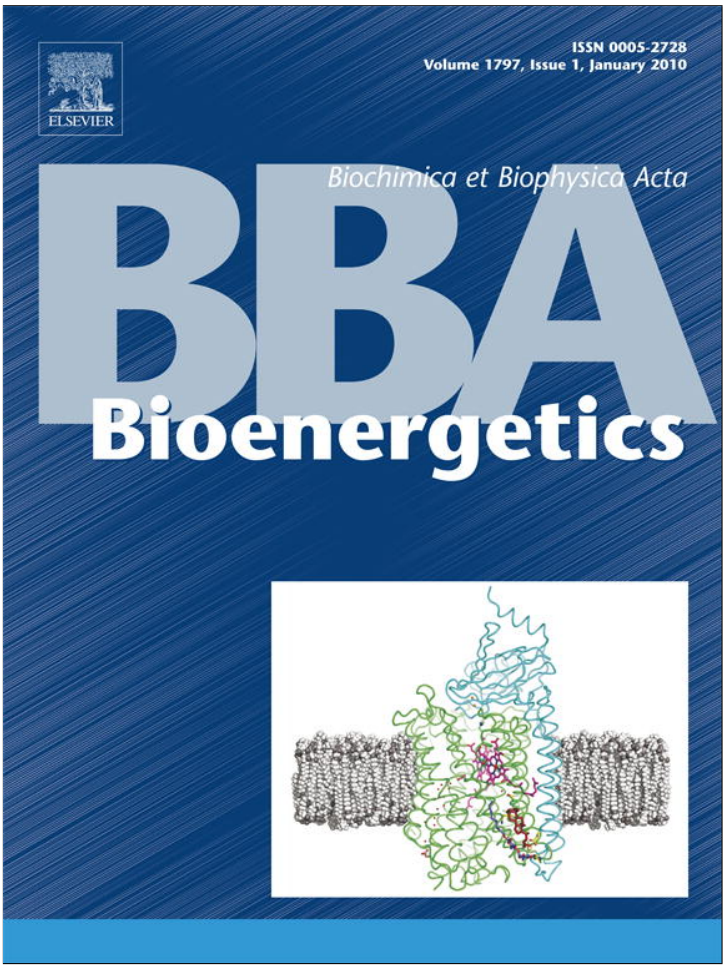

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Localization of Pcb antenna complexes in the photosynthetic prokaryote *Prochlorothrix hollandica*

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ABSTRACT

The freshwater filamentous green oxyphotobacterium *Prochlorothrix hollandica* is an unusual oxygenic photoautotrophic cyanobacterium differing from most of the others by the presence of light-harvesting Pcb antenna binding both chlorophylls *a* and *b* and by the absence of phycobilins. The pigment–protein complexes of *P. hollandica* SAG 10.89 (CCAP 1490/1) were isolated from dodecylmaltoside solubilized thylakoid membranes on sucrose density gradient and characterized by biochemical, spectroscopic and immunoblotting methods. The Pcb antennae production is suppressed by high light conditions (>200 μmol photons m^{-2} s^{-1}) in *P. hollandica*. PcbC protein was found either in higher oligomeric states or coupled to PS I (forming antenna rings around PS I). PcbA and PcbB are most probably only very loosely bound to photosystems; we assume that these pigment–protein complexes function as low light-induced mobile antennae. Further, we have detected α-carotene in substantial quantities in *P. hollandica* thylakoid membranes, indicating the presence of chloroplast-like carotenoid synthetic pathway which is not present in common cyanobacteria.

1. Introduction

A photosynthetic prokaryote *Prochlorothrix hollandica* (*P. hollandica*) is a member of a diverged polyphyletic group [1,2] of atypical cyanobacteria—green oxyphotobacteria [3], formerly called "prochlorophytes" (*pro* = before, *chlorophyta* = green plants) due to a suggestion that they are the progenitors of chloroplasts [4–7]. A revaluation of this hypothesis led to a conclusion that chloroplast evolved in multiple and independent events within the cyanobacteria lineage [1].

To date, three representatives of this group are described: two marine—*Prochloron didemni* (a coccoid extracellular symbiont of tropical ascidians) and *Prochlorococcus marinus* (free-living oblong picoplankton) and one freshwater—an unbranched filamentous phytoplankton *P. hollandica* [8]. The latter participates in water blooms in shallow eutrophic lakes in the Netherlands from where it was isolated in 1984 [9]. *Prochlorothrix* was also found in brackish water environment in the eutrophic Darss–Zingst estuary (southern Baltic Sea) [10].

The photosynthetic apparatus of green oxyphotobacteria bears some resemblance to that of chloroplasts. Typical cyanobacteria do not contain chlorophyll *b*, a characteristic pigment of light-harvesting complexes of higher plants and green algae. Cyanobacterial main light-harvesting system is composed of an assemblage of phycobiliproteins, binding a variety of phycobilins, and arranged in large pigment–protein complexes called phycobilisomes. Green oxyphotobacteria lack phycobilisomes, but similar to chloroplasts of higher plants and green algae, they produce intrinsic membrane light-harvesting complexes containing both chlorophyll *a* (Chl *a*) and *b* (Chl *b*) [3]. These chlorophyll molecules are bound to three types of Pcb (prochlorophyte chlorophyll *a*/*b*-binding) antenna proteins

Abbreviations: 2D SDS–PAGE, two-dimensional sodium dodecylsulfate–polyacrylamide gel electrophoresis; Cab, chlorophyll *a*/*b* binding; Car, carotenoid(s); Car/Chl, Car to Chl ratio; Chl *a*, chlorophyll *a*; Chl *a*/*b*, Chl *a* to Chl *b* ratio; Chl *b*, chlorophyll *b*; Chl, chlorophyll(s); CN–PAGE, colorless native–polyacrylamide gel electrophoresis; CP 43, CP 47, core antennae of PS II; D1, D2, reaction center proteins of PS II; DM, *n*-dodecyl-β-D-maltoside; DTT, dithiothreitol; F_m, maximal fluorescence yield; F_v, variable fluorescence yield; F_v/F_m, the ratio of variable to maximum fluorescence after dark adaptation, equivalent to the maximum quantum yield of photosystem II; HL, high light; HPLC, high-performance liquid chromatography; IsiA (CP 43′), iron stress-induced protein A also called CP 43′; kDa, kilodalton(s); LHC I, light-harvesting complex of PS I; LHC II, light-harvesting complex of PS II; LHC, light-harvesting complex; LL, low light; MES, 2-morpholinoethanesulphonic acid; MLL, moderate low light; Pcb, prochlorophyte chlorophyll *a*/*b* binding; Pheo, pheophytin; PMSF, phenylmethylsulphonyl fluoride; PS I, photosystem I; PS II, photosystem II; PS, photosystem; PVDF, polyvinylidene difluoride; SDS, sodium dodecylsulfate; SDS–PAGE, sodium dodecylsulfate–polyacrylamide gel electrophoresis; TM, thylakoid membrane(s); T−S, triplet-minus-singlet spectrum

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(PcbA, PcbB, PcbC) encoded by *pcb* genes. In *P. hollandica*, these *pcb* genes are tightly linked in a single operon and can be probably cotranscribed [11].

