, i.e. a detergent to Chl *a* ratio of 60:1 instead of 100:1. Third, the immunoblots presented by Brakemann et al. [16] show very faint signals in case of PSI, which might have led to an underestimation of the differences compared to the other fractions obtained.

Our results concerning the Fcp polypeptides found in the PSII fractions, Fcp2 and Fcp6, resemble the composition published for the trimeric FCPa complex of *C. meneghiniana*, grown under similar light and nutrient conditions [5]. Fcp6 is a homologue of LI818r-3 of *C. reinhardtii* and it is known that the mRNA levels of both increase abruptly upon illumination [36,37]. Currently, a photoprotective mechanism of LI818r-3 is discussed [38]. Since the expression of Fcp polypeptides in the FCP fraction and the PSII fraction were similar, it is supposed that parts of the FCPa complexes are closely associated with PSII.

The inner PSI antenna consisted of at least three different Fcp polypeptides with molecular weights of 19 kDa, 18 kDa, and approximately 17 kDa. The 18 kDa polypeptide is not represented by Fcp2 but by a polypeptide of the Fcp4 group. Strong reaction with the α-Fcp4 antibody, directed against the C-terminus of the known Fcp4 sequence, proved this point. However, LC-ESI-MS/MS analysis did not reveal Fcp4, but a homologous Lhca4 protein of *T. pseudonana*. Since the genome of *Cyclotella* is not sequenced, but several *fcp* 4 genes were identified [11], we have to assume that an Fcp4-like protein with high similarity to Lhca4 of *T. pseudonana* exists in *Cyclotella* as well. Also the re-solubilisation with NG plus OG could not remove this Fcp4-like polypeptide entirely from PSI, although it was also detected in the photosystem free $NGOG_1$ fraction. Furthermore, the immuno-reaction of the α-Fcp4 antibody in the PSI fraction was as strong as the reaction of the α-ccFcp antibody, in contrast to e.g. fraction B. Thus, the Fcp4 polypeptide seems to constitute the major fraction of the 18 kDa Fcp proteins in the PSI fraction and is strongly bound to the complex.

The 17 kDa Fcp signal was only visible in fractions D and $NGOG_2$. Thus, it is even more strongly bound to PSI compared to Fcp4. Since no gene encoding an Fcp of this size was found so far in *Cyclotella*, the shorter size could be due to degradation or to post-translational modifications. However, we could not identify any FCP-like protein in this band by LC-ESI-MS/MS (data not shown). Thus, the 17 kDa Fcp polypeptide is an Fcp protein due to the reaction with the α-ccFcp antibody, but further research will have to clarify whether its appearance is due to degradation, posttranslational modification or whether it is an Fcp protein not identified so far.

The 19 kDa Fcp polypeptide found in the PSI fraction could not be detected by any of the specific antibodies and neither by LC-ESI-MS/MS analysis (data not shown). In contrast to the FCP and PSII fractions, we can exclude Fcp6. The only other FCP described for *Cyclotella* with a similar molecular weight is Fcp5. From its sequence, Fcp5 can be placed into the same group as Fcp 1, 2, and 3, and it is the only constituent of the oligomeric FCPb complex. Since the 19 kDa polypeptide was completely removed from the PSI core fraction $NGOG_2$ (Fig. 4b), it is less tightly bound to PSI than Fcp4 and an interaction between PSI and Fcp5, i.e. FCPb complexes can be proposed.

From our data we conclude that *C. meneghiniana* possesses two different light harvesting antenna systems for PSI and PSII. This seems to be species independent, since high resolution SDS-PAGE and Western blots of PSI, FCP and PSII fractions isolated from *P. tricornutum* also showed that Fcp polypeptides of different molecular weight were associated with the two PS fractions. In *C. meneghiniana* the PSII light harvesting antenna resembles the polypeptide composition of the trimeric FCPa complex, whereas PSI binds an unknown 17 kDa Fcp polypeptide, an Fcp4 polypeptide, and the less tightly bound 19 kDa Fcp5, representing a possible linker to the FCPb oligomer. These data are supported by fluorescence measurements showing strong similarity between the isolated FCP complexes and the uncoupled antenna in the PSII fraction, but differences in the case for PSI. In the case of PSI it was shown that the associated Fcps are capable in transferring energy from Chl *c* towards two emitting Chl *a* pools. In FCP complexes, some of the Fcp polypeptides seem to be interchangeable upon changes in illumination [5]. A similar adaptation concerning the polypeptides more closely associated with the photosystems might also explain the differences to what was described for *C. cryptica* [16].

Still, attempts have to be made to characterise those Fcp polypeptides that could not be detected by specific antibodies. Identifying their sequences on genetic and on protein level will be necessary to complete our understanding of the organisation and re-organisation due to light adaptation of the light harvesting system in different species of diatoms.

Acknowledgements

Our work was supported by grants of the Deutsche Forschungsgemeinschaft to CB (Bu 812/4-1) and MM (Mi 373/3).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbabio.2009.04.006.

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