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Association of chlorophyll *a/b*-binding Pcb proteins with photosystems I and II in *Prochlorothrix hollandica*

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Abstract

Action spectra for photosystem II (PSII)-driven oxygen evolution and of photosystem I (PSI)-mediated H_2 photoproduction and photoinhibition of respiration were used to determine the participation of chlorophyll (Chl) *a/b*-binding Pcb proteins in the functions of pigment apparatus of *Prochlorothrix hollandica.* Comparison of the *in situ* action spectra with absorption spectra of PSII and PSI complexes isolated from the cyanobacterium *Synechocystis* 6803 revealed a shoulder at 650 nm that indicated presence of Chl *b* in the both photosystems of *P. hollandica*. Fitting of two action spectra to absorption spectrum of the cells showed a chlorophyll ratio of 4:1 in favor of PSI. Effective antenna sizes estimated from photochemical cross-sections of the relevant photoreactions were found to be 192±28 and 139±15 chlorophyll molecules for the competent PSI and PSII reaction centers, respectively. The value for PSI is in a quite good agreement with previous electron microscopy data for isolated Pcb–PSI supercomplexes from *P. hollandica* that show a trimeric PSI core surrounded by a ring of 18 Pcb subunits. The antenna size of PSII implies that the PSII core dimers are associated with ~14 Pcb light-harvesting proteins, and form the largest known Pcb–PSII supercomplexes.

Keywords: Photosystems I and II; Pcb protein; Action spectra; Antenna size

1. Introduction

The oldest group of oxygenic phototrophs, the cyanobacteria, perform photosynthesis by the tandem cooperation of two types of reaction centers, which generate photopotentials of oxygen and hydrogen electrodes in photosystem II (PSII) and photosystem I (PSI), respectively, and ensure a circuit from the unlimited electron source water to a low potential reductant for CO_2 fixation. The core complexes of the two photosystems were greatly conserved during the evolution of oxygenic photosynthesis in eukaryotes, algae and higher plants. In contrast, the composition and design of light-harvesting antenna complexes of PSI and PSII in prokaryotes and eukaryotes are dissimilar and flexible [1,2]. As light-harvesting antenna, the majority of cyanobacteria employ phycobilisomes, which are giant extramembrane pigment–protein complexes on the stromal side of thylakoids [3–5]. However, a few species of phycobilisome-lacking oxyphotobacteria contain as a substitute the intrinsic antenna complexes of chlorophyll (Chl) *a/b*(*d*)-binding light-harvesting Pcb proteins [6–9] that are related to CP43 subunit of PSII, along with the IsiA proteins induced in some phycobilisome-containing cyanobacteria in response to depletion of iron. The mixed group of Pcb-containing oxyphotobacteria includes species of ascidian simbionts *Prochloron didemni* and *Acaryochloris marina* (an epiphytic [10] and free-living [11] strains of *Acaryochloris* were recently also found), a freshwater filamentous species *Prochlorothrix hollandica* and several distantly related strains of the most abundant picoplanktonic photoproducers of global significance, *Prochlorococcus*.

The general structure of the photosynthetic apparatus of Pcb-containing oxyphotobacteria has been elucidated mainly by electron microscopy and single particle image analysis of isolated pigment–protein complexes [12–17]. The obtained

Abbreviations: PSI and PSII, photosystems I and II; Chl, chlorophyll

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data, together with genomic analysis, have shown that, depending on the different *pcb* genes content and expression (particularly, modulated by light adaptation and availability of iron in the medium), different species form either an 18-subunit circle of Pcb complexes around the PSI trimers [12,13,16,17], similarly to the IsiA–PSI supercomplex [18–20], or two of 4–5-subunit arcs of Pcb complexes flanking the PSII dimers [13–15].