Even though the Pcb proteins bind Chl *b*, they are not similar to the Chl *a*/*b* binding proteins of the LHC superfamily (encoded by *cab* genes) that act as outer light-harvesting antennae of plant chloroplast [12,13]. They are structurally and phylogenetically closely related to the IsiA (CP 43′) proteins of cyanobacteria, an iron deficiency-induced Chl *a*-binding antenna protein, and to the CP 43 protein, a Chl *a* inner antenna of photosystem II (PS II) [14,15]. The main dissimilarity among these six-helix trans-membrane proteins (CP 43, IsiA, and Pcb proteins) is in the length of the loop on lumenal side between the 5th and the 6th helixes [16].

To date, no crystal structure of the Pcb proteins has been published. Thus, many issues regarding structural and functional differences among Pcb antennae, as well as multiplication and specialization of Pcbs and their association with photosystems, still remain unresolved.

The two highly homologous *pcbA* and *pcbB* genes encode antenna polypeptides of about 32 kDa and 33 kDa, respectively, and they are more related to the IsiA lineage than the third, divergent, *pcbC* gene. The *pcbC* gene encoding a 38 kDa polypeptide is a result of an independent gene duplication involving an ancestral gene closely linked to CP 43 [11]. This fact can indicate a different function of Pcb proteins in association with photosynthetic complexes and the preceding studies suggest that there are specific Pcb antennae for each photosystem [17–19]. Furthermore, a phylogenetic study of the Pcb proteins and other members of the core complex antenna family (CP 47, CP 43, IsiA) showed that the PcbC/G of *P. marinus* SS120 and PcbC of *P. hollandica* each form a separate cluster with regard to the PcbA and PcbB of *P. hollandica*, respectively [20].

The number of *pcb* genes in a genome is different depending on the species, strain and ecotype. It is known that Pcb proteins in low light-adapted strains of *P. marinus* and *P. hollandica* form a light-harvesting antenna ring around photosystem I (PS I) core complex [18,21], similar to the rings of IsiA subunits around PS I under condition of Fe-deprivation [22,23]. On the other hand, in the high light-adapted ecotype *P. marinus* MED4 such mechanism of the antenna ring formation has not been observed [17].

Here, we present the results of a biochemical study of *P. hollandica* Pcb antennae. We aimed at obtaining information about light-harvesting strategy and the energy redistribution between photosystems. Our work was based on identification and localization of the Pcb proteins. We further tried to assess function of the single types of Pcb antenna. Influence of light conditions on the changes in the content of Pcb antenna proteins during a cell growth was also studied.

Our findings support the suggestion that the PcbA and PcbB antenna proteins function as a mobile unbound antenna of both photosystems in contrast to the PcbC proteins, which are bound to the PS I complex.

2. Material and methods

2.1. LL, MLL, and HL cultivation

Cells of *P. hollandica* SAG 10.89 (CCAP 1490/1) were grown in Erlenmeyer flasks containing 2 l or 5 l of BG-11 mineral medium [24], at 22 °C, under three different regimes of continuous illumination: low light (LL; 1–5 μmol photons m^{-2} s^{-1}), moderate low light (MLL; 20–30 μmol photons m^{-2} s^{-1}), and high light intensity (HL; 200–250 μmol photons m^{-2} s^{-1}), respectively.

2.2. TM isolation

Cells were harvested by centrifugation at 4000×*g* for 4 min and washed in buffer A (25 mM Hepes–NaOH, pH 7.0, 10 mM NaCl). The cells were broken in the buffer A with addition of PMSF (phenylmethylsulphonyl fluoride) at final concentration of 0.1 mM by three passages through a French press at 16,000 psi and then two times at 10,000 psi. Unbroken cells were spun down at 4000×*g* for 5 min. Thylakoid membranes were collected by spin at 60,000×*g* for 30–45 min and stored in the buffer A with 10% glycerol at −75 °C.

2.3. TM solubilization and ultracentrifugation

Membranes with chlorophyll concentration of 1 mg ml^{-1} were transferred into buffer B (25 mM MES, pH 6.5), solubilized with 1% mild detergent *n*-dodecyl-β-D-maltoside (DM) and incubated by mixing in the dark on ice for 20 min. Unsolubilized material was removed by centrifugation at 60,000×*g* for 30 min. The supernatant was loaded onto fresh 0–0.9 M continuous sucrose density gradient prepared by freezing and thawing centrifuge tubes filled with buffer C (25 mM MES, pH 6.5, 10 mM NaCl, 0.02% DM, and 0.45 M sucrose). The following centrifugation was carried out at 4 °C using a P56ST swing-out rotor (Hitachi) at 130,000×*g* overnight.

2.4. Gel filtration

Separate zones from sucrose density gradient centrifugation were harvested with a syringe and concentrated using Amicon filtrating devices with a cutoff of 10 kDa. For further purification and characterization, the zones were loaded onto Superdex 200 HR gel filtration column (Amersham Biosciences). Gel filtration was carried out using buffer D (50 mM MES, pH 6.5, 10 mM NaCl) supplemented with 0.02% DM (wt./vol.). Chromatograms were recorded at 435 nm.

2.5. Electrophoresis

Protein composition was determined by SDS-denaturating gel electrophoresis (SDS–PAGE) on 12.5% polyacrylamide gel [25] containing 6 M urea using Coomassie and/or silver staining.

Pigment–protein membrane complexes were analyzed by colorless native polyacrylamide gel electrophoresis (CN–PAGE) according to Schägger [26] using 4.5–14% linear gradient of acrylamide. During electrophoresis, protein complexes were visible without staining, forming green or orange-yellow bands (depending on the chlorophyll and carotenoid content). Apparent molecular weights were estimated by coelectrophoresis of high molecular weight protein standard (Amersham Biosciences).

For an analysis of separated native protein complexes, second dimension of sodium dodecyl sulfate electrophoresis 2D SDS–PAGE was used. The lane of interest (approximately 0.5×7.5 cm) of the CN–PAGE gel was cut out and incubated at room temperature for 15 min in 3 ml of preheated (50 °C) denaturing solution (25 mM Tris–HCl, pH 7.5, 4% SDS (wt./vol.), 0.1 M DTT (dithiothreitol) and 10% β-mercaptoethanol (wt./vol.)). Thereafter, the strip was briefly rinsed in distilled water to remove an excess of denaturating solution and placed horizontally on top of denaturing 12.5% SDS–PAGE gel [25] for separation of proteins in a second gel dimension. Fixation and staining of proteins in 2D SDS–PAGE gels was done with Coomassie or silver staining protocol. Where needed, 2D SDS–PAGE gels were electroblotted, and Pcb antennae proteins were detected by immunoblot technique.

