Biochimica et Biophysica Acta 1837 (2014) 1904–1912

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbabio

Evidence of functional trimeric chlorophyll a/c_2-peridinin proteins in the dinoflagellate *Symbiodinium*

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ARTICLE INFO

Article history:
Received 17 March 2014
Received in revised form 7 July 2014
Accepted 24 July 2014
Available online 19 August 2014

Keywords:
Symbiodinium
Chlorophyll a/c_2-peridinin-protein
Light-harvesting complex
Native mass spectrometry

ABSTRACT

The chlorophyll *a*-chlorophyll c_2-peridinin-protein (apcPC), a major light harvesting component in peridinin-containing dinoflagellates, is an integral membrane protein complex. We isolated functional acpPC from the dinoflagellate *Symbiodinium*. Both SDS-PAGE and electrospray ionization mass spectrometry (ESI-MS) analysis quantified the denatured subunit polypeptide molecular weight (MW) as 18 kDa. Size-exclusion chromatography (SEC) and blue native gel electrophoresis (BN-PAGE) were employed to estimate the size of native acpPC complex to be 64–66 kDa. We also performed native ESI-MS, which can volatilize and ionize active biological samples in their native states. Our result demonstrated that the native acpPC complex carried 14 to 16 positive charges, and the MW of acpPC with all the associated pigments was found to be 66.5 kDa. Based on these data and the pigment stoichiometry, we propose that the functional light harvesting state of acpPC is a trimer. Our bioinformatic analysis indicated that *Symbiodinium* acpPC shares high similarity to diatom fucoxanthin Chl *a/c* binding protein (FCP), which tends to form a trimer. Additionally, acpPC protein sequence variation was confirmed by *de novo* protein sequencing. Its sequence heterogeneity is also discussed in the context of *Symbiodinium* eco-physiological adaptations.

1. Introduction

Dinoflagellates are ubiquitous alveolate protists, closely related to apicomplexans and ciliates [1–3]. They have diverse morphology, genetics and different trophic modes (mixotrophic, heterotrophic and phototrophic). Photosynthetic dinoflagellates substantially contribute to the net primary production on Earth. Most of them are free-living species, while eight genera contain symbiotic representatives. Among them, *Symbiodinium* is the most commonly found genus of dinoflagellates in symbiosis with marine invertebrates and protists e.g. Cnidaria, which includes coral reef builders [4]. *Symbiodinium* provides corals their coloration and a variety of photosynthetically-produced nutrients; in return, corals supply the endosymbionts carbon dioxide, nitrate, phosphate and other inorganic substances that are essential for photosynthesis. Under certain conditions, when the photosynthesis of *Symbiodinium* is affected or impaired, and the endosymbiotic relationship cannot be maintained, corals start to expel *Symbiodinium*, leading to coral bleaching, which can significantly decrease the diversity of marine ecosystems. Coral bleaching can be caused by a number of biotic and abiotic factors, among which increased temperatures and solar irradiances are most extensively studied. Under thermal stress, the Photosystem II (PSII) of *Symbiodinium* is inhibited to protect the cell from reactive oxygen species generated by excess electrons, which originate from PSII charge separation [5–8]. During this process, energy donors to PSII, namely Light Harvesting Complexes (LHCs), are thought to partially disconnect from PSII to reduce the level of excitations funneled to it [5]. The cellular level of LHCs also drops [7], preventing further damage. Although *Symbiodinium* LHCs are of considerable significance, the molecular level understanding of these protein-pigment complexes is limited compared to that of their counterparts in higher plants, green algae, diatoms and photosynthetic bacteria.

