

RESEARCH PAPER

Characterization of a trimeric light-harvesting complex in the diatom *Phaeodactylum tricornutum* built of FcpA and FcpE proteins

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Abstract

Fucoxanthin chlorophyll proteins (Fcps), the light-harvesting antennas of heterokont algae, are encoded by a multigene family and are highly similar with respect to their molecular masses as well as to their pigmentation, making it difficult to purify single Fcps. In this study, a hexa-histidine tag was genetically added to the C-terminus of the FcpA protein of the pennate diatom *Phaeodactylum tricornutum*. A transgenic strain expressing the recombinant His-tagged FcpA protein in addition to the endogenous wild type Fcps was created. This strategy allowed, for the first time, the purification of a specific, stable trimeric Fcp complex. In addition, a pool of various trimeric Fcps was also purified from the wild-type cells using sucrose density gradient ultracentrifugation and gel filtration. In both the His-tagged and the wild-type Fcps, excitation energy coupling between fucoxanthin and chlorophyll *a* was intact and the existence of a chlorophyll *a*/fucoxanthin excitonic dimer was demonstrated using circular dichroism spectroscopy. Mass spectrometric analyses of the trimeric His-tagged complex indicated that it is composed of FcpA and FcpE polypeptides. It is confirmed here that a trimer is the basic organizational unit of Fcps in *P. tricornutum*. From circular dichroism spectra, it is proposed that the organization of the pigments on the polypeptide backbone of Fcps is a conserved feature in the case of chlorophyll *a/c* containing algae.

Key words: Diatoms, FcpA, His-tag, thylakoid, photosynthesis.

Introduction

Diatoms (Bacillariophyceae) are unicellular, eukaryotic algae which belong to the Heterokont group of algae. This group of algae was derived by a secondary endosymbiosis event between a red alga and a heterotrophic eukaryote (Bhattacharya and Medlin, 1998; Archibald and Keeling, 2002; Yoon *et al.*, 2002). Diatoms are major players in the biochemical cycles of carbon, nitrogen, silica, and phosphorus. By participating in these vital element cycles they have

a profound effect on the global climate both in marine and freshwater environments (Wilhelm *et al.*, 2006). Despite the fact that the general organization of the photosynthetic apparatus of diatoms, plants, and red algae is very similar, there are significant differences in membrane topology, and polypeptide and pigment composition. The thylakoid membranes are not differentiated into distinct granal and stromal regions but organized into bands consisting of three

Abbreviations: ASP, artificial seawater medium; β -DDM, β -dodecyl maltoside; Chl, chlorophyll; CD, circular dichroism; Ddx, diadinoxanthin; Dtx, diatoxanthin; Fx, fucoxanthin; Fcp, fucoxanthin chlorophyll protein; FCP, fucoxanthin chlorophyll protein complex; FcpA_{HIS}, His-tagged FcpA; HPLC, high pressure liquid chromatography; IMAC, immobilized metal affinity chromatography; LC-ESI-MS/MS, liquid chromatography-electrospray ionization-tandem mass spectrometry; LHC, light-harvesting complex; Ni-NTA, nickel nitrilo triacetate; NPQ, non-photochemical quenching; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; WT, wild type.

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thylakoids each (Gibbs, 1962). Furthermore, unlike in higher plants, no specific localization of photosystem (PS) I and PSII within the diatom thylakoid membrane system is known; they seem to be rather homogeneously distributed (Pysznik and Gibbs, 1992).

In contrast to higher plants and green algae, the light-harvesting complex (LHC) of diatoms contains chlorophyll (Chl) *c* instead of Chl *b* as the accessory Chl and fucoxanthin (Fx) instead of lutein as the major carotenoid. Since they are the major components of the LHC, the antennae are referred to as Fcps (Fucoxanthin chlorophyll *a/c* binding proteins). The major xanthophyll cycle pigments in diatoms are diadinoxanthin (Ddx) and its de-epoxidized form diatoxanthin (Dtx). The localization of these pigments within the Fcp antennae has been demonstrated and diatoxanthin in particular has been shown to play a critical role in the protective process of non-photochemical quenching (NPQ) under conditions of excess illumination (Lavaud *et al.*, 2003; Guglielmi *et al.*, 2005; Gundermann and Büchel, 2008; Goss and Jakob, 2010).

Fcps in diatoms are membrane intrinsic light-harvesting antenna complexes which are members of the LHC superfamily encoded by a conserved multigene family (Bhaya and Grossman, 1993; Green and Pichersky, 1994; Green and Durnford, 1996). They function either in light harvesting and/or photoprotection. Fcps share significant similarity in sequence and in predicted structure with plant LHCII. Fcps, like LHC polypeptides in plants, are inserted in thylakoid membranes by three membrane spanning α -helices. The similarity between these is especially strong in helix 1 and 3 (Green and Pichersky, 1994). However, mature Fcps have been found to be smaller in size than the plant LHCs due to shorter loops and termini, which, in turn, makes them more hydrophobic than plant LHCs.