2.6. Immunoblot analysis

For immunoblot analysis, proteins were separated by SDS–PAGE, transferred onto PVDF (polyvinylidene difluoride) membrane and detected using polyclonal antisera raised against the synthetic peptides of the PcbA, PcbB, and PcbC protein and against peptides 59–76 and 380–396 from the barley D1 and CP 47, respectively.

2.7. Pigment content and fluorescence emission spectra

The content of chlorophylls and carotenoids was determined according to Lichtenthaler [27]. Fluorescence emission spectra were measured at 77K using a Fluorolog-2 spectrofluorometer (Jobin Yvon, Edison, NJ, USA) with an excitation wavelength of 435 nm.

2.8. HPLC

Analysis of pigment composition in thylakoid membranes and seven zones harvested from the sucrose density gradient and purified by gel filtration was carried out by high-performance liquid chromatography (HPLC). The HPLC system consists of Pump Controller 600, Delta 600 injection system, and a PDA 996 detector (Waters, USA) on a reverse-phase ZORBAX ODS column (4.5×250 mm, 5 μm, non-end-capped), using an isocratic elution by a mixture of methanol–acetonitrile (85:15) for 15 min followed by a linear gradient to a mixture of methanol–hexane (4:1) for 2.5 min and an isocratic elution in methanol–hexane until the last pigment was eluted. The flow rate was 1 ml min^{-1}. Ratios of photosynthetic pigment molecules were estimated from areas under corresponding chromatogram peaks displayed at wavelengths according to their extinction coefficients. The molar extinction coefficients ε (dm^3 $mmol^{-1}$ cm^{-1}) were 79.24 and 38.87 for Chl *a* at 665 nm and Chl *b* at 652 nm, respectively [27]. The specific extinction coefficients ($A_\lambda^{1\%}$) were 2710, 2590, and 2480 for α-Car at 445 nm and for β-Car and Zea at 450 nm, respectively [28].

2.9. Differential spectroscopy

Optical differential absorption spectra were measured with a pump-and-probe based instrument described in detail elsewhere [29]. Xenon flash lamps were used for both pump and probe; sample and reference rays were dispersed in an imaging monochromator and detected by two 38-element photodiode arrays. The resolution of the instrument was 2.1 nm. Blue Corning 4-96 filter was used on the pump flash lamp. Cuvettes with 1 cm optical path length were used. By changing the delay between pump-and-probe flashes, kinetics of absorption changes were measured in the range of 3–400 μs. All data points were averages of 20 measurements. Samples were diluted by the buffer D to a maximum absorbance in the Qy region of 0.3–0.8 depending on the quantity of the sample available. For accumulation of reduced pheophytin, the sucrose density gradient zones were mixed with methyl viologen (to a final concentration of 10 μM) and dithionite (1 mg ml^{-1}). The dark spectra were collected after a 10-min dark equilibration. The light spectra were collected after 5 s of actinic illumination (light intensity ~2000 μmol photons m^{-2} s^{-1}).

2.10. Fluorescence yield

The instrument described above was also used for basic chlorophyll fluorescence yield measurements of whole *P. hollandica* cells. Halogen lamp (Fiber-Lite A3200, Dolan-Jenner, USA) giving light intensity of ~2000 μmol photons m^{-2} s^{-1} on a surface of the sample cuvette was used as saturating light; it was switched by a shutter with opening halftime of 0.5 ms (Uniblitz VMD-D1, Vincent Associates, USA). The halogen lamp was filtered by heat-reflecting Calflex (Balzers, Switzerland) filter and Corning 4-96 blue filter. Total fluorescence emission above 665 nm was detected. An undiluted cell culture was dark-adapted for 15 min prior to the fluorescence yield measurement.

2.11. Electron microscopy

Freshly prepared sucrose density gradient zones were immediately used for electron microscopy. The specimen was placed on glow-discharged carbon-coated copper grids and negatively stained with 2% uranyl acetate. Electron microscopy was performed with JEOL 1010 transmission electron microscope (JEOL, Japan) using 80 kV at 60,000× magnification.

3. Results and discussion

At first, we have searched for a connection between the expression of Pcb antennae and the light intensity used during cell cultivation. Cells of *P. hollandica* were cultivated under very low light (LL), moderate low light (MLL), and high light (HL) conditions.

The Chl *a*/*b* ratio was gradually increasing in parallel with the irradiance used during cultivation. The lowest Chl *a*/*b* ratio (7–8) was found in thylakoid membranes of LL-acclimated *P. hollandica*, while the HL culture exhibited the highest Chl *a*/*b* ratio (12–13). The Chl *a*/*b* ratio under the MLL conditions was around 10. This is in contrast to the study of Burger-Wiersma [30] where the reverse trend was observed. However, our results correspond well to the presence of Chl *b* binding Pcb pigment–protein complexes at different growing conditions as described further. We have checked the presence of each type of Pcb antennae in thylakoid membranes isolated from LL, MLL, and HL cultures using antibodies against the PcbA, PcbB, and PcbC proteins. The immunoblot analysis proved the presence of all types of Pcb antenna proteins in thylakoid membranes isolated from LL-acclimated *P. hollandica*; only PcbC antenna protein was detected in MLL-acclimated culture and no Pcb proteins were detected in HL-acclimated cell culture (Fig. 1). Our data indicate that when the intensity of light gradually changes from the high light to the low light conditions, PcbC antennae complexes are expressed first, followed by PcbA and PcbB expression during further deterioration of illumination conditions. The production of Pcb proteins is therefore strongly regulated by light intensity which is a common mechanism of an adaptive response to light-limiting conditions resulting in an increase/decrease in effectiveness of the light-harvesting process.

On the basis of our findings that only at low light-growing conditions all three Pcb antennae proteins are expressed, we have concentrated our following work on the LL culture only.

Pigment–protein complexes of *P. hollandica* grown at low light conditions were resolved into seven color zones (the upper one orange-yellow and the other green) on the sucrose density gradient after centrifugation of *P. hollandica* thylakoid membranes solubilized with DM (Fig. 2A). These zones (referred thereafter as zones 1–7) were harvested and subjected to further biochemical and spectroscopic analyses.