There are two major LHCs in *Symbiodinium*: the water-soluble peridinin-chlorophyll *a*-proteins (PCPs) and the thylakoid intrinsic chlorophyll *a*-chlorophyll c_2-peridinin-protein complex (acpPC). They both contain peridinins, the unique carotenoid to dinoflagellates, as a major photosynthetic pigment. PCP has no sequence similarity to other LHCs [9]. Because of its uniqueness, PCP has been the subject of intensive experimental and theoretical studies [10–23]. In general, dinoflagellate PCP proteins are varied in the aspects of the length, pigment content, sequence and spectroscopic

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http://dx.doi.org/10.1016/j.bbabio.2014.07.023
0005-2728/

properties [11–13,18,21]. Among different PCPs, the structures of MFPCP (main form PCP) and HSPCP (high-salt PCP) from *Amphidinium carterae* have been resolved to 2.0 Å and 2.1 Å, respectively by X-ray crystallography [11,18]. A higher resolution of 1.5 Å was recently achieved in recombinant PCPs [17]. Spectroscopic studies revealed an efficient peridinin-to-Chl *a* energy transfer, dominated by the pathway from the peridinin S_1/ICT (intramolecular charge-transfer) state to the Chl *a* Q_y state [22]. Genomic analysis of PCP genes and transcripts based on data from *Amphidinium carterae* [19], *Gonyaulax polyedra* [15], *Heterocapsa pygmaea* [10,20] and *Symbiodinium* sp. [14,16,21,23] revealed that PCP genes are nuclear-encoded, intronless and exist in tandem arrays.

Compared to PCP, acpPC is more abundant in cells [12]. This complex contains Chl *a*, Chl c_2, peridinin and diadinoxanthin, associated with a polypeptide of 18–20 kDa, the N-terminal region of which is related to the LHCs of higher plants [12,24,25]. No crystal structure of acpPC has been determined. Spectroscopic studies show that in the complex peridinins and Chl c_2 molecules can both feed excitations to Chl *a* [26–29]: energy transfer pathways to Chl *a* involve the peridinin S_2 state and S_1/ICT (intramolecular charge-transfer) states, and Chl c_2-to-Chl *a* efficiency is close to 100%. The role of diadinoxanthin within the complex is still unclear. It was predicted to be photoprotective, as acpPC tends to have more diadinoxanthins when grown under high light [30], and Polívka et al. did not detect any energy transfer from diadinoxanthin to Chl *a* or Chl c_2 [26]. However, the Chl *a* triplet quenching is inefficient in acpPC [28,29]. There are also indications that rather than diadinoxanthin, some peridinins may participate in photoprotection [28]. Recent findings from *Symbiodinium* genome projects revealed multiple LHC genes [31,32], providing a genetic basis for the previous report that sequence variations may exist at the protein level. Interestingly, unlike PCPs, which can have different pIs (isoelectric point), there is no literature showing acpPCs possess the same property. It is unclear why *Symbiodinium* or dinoflagellates in general have LHCs (PCPs and acpPCs) encoded by multigene families. This might help the marine algae to harvest photonic energy more efficiently, while adapting to a variety of habitats with low light intensities and poor nutrients [33].

In this study, we relied on mass spectrometry supplemented with traditional biochemical and spectroscopic methods to identify and characterize the major LHC acpPC in the dinoflagellate *Symbiodinium*. Mass spectrometry (MS) of intact protein complexes, often referred to as native MS, has emerged as a powerful tool to study the stoichiometry of protein assemblies and protein-ligand binding [34–42]. Typically, membrane proteins stabilized in detergent-containing solutions are ionized by nanoelectrospray (nanoES) and then transmitted into the mass spectrometer. Detergent micelles and solution molecules around proteins are gradually removed by applying collisional activation. The desolvated intact ionized proteins are subsequently separated in a time-of-flight (TOF) mass analyzer. To maintain the structure of the target protein while minimizing the interference of detergents, collisional activation is one of the key factors, as too much activation can unfold the protein, while insufficient activation results in poor detergent removal and desolvation [39]. To facilitate this process, membrane proteins are exchanged into ammonium acetate (as both ammonia and acetic acid are volatile and evaporate readily during electrospray) supplemented with a minimal amount of the detergent of interest (typically 2 x CMC, the critical micelle concentration) [39].

