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Biochimica et Biophysica Acta 1767 (2007) 1428–1435

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The monomeric photosystem I-complex of the diatom *Phaeodactylum tricornutum* binds specific fucoxanthin chlorophyll proteins (FCPs) as light-harvesting complexes

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Received 23 April 2007; received in revised form 11 September 2007; accepted 17 September 2007
Available online 26 September 2007

Abstract

A photosystem I (PSI)–fucoxanthin chlorophyll protein (FCP) complex with a chlorophyll *a*/P700 ratio of approximately 200:1 was isolated from the diatom *Phaeodactylum tricornutum*. Spectroscopic analysis proved that the more tightly bound FCP functions as a light-harvesting complex, actively transferring light energy from its accessory pigments chlorophyll *c* and fucoxanthin to the PSI core. Using an antibody against all FCP polypeptides of *Cyclotella cryptica* it could be shown that the polypeptides of the major FCP fraction differ from the FCPs found in the PSI fraction. Since these FCPs are tightly bound to PSI, active in energy transfer, and not found in the main FCP fraction, we suppose them to be PSI specific. Blue Native-PAGE, gel filtration and first electron microscopy studies of the PSI–FCP sample revealed a monomeric complex comparable in size and shape to the PSI–LHCI complex of green algae.
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Keywords: Photosynthesis; Fluorescence; Diadinoxanthin; Membrane protein; Electron microscopy

1. Introduction

Diatoms (*Bacillariophyceae*) represent one of the most successful taxonomic groups of approximately 100,000 species, which contribute close to one quarter of global productivity [1]. They inhabit ocean and fresh water environments as well as soil and have a strong impact on the biochemical cycles of carbon, nitrogen, phosphorus, and silica. Diatoms are eukaryotic, unicellular organisms that carry out oxygenic photosynthesis. The general features of their photosynthetic apparatus resemble those of higher plants and cyanobacteria, yet there are some important differences. The thylakoids of diatoms are not divided in grana and stroma lamellae but organised in bands consisting of three thylakoids each. Immuno-cytochemical experiments [2] showed randomly distributed photosystem (PS) I and light harvesting (LHC) complexes, i.e. no segregation of PSI and PSII could be detected so far. The LHC complexes of diatoms, fucoxanthin-chlorophyll-proteins (FCPs), bind Chl *c* instead of Chl *b* and fucoxanthin instead of lutein. Upon protein binding the absorbance of fucoxanthin is shifted to the red thus absorbing from 460 nm to 570 nm, a spectral range not used by higher plants and green algae. Still, sequencing from cDNA clones of *Cyclotella cryptica* and *Phaeodactylum tricornutum* proved the homology of FCP genes to LHC genes of higher plants [3,4], placing the FCPs into the family of *cab* proteins (chlorophyll *a* binding proteins). Furthermore, FCP genes could be classified into three groups. The first group, *fcp* 1–5 in *C. cryptica* and *fcp* A–F in *P. tricornutum*, is closely related to FCPs of brown algae. The second group, *fcp* 4 of *C. cryptica* and a homologue in *Thalassiosira pseudonana* [5], is related to *lhca-R1/2*, the only intrinsic light harvesting protein in red algae

Abbreviations: BN-PAGE, blue native polyacrylamide gel electrophoresis; Chl, chlorophyll; DDM, β-dodecyl maltoside; EST, expressed sequence tags; FCP, fucoxanthin chlorophyll protein; HPLC, high pressure liquid chromatography; IEX, ion exchange chromatography; LHC, light harvesting complex; PS, photosystem; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; TEM, transmission electron microscopy

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doi:10.1016/j.bbabio.2007.09.004

and to membrane intrinsic antenna proteins in cryptophytes. The third group is related to LI818 of *Chlamydomonas reinhardtii*, and is represented by the genes *fcp* 6, 7, and 12 of *C. cryptica*. From *Cyclotella meneghiniana* it is known that FCPs assemble into two different oligomeric states, trimers that consist mainly of 18 kDa subunits and higher oligomers (hexa- or nonamers), which are built of 19 kDa subunits [6,7]. However, no specific association of these complexes to photosystem I or II could be proven so far.

Although EST data for the diatoms *T. pseudonana* and *P. tricornutum* [8] and the complete plastome sequences of *Odontella sinensis* [9], *P. tricornutum*, and *T. pseudonana* are available (GenBank accession nos.EF067920,EF067921), the knowledge about structure and function of other proteins involved in photosynthesis is still little. During the 1980s, mostly photosystem I cores without associated antenna complexes were isolated from diatoms [10–12]. Later, more gentle solubilisation conditions lead to functional photosystem I complexes with bound light harvesting proteins [13]. However, with respect to polypeptide composition and pigment content the FCPs associated with PSI were indistinguishable from the main antenna complexes. The authors concluded that only one antenna system exists in diatoms, which serves both photosystems, like also shown for the closely related Phaeophyceae (brown algae) [13]. This is in line with a recent report by Brakemann et al. [14], who demonstrated the occurrence of *fcp* 2 and *fcp* 4 peptides of *C. cryptica* in both the PSI and the PSII fraction isolated by sucrose gradient centrifugation. However, for other members of the Chl c containing group of algae like cryptophytes [15] and xanthophyceae [16] specific antenna proteins of PSI were described recently.