A recent electron microscopy study of isolated pigment–protein complexes from a filamentous cyanobacterium *Prochlorothrix hollandica* [17] visualized the PcbC–PSI supercomplexes but, unlike to the low-light-adapted strain *Prochlorococcus* CC120 [13], no evidence for association of the PcbA and PcbB proteins with PSII was obtained despite their presence in the photosynthetic membranes of the species. This is also evidenced by conflicting results of earlier studies of the organization of photosynthetic apparatus in *P. hollandica* that argued for interaction of Pcb antenna complex either solely with PSII [21,22] or mainly with PSI [23–25]. It should be kept in mind, however, that the quality of biochemical procedures and preparations is of crucial importance in interpreting the reported data, and restricts clarification of the native organization of photosynthetic apparatus.

To resolve these discrepancies, we present here the results of *in situ* functional analysis of photosynthetic apparatus in *P. hollandica* by action spectra and photochemical cross section measurements of partial reactions of photosynthesis that reveal the association of Pcb antenna with both PSI and PSII. Previously, the same approach was used for the study of different photosystems [26] and, particularly, for the first time showed the presence of additional antenna complex in PSII of *A. marina* [27] which was lately isolated and identified as a Pcb protein [28,15].

2. Materials and methods

2.1. Cultures and growth conditions

4–5 days cultures of *Prochlorothrix hollandica* (PCC 9006) were grown in BG11 medium modified by $FeSO_4$ substitution for ferrous citrate, and a five-fold decrease in $NaNO_3$ content [29]. Culture was maintained at 18 °C in 50 ml glass vessels under continuous illumination of ~20 μmol quanta m^{-2} s^{-1} from white fluorescence lamps. Several experiments were also performed with the cultures grown at ~5 and 150 μmol quanta m^{-2} s^{-1}.

2.2. Photochemical activity measurements

Polarographic measurements of the flash-induced gas exchange reactions related to both photochemical activity of PS II (O_2 evolution) and PS I (photoinhibition of aerobic respiratory O_2 uptake and anaerobic H_2 photoproduction), their action spectra and photochemical cross-sections were performed as previously described [30,27]. A bare platinum electrode at a potential −0.7 or 0 V vs. Ag/AgCl reference electrode was used to measure photoinduced O_2 and H_2 gas exchange rates, respectively. A sample of 20 μl cell suspension, with optical density of 0.1–0.2 at 675 nm and Chl equivalent layer density of 1.3–2.6 μg cm^{-2}, was placed on a 6 mm platinum disc in a groove of 0.65 mm depth, covered by a cellophane membrane forming the assay microchamber. The surrounding chamber of ~30 ml with a reference Ag/AgCl electrode was filled up by a solution of 50 mM KC1 and 50 mM Na-phosphate buffer, pH 7.0. The samples in the setup were illuminated by one or two of the three kinds of light sources: a monochromator, a "cool light" projection system through interference or cut-off filters with a fiber-optic illuminator OVS-2 (Russia), and an ISS-100-3M (Russia) xenon flash tube generating 1.8 μs flashes through a range of interference filters. The intensities of the light beams on the electrode were varied by neutral density filters and measured by a calibrated thermoelement, RTN-20S (Russia). O_2 evolution upon actinic light was usually recorded in the presence of a continuous illumination at >700 nm from the fiber-optic illuminator that ensured sufficient PSI activity for saturation of photoinhibition of respiration. As the individual characteristics, photoinhibition of respiration was recorded in the same sample after addition of 10 μM diuron. H_2 photoevolution measurements were carried out under anaerobic conditions after 2 h dark adaptation of cells in the medium equilibrated with pure argon.

The action spectra measurements were carried out using cycles of intermittent 1 s illumination followed by 20–60 s dark period between the successive wavelengths at 2.5–10 nm intervals. The spectral half-width of the monochromatic beam was 1–6 nm. Its intensity varied in the ranges 0.1–0.25 nE cm^{-2} s^{-1} in the spectral range of 400–720 nm. Action spectra were corrected for any nonlinearity of the light energy-dependent curves by estimation of the energies at different wavelengths producing the same photochemical yields as at a control wavelength, usually at 675 nm. Correction for time-dependent changes during spectral scanning was routinely introduced by measuring the ratio of signals at the given wavelength versus the signal at 675 nm. The spectra were corrected also for the spectral coefficient of light reflection by platinum surface through the settled cells.