In this work, we utilized native MS to measure the size of acpPC, a membrane-bound LHC of unknown structure, solubilized with n-dodecyl-β-D-maltoside (DDM). We successfully maintained the native state of this complex and obtained a MW that is consistent with the results from traditional biochemical methods such as size exclusion chromatography (SEC) and blue native PAGE (BN-PAGE). Along with the pigment stoichiometry reported by our group elsewhere [29], we conclude that acpPC is probably a trimeric complex.

2. Materials and methods

2.1. Algal culture and protein preparation

Symbiodinium sp. CS-156 cells were cultured in f/2 media under a 14 h:10 h cycle of light:dark at 25 °C with gentle stirring and air-bubbling. Illumination was provided by a white color fluorescent lamp at an intensity of 80 μmol photon•m^{-2}•s^{-1}. The culture in late exponential phase was harvested by centrifugation at 8000 ×g for 10 min at 4 °C, and acpPC was purified as previously described with minor modifications [29]. Briefly, HiTrap Q HP (GE Healthcare) column fractions with highest A_{672nm}:A_{280nm} ratios were pooled, dialyzed against 20 mM sodium phosphate pH = 7 0.15 M NaCl, concentrated, passed through a 0.2 μm filter, and applied to a HiPrep Sephacryl S-100 HR (GE Healthcare) column equilibrated with the same buffer with 0.02% DDM.

2.2. Protein identification: spectroscopic characterization

The acpPC sample was in 20 mM sodium phosphate pH = 7 and 0.02% DDM. Steady-state absorption spectrum of acpPC was recorded at room temperature using a Perkin-Elmer Lambda 950 UV-Vis spectrophotometer. Circular dichroism (CD) spectra of acpPC were taken at room temperature using 0.1 cm optical path length, 1 nm bandwidth, and 0.5 s response time in the range of 260–750 nm in a Jasco J-815 CD spectrometer. All CD spectra are averages of 8 sequentially recorded spectra.

2.3. Characterization of the oligomeric state

To analyze the oligomeric state of acpPC, the size of the polypeptide and native protein needs to be determined. We first estimated the protein size by SDS-PAGE, size exclusion chromatography and non-denaturing electrophoresis, and then determined the accurate size by mass spectrometry (LC/MS and native MS).

SDS-PAGE was conducted according to [43] with minor modifications. Briefly, acpPC was denatured by adding acetone (1:5, v/v), followed by 2 h incubation at −20 °C and centrifugation. The supernatant was discarded and the pellet was resuspended in 15 μl of buffer A/4. SEC was performed on a Bio-rad FPLC system with a Superdex 75 10/300 GL column (GE Healthcare), which was calibrated with bovine serum albumin (BSA, 67 kDa), β-lactoglobulin (BLG, 36.6 kDa), ribonuclease A (RNase A, 13.7 kDa) and vitamin B_{12} (1355 Da). Blue dextran (~2 MDa) was used to determine the void volume of the column. All calibrants were purchased from Sigma Aldrich. 200 mM ammonium acetate pH = 5 supplemented with 0.02% DDM was used as the mobile phase at a flow rate of 0.4 mL/min. Elution profiles were recorded using a UV absorbance detector (Bio-Rad) at 280 nm for calibrants, 280 and 672 nm for acpPC. Nondenaturing (BN-PAGE) electrophoresis was run according to Schägger and von Jagow [44] with minor modifications. Briefly, 0.01% Coomassie G-250 (Sigma Aldrich) was added to the cathode buffer A instead of 0.02% Serva blue G. The gel with the separation gradient of 5–16% was run at 100 V at 4 °C for 30 min, then the blue cathode buffer was changed to the clear one without any dye, and the gel was run at the same conditions until the dye front reached the bottom of the gel.