Gel filtration analysis (Fig. 2B) confirmed different sizes of particles in the individual sucrose density zones. Retention times decreased towards zone 7 (13 min); the longest retention time (23 min), indicating a content of the smallest particles, was observed for zone 1. This zone also had (besides the main peak at 23 min) a shoulder at 25 min. Zone 2 had a peak at 21 min with a shoulder at 25 min similar to the one in zone 1. Zones 3, 4, and 5 had peaks with a retention time of about 17.5 min, but only zone 5 had an additional peak at 16 min. Zone 6 had the maximum at 15 min; the peak was,

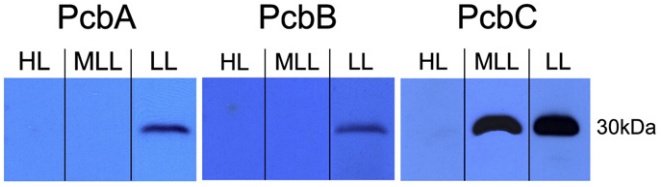

Fig. 1. Immunoblot analysis of Pcb proteins in the thylakoid membranes isolated from high light (HL), moderate low light (MLL), and low light-acclimated (LL) *P. hollandica* cells. Proteins of thylakoid membranes were electrophoresed under denaturing conditions and electroblotted to PVDF membrane. Pcb proteins were immunodetected using polyclonal antisera raised against the PcbA, PcbB, and PcbC proteins.

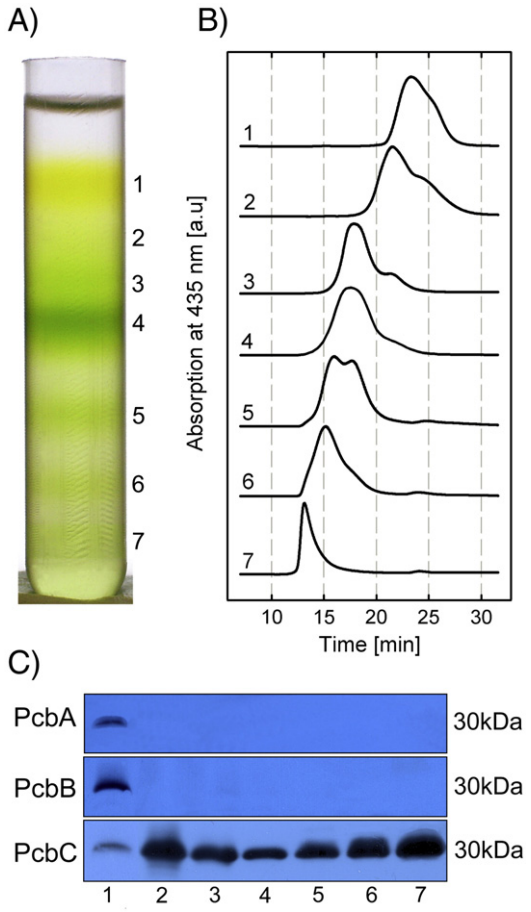

Fig. 2. Biochemical analysis of the pigment–protein complexes of TM from *P. hollandica*. (A) Sucrose density gradient ultracentrifugation of *P. hollandica* thylakoid membranes. The thylakoid membranes were solubilized with mild detergent (DM) and separated on a linear sucrose density gradient into seven zones (1–7). (B) Gel filtration chromatography elution profiles of seven zones (1–7) harvested from sucrose density gradient. Chromatograms were detected at 435 nm. (C) Immunodetection of individual types of Pcb proteins in the seven zones resolved on the sucrose density gradient using antibodies against Pcbs.

however, broadened to the longer retention times with a shoulder at about 17.5 min. Zone 7 had a single peak with retention time of 13 min. All zones exhibited, to some extent, a small peak at long retention times around 25 min.

The retention times from gel filtration chromatography correspond well with the number of bands and relative mobility of pigment protein complexes separated by CN–PAGE (see later in the text).

Using specific antibodies, we have searched for individual types of Pcb proteins in sucrose density gradient zones containing different pigment-protein complexes. The PcbA and PcbB antenna proteins were immunodetected only in zone 1 which contained mainly free pigments. PcbC protein was detected in all seven zones; however, its amount in zone 1 was significantly lower compared to zones 2–7 (Fig. 2C). The absence of PcbA and PcbB proteins in the zones containing photosystem complexes suggests their very loose binding to photosystems. We suppose that these antennae serve as mobile light-harvesting complexes that may be easily decoupled from photosystems and thus regulate energy flow towards photosystems depending on the environmental light conditions.

SDS–PAGE analysis of the seven sucrose density gradient zones is shown in Fig. 3A. Electrophoresis of zone 1 exhibited poor protein separation quality, probably due to high content of carotenoids, lipids, and polysaccharides. Consequently, a weak band of Pcb antennae (close to 30 kDa) was slowed down during electrophoresis and

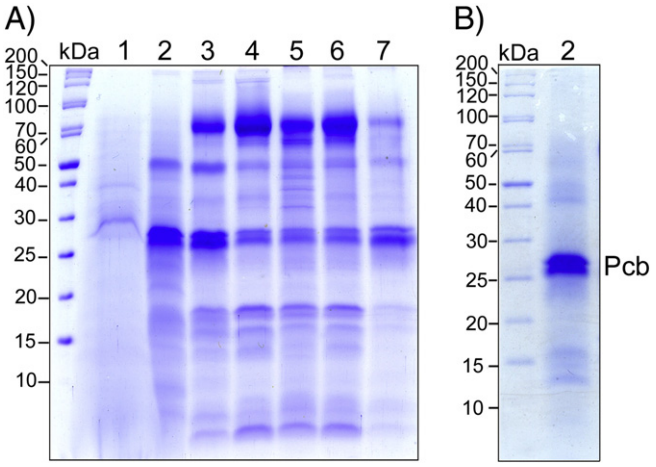

Fig. 3. SDS–PAGE analysis of the pigment-protein complexes of *P. hollandica* fractionated on the sucrose density gradient. (A) Lanes 1–7 represent zones resolved after sucrose density gradient ultracentrifugation of DM solubilized thylakoid membranes of *P. hollandica*. An amount of sample corresponding to 5 μg of Chl per lane was loaded into each well. Proteins were separated on a 12.5% denaturing gel containing 6 M urea and visualized by Coomassie staining. Apparent molecular weights were estimated by coelectrophoresis of a low molecular weight protein standard (Fermentas). (B) SDS–PAGE of zone 2 containing PcbC proteins purified by a gel filtration.

appeared to have higher molecular mass. Some protein bands with molecular mass of more than 50 kDa were also present in zone 1. Two dominant bands located around 27 and 28 kDa and matching the size of Pcb antennae were present in zones 2–7, with the highest content in zones 2, 3, and 7. Zones 3–7 also contained major proteins of the heterodimer PsaA/PsaB of PS I reaction center and low molecular weight proteins with molecular masses of less than 20 kDa, probably small PS I subunits.