All known Fcps have a molecular weight between 17–23 kDa (Fawley and Grossman, 1986; Caron and Brown, 1987) and are classified into three major groups (termed I, II, and III) based on sequence analyses. Group I represents the major Fcps in diatoms and it includes the genes *fcp1-3* and *fcp5* from *Cyclotella cryptica* and *fcpA-F* from *Phaeodactylum tricorutum*, which are hypothesized to serve in the function of light harvesting (Durnford *et al.*, 1996; Eppard *et al.*, 2000; Green, 2003; Bowler *et al.*, 2008). Group II is represented by the genes *fcp4* of *C. cryptica* and its homologues *lhca* and *lhcr* in *Thalassiosira pseudonana* and *P. tricorutum*, respectively (Armbrust *et al.*, 2004). It was shown recently that in *Cyclotella meneghiniana* *fcp4* encodes PSI specific antenna proteins (Veith *et al.*, 2009). LI818 is a light-inducible protein found in the green alga *Chlamydomonas reinhardtii*. The respective homologue genes, i.e. *fcp6*, *7*, and *12* in *C. cryptica* belong to Group III of the Fcps, which are proposed to function in light protection (Richard *et al.*, 2000; Beer *et al.*, 2006; Zhu and Green, 2008; Peers *et al.*, 2009). The genome of *P. tricorutum* also contains sequences which represent light-inducible proteins similar to LI818, which are denoted as Lhex proteins (Bowler *et al.*, 2008).

Biochemical investigations have shown that the major antenna proteins of PSII in plants are trimerically arranged

Lhcb1–b3 proteins, while the minor antennae consist of Lhcb4–b6 proteins (also termed as CP24, CP26, and CP29 proteins). It has also been confirmed at a structural level by high resolution X-ray diffraction studies that the LHCII in higher plants is a stable trimer (Liu *et al.*, 2004). Unfortunately, such a wide array of information regarding the biochemical identities and structural organization of Fcps is not yet available for diatoms; however, this has started to change in recent years. Guglielmi *et al.* (2005) reported the isolation of an Fcp trimer from *P. tricorutum* by applying a rapid sequence of thylakoid solubilization and subsequent gel filtration. Büchel (2003) reported the presence of two different Fcp fractions in *C. meneghiniana*; which were different in molecular weight and oligomeric status. Furthermore, Beer *et al.* (2006) identified the subunits of the higher oligomer FCPb and the trimer FCPa in *C. meneghiniana*. Lepetit *et al.* (2007) recently showed that, in *P. tricorutum*, the basic stable unit of organization of Fcps is a trimer. It was speculated that the co-existence of higher oligomers and trimers of Fcps in *P. tricorutum* was unlikely. In addition, FcpA–F were identified in the main antenna complex fraction by mass spectrometry with FcpC, D, and E having the strongest signals, while no subpopulation of trimers could be isolated and characterized. The high degree of similarity in molecular weights and possibly also in pigmentation among all Fcps is so far the biggest hurdle for the purification of different Fcp complexes or single Fcps. The availability of the genome (Bowler *et al.*, 2008) and genetic tools for stable genetic transformation of *P. tricorutum* (Apt *et al.*, 1996; Zaslavskaja *et al.*, 2000; Kroth, 2007; Saut *et al.*, 2007; Maheswari *et al.*, 2008) makes this diatom a very suitable model organism to study the organization of the photosynthetic apparatus. Moreover, such tools allow adding affinity tags to any chosen Fcp protein, enabling their purification via affinity chromatography.

In order to elucidate possible differences in function between different Fcps and to examine the molecular organization of Fcp complexes, specific Fcps, either as single polypeptides or as a complex in a highly pure and native state, have to be obtained. Here, the aim was to study FcpA in more detail and thus the gene of *P. tricorutum* was genetically manipulated by tagging the 3' terminus with a nucleotide sequence encoding a hexa histidine (His) tag. The transgenic strain expressing the His-tagged FcpA protein could then be used to isolate an Fcp complex containing a specific *fcp* gene product, FcpA_{His}, for the first time. Data are presented here concerning the purification and biochemical, as well as spectroscopic, characterization of the pure His-tagged FcpA complex in comparison with the wild-type (WT) Fcp pool in *P. tricorutum*.

Materials and methods

Cloning of a construct for expression of recombinant FcpA_{His} and transformation of P. tricorutum

The complete nucleotide sequence for *fcpA* was available at the EMBL–EBI database with gene ID number Z24768 as deposited

by Bhaya and Grossman (1993) and can also be found in the US Department of Energy's Joint Genome Institute *P. tricornutum* genome database (<http://genome.jgi-psf.org/Phatr2/Phatr2.home.html>) (Bowler et al., 2008) with protein ID 18049. Genomic DNA was isolated from wild-type cells (UTEX culture collection, strain 646). Using the following oligonucleotide primers, the coding region of the *fcpA* gene was amplified by PCR: (F) 5'-GA(GATATC)ATTCAAGATGAAATTTGC-3' and (R) 5'-A(GTCGAC)TTAATGGTGATGGTGATGGTGAGGAAGGATAG-3'. Primer (F) inherently contained an EcoRV restriction site whereas the reverse primer (R) was designed to introduce a Sall restriction site and simultaneously to generate a fragment encoding a His-tag at the 3' terminus of the gene. The restriction sites within the primers are indicated in parentheses while the underlined sequence codes for six histidine residues. This insert was introduced in the multiple cloning site of the *P. tricornutum* transformation vector pPha-T1 (Genbank accession number AF219942) as developed by Zaslavskaja et al. (2000), by digesting the vector and the PCR fragment with EcoRV and Sall and subsequently ligating the construct into the vector. The resulting plasmid pPha-T1FcpAHis, carries a His-tagged *fcpA* gene under the control of the native *fcpA* promoter (Fig. 1). It was allowed to amplify in *E. coli* under ampicillin pressure. Plasmid DNA was isolated and used for the transformation of WT *P. tricornutum* cells.

