Association of chlorophyll \( a/c_2 \) complexes to photosystem I and photosystem II in the cryptophyte *Rhodomonas CS24*

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**Abstract**

Photosynthetic supercomplexes from the cryptophyte *Rhodomonas CS24* were isolated by a short detergent treatment of membranes from the cryptophyte *Rhodomonas CS24* and studied by electron microscopy and low-temperature absorption and fluorescence spectroscopy. At least three different types of supercomplexes of photosystem I (PSI) monomers and peripheral Chl \( a/c_2 \) proteins were found. The most common complexes have Chl \( a/c_2 \) complexes at both sides of the PSI core monomer and have dimensions of about \( 17 \times 24 \) nm. The peripheral antenna in these supercomplexes shows no obvious similarities in size and/or shape with that of the PSI–LHCl supercomplexes from the green plant *Arabidopsis thaliana* and the green alga *Chlamydomonas reinhardtii*, and may be comprised of about 6–8 monomers of Chl \( a/c_2 \) light-harvesting complexes. In addition, two different types of supercomplexes of photosystem II (PSII) dimers and peripheral Chl \( a/c_2 \) proteins were found. The detected complexes consist of a PSII core dimer and three or four monomeric Chl \( a/c_2 \) proteins on one side of the PSII core at positions that in the largest complex are similar to those of Lhcb5, a monomer of the S-trimer of LHClI, Lhcb4 and Lhcb6 in green plants.

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1. Introduction

Oxygenc photosynthesis occurs not only in green plants, but also in many different types of algae and cyanobacteria that live in large quantities in both salt- and freshwater habitats. All these organisms have very similar photosynthetic reaction centers in the two photosystems, i.e., photosystem I (PSI) and photosystem II (PSII). In addition, they have very similar photosynthetic reaction centers in the two photosystems, i.e., photosystem I (PSI) and photosystem II (PSII). In addition, they have very similar photosynthetic reaction centers in the two photosystems, i.e., photosystem I (PSI) and photosystem II (PSII). In addition, they have very similar photosynthetic reaction centers in the two photosystems, i.e., photosystem I (PSI) and photosystem II (PSII).

Some types of cyanobacteria contain membrane-intrinsic antenna proteins belonging to the family of core antenna proteins, i.e., the prochlorophyte chlorophyll \( a/b \) binding proteins (Pcb’s) and/or IsiA, the chlorophyll \( a \) binding protein that is expressed in cyanobacteria when they grow under conditions of iron limitation or other types of oxidative stress [3,4].

In this work, we focus our attention on the occurrence and structure of supramolecular associations of the two photosystems in a cryptophyte alga. Cryptophytes are eukaryotic algae that are unique because they utilize both membrane-extrinsic phycobiliproteins and membrane-intrinsic Chl \( a/c_2 \) light-harvesting proteins belonging to the LHC superfamily in their light-harvesting apparatus. The phycobiliproteins differ from those in cyanobacteria and red algae in their occurrence of only one type of phycoerythrin or phycocyanin, which are also differently organized [5]. *Rhodomonas CS24* contains phycoerythrin 545 (PE545), a heterodimeric \((α1β)^2\) complex consisting of 4 polypeptides with a total mass of 60 kDa. The protein has been crystallized [6] and its structure has been solved at 0.97 Å resolution by X-ray diffraction (protein data base entry 1XGO) [7]. These phycobiliproteins are not organized into large phycobilisomes bound at the stromal side of the photosynthetic membrane, like in cyanobacteria and red algae, but they are located at the luminal side of the membrane. Some electron microscopy studies have suggested that within the lumen the phycobilin proteins assemble into stacks or rows, although there is no real

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The chloroplast of cryptophytes is related to that of red algae [8]. Sequencing of the plastid genome of the cryptophyte *Guillardia theta* indicated the presence of PSI subunits Psα–A–E, Psα–F and Psα–I–M and the PSI subunits Psβ–A–F, Psβ–H–L, N, T, V, and X [8], but otherwise cryptophytes have not been extensively studied genetically.

The intrinsic LHC antenna of cryptophytes appears to consist of several proteins. Originally, a *Rhodomonas* light-harvesting complex of approximately 55 kDa was detected in SDS gels, which was comprised of PSII show a higher chlorophyll that of cyanobacteria and the core complex of green plants [1,14].