Absorption spectra were measured on a Specord M40 (Germany) spectrophotometer. Chlorophyll concentrations in dimethylformamide extracts of cells were determined from the equations of Porra et al. [31].

2.3. Effective antenna size measurements

Photochemical cross-sections of PS II and PS I were measured from the energy-saturation characteristics of O_2 and H_2 photoevolution (or photoinhibition of respiration) upon the single-turnover flashes according to the method described earlier [32,26]. A xenon flash lamp (ISS-100-3M, Russia) provided white light saturating flashes (1.8 μs and 5 μs at one-half and one-tenth peak intensity, respectively) which were attenuated by calibrated sets of neutral density filters to determine the saturation behavior of PS-specific signals. The flashes were spaced 10 and 60 s apart during the O_2 evolution (under continuous background illumination of a weak light >700 nm) and H_2 photoproduction (or photoinhibition of respiration) measurements, respectively. Monochromatic light curves at 596 and 625 nm at intensities below saturation were measured with interference filters (Karl Zeiss, 10-nm half-band width) and normalized to the white light saturation curves to convert the relative white flash energy scale into an absolute monochromatic energy scale using a calibrated thermoelement (RTN-20S, Russia). At 596–625 nm, the spectral region of low absorption and rather close values of Chl *a* and Chl *b* absorbance, the cross-sections of photochemical reactions correspond to the number of Chl (*a*+*b*) molecules per one reaction center.

Effective antenna sizes of the photosystems were calculated from the photochemical cross sections of partial reactions using the in situ values of an optical cross section for a single chlorophyll molecule, 0.30±0.01 (596 nm) and 0.55±0.02 $Å^2$ (625 nm), estimated in the isolated PS I and PS II core complexes from *Synechocystis* PCC 6803 [33–35] and *Thermosynechococcus elongatus* [36].

3. Results

3.1. Energy migration from the Pcb antenna to PSI and PSII

The examined cultures of *P. hollandica* consistently showed a low content of Chl *b* in the cells with a Chl *a*/Chl *b* ratio of 11.5±2 (Fig. 1), which was not appreciably changed by our light growth conditions (cf. [37]). This might complicate spectral analysis of a contribution of a low Chl *b*-containing Pcb antenna to photosystems I and

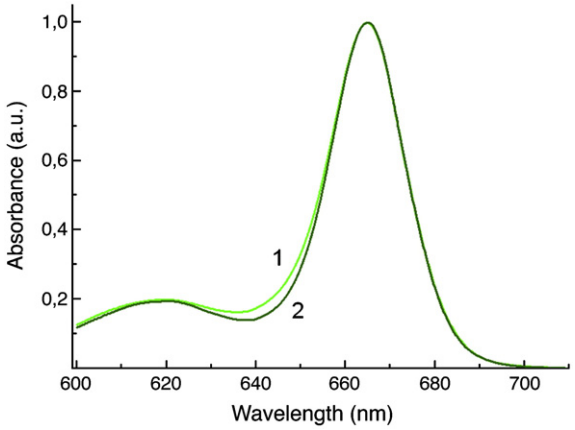

Fig. 1. Absorbance spectra of chlorophyll extracts in *N,N′*-dimethylformamide from cells of *P. hollandica* (1) and *Synechocystis* PCC6803 (2). The spectra are normalized to the amplitudes at 665 nm. For the Pcb-containing species, a calculated Chl *a*/Chl *b* ratio was ~12.

II. Thus, for a better resolution of the Chl *b* bands in the action spectra, we used as references the absorbance spectra of isolated PSI trimer and PSII core complexes from *Synechocystis* 6803 recorded in the previous studies [33–35].