Since zone 2 of sucrose density gradient was at first contaminated by diffusely dispersed zone 1, we have further purified it by gel filtration to remove contaminants from zone 1. Subsequent SDS–PAGE protein analysis revealed it was almost pure PcbC protein isolation (Fig. 3B).

The zones of sucrose density gradient were characterized spectroscopically by their chlorophyll *a* to *b* (Chl *a*/*b*) ratio and carotenoids to chlorophyll (Car/Chl) ratio. The lowest Chl *a*/*b* ratio (~4), corresponding to the highest presence of Pcb antenna proteins, was found in zone 2. This ratio is in agreement with other studies on Pcb antennae [31,32]. The highest Chl *a*/*b* ratio (~10), indicating low Pcb content, was detected in zone 6. Zone 7 had the value of Chl *a*/*b* ratio of about 7. The decrease of the ratio in zone 7 compared to zone 6 corresponded to the presence of PS I supercomplexes with attached Pcb antennae. Regarding the Car/Chl ratio, the highest value (~0.55) was detected in the upper orange-yellow zone 1; the other zones exhibited about 4 times lower ratios.

The pigment composition of TM and of sucrose density gradient zones was determined by reverse-phase HPLC. The chromatogram of pigments extracted from TM (Fig. 4) consisted of zeaxanthin, chlorophyll *b*, chlorophyll *a* and three carotene peaks: β-carotene, *trans*-α-carotene, and *cis*-α-carotene. The molar ratio of the photosynthetic pigment molecules in the thylakoid membranes was calculated to be 22 (Chl *a*) : 3 (Chl *b*) : 3 (Zea) : 2 (β-Car) : 1 (α-Car). The Chl *a*/*b* ratio determined by HPLC corresponded to the Chl *a*/*b* ratio obtained spectroscopically [27].

The pigment molar ratio of purified PcbC antennae (zone 2) was calculated to be 17 (Chl *a*) : 4 (Chl *b*) : 1 (Zea) : 2 (β-Car) : 1 (α-Car). In comparison, IsiA protein binds 16 Chl *a* molecules, 1 Zea molecule, 2 β-Car molecules, and 1 echinenone molecule [33]. The number of chlorophyll molecules in Pcb protein is a little higher compared to IsiA and CP 43 [34]; however, in the case of IsiA, the carotenoid content of

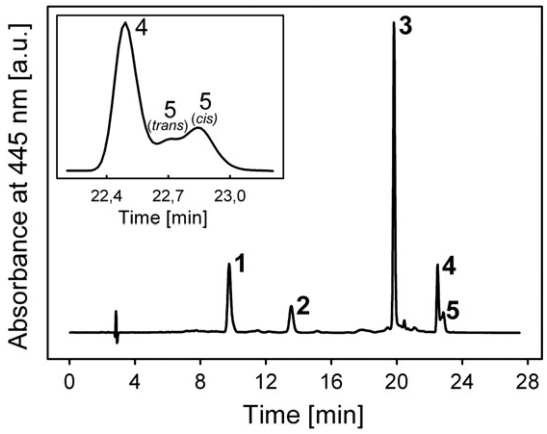

Fig. 4. HPLC chromatogram of pigment extracted from *P. hollandica* thylakoid membranes, showing zeaxanthin eluted at ~10 min (peak number 1), chlorophyll *b* at ~13.5 min (2), chlorophyll *a* at ~20 min (3), β-carotene at ~22.5 min (4), and α-carotene at ~22,9 min (5). Inset shows detailed resolution of the carotene elution region, two isoforms of α-carotene: *trans*-α-carotene at ~22.7 min (*trans* 5) and *cis*-α-carotene at ~22.9 min (*cis* 5) are visible. Detection wavelength was 445 nm.

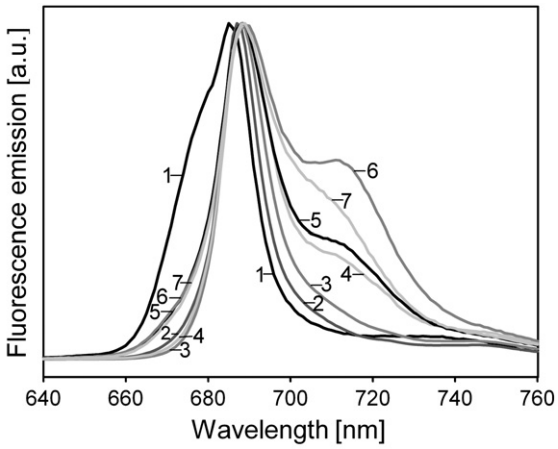

Fig. 5. 77K fluorescence emission spectra of the sucrose density gradient zones. Spectra were excited at 430 nm and normalized to their fluorescence maxima.

PcbC differs only in the presence of the molecule of α-Car instead of echinenone.

Regarding sucrose density gradient zones, the pigment composition differed significantly only in the case of zone 1, where zeaxanthin was the dominant pigment (about 66% of the total pigment content). The high content of zeaxanthin in zone 1 may originate from cytoplasmic membrane "S-layer" on the outermost surface of cells. In *P. marinus*, a notable portion of zeaxanthin was found associated with thylakoid fractions, and after fractionation, most of it was generally retrieved in the uppermost bands of sucrose density gradient [35]. Large part of zeaxanthin pool was previously found associated with water-soluble acidic 56 and 58 kDa proteins, which can represent up to 3% of the total cellular proteins under HL conditions [36]. Our silver-stained SDS–PAGE gel of zone 1 shows a band with an apparent molecular weight of ~55 kDa (data not shown). It is possible that the carotenoid-binding complexes were copurified alongside thylakoid membranes but an abundant RuBisCo is also a common contaminant in thylakoid fractionation. Zeaxanthin content in other zones ranged from 2% to 4% of total pigment content.

Besides zeaxanthin and β-carotene as the major carotenoids, we have identified both *cis* and *trans* isomer of α-carotene in thylakoid membranes (see inset of Fig. 4) and in all sucrose density gradient zones, in almost constant proportion to β-carotene. In *P. marinus*, α-carotene is one of the main carotenoid pigments [35], whereas no α-carotene was previously detected in *P. hollandica* [37]. The presence of α-carotene suggests that not only *P. marinus* but also *P. hollandica* possesses the lycopene ε-cyclase, key enzyme of the α-carotene and lutein biosynthesis pathway, absent in other cyanobacteria [38]. The relative amount of α-Car was about 3/4 of the β-Car content in zone 1 and about 1/2 in other zones of sucrose density gradient.

The low-temperature fluorescence emission spectra of all zones (Fig. 5) revealed only one emission peak at 686–688 nm in zones 1, 2, and 3. In zone 1, a shoulder at 680 nm was present, corresponding to a bulk of free chlorophylls. Zones 4, 5, 6, and 7 showed two peaks, at 688 nm and at 714 nm, where the long-wavelength emission characterizes a spectral form of PS I [18,31]. Relative to the 688 nm emission (distinct in all four zones), the 714 nm peak was most pronounced in zone 6 and was least apparent in zone 4.