PSI is a huge membrane protein complex consisting of 12 protein subunits in cyanobacteria and 14 protein subunits in higher plants. In the latter, PSI supercomplexes with four light harvesting complex (LHCI) subunits are formed. This supercomplex coordinates approximately 200 cofactors [17,18]. Structural analysis has shown that the plant supercomplex is monomeric. In contrast, cyanobacterial PSI is trimeric and only under iron starved conditions supercomplexes with intrinsic antenna proteins are formed [19]. No biochemical data are available about the oligomeric state of PSI in diatoms. Unfortunately, sequence comparison of PSI subunits between diatoms and higher plants or cyanobacteria leads to ambiguous results. In contrast to higher plants the *P. tricornutum* plastome contains subunit PsaM, which is supposed to enable trimerisation of PSI in cyanobacteria, but sequence identity is only 50%. The nuclear encoded psaG, binding the LHCI belt in higher plants, was not identified in diatoms so far. The same holds for psaH, the subunit hindering trimerisation in higher plants. The lack of these subunits argues for a cyanobacterial structure of PSI, but the annotation of nuclear genes in diatoms is far from complete. In contrast, the existence of a membrane intrinsic antenna like in higher plants might argue for a monomeric structure of PSI.

Here we present data on the organisation of PSI in diatoms. In order to elucidate the interaction with FCP polypeptides and the oligomeric state of PSI we isolated a PSI–FCP supercomplex. This complex was analysed spectroscopically and biochemically and we present first images of this complex obtained by transmission electron microscopy (TEM).

2. Materials and methods

2.1. Culture

Batch cultures of *P. tricornutum* were grown at 18 °C for 10–14 days on a shaker in ASP medium [20] under a 16-h light (40 μmol m^{-2} s^{-1}) to 8-h dark cycle.

2.2. Preparation of thylakoids

2.2.1. P. tricornutum

Thylakoids were prepared as described in [6] with slight modifications. Harvested cells were resuspended in homogenisation buffer (HB; 10 mM HEPES, 2 mM KCl, 5 mM EDTA, 1 M sorbitol at pH 7.4) and broken by a double frenchpress cycle (Polytec/Thermo) at 20,000 psi. To avoid contamination with unbroken cells, the homogenate was spun for 2 min at 3000×*g* and the supernatant was collected. The pellet was resuspended in HB and centrifuged as described above. Both supernatants were combined and the centrifugation step repeated. The final supernatant was then ultracentrifuged for 20 min at 206,000×*g*. The pellet was resuspended in washing buffer (WB; 10 mM HEPES, 2 mM KCl, 5 mM EDTA at pH 7.4) and spun for 20 min at 163,000×*g*. After resuspending the pellet in WB the chlorophyll content was determined according to [21].

2.2.2. Pisum sativum

Plants were grown on vermiculit at room temperature without special light conditions. Thylakoids were isolated from 10 to 14 days old plants according to [22]. The chlorophyll concentration was determined as described by [23].

2.2.3. Synechocystis sp. PCC 6803

Synechocystis sp. PCC 6803 (a kind gift of G. Sandmann, Frankfurt University) was grown under continuous light (35 μmol m^{-2} s^{-1}) at 28 °C in BG11 medium [24] supplemented with 5 mM TES (pH 8.0) and bubbled with 1.5% CO_2 in air for 4 days. 24 h prior to cell harvesting 100 mg/l penicillin G was added [25]. During the preparation of the membranes, sample and buffers were kept on ice and under low illumination. Cells were pelleted at 5000 rpm for 15 min in a Herolab HiCen 21 C centrifuge (rotor A6.9) and resuspended in 15 ml buffer A (50 mM Mes, pH 6.0, 10 mM $MgCl_2$, 5 mM $CaCl_2$, 25% glycerol) [26]. 200 mg lysozyme was added and the cell solution was incubated on a shaker for 30 min at 30 °C. Cells were then broken by five passages through a frenchpress at 20,000 psi. To remove unbroken cells the solution was spun at 3000 × *g* and 4 °C for 2 min. The supernatant was collected and centrifuged again. The resulting supernatant was then ultracentrifuged for 20 min at 163,000 × *g* and 4 °C. The pelleted membranes were resuspended in 10 mM Mes (pH 6.0) and the ultracentrifugation step was repeated. The final pellet was resuspended again in 10 mM Mes (pH 6.0) and the Chl *a* concentration was determined according to [21]. The sample was frozen in liquid nitrogen.

2.3. Separation of pigment protein complexes

2.3.1. Ion Exchange Chromatography (IEX)

Thylakoids of *P. tricornutum* were solubilised with 20 mM β-dodecyl maltoside (DDM) as described elsewhere [7]. For the separation of membrane complexes the solubilised thylakoids were loaded onto an ion exchange column (DEAE Toyopearls 650D (TOSOH)) connected to an Äkta Purifier P-900 (Amersham Bioscience). Fractions were eluted using a NaCl gradient (86 mM to 90 mM) in buffer 1 (25 mM Tris, 2 mM KCl, 0.03% DDM at pH 7.4) followed by a wash at 0.75 M NaCl in buffer 1. Elution was controlled spectroscopically at three different wavelengths (λ_1=280 nm, λ_2=437 nm, λ_3=530 nm). The harvested fractions were concentrated using filtration devices (Amicon) with a 30 kDa cutoff filter.