The *in situ* photochemical activities of partial reactions of photosynthesis in *P. hollandica* were conveniently monitored by the PSII-driven O_2 evolution and PSI-mediated photoinhibition of respiration (in the presence of 10 μM diuron) or anaerobic H_2 photoproduction, like in other oxyphotobacteria [27,35,38]. Capabilities for H_2 photoproduction have been reported for many photosynthetic microorganisms [39,40], but was found for the first time in *P. hollandica*. This is not unexpected in light of the earlier established ability of the species to perform sulfide-dependent anoxygenic photosynthesis [41], and the presence of the gene for a bidirectional FeNi-hydrogenase in its genome (Genbank accession number AAB53705). However, the maximum rate of H_2 photoproduction was very low, ~1% of the light-saturated photosynthetic O_2 evolution, like in many cyanobacteria [39,40].

In Fig. 2, the *in situ* action spectra of the partial reactions of photosynthesis in the *P. hollandica* cells are compared with the absorption spectra of the Chl *a*-only-binding complexes of PSI and PSII from *Synechocystis* 6803 in the spectral region of 580–720 nm. The data clearly show a marked contribution of energy migration from Chl *b* (a shoulder at 650 nm) and a Chl *a*-670 spectral form to PSII of *P. hollandica* (Fig. 2A). These pigments obviously belong to a Pcb antenna complex of PSII, and their absorbance maxima are shifted to shorter wavelengths by ~3 nm relative to the core complex. The Chl *a*/Chl *b* ratio of ~5 in the Pcb–PSII supercomplex can be roughly estimated from the relative height of Chl *b* band in the action spectrum.

A contribution of the Pcb antenna is also visible in the PSI action spectrum of *P. hollandica* (Fig. 2B) as a small increase of photochemical efficiency around 650 and 670 nm relative to the absorption spectrum of isolated PSI trimer complex. The lesser pronounced contribution of the Pcb antenna in PSI compared to PSII is explained mainly by the ~2.6 times larger core antenna of the former. Thus, the Chl *a*/Chl *b* ratio in the Pcb–PSI supercomplex roughly estimated from the contribution of Chl *b* to action spectrum should be at ≥10.

To determine the proportions of chlorophyll in each photosystem, the sum of action spectra of PSI and PSII was fitted to the absorption spectrum of whole cells of *P. hollandica* (Fig. 3) by a regression analysis of the least-squares method. Like in other cyanobacteria [35,38], the dominating part of Chl in *P. hollandica*, ~80%, is shared by PSI complexes and only ~20% belongs to PSII. The large excess of PSI units, as well as a possible presence in the membranes of a PSI fraction without Pcb antenna, adequately explains the low Chl *a*/Chl *b* ratio in the whole cells.

3.2. Effective antenna sizes of the Pcb–PSI and Pcb–PSII supercomplexes in situ

With a one-turnover flash illumination, the experimentally determined values of photochemical cross sections for the PSII-driven O_2 evolution in *P. hollandica* were 42±5 and 76±8 Å^2 (n=7) at 596 and 625 nm, respectively. For the PSI-mediated photoinhibition of respiration (n=6) and anaerobic H_2 photoproduction (n=2), the average values were 57±9 and 101±15 Å^2 at 596 and 625 nm, respectively. Using the