Transformation of *P. tricornutum* with pPha-T1FcpAHis was carried out according to Apt et al. (1996) and Zaslavskaja et al. (2000) with minor modifications. WT cells plated on Artificial Seawater medium (ASP) agar (Provasoli et al., 1957) were bombarded using the Bio-Rad Biolistic PDS-1000/He Particle delivery system (Bio-Rad laboratories, Hercules, CA) fitted with a 1350 psi rupture disc. 1 µg plasmid DNA was coated onto M17 tungsten particles (Bio-Rad) and used for bombardment. Bombarded cells were illuminated continuously for 24 h before plating them on ASP agar plates supplemented with 75 µg ml⁻¹ zeocin to select possible transformants. Clones which could survive zeocin selection were screened for expression of the His-tagged recombinant protein at the mRNA level (data not shown). The recombinant clones now contained one or more copies of the His-tagged *fcpA* gene along with a background of all other WT *fcp* genes. The clone FcpA1.2 was observed to have the highest expression level and was chosen for further work. The presence of the His-tagged gene within this clone was again confirmed by PCR and by sequence analyses (data not shown). It was cultured and subcultured regularly in ASP medium containing zeocin under low light conditions for a period of 6 months before His-tagged FcpA was isolated.

Culture conditions

P. tricornutum Böhlin WT (UTEX culture collection, strain 646) and the mutant, FcpA1.2, were grown in ASP medium for 10 d at 18 °C under constant aeration by bubbling sterile air, and at a light intensity of 40 µE m⁻² s⁻¹ and a 16/8 h light/dark cycle.

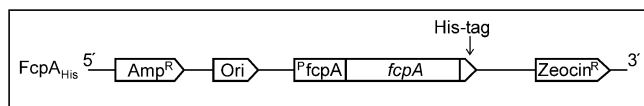


Fig. 1. Schematic representation of the *fcpA* gene under the control of the *fcpA* promoter (P^{fcpA}) and position of the histidine-tag in the constructed plasmid pPha-T1FcpAHis. The sequence encoding a six histidine residue was inserted at the 3' end of the coding region of the *fcpA* gene. The transformation vector pPha-T1 carries a gene for Ampicillin (Amp) resistance upstream of the *fcpA* gene and for Zeocin resistance downstream of the *fcpA* gene. The origin of replication (Ori) for amplification in *E. coli* is also found upstream of the *fcpA* gene.

Thylakoid preparation

Cells were harvested from 10-d-old cultures at a Chl a concentration of approximately 10–15 mg l⁻¹ within 1–2 h after the onset of light, and thylakoids were prepared as described in Veith and Büchel (2007) with a few minor changes. Harvested cells were resuspended in homogenization buffer (10 mM HEPES, 2 mM KCl, 5 mM EDTA, 1 M sorbitol, pH 7.4) and broken by repeated French Press cycles (Polytec/Thermo) for six times at a pressure of 20 000 psi. The cell lysate was spun for 2 min at 4 °C at 3000 g to avoid contamination with unbroken cells. The pellet was resuspended in homogenization buffer and centrifuged again at 3000 g for 2 min at 4 °C. The supernatants from both centrifugation steps were collected and ultracentrifuged for 40 min at 206 000 g. The pelleted thylakoid membranes were resuspended in washing buffer A (10 mM HEPES, 2 mM KCl, pH 7.4) and ultracentrifuged once again for 40 min at 163 000 g. The pellet of thylakoid membranes was resuspended in washing buffer A and the chlorophyll content was determined according to Jeffrey and Humphrey (1975).

Purification of Fcps

Sucrose density gradient ultracentrifugation: Thylakoids of the WT and the mutant were solubilized with 15 mM β-dodecyl maltoside (β-DDM) at a concentration of 0.25 mg ml⁻¹ Chl a (detergent: Chl a, 30:1) in a 2 ml end volume for 20 min under constant shaking on ice and loaded onto a discontinuous sucrose density gradient and ultracentrifuged at 141 000 g (Rotor: Beckmann SW28) for 22 h at 4 °C. The sucrose gradient consisted of seven steps of sucrose solutions made in washing buffer A supplemented with 0.03% β-DDM and were layered in the order of 25%, 17.5%, 15%, 12.5%, 10%, 7.5%, and 5%. The brown-coloured Fcp band localized between 10% and 12.5% sucrose. It was harvested carefully to avoid any contamination with the green photosystem fraction which was localized at the interface of the 15% sucrose and 17.5% sucrose density bands. Fcp fractions from the WT and mutant were then concentrated in concentration devices with 30 kDa cut-off (Amicon) and were frozen under liquid nitrogen and stored at -80 °C until further purification.

Gel filtration: For further purification of Fcps in the WT, the Fcp band obtained from sucrose density gradient ultracentrifugation was applied to a self-packed gel filtration column (Superdex 200, GE Healthcare) connected to an ÄKTA purifier P-900 (Amersham Biosciences). Flow-rate was maintained at 0.5 ml min⁻¹ during the run and absorbances of the eluates were analysed at 437 nm, 280 nm, and 675 nm. The column was calibrated with FCPa and FCPb complexes from *C. meneghiniana* since their oligomeric status and molecular weights had previously been established allowing their use as molecular weight markers (Büchel, 2003; Beer et al., 2006). The major peak fractions were collected and concentrated in 30 kDa cut-off concentration devices. This pure WT Fcp pool was immediately analysed by spectroscopy and then frozen in liquid nitrogen and stored at -80 °C for further analyses.

Nickel-NitriloTriacetate (Ni-NTA) IMAC: For the transgenic strain, the concentrated Fcp band from the sucrose density gradient was applied to a self-packed Ni-NTA column (Chelating Sepharose Fast Flow, Amersham Biosciences) pre-equilibrated with binding buffer (25 mM phosphate, 150 mM KCl, 5 mM imidazole, 0.6 mM β-DDM, pH 7.4) and allowed to incubate for 1 h at 4 °C for binding of the His-tagged proteins to the column material. The column was washed with washing buffer B (25 mM phosphate, 50 mM KCl, 0.6 mM β-DDM, pH 7.4) at 0.5 ml min⁻¹ flow-rate until the absorbance at 672 nm was less than 0.005 absorbance units. His-tagged FcpA (FcpAHis) was subsequently eluted using elution buffer (25 mM phosphate, 15 mM KCl, 400 mM imidazole, 0.6 mM β-DDM, pH 7.4) in 0.5 ml aliquots, which were concentrated using 30 kDa microcon concentration devices (Amicon).