2.3.2. Gel filtration

Solubilised thylakoids (0.5 mg Chl in 2 ml) or complexes purified by IEX (0.01 mg Chl in 100 μl) of *P. tricornutum* were applied to a Superdex 200 (GE

Healthcare) gelfiltration column 10/300 GL (Amersham Biosciences) connected to an Äkta Purifier P-900 (Amersham Biosciences). Elution was carried out with buffer 1 at a flow rate of 0.5 ml/min and controlled spectroscopically at two different wavelengths (λ_1=437 nm, λ_2=700 nm). Collected fractions were analysed spectroscopically.

In case of *Synechocystis* sp. PCC 6803, thylakoids (400 μl at 1 mg Chl/ml) were solubilised 20 min on ice in the dark with 20 mM DDM in buffer 1. After centrifugation for 5 min in a tabletop centrifuge at 13.000 rpm and 4 °C, the supernatant was loaded onto the gelfiltration column. Run conditions were the same as described for *P. tricornutum.*

2.4. Characterisation of purified complexes

2.4.1. Electrophoresis

10% denaturing Tris–Tricine gels were cast according to [27]. Samples were denatured in rotiload (Roth) for 30 min and gels were stained with Coomassie Blue G 250 after the run. Roti-Mark prestained (Roth) was used as molecular marker.

Blue Native PAGE (BN PAGE) was carried out as described by [28] with slight modifications introduced by [29]. In short, pea thylakoids were adjusted to 2 mg chlorophyll/ml in resuspension buffer (RB, 20% glycerol and 25 mM Bis–Tris–HCl, pH 7.0) and solubilised with an equal volume of RB including 2% DDM for 30 min on ice. After centrifugation at 13.000 rpm for 5 min in a tabletop centrifuge the supernatant was diluted by adding 1/10 of volume of sample buffer (SB, 100 mM Bis–Tris–HCl, pH 7.0, 0.5 M 6-amino-n-caproic acid, 30% glycerol) and sample equal to 15 μg of Chl was loaded onto 5%–13.5% gradient gels. In case of *P. tricornutum*, isolated fractions from the ion exchange chromatography (IEX) were mixed with SB to yield a final concentration of 0.3 mg Chl/ml and 10 μl were loaded directly onto the gel. Electrophoresis was performed in a Hoefer Mighty Small electrophoresis unit run at 150 V–200 V at 4 °C for several hours.

2.4.2. Western Blots

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 (25 mM 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). For slot blots, samples were directly applied to PVDF membranes. Immunodetection was performed using the ECL Plus kit (Amersham Biosciences) according to the manufacturer's instructions. Antibodies directed against higher plant PSI (α-psaA/psaB), against higher plant reaction centre II protein D2 (α-psbD) and against all FCP polypeptides of *C. meneghiniana* (α-ccFCP) were kind gifts of J. Feierabend (University of Frankfurt) and E. Rhiel (University of Oldenburg), respectively.

2.4.3. HPLC (high pressure liquid chromatography)

Samples were precipitated in 90% methanol (final concentration) and supernatants were analysed. The amount of pigments was quantified according to [30].

2.4.4. Absorbance spectra and P700 quantification

All spectra were recorded at room temperature with 1 nm bandpass and 1 cm optical pathlength using a Jasco spectrophotometer (V 550). Absorbance spectra were taken between 350 nm and 750 nm.

Difference spectrum measurements of oxidised minus reduced samples of the PSI fraction were performed with 0.025 mM $K_3[Fe(CN)_6]$ as oxidant and 0.7 mM Na-ascorbate as reductant. Spectra were recorded from 650 nm and 750 nm and a molar extinction coefficient of 64 mM^{-1} cm^{-1} [31] was used for calculation of the P700 concentration.

2.4.5. Fluorescence spectra

Fluorescence spectra were measured in a Jasco FP-6500 fluorometer at room temperature and 77 K using bandpasses of 3 nm both on emission and excitation side. Emission spectra were taken upon excitation at λ_{ex}=465 nm or λ_{ex}=560 nm and measured from λ_{em}=600 nm to λ_{em}=800 nm. For the excitation spectra, emission was recorded at λ_{em}=675 nm upon excitation from λ_{ex}=400 nm to λ_{ex}=600 nm. For direct comparison of different fractions, samples were adjusted either to the same Chl *a* concentration (absorbance of 0.03 at the Q_Y band of Chl *a* ≈ 0.33 μg/ml of Chl *a*) or to a similar fucoxanthin concentration (identical absorbance at 560 nm).

2.4.6. Electron microscopy

A droplet of sample (8 ng/ml Chl) was placed on a recently prepared plain carbon grid. After 30 s incubation, specimen were negatively stained for 45 s with 2% uranyl acetate and grids were washed twice with distilled water afterwards. Images were taken using a Phillips CM12 electron microscope at 120 kV at a magnification of 60,000×. Micrographs were scanned with a Super Coolscan 9000 ED (Nikon) with a resolution of 100 pixel/16 nm at specimen level. Alternatively, images at a magnification of 110,000× were taken with a CCD camera (Gatan Erlangshen ES5W).