Recently, thylakoid membranes of the cryptophyte *Rhodomonas* sp. were solubilized with the mild detergent n-dodecyl-β-D-maltoside (β-DM) and subjected to sucrose density gradient centrifugation [10]. The resulting gradiants showed six pigment-bearing bands. Two of the bands showed characteristics of light-harvesting complexes, other bands could be attributed to PSI and PSII. Up to 10 different light-harvesting proteins could be identified, some of which are specific for PSI, others for PSII. The polypeptides of the light-harvesting complex of PSI show a higher chlorophyll a/a ratio than the antenna proteins of PSI [10]. Recently two LHC genes of *Rhodomonas* CS24 were sequenced [11] and most likely the folding of the proteins expressed by these genes is similar to that of the main light-harvesting complex of green plants (LHClI) that is structurally resolved at 2.5 Å [12,13].

Electro-microscopy analysis indicates that the PSI core complex from the red alga *Porphyridium cruentum* is structurally very similar to that of cyanobacteria and the core complex of green plants [11,14]. Supernumerary associations of PSI and PSII with peripheral membrane-intrinsic antenna proteins have not yet been studied in cryptophytes and other non-green algae. In this study, we have prepared photo-synthetic supercomplexes from membranes of the unicellular cryptophyte *Rhodomonas* CS24 and demonstrate by electron microscopy and single-particle image analysis that both photosystems form super-complexes with several Chl a/c$_2$ proteins that are unique in size and shape and are different from those of green plants, green algae and cyanobacteria.

2. Materials and methods

2.1. Harvesting cells

Cells were pelleted by centrifugation at (4000 g, 15 min, 4 °C), the resulting pellet was resuspended in Heps/KCl buffer at pH 7.8 and 1 mM of PMSF (phenylmethane-sulphonylfluoride). After the addition of 0.1 mg/ml lysozyme and 30 min incubation at 4 °C, the cells were disrupted using a French Press twice at 1000 psi. After the removal of undissrupted cells the lysate was centrifuged for 10 min at 4 °C, at 7000 × g, the resulting pellet was discarded. The thylakoids were collected by ultra-centrifugation of the supernatant was pushed through a 0.45 μm filter to remove large fragments. The solubilized fractions were subjected to gel filtration chromatography using a MALDI-TOF/TOF Proteomics Analyzer (Applied Biosystems, Foster City, CA, USA), prior peptide separation on a nanoscale liquid chromatography system. Peptides with signal-to-noise level above 40 were selected for MS/MS analysis. A detailed protocol is included as Supplementary material. Protein identification was carried out using Protein Pilot (version 2.0, Applied Biosystems), searching against the *Rhodomonas salina* mitochondrial protein sequence database [16] allowing for modification by amino acid substitution. The *R. salina* protein sequences were extracted from the UniProtKB/TrEMBL database. Protein identifications with confidence greater than 99.0%, based on 1 peptide identified with a probability higher than 99% or at least 2 peptides identified independently with 99% probability, were accepted.

2.5. Electron microscopy

Samples were negatively stained with 2% uranyl acetate on glow discharged carbon-coated copper grids. Electron microscopy was performed on a Philips CM120 electron microscope equipped with a LaB$_6$ tip operating at 120 kV. Images were recorded with a Gatan 4000 SP 4 K slow-scan CCD camera at 80,000 magnification at a pixel size (after binning the images) of 0.375 nm at the specimen level with GRACE software for semi-automated specimen selection and data acquisition [17]. Single-particle analysis of a data set of 60,000 particles was performed using GRIP (Groningen Image Processing) software, including multireference and no-reference alignments, multivariate statistical analysis, and classification, performed as described previously [18].

2.3. Spectroscopy

The isolated fractions were diluted in 20 mM Bis–Tris pH 6.5, 10 mM NaCl, 0.03% β-DM and 60% v/v glycerol to an OD at 680 nm of 0.1 cm$^{-1}$ for fluorescence measurements and 1 cm$^{-1}$ for absorption measurements. A Unax Cryostat with a gas/flow temperature controller was used to vary the temperature between 6 and 280 K. Absorbance and fluorescence emission measurements were recorded on home-built spectrometers, the latter using a 0.5 μm imaging spectrograph and a CCD camera (Chromex Chromcam I). Broadband excitation was provided by a tungsten halogen lamp (Oriel) and 420 nm interference filter (bandwidth ~15 nm). The spectral resolution of the detection setup was 0.2 nm. The recorded emission spectra were corrected for the wavelength sensitivity of the detection system.