Characterization of FcpA

Gel filtration: To analyse the oligomeric state, FcpA_{His} corresponding to ~2.5 µg Chl *a* was applied to a self-packed column (Superdex 200, GE Healthcare) connected to an ÄKTA purifier P-900 (Amersham Biosciences). Run conditions were identical to those used for the WT.

Electrophoresis: 15% TRIS-Tricine denaturing gels were made according to Schägger and von Jagow (1987). Samples were denatured with Rotiload (Roth) for 15 min and gels were stained with Coomassie Blue G 250 or were silver stained.

Western blot: Unstained gels were rinsed briefly with double-distilled water and blotted onto PVDF membranes (Roth) according to Veith and Büchel (2007). Immunodetection was carried out using ECL detection solutions (Amersham Biosciences) after decorating either with antibodies made against all Fcp polypeptides from *C. meneghiniana* isolated as described in Büchel (2003); α -Fcp, or with a monoclonal His antibody; α -His (Sigma).

Mass spectrometry (MS): The band of interest was cut out from a silver-stained gel and freeze-dried. A tryptic digest and nano LC-ESI-MS/MS analysis of the peptides were carried out according to Veith *et al.* (2009) without changes.

HPLC: Cells were pelleted at 5000 rpm and lyophilized. They were then suspended in extraction solution (1:2; cell volume:extraction volume) of 90% methanol buffered with 1 mM TRIS-HCl (pH 7.5), and disrupted mechanically in the presence of glass powder by an homogenization pistil followed by sonication for 30 s. Cells were then allowed to stand on ice for 1 min. This process was repeated 10 times after which the solution was spun at 13 000 rpm for 15 min.

In the case of Fcps isolated from the WT and mutant, samples were precipitated directly in 90% final concentration of methanol and spun briefly. The supernatants were analysed for pigment content by HPLC and the amounts of pigments were quantified according to Papagiannakis *et al.* (2005).

Spectroscopic analyses: Absorbance spectra were recorded with a spectrophotometer (Jasco V550) using a bandpass of 1 nm and 1 cm optical path length at room temperature. Samples were adjusted to an absorbance of 0.03 at the Q_Y band of Chl *a*, which corresponded to a Chl *a* concentration of ~0.3 µg ml⁻¹. Fluorescence spectra were recorded at room temperature in a fluorescence spectrometer (Jasco FP6500) with a bandpass of 3 nm both on the excitation and emission side and at 0.1 nm data pitch. Emission spectra were recorded between 600–800 nm when the samples were illuminated at $\lambda_{\text{ex}}=465$ nm to preferentially excite Chl *c*. Excitation spectra were recorded at $\lambda_{\text{em}}=675$ nm upon excitation from $\lambda_{\text{ex}}=400$ –600 nm. A rhodamine-B spectrum was used as a reference for the correction at the excitation side and the photomultiplier was corrected using a calibrated lamp spectrum. Circular dichroism (CD) spectra were measured at room temperature using 1 cm optical pathlength, 2 nm bandwidth, and a 2 s response time in the range of 370–750 nm in a CD spectrometer (Jasco 810). Concentration of the samples was adjusted to ~5 µg ml⁻¹ of Chl *a*. Five spectra were recorded sequentially in each case to improve the signal-to-noise ratio and the data presented herewith are an average of these sequential spectra.

Results and discussion

Purification of a single Fcp and its biochemical characterization was the primary goal of this study. The availability of the *fcpA* gene sequence in *P. tricornutum*, a suitable

vector (pPha-T1), and a method for its stable transformation, made it possible to create a transgenic strain of *P. tricornutum* in which the *fcpA* gene carries a fragment encoding a hexa-histidine tag on the 3' terminus of the gene. Expression of the modified gene is under the control of the native promoter of the gene (FcpA promoter; Zaslavskaja *et al.*, 2000) in the transformed cell lines, thereby avoiding artificial overexpression of FcpA_{His}. The modified gene also encodes the complete FcpA plastid targeting presequence with a conserved sequence at the predicted signal peptide cleavage site (Kilian and Kroth, 2005; Gruber *et al.*, 2007). It has been shown previously, with the presequence of the γ subunit of the *P. tricornutum* plastid ATP synthetase, that preproteins are also targeted correctly to the plastid when a His-tag has been engineered to their C-terminus (Apt *et al.*, 2002). Targeting of the FcpA_{His} gene product to the thylakoid membranes should therefore function analogously to the WT FcpA gene product. The Fcp complex, isolated by the use of this His-tag, is compared here with the pool of WT Fcp complexes.

Purification of Fcps

Discontinuous sucrose density gradient ultracentrifugation was carried out for both the WT and the transgenic strain as a prepurification step to separate the photosystem fractions from the Fcp pool. A fractionation pattern similar to that reported by Guglielmi *et al.* (2005) and Lepetit *et al.* (2007) was observed within the gradients in both cases (Fig. 2). A golden-olive coloured fraction was localized at the 7.5% sucrose step. It was mainly found to contain free pigment based on spectroscopic and SDS-PAGE analyses and was thus discarded. A green-coloured fraction containing photosystem(s) was fractionated at the interface of the 17.5% and the 25% sucrose step and was also not considered further. The brown-coloured fraction which occurred between the 12.5% and 15% sucrose steps was found to be enriched in Fcp and was harvested for further purification. This Fcp fraction contained about 65–70% Chl *a* of that found in thylakoids, both in WT and the mutant. The WT Fcp fraction from sucrose density gradients was further purified by gel filtration. Figure 3 shows the profile of the WT Fcp band from the sucrose density gradient applied to a gel filtration column. A major peak was observed at 16.6 ml followed by a tail peak (20–29 ml). Fractions were collected from the major peak (16.6 ml) and used as the WT Fcp pool after spectroscopic confirmation that the energy transfer was intact (see below).