3. Results

3.1. Isolation of the complex

The separation of thylakoids from *P. tricornutum* via IEX resulted in the elution of four prominent pigmented fractions named I–IV (Fig. 1). Fraction I turned out to consist mainly of free pigment (data not shown) and some free proteins, e.g. probably ATPase subunits as reported by Amunts et al. [32]. Fraction II and III showed similar Chl *a* absorption but absorption of fucoxanthin was lower in fraction II. Thus, fraction II should resemble a photosystem and could be easily distinguished from the brown fraction III, the main FCP fraction. Fraction IV was eluted during the washing step at 0.75 M NaCl. According to the ratio between Chl *a* absorption and absorption in the UV, this band contained most of the unpigmented proteins. Immunodetection revealed that PSII is found in this fraction as well (Fig. 2). As we were most interested in PSI and FCPs this fraction was not considered further.

3.2. Polypeptide composition

Fig. 2a shows a denaturing SDS-PAGE of fraction II (lane 1) and fraction III (lane 2). A double band around 65 kDa as well as several bands in the FCP region between 15 kDa and 20 kDa

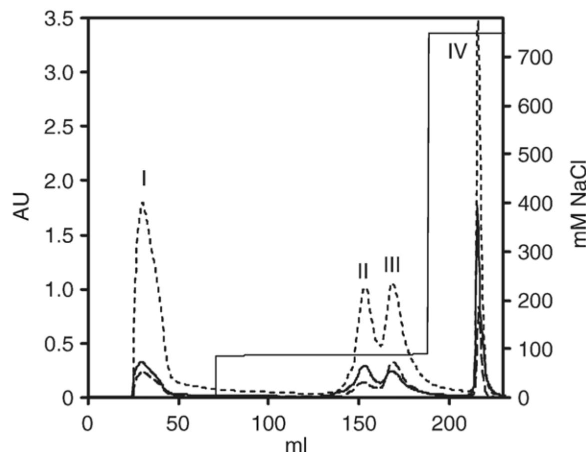

Fig. 1. Separation of pigmented proteins from solubilised thylakoids of *P. tricornutum* by IEX. The dotted line shows the absorption at 437 nm, the dashed line the absorption at 530 nm and the solid line the absorption at 280 nm. The slim line represents the NaCl gradient used for elution (86–90 mM) and washing (750 mM). Peaks were numbered (I–IV) in the order of elution.

were observed in fraction II, whereas fraction III was characterised by a prominent single band of 18 kDa, the expected molecular mass of FCP. Western blots with antibodies against the PsaA/B core subunits of higher plants (α-psaA/B) confirmed the presence of PSI in fraction II (Fig. 2b). This fraction was virtually free of PSII as demonstrated in Fig. 2c by the lack of reaction with an antibody directed against D2 (α-psbD). Using an antibody against all FCP polypeptides from *C. cryptica* (α-ccFCP) only one FCP signal could be observed in fraction III. Surprisingly, two bands with molecular masses different from those of the main FCP fraction were detected in fraction II (Fig. 2b).

3.3. Characterisation of the complex

Difference spectra of fraction II were recorded to detect the redox dependent absorption changes of P700. The peak maximum observed at 700 nm proved that fraction II indeed contained a functional PSI. From three independent measurements a ratio of 210 (±6) Chl/P700 was calculated.

Comparison of fraction II and fraction III by HPLC resulted in the following pigment ratios (see Table 1). Whereas fraction III had similar values compared to FCPs from *C. meneghiniana* [7], fraction II differed significantly from that. The fucoxanthin/Chl *a* ratio was reduced to 0.5 and the Chl *c*/Chl *a* ratio was only 0.1. Nonetheless, the ratio between Chl *c* and fucoxanthin remained the same in both fractions (0.2). These data indicate that FCP complexes with a similar Chl *c* to fucoxanthin ratio are present in fraction II as well. However, the amount of diadinoxanthin (the pigment that replaces the function of violaxanthin in non-photochemical quenching in diatoms) differed significantly. The diadinoxanthin to fucoxanthin ratio was increased by a factor of four in fraction II compared to fraction III. From the PSI crystal structure of cyanobacteria it is known that 96 chlorophylls and 22 carotenoids are bound per PSI monomer [33]. Fraction II contained ten times more Chl *a* than ß-carotene, which implies that almost half of the Chl *a* is bound to FCPs associated with the PSI complex.

Table 1
Pigment ratios of fraction II and III

	Fucoxanthin	Chl c	Diadinoxanthin	β-carotene
Pigment per Chl a (mol/mol)				
Fraction II	0.505±0.055	0.080±0 017	0.024±0.008	0.093±0.035
Fraction III	1.054±0.088	0.233±0.053	0.013±0.003	0.054±0.008
Pigment per fucoxanthin (mol/mol)				
Fraction II	1	0.158±0.026	0.048±0 011	0.182±0.060
Fraction III	1	0.220±0.033	0.012±0 002	0.052±0.011

Values were calculated on the basis of Chl *a* (upper part of the table) or fucoxanthin (lower half of the table) content. Ratios are given as mean±standard deviation of 6 measurements on 4 independent preparations (fraction II) and of 4 measurements on 2 preparation (fraction III), respectively.