The yield of chlorophyll triplets expressed as amplitude of the 672 nm chlorophyll bleaching in individual zones is shown in Fig. 6. The triplet yield gradually decreased from zone 2 towards zone 6 and then increased in zone 7. The yields of carotenoid triplet showed similar trends (data not shown). Overall trend of triplet yields in individual zones is therefore very similar to the Chl *a*/*b* ratio trend which suggests that the observed T − S spectra originate mainly in the Pcb antennae. Maximum triplet yield observed in zone 2 supports our interpretation that this zone contains mostly pure Pcb antennae. The increase of triplet yield in zone 7 versus zone 6 also confirms that zone 7 contains more Pcb antennae than zone 6.

The ratios of carotenoid versus chlorophyll signal in T − S spectra were very similar in zones 2–7 (~2) which confirms that the triplets are formed in the same intact structure. Identical relaxation properties of chlorophyll and carotenoid triplets in zones 2–7 further assert that PcbC antennae in these zones have not been damaged by the isolation procedure. The relatively high yield of Chl and also Car triplets in zone 1, as well as a low Car/Chl triplet ratio of 0.8 in this zone, may be attributed to the mixture of some Pcb antennae and free pigments.

The inset of Fig. 6 shows a triplet-minus-singlet (T − S) difference absorption spectrum of zone 2, measured 3 μs after actinic flash. In the blue region, the spectrum had positive peaks at 480 and 520 nm and a negative peak at 425 nm and corresponds well with known T − S spectra of other photosynthetic antennae (e.g., LHC I by [39] or LHC II by [40]). The blue region of T − S spectrum indicates that the triplet state of carotenoid was formed by an actinic flash. In the red region, characteristic bleaching of chlorophyll was visible at 672 nm. This bleaching showed the presence of chlorophyll molecules which are in the triplet state following the actinic flash.

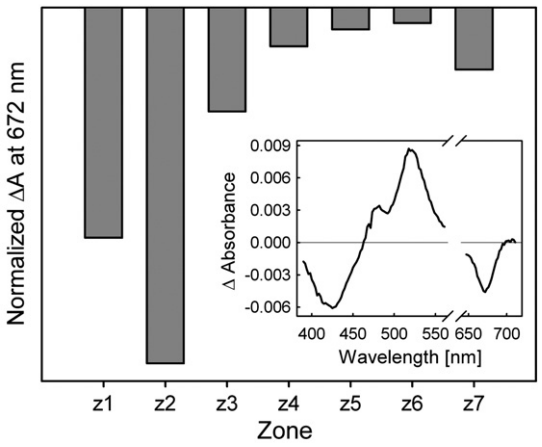

Fig. 6. Normalized yield of chlorophyll triplet state from sucrose density gradient zones and T − S spectrum of zone 2 (inset). Absorbance changes at 672 nm detected 3 μs after actinic flash were normalized by sample absorption at 673 nm. Inset: T − S spectrum of zone 2 detected 3 μs after actinic flash, average of 20 repetitions is shown, this sample had $A_{673} = 0.31$.

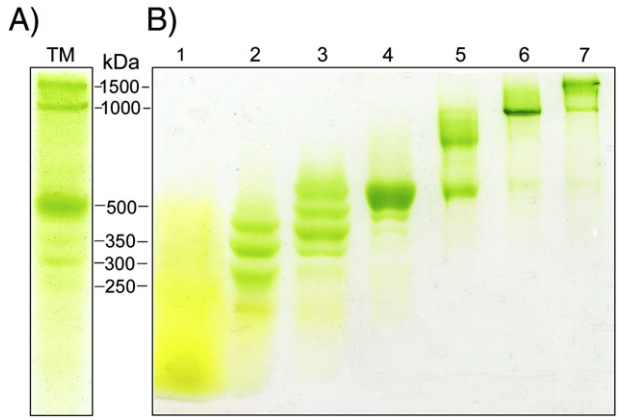

Fig. 7. CN–PAGE analysis of the pigment-protein complexes of *P. hollandica* fractionated on the sucrose density gradient. (A) Colorless native electrophoresis of pigment–protein complexes of thylakoid membranes from *P. hollandica*. Thylakoid membranes were solubilized with DM in 1:40 (Chl/DM; wt./wt.) ratio. (B) CN–PAGE of pigment–protein complexes fractionated by sucrose density gradient centrifugation. Pigment–protein complexes were separated on a 4.5–14% gradient non-denaturing polyacrylamide gel. Apparent molecular weights were estimated by coelectrophoresis of a high molecular weight protein standard (Amersham Biosciences). An amount of sample corresponding to 10 μg of Chl per lane was loaded.

The carotene- and chlorophyll-originated peaks of Fig. 6 inset (525 and 672 nm, respectively) had identical kinetic properties, relaxing with biexponential kinetics with characteristic times of 1.3 and 8.4 μs. Approximately 80% of the absorbance change relaxation is explained by the faster component.

Using CN–PAGE, thylakoid membranes were separated into six green bands with apparent molecular masses of 1500, 1000, 500, 350, 300, and 250 kDa, respectively (Fig. 7A). CN–PAGE of individual zones from sucrose density gradient is shown in Fig. 7B. It is obvious that the green bands separated from thylakoid membranes appear also in the individual zones. On top of that, two green bands with molecular mass of about 800 and 900 kDa appeared in zone 5.

CN electrophoresis of zone 1 (Fig. 7B) always resulted in an orange-yellow "smear" in the whole line without any visible bands. This uppermost sucrose density gradient zone consists of free pigments (mainly carotenoids) trapped in detergent micelles, PcbA and PcbB antennae, traces of PcbCs and probably carotenoid-binding proteins and lipopolysacharides [36].

To further characterize complexes separated by sucrose density gradient followed by CN–PAGE, we have combined these methods with second dimension electrophoresis and immunoblotting; the results are summarized in Fig. 8.

Zone 2 was separated by CN–PAGE into three green bands with apparent molecular masses of about 350 kDa, 300 kDa, and 250 kDa, respectively, and one orange-yellow band of about 165 kDa. Resolution of the three green bands on the second gel dimension (Fig. 8A) revealed very abundant bands at 27 and 28 kDa, representing Pcb antenna proteins. Only PcbC antenna proteins were detected in these three green bands (350 kDa, 300 kDa, and 250 kDa) by immunoblotting. Using electron microscopy and negative staining of zone 2 protein content, we were able to observe particles of the Pcb antenna in different oligomeric state with different size and shape (not shown). This strongly supports the interpretation that the CN–PAGE green bands at 350 kDa, 300 kDa, and 250 kDa are composed of different oligomeric states of PcbC antennae protein complexes. The lowest orange-yellow band (165 kDa) on CN–PAGE gel of zone 2 dissociated on 2D SDS–PAGE into several small proteins with molecular masses of less than 23 kDa.