For the transgenic strain, the His-tagged FcpA was further purified from the Fcp fraction collected after sucrose density gradient centrifugation using IMAC.

Polypeptide analysis

Denaturing SDS-PAGE was carried out to analyse the purity of the FcpA_{His} and to estimate the relative molecular mass of its polypeptide(s). The predicted molecular mass of FcpA_{His} is 18.9 kDa. Figure 4A presents a Coomassie-stained gel loaded with WT Fcp (lane 1) and FcpA_{His}

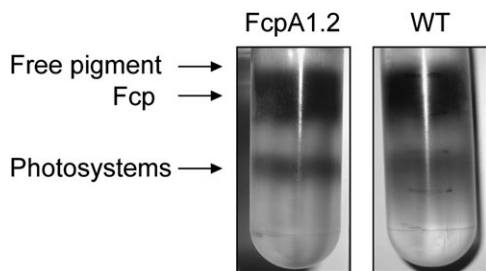


Fig. 2. Fractionation of thylakoids, on a sucrose density gradient after solubilization with 15 mM β -DDM (detergent:Chl *a*, 30:1). The left tube shows the results of fractionation of the transgenic strain, FcpA1.2, whereas the fractionation of the WT thylakoids under identical conditions is shown on the right.

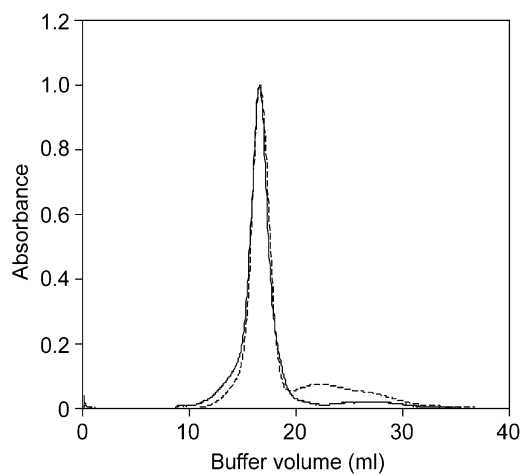


Fig. 3. Elution profile of gel filtration of FcpA_{His} (solid line) and of WT Fcp (dashed line) fractions obtained from sucrose density gradient centrifugation. Absorbance was measured at 437 nm.

(lane 2). A single prominent band around 19 kDa proved that contaminants like photosystem fractions were absent in the FcpA_{His} fraction. WT Fcp had been loaded at the same concentration of Chl *a* as the FcpA_{His}, however, signals were stronger in the case of the mutant Fcp. Since this turned out to be reproducible, independent of the batches of preparation used, it was concluded that WT Fcp contained lower amounts of polypeptides than the FcpA_{His}, i.e. the FcpA_{His} fraction contains fewer pigments per polypeptide (see also the HPLC analysis described below).

Samples were also analysed by immunoblots with antibodies either against the His-tag (α -His) or against all Fcps of *C. meneghiniana* (α -Fcp). His-tagged PsbS protein from higher plants expressed in *E. coli* with a predicted molecular mass of 24 kDa was used as a positive control for the anti-His antibody. In cases where the blot was developed with α -Fcp (Fig. 4B), signals were detected in the fractions from WT Fcp and also in the FcpA_{His} fraction, confirming that the purified product consisted of Fcp polypeptides in each case. As expected, only a single sharp signal was seen at 19 kDa in the FcpA_{His} sample when developed with α -His (Fig. 4C) and none in the WT Fcp, confirming the presence of the His-tagged protein.

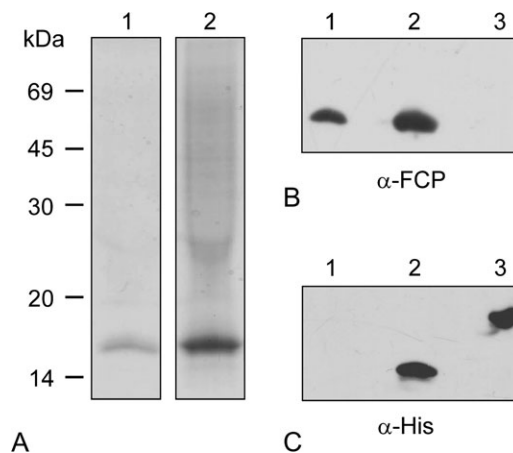


Fig. 4. Coomassie-stained SDS-PAGE (A) and Western blots (B, C) of WT Fcps (lanes 1) and FcpA_{His} containing fractions isolated using IMAC (lanes 2). In (A), samples corresponding to 0.5 μ g Chl *a* were loaded. Adjacent on the left the molecular weights in kDa of marker proteins are given. The Western blot in (B) was developed with an α -Fcp antibody, whereby His-tagged PsbS was loaded in lane 3 as a negative control. The Western blot in (C) was developed with an α -His antibody, whereby His-tagged PsbS in lane 3 now served as a positive control.