2.4. Blue native PAGE and mass spectrometry

The fractions of the first peak eluted during size exclusion chromatography were separated by one dimension gradient (3.75–13% blue native polyacrylamide gel electrophoresis (BIN-PAGE) [15]. Subsequently, the separated protein complexes were subjected to in-gel trypsin digestion and separately analyzed by mass spectrometry using a MALDI-TOF/TOF Proteomics Analyzer (Applied Biosystems, Foster City, CA, USA), prior peptide separation on a nanoscale liquid chromatography system. Peptides with signal-to-noise level above 40 were selected for MS/MS analysis. A detailed protocol is included as Supplementary material. Protein identification was carried out using Protein Pilot (version 2.0, Applied Biosystems), searching against the *Rhodomonas salina* mitochondrial protein sequence database [16] allowing for modification by amino acid substitution. The *R. salina* protein sequences were extracted from the UniProtKB/TrEMBL database. Protein identifications with confidence greater than 99.0%, based on 1 peptide identified with a probability higher than 99% or at least 2 peptides identified independently with 99% probability, were accepted.

3. Results

3.1. Gel filtration chromatography

We solubilized thylakoid membranes from the cryptophyte *Rhodomonas* CS24 by a short treatment with the mild detergent β-DM and separated the solubilized fraction by a single gel filtration chromatography step, with the aim to keep possible supercomplexes of PSI and/or PSII with peripheral membrane-intrinsic Chl a/c$_2$ antenna proteins intact. With similar procedures PSI–LHClI supercomplexes from plants [18] and large PSII–LHClI supercomplexes from the green alga *Chlamydomonas* [19] were shown to stay intact.

Fig. 1 shows the chromatogram, recorded at 400 nm. Four protein peaks are discerned, at elution times of 46, 57, 63 and 67 min. The A$_{400}$/A$_{460}$ ratio is representative of the Chl c/a ratio present in the different fractions. A significantly lower Chl c content is thus recorded for the first fraction with respect to the later fractions. The A$_{700}$/A$_{675}$ ratio represents the relative amount of long-wavelength absorbing Chl a present in the fractions. In green plants, this ratio is normally used to
indicate the relative amount of PSI and LHCl in the fractions, since PSII and LHCl hardly absorb at 700 nm. Fig. 1 shows that this ratio is 0.1 in the first fraction and lower in all subsequent fractions. For comparison, this ratio is about 0.3 for green plant PSI supercomplexes [20]. From a time-resolved study on Rhodomonas CS24 intact algae it was however concluded that the amount of long-wavelength absorbing (red) pigments in PSI of Rhodomonas is very small [21]. Because at an elution time of 46 min only large complexes are expected to elute from the columns, we conclude that the first fraction is very likely enriched in PSI supercomplexes and may contain some PSII supercomplexes as well. We used this fraction (designated Fraction 1 in the following) for all further experiments. The later fractions, eluting at 57, 63 and 67 min, are probably enriched in Chl $a/c_2$ antenna units in different aggregation states, most likely monomers (67 min), dimers (63 min) and trimers or tetramers (57 min).

### 3.2. Low temperature spectroscopy

In Fig. 2, the room temperature absorption spectrum of Fraction 1 is shown. The spectrum reveals the Chl $a$ QY absorption maximum at 679 nm, which is typical for PSI in green plants and cyanobacteria (see, e.g. [22]). In plants, the absorption by PSII core is known to be blue-shifted with respect to that of PSI, with absorption maximum at 674 nm [23]. In the Soret region, the Chl $a$ absorption maximum is observed at 437 nm. The Soret region reveals a small contribution of Chl $c$ at 467 nm. The QY absorption contribution of Chl $c$ around 630 nm is hidden by the broad Chl $a$ QX absorption around that wavelength. The absorption band at 493 nm is assigned to carotenoids, which are mainly alloxanthins in Rhodomonas CS24.