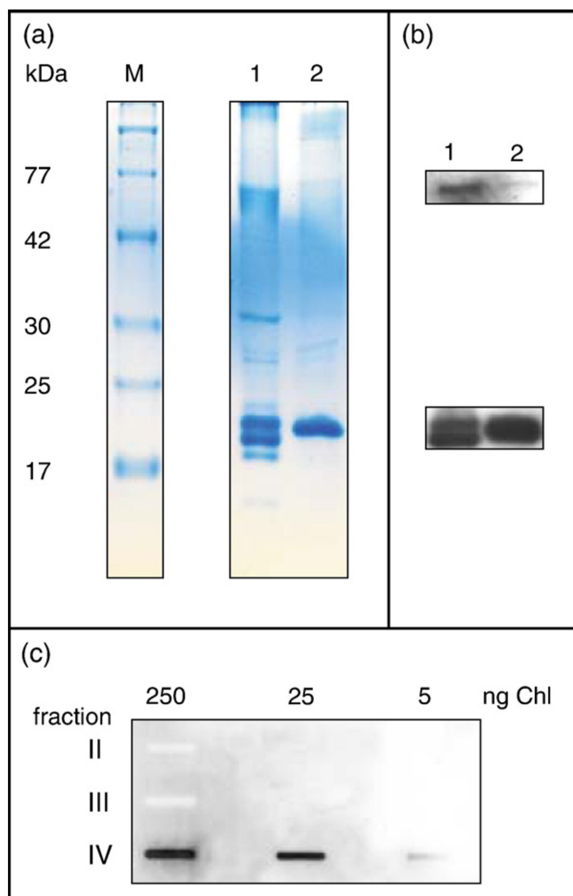

Fig. 2. SDS-PAGE and western blots of the isolated fractions. Panel a shows a Coomassie stained SDS-PAGE of fraction II (lane 1), fraction III (lane 2) and marker proteins (M). In panel b western blots demonstrate the reaction of the antibodies α-PsaA/B (upper panel) and α-ccFCP (lower panel). In panel c the reaction of different fractions with an antibody against PSII (α-psbD) is depicted. The different amounts of Chl loaded in each lane are indicated in the figure.

HPLC data were supported by further spectroscopic analysis. The absorbance spectrum (Fig. 3a) showed Chl *a* absorption with a maximum of the Q_Y band at 676 nm and β-carotene absorbance around 500 nm. Only traces of Chl *c* and fucoxanthin could be observed as shoulders around 465 nm and between 475 nm and 580 nm. In contrast, fraction III resembled a typical FCP spectrum with pronounced shoulders of Chl *c* and fucoxanthin and a maximum at 672 nm [6,11,34].

To clarify if fucoxanthin and Chl *c* are decoupled contaminants, are belonging to co-eluted but intact FCP complexes or are arranged in FCPs bound to the PSI complex in fraction II, fluorescence spectra were carried out. Fluorescence excitation spectra of fraction II and III normalised at 440 nm demonstrated an energy transfer from the pigments Chl *a*, Chl *c* and fucoxanthin to a final Chl *a* emitter in both samples (Fig. 3b). Although Chl *c* and fucoxanthin are hardly visible in the absorbance spectra of fraction II due to their minor amount, their presence and functionality became visible in the excitation spectra. Thus, hardly any of these pigments seem to be decoupled from their proteins. However, the fact that the excitation spectrum does not resemble the absorbance spectrum of fraction II but that of a FCP complex argues for at least some co-elution of unbound FCPs.

By running fraction II on an analytical gel filtration column, the presence of free FCPs could be demonstrated (Fig. 4a). However, the purified PSI containing fraction still contained

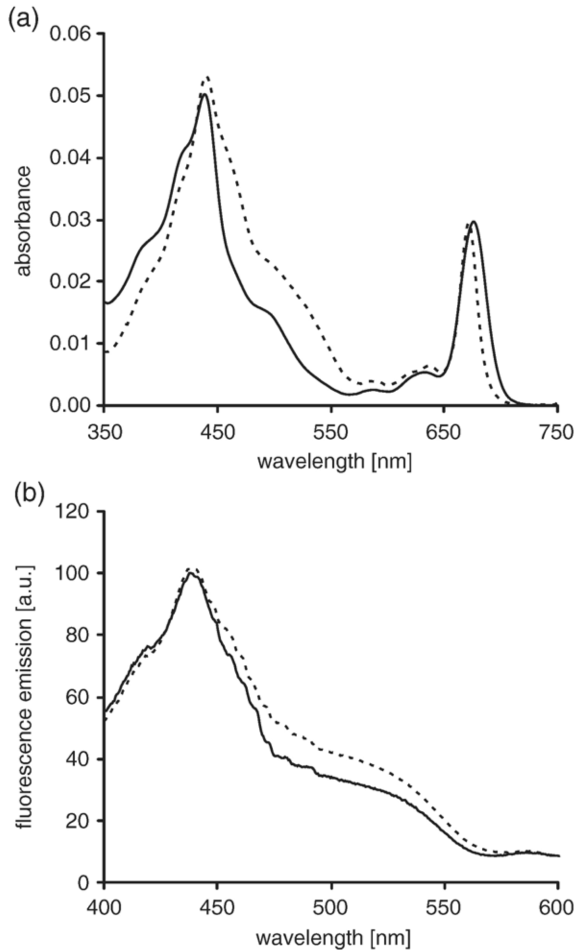

Fig. 3. Room temperature absorbance and fluorescence excitation spectra. In panel a absorbance spectra of fraction II (solid line) and fraction III (dotted line) adjusted to an identical absorption at the Q_y band of Chl *a* are depicted. The excitation spectra (b) of the same samples were recorded at 675 nm and normalised at 440 nm.