Oligomeric state

FcpA_{His} was subjected to analytical gel filtration to investigate the oligomeric state as well as the homogeneity. Figure 3 shows the gel filtration profile of FcpA_{His} where a single sharp peak was observed at 16.6 ml. An additional broad but minor peak was observed between 24–27 ml and spectroscopic investigations showed that the former peak was composed solely of Fcps and the latter was devoid of them. After spectroscopic investigations, the latter peak was assumed to be free pigment and was not considered further. It has been shown for Fcps from *C. meneghiniana* that the xanthophyll cycle pigments are lost more easily when they are purified via ion exchange chromatography as compared to those purified by sucrose gradient (Beer *et al.*, 2006). It is therefore assumed that a part of the latter (free pigment) peak could have been pigments originally associated with the FcpA_{His} complex. However, the loosely bound pigments decoupled from the complex during the washing conditions on the IMAC as well as during gel filtration. The WT Fcp fraction of *P. tricornutum* was also eluted from the same column under identical conditions at 16.6 ml (see above). Peak fractions in both WT Fcp and FcpA_{His} were analysed for energy transfer and it was confirmed that the Fcps were intact (see below). Trimeric FCPa complex from *C. meneghiniana* also eluted at 16.5 ml under identical conditions (K. Gundermann, personal communication). The similarity in the elution volume thus related the oligomeric state of the trimeric FCPa complex from *C. meneghiniana* (Büchel, 2003; Beer *et al.*, 2006) and the isolated FcpA_{His} complex along with the WT Fcp from *P. tricornutum*. Neither higher oligomers nor monomers were observed in the samples. Thus, it was concluded that FcpA_{His} in *P. tricornutum* is part of a trimeric complex. Furthermore, we

also confirmed that all Fcps in the WT sample were also trimeric under this particular condition of solubilization of thylakoids.

To date FCP complexes in different oligomeric states have been isolated both from the centric diatom *C. meneghiniana* and from the pennate diatom *P. tricornutum*. Co-existing trimeric FCPa complexes and higher oligomeric FCPb complexes were isolated and characterized from *C. meneghiniana* (Büchel, 2003; Beer *et al.*, 2006). In *P. tricornutum*, it was shown that there exists an antenna complex FCPo in a higher oligomeric state and a trimeric FCP complex, comparable to FCPb and FCPa in *C. meneghiniana*, respectively. The major difference as compared to the antenna complexes from *C. meneghiniana* was that at least *in vitro* the FCPo and FCP complexes were never co-existent (Lepetit *et al.*, 2007). FCPo complexes were obtained by mild solubilization of thylakoids (detergent:Chl, 5:1), while the trimeric FCP complexes were purified using relatively harsher conditions (detergent: Chl, 20:1 and 40:1). In our case the detergent:Chl *a* ratio was set to 30:1, explaining that both the complexes of FcpA_{His} and the WT Fcp pool were trimeric. From our observations, we can support the hypothesis that a trimer is the basic stable unit of organization of all Fcps in *P. tricornutum*.

Polypeptide composition of trimers

MS was used to identify the polypeptides which built up the FcpA_{His}-containing complex. Trypsin digestion of the 19 kDa band from a silver-stained gel followed by nano LC-ESI-MS/MS led to the identification of several peptide sequences. These were used to identify proteins within a combined diatom database as described in Veith *et al.* (2009). Two Fcp proteins were unambiguously identified (Table 1). These were Lhcf1 (JGI protein ID: 18049) and Lhcf5 (JGI protein ID: 30648) from *P. tricornutum*, which are also annotated as FcpA and FcpE, respectively. The presence of just these two gene products is in contrast to the WT fraction, in which Lepetit *et al.* (2007) demonstrated the presence of FcpA, B, C, D, E, and F using mass spectrometry as well. Thus, the FcpA_{His} complex is a specific trimer made up of FcpA and FcpE subunits. Since the applied LC-ESI-MS/MS technique is not a direct quantitative approach, further work needs to be done to elucidate the quantitative contributions of these two polypeptides in the trimer.

Spectroscopic characterization and pigment analysis

The pigment composition of the trimeric His-tagged complex and the WT Fcp pool was compared by HPLC analyses, which resulted in some differences. In both cases the Chl *c* content was similar with about 0.36–0.38 mol per mol Chl *a* (Table 2). However, the Fx content in the case of the His-tagged complex was about 1.2 mol mol⁻¹ of Chl *a*, while in WT Fcp the content was about 1.5 times higher. To our knowledge, there is no literature report of such high values for Fx in Fcps of *P. tricornutum*, except for one of

Table 1. Nano LC-ESI-MS/MS analysis of a FcpA_{His} containing complex

JGI-Phatr2, Joint Genome Institute-vs2 of the *P. tricornutum* genome. *z*, charge. *X*_{corr}, cross-correlation factor. Mo, oxidized Met. * The peptide is present in both models.

Protein ID (JGI-Phatr2)	Name	Identified peptides	<i>z</i>	<i>X</i> _{corr}
18049	Lhcf1 (FcpA)	NNYLDFGWDTFSEDK	2	4.55
		NNYLDFGWDTFSEDKK	2	3.99
		DITGGFEVGDFFR*	2	3.93
30648	Lhcf5 (FcpE)	NDFIDFGWDSFDEETK	2	4.04
		DITGGFEVGDFFR*	2	3.93
		ISMOLAVAGYLVQENGIR	2	3.24

Table 2. HPLC analysis of whole cells and isolated Fcp complexes

Pigment composition is indicated on mol mol⁻¹ Chl *a* basis and values represent average and standard deviations of three to five independent measurements.