Fig. 3 displays the 4 K absorption spectrum of Fraction 1 and its second derivative. Chl $a$ absorption bands are observed at 662, 666, 675 and 679 nm as well as shoulders around 681.5, 687 and 695 nm. Note the almost complete absence of red pigments, with absorption wavelength longer than 700 nm. This spectrum resembles that of PSI core complexes from organisms without many red Chls [22,24]. The peaks at 679, 687 and 695 are typical for PSI core complexes because...
PSII usually does not have strong absorption bands at 687 and 695 nm. We conclude from this observation that PSI dominates Fraction 1, but that small amounts of PSII can not be excluded.

Fig. 4 shows the recorded emission spectra of Fraction 1 at different temperatures upon excitation at 420 nm. At 6 K, the emission maximum is observed at 702 nm and shows a shoulder at wavelengths above 700 nm. At 77 K, the cryptophyte PSI emission is observed to consist of a band at 687.5 nm with a shallow shoulder at wavelengths above 700 nm. There is no obvious 695 nm emission maximum in Fraction 1 at 77 K (Fig. 4), which is expected if PSII is present in significant amounts. A clear 695 nm emission band was observed in Rhodomonas CS24 cells at this temperature (data not shown). These data confirm that Fraction 1 consists predominantly of PSI supercomplexes and only to a small extent can it be contaminated with PSII.

### 3.3. Blue native PAGE and mass spectrometry analysis

The four protein fractions corresponding to the first peak obtained after size exclusion chromatography were separated by blue native poly acrylamide gel electrophoresis (BN-PAGE). The gel showed that the first two fractions were enriched in supercomplexes (Fig. 5). To probe the composition of the separate complexes, the three high-molecular weight bands were excised from the BN-PAGE and analyzed by LC-MALDI-MS/MS. The obtained spectra were matched against the protein sequence database of the closely related organism Rhodomonas salina. The complete list of the matched peptides is presented in the Supplementary material (Table S1). Based on this approach the presence of Psa-A, B, C, D, F, L, as well as the presence of PSII Psb-B and D subunit, was confirmed in all three high-molecular weight bands with a confidence probability higher than 99% (Table 1). These results indicate that both PSI and PSII are present in the purified fraction (Fraction 1) which was further characterized by single-particle electron microscopy.

### Table 1

Components of PSI and PSII complexes identified by mass spectrometry

<table>
<thead>
<tr>
<th>Total number</th>
<th>Probability</th>
<th>Accession number</th>
<th>Protein name</th>
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<tr>
<td>23.55</td>
<td>99%</td>
<td>A6MVZ7</td>
<td>Photosystem I P700 chlorophyll a apoprotein A1 (Psa-A)</td>
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<tr>
<td>18.9</td>
<td>99%</td>
<td>A6MVZ7</td>
<td>Photosystem I P700 chlorophyll a apoprotein A2 (Psa-B)</td>
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<tr>
<td>10</td>
<td>99%</td>
<td>A6MVZ1</td>
<td>Photosystem I subunit II (Psa-D)</td>
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<tr>
<td>6.1</td>
<td>99%</td>
<td>A6MVZ1</td>
<td>Photosystem II CP47 chlorophyll apoprotein B (Psb-B)</td>
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<tr>
<td>6</td>
<td>99%</td>
<td>A6MVZ1</td>
<td>Photosystem II protein D2 (Psb-D)</td>
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<tr>
<td>6</td>
<td>99%</td>
<td>A6MVZ8</td>
<td>Photosystem I subunit XI (Psa-L)</td>
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<td>Photosystem I iron-sulfur center subunit VII (Psa-C)</td>
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<td>99%</td>
<td>A6MVU7</td>
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<tr>
<td>2.2</td>
<td>99%</td>
<td>A9BKV6</td>
<td>Ranbpm</td>
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**Fig. 6.** Single-particle electron microscopy of PSI and PSII projections. (A,B) Projection maps of the Rhodomonas PSI supercomplex as in the plane of the membrane, consisting of a PSI core plus additional antenna; averages of 1024 particles, respectively; (C,D) Projection maps of the Rhodomonas PSI supercomplex, in slightly tilted position, averages of 2048 particles, respectively; (E) Green plant PSI–LHCII supercomplex; updated from [25]; (F) Smaller Rhodomonas PSI complex, consisting of a PSI core and a peripheral antenna on the lower side, average of 1024 particles; (G,H) PSI supercomplexes consisting of a dimeric core complex in the upper part and a variable peripheral antenna on the lower side; averages of 512 particles; (I) Dimeric PSI core complex, without imposed two-fold symmetry; average of 1024 particles. Red dots in frames A–F mark equivalent positions in the PSI complexes; the green triangle in frame E marks the position of the LHCII trimer in the green plant PSI supercomplex.
3.4. Electron microscopy analysis