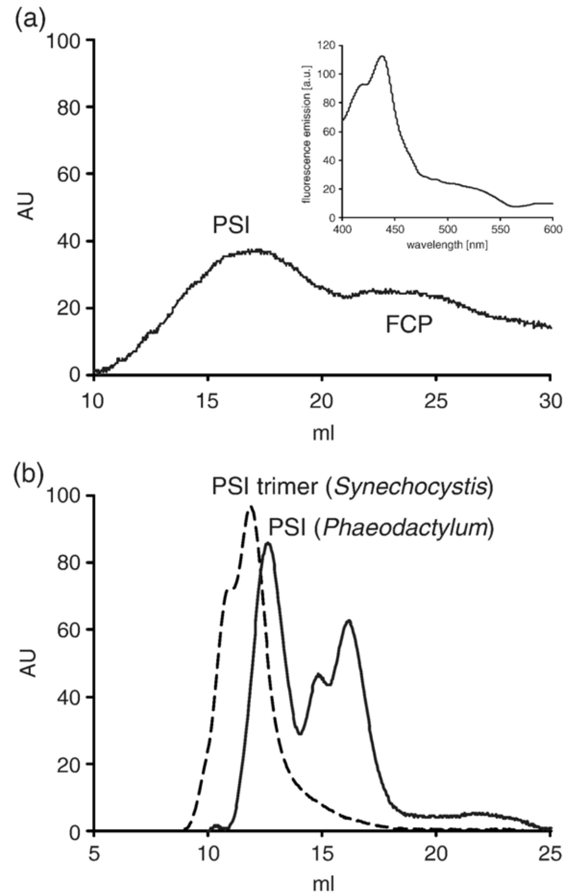

Fig. 4. Gel filtration of PSI complexes and thylakoid membranes. Panel a shows a run of Fraction II on a gel filtration column in order to separate unbound FCP. The run was recorded at 434 nm and the identity of the fractions as revealed by their absorbance spectra is indicated. The inset demonstrates the fluorescence excitation spectrum of the PSI–FCP complex devoid of detached FCPs recorded at 675 nm. In panel b solubilised thylakoids of *P. tricornutum* (solid line) were directly applied to a gel filtration system. For comparison the elution of cyanobacterial PSI (dashed line) is shown as well. Both elution profiles were recorded at 700 nm.

FCPs. This is demonstrated by a similar absorbance spectrum compared to fraction II (data not shown) and excitation energy transfer from fucoxanthin and Chl *c* to Chl *a* (inset Fig. 4a).

To elucidate whether bound FCPs transfer the absorbed energy to PSI, fluorescence emission spectra were recorded. A distinction between energy emitted by Chls of FCPs or energy coming from the PSI core chlorophylls is possible because of the high fluorescence yield of FCP complexes compared to the yield of PSI cores [13]. If FCPs are fully coupled to PSI they should fluoresce less compared to free FCPs given an identical absorbance of excitation energy and an identical amount of emitters. Both conditions are impossible to fulfil simultaneously for samples of different pigment composition. Thus, two experiments were carried out. Emission spectra of fraction II and III upon excitation of fucoxanthin at 560 nm were recorded at either the same Chl *a* concentration or at the same fucoxanthin absorbance at 560 nm. In the latter case, i.e. under conditions of identical absorbance of FCPs in both samples but higher emitter concentration in fraction II, the fluorescence yield was quenched significantly by 64% in fraction II compared to free FCPs (Fig. 5). This value was

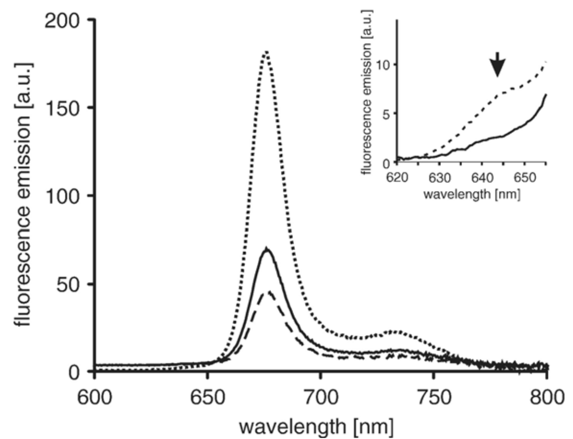

Fig. 5. Room temperature fluorescence emission spectra of the isolated complexes. Spectra of fraction III (FCP, dotted line), fraction II adjusted to the same absorption at 560 nm (solid line) and to the same Chl *a* concentration (dashed line) as fraction III were recorded upon excitation at 560 nm. The inset compares the emission of fraction II upon excitation at 560 nm (solid line) and 465 nm (dotted line).

further increased to 77% if the emitter concentration (Chl *a*) was adjusted. No numbers are available for the precise differences in fluorescence yield of the final Chl *a* emitters of FCPs and PSI. Therefore no estimate can be given which percentage of FCPs is energetically coupled to PSI in fraction II. However, the obvious quench in both experiments argues for energy transfer from fucoxanthin in some of the FCPs to Chl *a* of PSI in fraction II.