mol mol ⁻¹ Chl <i>a</i>	Fx	Chl <i>c</i>	Ddx+Dtx
WT cells	0.782±0.136	0.219±0.013	0.124±0.021
FcpA1.2 cells	0.806±0.052	0.232±0.030	0.120±0.008
WT Fcp	1.710±0.137	0.369±0.027	0.207±0.020
FcpA _{His}	1.199±0.090	0.376±0.027	0.055±0.040

the fractions obtained by Guglielmi *et al.* (2005) using gel filtration. In order to obtain large amounts of protein, the cultures used here were grown under low light by bubbling sterile air (see the Materials and methods), allowing the cells to multiply faster than in shaking cultures (data not shown), and in addition to grow to a high density (10–15 mg Chl *a* l⁻¹). At this Chl *a* concentration, the cultures appeared dark brown in colour, indicating that the amount of fucoxanthin, the major carotenoid inside the FCPs, was up-regulated. To evaluate this hypothesis, whole cells were measured as well and an unusually high content of Fx was found in both the WT and the transgenic strain (0.78–0.80 mol Fx mol⁻¹ Chl *a*) compared with the value of 0.68 published for cells not grown to such densities (Lavaud *et al.*, 2003). In addition, pigmentation of Fcps in *P. tricornutum* was usually studied using Fcp complexes purified either by gel filtration or by sucrose density centrifugation (Guglielmi *et al.*, 2005; Lepetit *et al.*, 2007), whereas a combined approach was used in this study for the WT Fcp pool, i.e. a second purification step that should reduce contamination. However, it cannot be ruled out that some contamination with Fx from the free pigment fraction took place when using these two, still very gentle, preparation methods sequentially.

FcpA and FcpE belong to group I of Fcps which are thought to be the classical light-harvesting Fcps. It was therefore unexpected to find lower amounts of fucoxanthin in the FcpA_{His} complexes compared with the WT Fcp pool. It is known that the xanthophyll cycle pigments can be lost

from the antenna complexes more easily than Fx, Chl *c* and Chl *a* due to their probable peripheral binding site (Beer *et al.*, 2006). It was therefore not surprising that the concentrations of Ddx+Dtx were higher in the WT Fcp fraction than in the FcpA_{HIS} complex (Table 2), because WT Fcp was not subjected to the slightly harsher condition of IMAC during purification. In the case of the trimeric FcpA_{HIS} complex, the pigment data include only the pool of pigments tightly associated with the polypeptides, which seem to be the 'core' pigments quite similar in all FCP complexes isolated so far. Thus, a characterization of more individual Fcps is needed to prove that the different Fcps can bind different pigments in different stoichiometries.

Figure 5A shows a comparison between room temperature absorbance spectra of WT Fcp, the FcpA_{HIS} complex, and FcpA_{HIS} after subsequent gel filtration. These spectra are very similar to those reported for Fcps from other diatoms (Büchel, 2003; Lepetit *et al.*, 2007). In all cases, the absorption maxima at the Q_Y region of Chl *a* were consistently found at 672 nm. A small peak at 636 nm represented the Q_Y band of Chl *c* in each case. In the Soret region, the absorption maximum was observed at 441.5 nm for the WT Fcp whereas the FcpA_{HIS} complex showed a slight blue shift of 1.5 nm. This minor hypochromic shift was attributed to the conditions of the IMAC and the subsequent gel filtration, and therefore to a loss of loosely bound pigments. This became more obvious by a significant decrease in the absorbance in the Soret region in FcpA_{HIS} complexes compared with WT Fcp by ~25%. The loss of pigments was even more pronounced in the case of a shoulder at 486 nm, which is present in WT Fcp and absent in the FcpA_{HIS} trimer. This shoulder probably reflects on the higher amount of xanthophyll cycle pigment pool and/or fucoxanthin in the WT, owing to the fact that it has a pool of different Fcps. This observation was supported by the pigment analysis that the WT Fcp and the pure FcpA_{HIS} complex have different amounts of associated pigments (see above).

Fluorescence excitation spectra (Fig. 5B) for both WT Fcp and the trimeric FcpA_{HIS} complex demonstrated that the isolated complexes were intact with respect to energy transfer from Chl *c* and Fx to Chl *a*. It also showed that the additional pigments seen in absorbance spectra of the WT Fcp compared with FcpA_{HIS} (Fig. 5A) were not involved in the transfer of excitation energy to Chl *a*. Thus, the additional pigments found by HPLC in the WT complexes, i.e. fucoxanthin, diadinoxanthin, and diatoxanthin, most probably represent pigments not properly working in excitation energy transfer. Excitation at 465 nm (Soret band of Chl *c*) to record the fluorescence emission resulted in one sharp peak at 675.1 nm and at 675.7 nm for the FcpA_{HIS} complex and the WT Fcp, respectively (Fig. 5C). A very small shoulder at 636 nm was observed in the case of FcpA_{HIS} purified only via IMAC suggesting that a small amount of Chl *c* was decoupled from the complex or that this shoulder arose due to the presence of free Chl *c* on the IMAC column. This small Chl *c* emission disappeared after gel filtration and indirectly proved that whatever Chl *c*