CN–PAGE of zone 3 yielded five green bands in the size range of 500 to 300 kDa. In the second dimension, zone 3 comprised of small portion of PS I monomers (CN–500 kDa), and antenna oligomers of PcbC similar as in zone 2 (CN–350–250 kDa). 2D SDS–PAGE/immunoblotting evidenced that the PcbC proteins are not associated with PS I monomer in this zone, and that the PcbC oligomers are copurified with PS I monomers by sucrose density gradient (Fig. 8B). It appears that zone 3 contains a mixture of zones 2 and 4 content.

Zone 4 was much more enriched with monomeric PS I (CN–500 kDa) with only traces of PcbC antennae oligomers compared to zone 3. Similar to zone 3, PcbC complexes of zone 4 are not bound to the PS I here (Fig. 8C).

In zone 5, the 500 kDa band was accompanied with a 800 kDa band and two minor bands around 900 kDa and 1000 kDa. As shown by 2D SDS–PAGE/immunoblot analyses in Fig. 8D, zone 5 is a mixture of PS I monomers (CN—500 kDa) with a small number of Pcb subunits and probably higher oligomeric states of PS I with attached Pcb proteins (CN—800–900 kDa) and antenna-free PS I trimers (CN—1000 kDa). Negatively stained electron micrographs confirmed that zone 5 is a mixture of several types of complexes. Particles of PS I monomers and pieces of loose Pcb antennae in a rod-like and hooped shapes with a length of 20–40 nm were the most abundant particles. The electron microscopy images also contained particles that resemble PS I monomers and higher oligomeric state of PS I with pieces of attached Pcb proteins and a few PS I trimers (not shown).

CN electrophoresis of zone 6 revealed only one intense focused band (CN—1000 kDa), pointing to a presence of relatively pure trimeric PS I with minimal contamination by free Pcb subunits and monomeric PS I (Fig. 8E).

Zone 7 corresponds to trimeric PS I complexes surrounded by a ring of 18 subunits of the PcbC proteins (CN—1500 kDa) as described by Bumba et al. in a previous study [18]. Nevertheless, the presence of CN—1000 kDa band on the CN electrophoresis shows a relatively high content of antenna-free PS I trimers (Fig. 8F).

The presence of various complexes in the sucrose density gradient zones, such as PS I monomers and trimers with no or little PcbC subunits attached or the presence of PcbC oligomers, may be a consequence of the detergent solubilization procedure used, which, although very mild, may have affected the supramolecular structure of the thylakoid membrane complexes. However, an interesting recent study has revealed that IsiA can assemble into numerous types of single or double rings surrounding PS I [41]. PS I monomers with double IsiA rings and also PS I-free IsiA complexes were described, implicating high variability of the IsiA–PS I interactions. Similarly, another study [42] showed that in a freshwater cyanobacterium *Synechocystis*, the trimeric conformation of PS I is not required for the IsiA proteins to functionally couple with this photosystem. Based on the structural similarity between IsiA and Pcb proteins, it is possible that Pcb proteins can assemble in a similar way and form diverse structures of PcbC oligomers or various types of PcbC–PS I complexes, as we have observed in the zones 2, 3, 4, and 5. Detection of PS I monomers coupled with PcbC antennae may also imply that the link between PcbC and PS I is fairly strong and may persist even after the PS I monomers are released.

Detection of PS II in cell cultures of *P. hollandica* during our work proved itself to be difficult. We have checked the state of PS II in whole cells of *P. hollandica* grown at low light by measuring variable chlorophyll fluorescence yield. The detected F_v/F_m ratio, during a multiple turnover saturating flash, of 0.74 indicates the presence of functional PS II complexes. However, when searching for active PS II complexes in sucrose density gradient zones by the method of reduced pheophytin accumulation, we were not able to detect any absorption changes attributable to the accumulation of the $Pheo^-$. This indicates that the presence of active PS II particles in zones 1–7 after solubilization and sucrose density gradient was probably under the detection limit of this method.

No clear bands that correspond to PS II proteins were present in SDS–PAGE gels of the sucrose density gradient zones. We have also searched for PS II by immunoblotting with antibodies reactive with D1

and CP 47 proteins. D1 was found in the thylakoid membranes and zones 6 and 7, with slight reaction in zone 5 (data not shown), whereas CP 47 was not detected in isolated thylakoids or in any of the sucrose density gradient zones. As the used antibody was raised against the barley CP 47, the most probable explanation for this result is its inability to react with the protein from *P. hollandica.*

We were able to detect some PS II proteins in 2D SDS–PAGE gels of *P. hollandica* thylakoid membranes and compare them with gels from