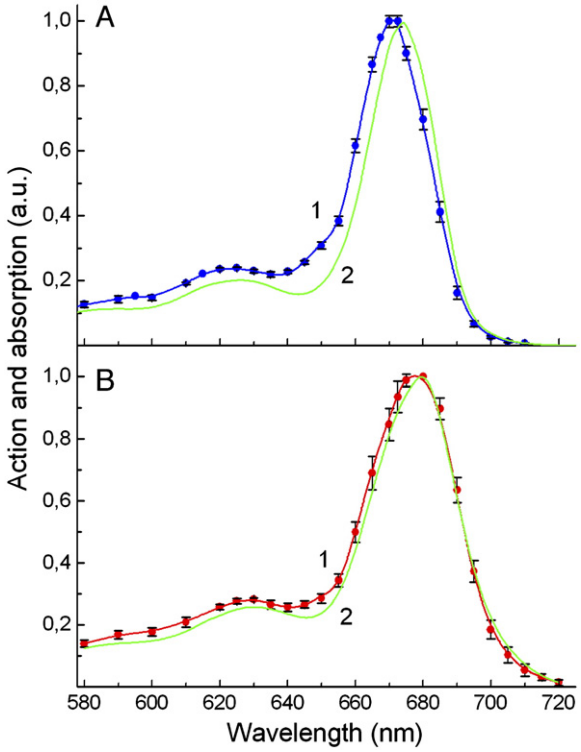

Fig. 2. Comparison of action spectra for the partial reactions of photosynthesis in whole cells of *P. hollandica* with absorption spectra of isolated core complexes of the two photosystems in *Synechocystis* PCC6803. (A) Average action spectrum of the PSII-driven O_2 evolution (1) from eight different assays, and absorption spectrum of the isolated PSII core complex (2) with the adjusted maximum absorption of 37% (A_{674}=0.2). (B) Average action spectrum of the PSI-mediated reactions (1) of H_2 evolution (two assays) and photoinhibition of respiration (four assays), and absorption spectrum of the isolated trimeric PSI complex (2) with the adjusted maximum absorption of 37% (A_{680}=0.2).

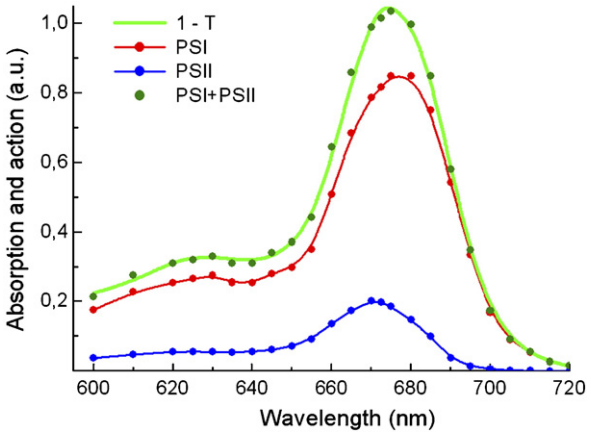

Fig. 3. Action spectra of PSI and PSII, adjusted to fit the absorption spectrum of *P. hollandica* cells. Photochemical activities of PSII (O_2 evolution) and PSI (photoinhibition of respiration) were sequentially measured in the same sample with maximum absorption of 31% (A_{680}=0.16).

estimated values of optical cross sections of a single chlorophyll molecule in the isolated PSI and PSII core complexes, 0.30 and 0.55 Å^2 at 596 and 625 nm, respectively (from data [33–36]), the obtained photochemical cross sections were converted to effective antenna sizes of 192±28 and 139±15 Chl (*a*+*b*) for the Pcb–PSI and Pcb–PSII supercomplexes, respectively.

The determined values of photochemical cross-sections at the selected wavelengths allowed conversion of action spectra of the two supercomplexes in *P. hollandica* into absolute units (Fig. 4). Comparison of the absolute spectra in the range 400–720 nm shows close functional absorptions of PSI and PSII in the region of the Psb bands at 650–670 nm, but with significant predominance of PSI absorption in the spectral regions of the PSI core-associated β-carotene around 500 nm and the long-wavelength forms of Chl *a* at >680 nm. It should be also noticed that the ratio of the blue Soret to the red absorption peaks is appreciably diminished for PSII compared to PSI possibly as a result of selective screening by photochemically inactive carotenoids of the PSII location site, the stacked regions of photosynthetic membranes in *P. hollandica*.

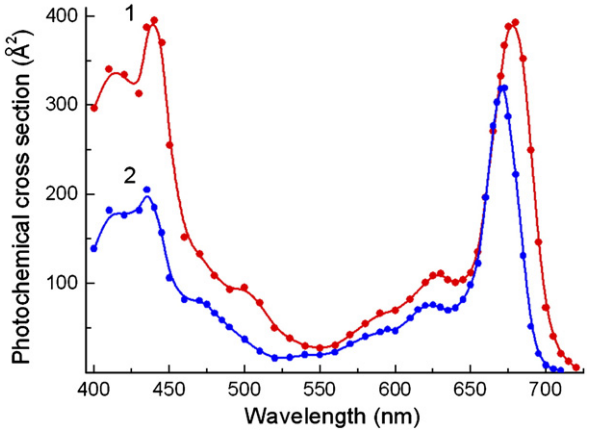

Fig. 4. Absolute action spectra of Pcb–PSI (1) and Pcb–PSII (2) units in *P. hollandica* cells. Photochemical cross sections in the spectral range of 400–720 nm were recalculated from the average action spectra normalized to the determined values of 57 and 42 Å^2 at 596 nm for Pcb–PSI and Pcb–PSII supercomplexes, respectively.