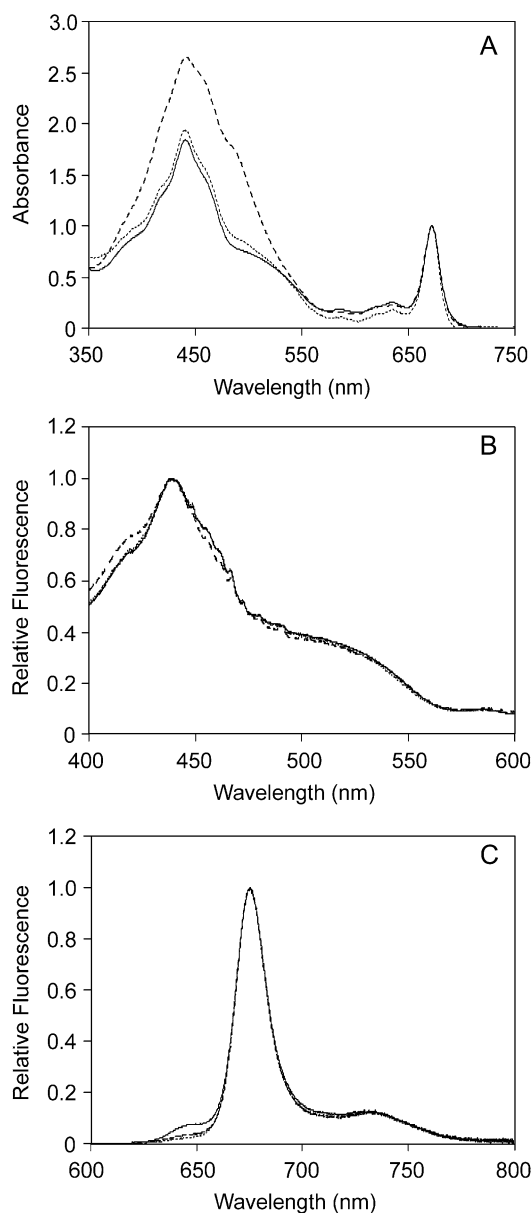


Fig. 5. Absorption spectra (A), fluorescence excitation spectra measured at 675 nm (B) and fluorescence emission spectra (C) recorded upon excitation at 465 nm were recorded using WT Fcp purified on gel filtration (dashed line), FcpA_{HIS} after IMAC (solid line) and FcpA_{HIS} further purified on gel filtration (dotted line) at a Chl concentration of ~0.3 $\mu\text{g ml}^{-1}$, and normalized to the Q_Y absorption (A), the maximum in the Soret (B) and the maximal Chl *a* emission (C).

emission was observed after IMAC was only due to free Chl *c* independent of the FcpA_{HIS} complex. The additional gel filtration in FcpA_{HIS} was helpful to determine the oligomeric state and to confirm that the complex was extremely stable and functional even after multiple series of purification processes.

The CD spectra of WT Fcps as well as FcpA_{HIS} complex were observed to be very similar to each other (Fig. 6). They also resembled the previously reported CD spectra from *P. tricornutum* (Lepetit *et al.*, 2007) and also from *C. meneghiniana* (Büchel, 2003) concerning the spectral

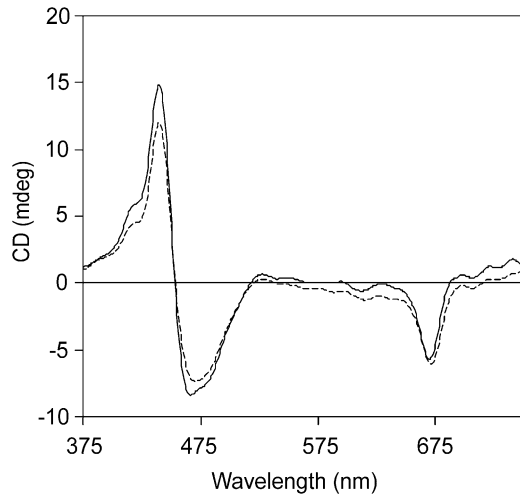


Fig. 6. Circular dichroism spectra of WT Fcp (dashed line) and of FcpA_{His} (solid line). Concentration of both samples was adjusted to 5 $\mu\text{g ml}^{-1}$ Chl *a*.

structure. A prominent single negative band was observed at (–)669 nm in the case of FcpA_{His} and at (–)670 nm for the WT Fcps which was attributed to Chl *a*. This peak showed a 3 nm blue shift as compared to the Q_Y band of Chl *a* in the absorbance spectrum which might suggest the existence of a heterogeneous pool of Chl *a* in Fcps (Mimuro et al., 1990). As this was a single band, it was concluded that no Chl *a*–Chl *a* excitonic interactions exist in Fcps of *P. tricornutum*. Minor negative bands were observed at (–)612 nm and (–)615 nm for FcpA_{His} and WT, respectively, corresponding either to the Q_x absorbance of Chl *a* or to the Q_Y absorbance of Chl *c*. A huge split signal was observed in the Soret region at (–)467 nm and (+)440 nm for FcpA_{His}, resembling the spectral fingerprint observed earlier for Fcps in *P. tricornutum* (Lepetit et al., 2007), clearly indicated the existence of a Chl *a*-carotenoid excitonic dimer. This again established the intactness of the isolated complex. Lepetit et al. (2007) could not find any differences in the CD spectra of Fcps in *P. tricornutum* in higher oligomeric or in the trimeric state, like those observed in the case of *C. meneghiniana* earlier (Büchel, 2003). The similarity in the spectral shape of FcpA_{His} and WT Fcps in our case to that of the FCPo and FCP complex suggests that the arrangement of pigments on the polypeptide backbone in all Fcps is similar in *P. tricornutum*. Furthermore, the common split signal in FCP complexes of *C. meneghiniana* and *P. tricornutum* indicate that the Chl *a*-fucoxanthin excitonic dimer is probably a conserved feature of the Fcps in diatoms.

In summary, it was possible to isolate for the first time a trimeric FCP complex containing a specific gene product, FcpA. The pigments bound to this complex were functional in excitation energy transfer. The complex was found to be composed of FcpA as well as FcpE, which indirectly demonstrated that the other well expressed polypeptides in *P. tricornutum*, mainly FcpB, FcpC, and FcpD, have to be localized in different trimers. Thus, this is the first report on different subpopulations of Fcp complexes in *P. tricornutum*.

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