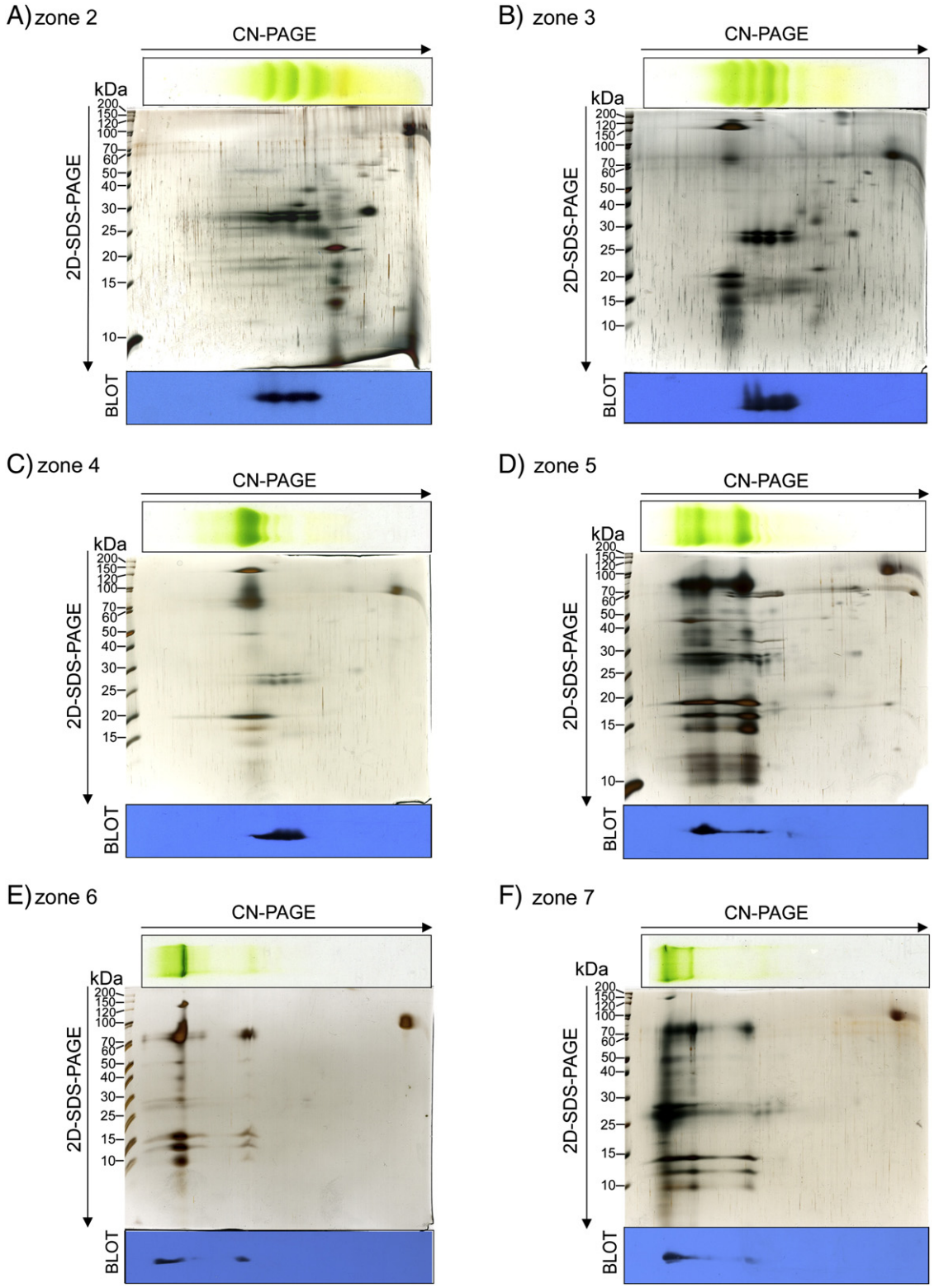

Fig. 8. Two-dimensional protein analysis of the pigment–protein complexes of *P. hollandica* and localization of the Pcb antenna proteins. CN/SDS–PAGE/immunoblot analysis of pigment–protein complexes of sucrose density gradient zones 2–7 (A–F). TM membranes solubilized with DM were resolved on linear sucrose density gradient and pigment-protein complexes were separated on a 4.5–14% CN–PAGE gel. Lanes of interest were cut out from the gel, incubated with solubilization buffer, rotated 90° counterclockwise, and run on a 12.5% SDS–PAGE gel in the second dimension. Thereafter, 2D SDS–PAGE gels were electroblotted onto PVDF membrane and a position of PcbC antenna proteins was confirmed with antibodies.

a mutant of *Synechocystis* without PsbO and PsbV proteins which is characterized by depletion in PS II complexes (data not shown). Thylakoid membranes were solubilized with 1% DM as in the case of the sucrose density gradient preparation. Traces of all four characteristic PS II proteins (CP 43, CP 47, D1, and D2) were detectable on 2D SDS–PAGE gel of *P. hollandica* in the region of the high molecular weight complexes (CN—1500 kDa), however, in much lower amount compare to PS II complexes in the mutant of *Synechocystis*. Further, a slight amount of PS II monomer was visible alongside PS I monomer (CN—500 kDa), and a little below some free RC 47 (PS II without CP 43) were also detected. This is consistent with the data obtained by immunoblotting of the sucrose gradient zones, that PS II is present in the region of supercomplexes with high molecular masses, zones 6 and 7, and to some extent also zone 5.

In our studies, we have used a standard method of solubilization with mild detergent dodecyl maltoside, which was successfully applied for fractionation of thylakoid membranes from the other two green oxyphotobacteria species [35,43]. In previous studies of van der Staay et al. [32], PS II fractions were isolated from *P. hollandica* using Zwittergent as the detergent. However, even with this method, we were not able to isolate any PS II fraction from our cells. Thus, it is highly probable that PS II was not degraded during our isolation procedure and that its negligible presence in our preparations is due to its low expression in our growing conditions. Burger-Wiersma et al. [30] observed an increase in the relative PS I to PS II ratio in LL-grown cells. As we were using LL-grown cells in most of our analyses, the problems with the PS II detection were most likely caused by an extremely low content of PS II in the analyzed thylakoids.

Previously, two different models of the thylakoid membranes organization in *P. hollandica* were proposed [32,44–47] that present opposing views on the organization and function of antennae complexes. The studies by van der Staay et al. [32,44] showed the major Pcb antenna to be associated with PS II while no antenna were found associated with PS I.

On the basis of these data, van der Staay et al. have proposed a model in which under dark, non-phosphorylated conditions (state 1) all Pcb proteins are assembled into one antenna complex associated with PS II. Under high light conditions (state 2), the 38 kDa antenna, presumably PcbC, gets phosphorylated and is subsequently uncoupled from the rest of antenna complex to protect PS II from overexcitation. According to their fluorescence data, the phosphorylated 38 kDa antenna does not migrate into the unstacked membranes but remains in the grana membranes.

The model published by Post et al. [46,47] claims that in state 1 the bulk of Pcb antennae, composed largely of the 30 and 35 kDa proteins, is shared by both photosystems in the form of supercomplex. Upon phosphorylation (state 2) of the major 35 kDa antenna protein, the bulk antenna is decoupled from PS II centers which are thereby detached from the major light-harvesting apparatus and the energy transfer to PS I is enhanced. The model predicts that a minor 33 kDa Pcb antenna, that copurifies with PS II, serves as a light-harvesting antenna to PS II and major antennae 30 and 35 kDa proteins associate predominantly with PS I.

We have found PcbC–PS I supercomplex to be the major photosynthetic supramolecular complex of *P. hollandica*. We have also detected traces of complete PS II in the region of high molecular complexes together with PcbC–PS I supercomplex. By comparison of our findings with the previous models of Pcb antenna organization, we suggest that the major antenna of light-harvesting system of *P. hollandica* is the PcbC antenna. We cannot exclude that it could be also the shared antenna that serves both photosystems as described in Post et al. model. However, on the basis of our data, we cannot postulate that the PcbC antennae are attached to the PS II.

In our mild detergent preparation, we have found PcbA and PcbB antennae only in uncoupled state, mainly copurified together with free pigments. This finding indicates that PcbA and PcbB proteins are very loosely bound antennae, which can probably serve as auxiliary light-harvesting complexes for both photosystems complementary to the major PcbC antennae. It is also possible that one or both PcbA and PcbB antennae can serve as preferable antenna for PS II and that they are much loosely coupled to the PS II than are PcbC antennae to the PS I trimers.

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