4. Discussion

The *in situ* measurements of absolute action spectra unambiguously revealed the functional association of Pcb antenna complexes with the competent photosynthetic units of both PSI and PSII in the whole cells of *P. hollandica*. In essence, this reconciles the controversial earlier data of two research groups [21–25] based mainly on biochemical analysis of the photosynthetic membranes. The recent studies of Bumba et al. [17] once again underline the limitations of preparative procedures in isolation of the supercomplexes, showing only the 18-mer Pcb–PSI, even though the presence of Pcb–PSII could be indirectly deduced. Attempts to isolate the phycobilisome–PSI supercomplex that is clearly revealed by action spectrometry in the whole cells of cyanobacteria [26,42] have met with similar failure.

The *in situ* measurements of absolute antenna sizes gave an average value of ~190 and ~140 chlorophylls in the Pcb–PSI and Pcb–PSII supercomplexes, respectively. Taking into account the content of 96 and 36 Chls in the cores of PSI

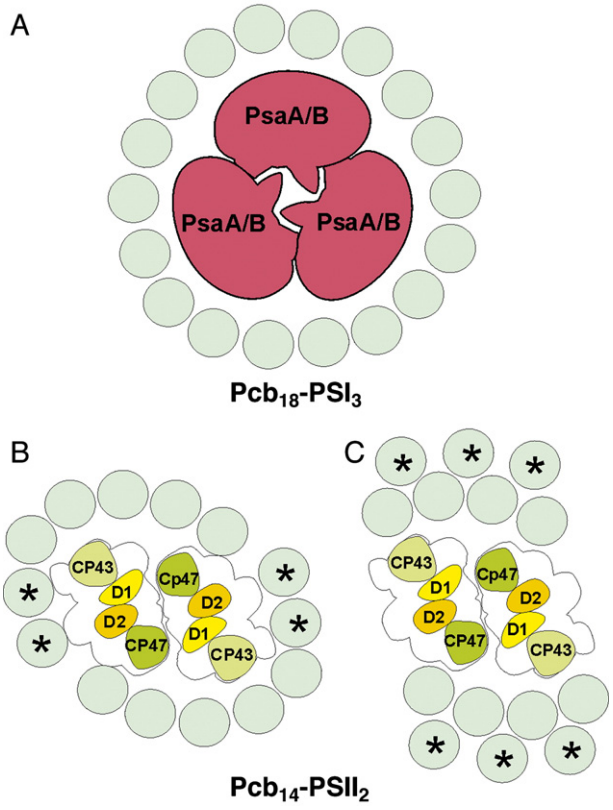

Fig. 5. A tentative model of the organization of photosynthetic units in *P. hollandica* based on the determined absolute antenna sizes *in situ* and the electron microscopy maps of isolated Pcb–PSI supercomplexes (A) from the same species [17] and Pcb–PSII supercomplexes (B, C) from other oxyphotobacteria [13,14] (the asterisks show supposed positions of the extra Pcb subunits in two possible configurations of the supercomplex in *P. hollandica*).