Electron microscopy was performed on freshly prepared Fraction 1. A set of 60,000 single-particle projections was collected from electron micrographs and was statistically analyzed and classified into several groups, of which the best resolved classes are shown in Fig 5. Based on similarities with 2D maps of green plant, algal and cyanobacterial PSI and PSII supercomplexes, the 2D projection maps of Fraction 1 can be clustered into two groups, of which one can be assigned to PSI (Fig. 6A–D) and the other to PSII complexes of variable size and shape (Fig. 6G–I).

The first group consists most likely of two different types of PSI–Chl a/c2 supercomplexes. One is a PSI core complex with a peripheral antenna part at the lower side (Fig. 5F), the other is a core complex with additional antennas on both sides (Fig. 5A–D). The monomeric core part is similar to the PSI core from green plants, as can be seen from equivalent positions (visualized by red dots in Fig. 6A–E) in the maps of the PSI–Chl a/c2 supercomplexes of Rhodomonas CS24 (Fig. 6A–D) and from the PSI–LHCIII supercomplex from A. thaliana (Fig. 6E) [26]. The small differences between the maps of Figs. 5A, B, C, and D may have to do in part with different positions of the complexes on the EM support film. The maps of Fig. 5C and D appear smaller, probably because of a tilt away from the membrane plane. But it also seems that there is variation on the upper left side. In the map of Fig. 6A there is an additional mass at this position, which appears to be absent in the other maps, especially in the map of Fig. 6D. The maximal dimensions of the PSI–Chl a/c supercomplexes in Fig. 5A are about 17×24 nm; including the detergent boundary layer. This is significantly larger than the 17×19 nm dimensions of PSI–LHCI complex from plants, which has four Lhca proteins at the side of the PSI-F/J units [20]. The dimensions are about equal to the 17×25 nm of the PSI–LHCIII supercomplex, which binds, besides the four Lhca proteins a single LHCIII trimer at the PSI core site consisting of the PSI-A, -H, -L and -K subunits [26] and the PSI complex from Chlamydomonas which measures 19×21 nm in projection [19]. This comparison indicates that PSI of cryptophyte Rhodomonas CS24 may have a peripheral antenna of about 6–8 Chl a/c2 proteins.

The second group is clearly related to PSII. Of the three obtained classes within this group, one is a “standard” dimeric PSII core complex, because its features and dimensions (19×14 nm) are very similar to those of green plants and cyanobacteria (Fig. 6I). In addition, two larger complexes were found (Fig. 6G,H), with an additional peripheral antenna that is larger in the projections of Fig. 5G than in those of Fig. 5H. The larger complexes have maximal dimensions of 17×17 and 17×16 nm. Because EM projections of membrane proteins, such as the spherical to oval-shaped Rhodomonas PSI projections, are difficult to assign we can not give a precise ratio between PSI and PSII complexes. The number of recognizable PSII complexes was, however, substantially lower than of PSI; in one batch it was less than a few percent, in another less than 20%. This indicates that PSI particles dominate Fraction 1, in agreement with the spectroscopy data discussed above.

4. Discussion

Statistical analysis and classification of a large set of EM projections of solubilized photosynthetic membrane proteins of Rhodomonas CS24 shows, for the first time in cryptophyte algae, the presence of photosynthetic supercomplexes.