Exciting Chl c at 465 nm resulted in similar spectra with the exception of a small Chl *c* peak around 630 nm probably due to minor losses of function during purification (inset Fig. 5). This peak was absent in the spectra recorded upon excitation of fucoxanthin. Thus, as already seen for *C. meneghiniana* [30], fucoxanthin seems to transfer its absorbed energy directly to Chl *a*. At 77 K the fluorescence bands got more sharpened, whereas the overall pattern remained the same (data not shown).

In contrast to our data, two publications about PSI complexes from diatoms isolated by sucrose gradient centrifugation [13,14] reported an additional emission maximum of PSI at 715 nm despite almost identical absorbance spectra. The polypeptide composition resembled the one shown in Fig. 2a as well. As pointed out above, PSI has an extremely low fluorescence yield. Thus, usually high concentrations are used for fluorescence measurements due to the limitations of the photomultipliers. In this case, re-absorption processes can enhance the fluorescence emission at long wavelengths. In order to check this we carried out emission spectra using a 10 fold concentrated sample and could indeed find an additional shoulder at 705 nm besides an enhancement of the 735 nm emission (data not shown). Thus, the PSI–FCP complex of *P. tricornutum* has two emission bands, whereby the one at longer wavelength becomes only visible if the conditions for measurements allow for re-absorption to occur.

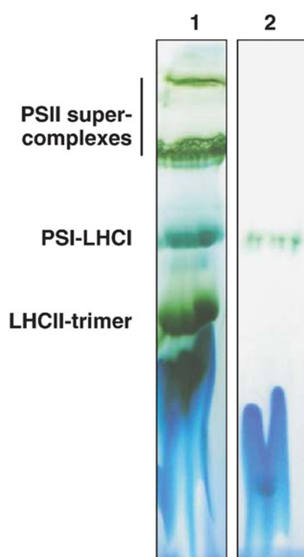

Fig. 6. BN-PAGE of pea thylakoids and the PSI–FCP complex purified from *P. tricornutum*. Fraction II was re-run on a BN PAGE (lane 2) and compared to the composition of solubilised pea thylakoids run under the same conditions (lane 1). The bands found in pea thylakoids are labelled according to [28,29].

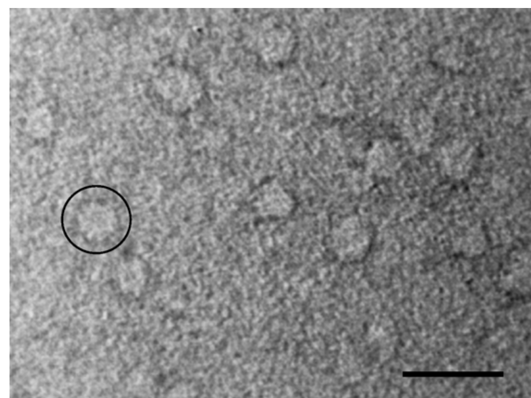

Fig. 7. Electron micrograph of negatively stained particles of the purified PSI–FCP complex. One top view of the complex is enclosed by a circle. The scale bar represents 50 nm.

3.4. Higher organisation of the complex

Since the comparison of protein sequences is insufficient to predict the organisation of the PSI–FCP complex of diatoms, a BN-PAGE of the isolated complex was carried out. As can be seen from Fig. 6 the major band of the isolated complex runs at the same height as the corresponding PSI–LHCI complex from pea thylakoids [35,36]. To further validate the monomeric state of the complex, gel filtration was carried out in comparison with cyanobacterial, trimeric PSI. To rule out any artificial monomerisation by IEX, solubilised thylakoids instead of isolated complexes were used. Fractions were identified according to literature using their absorption spectra. As can be seen from Fig. 4b, the cyanobacterial complex is significantly larger in size compared to the complex from *P. tricornutum*. Therefore, we conclude that the isolated PSI–FCP complex, at least under the conditions of native gel-electrophoresis or gel filtration, is a monomeric complex.

To support this assumption we performed electron microscopy studies with negatively stained particles of the complex isolated by IEX. A micrograph demonstrating top views is shown in Fig. 7. Particles could be compared in size and shape to monomeric PSI–LHCI particles of green algae [37], with a diameter of 21.1±3.2 nm (n=32). In images showing side views, artificial stacking of several particles could also be observed (data not shown). Trimeric or particles of even higher oligomeric state could not be found.

4. Discussion

During the last years detailed structural as well as spectroscopic information about PSI supercomplexes has accumulated. The PSI crystal structures of *T. elongatus* [33] and pea [17,18], as well as several structures derived from electron microscopy of green algae [37,38] and prochlorophytes [39] demonstrated the differences in the organisation of the light harvesting complexes but also showed the similarity of the core complexes. Based on the 2.5 Å crystal structure of cyanobacteria it was possible to built a theoretical model of plant PSI [18,40] revealing that the core

structure of a protein complex as complicated as PSI has been conserved over more than a billion years.