[43] and PSII [44], respectively, and the estimated value of 15 Chls linked to Pcb [9], these sizes correspond to ~6 and ~7 subunits of Pcb per PSI and PSII, respectively. The obtained ratio of 6 Pcb/PSI is in good accordance with the visible structure of a ring of 18 Pcb subunits around the trimeric PSI in *P. hollandica* [17] (Fig. 5A), like in other Pcb-containing species [12,13,16] and similarly to the design of IsiA–PSI supercomplexes of some Fe-limited cyanobacteria [18–20]. At the same time, the obtained ratio of ~7 Pcb/PSII for *P. hollandica* noticeably exceeds a stoichiometry of the visible interaction of 8 Pcb in *Prochlorococcus* MIT 9113 [13] and 10 Pcb in *Prochloron didemni* [14] with the dimeric PSII or 16 Pcb with the tetrameric PSII in *Acaryochloris marina* [15]. Since electron microscopy data for Pcb-PSII in *P. hollandica* are still missing, any spatial models of the complex configuration may be only speculative. From a general consideration, the ratio of ~7 Pcb/PSII implies the presence either nearly closed loops of Pcb around the PSII dimers (Fig. 5B) or bilayer arcs of Pcb on the opposite flanks of the supercomplexes (Fig. 5C), in the membranes of *P. hollandica*. Regarding the first mode, interestingly, the binding sites of the extra Pcb subunits in PSII may correspond to those of loosely-bound LHCII trimers of LHCII–PSII supercomplexes from higher plants [45]. On the other hand, the second possible configuration is similar to the bilayer arrangement of LHCI in the LHCI–PSI supercomplex isolated from *Chlamydomonas reinhardtii* [46]. In any case, the difficulties in isolating of the Pcb–PSII supercomplexes are probably from weakness and lability of the partner associations. Nevertheless, the earlier reported characteristics of isolated Pcb–PSII fraction with a Chl *a*/Chl *b* ratio of ~5 and a Chl (*a*+*b*)/cytochrome *b*-559 ratio of 165 [21] agree quite well with the determined *in situ* functional antenna size.

P. hollandica has three *pcb* genes, *pcbA*, *pcbB* and *pcbC* [8,9], encoding the three Pcb antenna proteins, and it known that the PcbC protein is rather distantly related to the PcbA/PcbB within this family. There is increasing evidence that the PcbA/PcbB subgroup in different Pcb-containing oxyphotobacteria is preferentially associated with PSII whereas PcbC (and PcbG) interacts with PSI [13,16,17]. Thus, in line with the assumption of Bumba et al. [17] and the structural data for other Pcb-containing oxyphotobacteria [13,16], it is reasonable to attribute the action spectra of O_2 and H_2 evolution in *P. hollandica* to functioning PcbA/B–PSII and PcbC–PSI supercomplexes, respectively. In *Prochloron didemni*, only a Pcb–PSII supercomplex was structurally characterized [14] without identification of the relevant gene product. However, the findings about the presence of both *pcbA* and *pcbC* genes in its genome [8] and some early biochemical data on isolation of Pcb–PSI fractions (reviewed in [25]) give grounds to suppose the existence of the PcbC–PSI supercomplex in the species too.

If so, the origin of the specialization as well as the key structural (and hence functional) differences of the Pcb proteins are intriguing. There are still no crystallographic data for Pcb and IsiA proteins to reveal details of their structures. At first glance, the oligomeric state of 18 Pcb (IsiA) subunits around the trimeric PSI is predetermined by a requirement of a particular shape and size of subunit to adjust the ring around PSI at a distance of the effective energy transfer, and is based on specific binding of either the subunits or the constituents of supercomplex. However, recent studies of the variations of IsiA in response to prolonged iron deficiency and to mutations affecting the small peripheral subunits and trimerization of PSI (reviewed in [20]) have surprisingly high flexibility for the Isi–PSI interactions and variable structures of PSI monomers with double IsiA rings as well as PSI-free IsiA supercomplexes. Obviously, it remains to be established how these structural variations affect functional characteristics of the cells under physiological conditions, though some preliminary measurements showed a constant effective antenna size of ~200 Chls of IsiA–PSI in situ during the iron starvation of *Synechocystis* 6803 (V. Boichenko, I. Elanskaya and I. Stadnichuk, unpublished data). Therefore, the many aspects of the structure and functions of the intrinsic Pcb and IsiA antenna complexes, particularly in selective associations with PSI and PSII need further profound research.

Acknowledgments

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