The first group consists of monomeric PSI–Chl a/c2 supercomplexes (the particles shown in Fig. 6A–D) from Rhodomonas are significantly larger than those of green plants and have a similar surface area as the PSI–LHCI–LHClII supercomplex found in Arabidopsis under state 2 conditions [25]. In this complex, a total of seven Chl a/b complexes is bound to PSI: four LHCI proteins are bound at the PSI-F/J side of the complex, while one trimeric LHCl complex is bound at the side of the PSI-A, -H, -L and -K subunits. At the present resolution, the cryptophyte data do not allow an unambiguous assignment of the number of Chl a/c2 at each side of the photosystem. The lower antenna part seems smaller and may contain 2–3 copies of Chl a/c2 antenna complexes, whereas the upper part is bigger, especially for the particles in Fig. 6A, and may contain 4–5 copies, bringing the total number of copies to about 6–8. It is unlikely that some of the Chl a/c2 antenna complexes appear as a (hetero)trimer attached to the PSI core, because the distance from the PSI core to the LHCIII edge in the PSI–LHClII complex from Arabidopsis (Fig. 5E) is significantly larger than the corresponding distance in the PSI complexes from Rhodomonas (Fig. 6A–D). However, the lack of higher resolution does not exclude the presence of trimer(s) at the moment. We note that the gel filtration chromatography suggests the presence of three different aggregation
states of the Chl a/c2 complexes, though details on the aggregation state of these complexes are not known.

The second group consists of a number of PSII–Chl a/c2 supercomplexes. The core complex part of the EM projection map of Rhodomonas PSII does not significantly differ from its counterpart in cyanobacteria [30], green plants [1] and red algae [14]. This suggests that the major PSII subunits, including the extrinsic subunits, are present and should be structurally homologous, and also that PSII forms very similar types of dimers in all these organisms. There is no information on the extrinsic subunits, but in the genome of the distantly related red alga C. meraloa there is a set of three extrinsic subunits PsbO, P and Q [8]. The number of the Chl a/c complexes associated to the core part is estimated by modeling the high-resolution structures of PSI and monomeric LHClII into the EM projection maps (Fig. 7A). This suggests that the largest particle (Fig. 6G) likely binds four copies of light-harvesting complexes on one side (Fig 7A) and that the smaller one (Fig. 6H) may bind only three copies (not shown). They flank the PSII core in about the same way as the minor antenna complexes Lhcb6, Lhcb4, one single Lhcb monomer from the LHCl S-trimer and Lhcb5 do in green plant C3S3M3 PSII supercomplexes (Fig. 6B). The Rhodomonas PSII supercomplexes are also structurally similar to PSI supercomplexes of the Prochlorococcus species with four Pcb antenna proteins attached at each side of the PSI core dimer [31] and to those of Prochloron didemni which bind up to 5 Pcb complexes at each side [32]. This is remarkable, because the Pcb proteins belong to the core complex family of light-harvesting proteins, which is completely unrelated to the Cab family of light-harvesting proteins to which the Chl a/c2 proteins of cryptophytes belong. Because the core part of Rhodomonas PSI is dimeric, one would expect to find particles that bind four copies of antenna complexes on both sides of the core as well. However, we could not find even small numbers of such complexes. This could hint to a conformational change in the dimer configuration, but this seems to be unlikely because the high-resolution X-ray structure of cyanobacterial PSI does not show such deviations. There exist, however, similar examples. In the mitochondrial supercomplex, consisting of dimeric Complex III (cytochrome bc1 complex) and Complex I, there is a place for the attachment of two Complex I particles, but only one Complex I site is occupied [33]. Regardless of this uncertainty, the data unambiguously show that all Chl a/c2 proteins bound to PSII are not trimeric. They are either monomeric, or have a dimeric arrangement similar to that of LHCl in green plants [28,29].

It is interesting to compare the PSI and PSII supercomplexes from Rhodomonas observed here with those from diatom algae. Diatoms are also eukaryotic algae with Chl a/c proteins as peripheral antennae. Recent results showed that in the diatom Phaeodactylum tricornutum PSI is associated with a number of Chl a/c proteins [35]. EM images revealed almost circular complexes with a diameter of about 21 nm, which points to a slightly larger complex than here for Rhodomonas and in [19] for C. reinhardtii. Nagao et al. were able to isolate a PSI complex with a significant number of associated Chl a/c proteins [36]. A structural analysis was not performed, but based on the available biochemical data a significantly larger PSII–LHCl supercomplex is expected than observed here for Rhodomonas.

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