Diatoms, another group of photosynthetic organisms that evolved very quickly during the last 250 million years [41], possess the same PSI subunits as cyanobacteria or higher plants, but differ considerably in the accessory pigment composition of their LHCs (FCPs), with Chl *c* and fucoxanthin bound. Since there is no structural information about the photosynthetic apparatus we developed a method to isolate a PSI–FCP complex from thylakoids of the pennate diatom *P. tricornutum*. SDS-PAGE and Western Blot analysis revealed that the PSI–FCP fraction contained two FCP polypeptides of different molecular weight compared to the main FCP fraction. The band pattern obtained strongly resembles the data from Berkaloff et al. [13] but these authors did not elucidate the polypeptide composition any further. Here we could for the first time identify FCP polypeptides in *P. tricornutum*, which differ from the main FCP pool and are more tightly bound to PSI. In addition, these FCPs were functionally active as proven by fluorescence spectra.

Unfortunately, little is known about different classes of FCPs in *P. tricornutum*. Whereas in *C. cryptica* and *T. pseudonana* three different groups were found on gene level, for *P. tricornutum* only the genes *fcp* A–F (group 1) are described. However, it is possible to find *fcp* sequences of group 2 and group 3 in the *P. tricornutum* EST data bank [8] (e.g.: GenBank accession nos.CD384635, CD380971). Since the antibody was generated against all FCP polypeptides of *C. cryptica* it is impossible to conclude which *fcp* genes of *P. tricornutum* encode the FCPs bound by PSI. *fcpA–F* sequences were derived from cDNAs, i.e. from highly expressed genes and thus are most probably present in the main FCP of *P. tricornutum*. Polypeptides of the same molecular mass, i.e. 18.0–18.2 kDa, are missing in the PSI–FCP fraction. No distinction is possible between FCPs of group 2 and group 3 in *P. tricornutum* since for both the predicted mass is above 20 kDa, but at least one of them is bound by PSI. In addition, a smaller FCP polypeptide is bound as well, which is either not described on gene level so far or is post-transcriptionally modified.

Besides the analysis of polypeptides we were able to proof the specific activity of the PSI reaction centre P700 by difference spectra measurements. Our calculations gave a ratio of approximately 200 chlorophylls per PSI which is in good agreement with PSI–LHCI preparations. Furthermore we could show that the ratio of Chl *c* and fucoxanthin (1:5) in the isolated PSI and FCP complexes is similar to the ratio measured in purified FCPs from *C. meneghiniana* [30]. This led to the assumption that the FCPs bound by PSI are comparable to the major FCP fraction concerning these pigments. However, the concentration of diadinoxanthin in relation to fucoxanthin is increased compared to the major FCP.

The PSI–FCP complex has short wavelength emitting chlorophylls at room temperature which are accompanied by species emitting at longer wavelengths. The latter have an even lower fluorescence yield and thus become only visible if significant re-absorption takes place. Since the FCP also emit at about the same wavelength but have a much higher fluorescence yield, energy coupling between FCPs and PSI can be proven. A significant decrease in fluorescence of the PSI–FCP fraction compared to free FCP of either the same chlorophyll or similar fucoxanthin concentration indeed demonstrated an energy coupling between both complexes. In higher plants, some of the LHCI complexes are distinguished by their long wavelength emission at low temperature [42]. This feature is completely absent in the PSI antenna system of diatoms.

Chl *c* emission at 630 nm only occurred upon direct excitation of Chl *c* at 465 nm. Exciting fucoxanthin at 560 nm resulted in the disappearance of the 630 nm emission. Thus, the excitation energy transfer from fucoxanthin to Chl *a* is directly coupled and excludes Chl *c* as shown for the main FCP complexes from *C. meneghiniana* [30].

Sequence comparison points to more cyanobacterial than higher plant features in the PSI of diatoms but no biochemical evidence is available. Our data by BN-PAGE, gel filtration and electron microscopy reveal that the PSI–FCP complex is more likely a monomeric complex. Approximately 100 chlorophylls are coordinated by PSI core complexes and there is evidence for only four Chl *a* and one Chl *c* molecules per FCP monomer [30]. As we measured 200 chlorophylls in the isolated PSI–FCP complex we have to consider 100 chlorophylls that are either bound directly by FCPs or function as linker chlorophylls between the subunits as demonstrated for higher plant PSI supercomplexes. This idea is supported by the fact that only half the amount of β-carotene is found in PSI–FCP complexes compared to the PSI core of *T. elongatus*. Thus, a significantly higher number of FCP monomers have to be bound by PSI of diatoms compared to the LHCI tetramer of higher plants, even if one takes into account the contamination by some unbound FCPs in our preparations. Germano et al. [38] estimated a number of 14 LHCI monomers to be bound to PSI of *Chlamydomonas*, which seems to be a realistic number for our preparation as well. However, as long as no information is available about the existence and the amount of linker chlorophylls, no real estimation can be given. Currently we are starting to perform single particle analysis of this complex to solve this problem.

Acknowledgements

The authors kindly acknowledge financial support by the EU (Intro2, MRTN-CT-2003-505069). We would like to thank K. Pieper for technical assistance.

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