To determine the accurate mass of the apoprotein, the denatured acpPC protein sample was analyzed by a Synapt G2 Q-IM-TOF mass spectrometer coupled with a NanoAcuity UPLC (Waters) as previously described [45] with minor modifications. The pellet obtain by acetone precipitation (see last paragraph for details) was resuspended in 5% acetonitrile 0.1% formic acid. The protein sample was directly loaded onto a home-packed C18 column (Magic, 0.075 mm × 50 mm, 5 μm, 200 Å, Michrom Bioresources) by a six-port injection valve (IDEX Health & Science). The gradient was delivered by NanoAcuity UPLC (0–2 min, 5% solvent B; 2–15 min 5–95% solvent B. Solvent A: water, 0.1% formic acid; Solvent B: acetonitrile, 0.1% formic acid) at flow rate 1 μL/min. The protein spectrum was acquired at sensitive mode ("v" mode) with

capillary voltage of 1.8 kV, cone voltage 30 V and source temperature 100 °C.

The size of the native protein was determined by native electrospray ionization mass spectrometry (native ESI-MS). The acpPC sample was concentrated by a MWCO filter (10 kDa, Vivaspin, GE Healthcare) and then buffer-changed to 200 mM ammonium acetate pH = 5 containing 0.02% DDM with a Micro Bio-Spin 6 chromatography column (MW exclusion limit 6 kDa, Bio-Rad). 5 μL was loaded into an offline electrospray capillary (GlassTip 2 μm ID, New Objective). The sample solution was injected to a hybrid ion-mobility quadrupole time-of-flight mass spectrometer (Q-IM-TOF, SYNAPT G2 HDMS, Waters). The instrument was operated under gentle nanoESI conditions in sensitivity mode ("v" mode): capillary voltage 1.6–2.1 kV, sampling cone 60 V, extraction cone 2 V, source temperature 30 °C, and source gas flow 20 mL/min. The initial trap and transfer collision energies were 10 and 5 V respectively. The collision energy was tuned during the experiment up to 150 V to remove tightly bond detergents. Each spectrum was acquired from m/z 300–10,000 every 1 s. The instrument was externally calibrated up to 10,000 m/z with the clusters produced by ESI of a NaI solution. The peak picking and data processing was performed in MassLynx (v 4.1, Waters).

2.4. Peptide sequencing of tryptic digested acpPC

The Coomassie blue-stained band from SDS-PAGE of purified acpPC was excised and in-gel digested with trypsin as previously described [46] with minor modifications. After dithiothreitol reduction and iodoacetamide alkylation, 20 μg/mL trypsin (Promega) in 36 mM NH_4HCO_3, 8.1% acetonitrile, 0.1 mM HCl was added to the gel pieces. After incubation at 37 °C for 12 h and centrifugation, the supernatant was collected, and the gel pellet was extracted with 1% trifluoroacetic acid in 60% acetonitrile for 30 min. Supernatants were then combined and dried by vacufuge. The trypsin digested PCP sample was reconstituted in water with 0.1% formic acid (25 μL). The sample was analyzed by LC-MS/MS using two instruments, a Waters Synapt G2 Q-IM-TOF and a Thermo LTQ Orbitrap (Thermo-Scientific). The data dependent mode was used in the LC-MS/MS experiment at Orbitrap as previously described [47] with minor modifications. The MS^E mode was used in the LC-MS/MS experiment on the Synapt G2 [48]. The tryptic digested PCP sample (5 μL) was loaded onto a homemade silica capillary column that was custom packed with C18 reverse phase material (Magic, 0.075 mm × 150 mm, 5 μm, 120 Å, Michrom Bioresources). The gradient was supplied by a Waters NanoAquity UPLC and run from 5% solvent B (acetonitrile, 0.1% formic acid) to 50% solvent B over 60 min, then to 95% solvent B for 2 min at 400 nL/min flowed by a re-equilibration step with 100% solvent A (water, 0.1% formic acid). The flow was directed by a nanospray source (Waters). In the MS^E continuum mode, ions were dissociated in the trap region by ramping the trap collision energy from 14 to 40 V. Spectra from 50 to 2000 m/z were acquired with scan time 1 second for 70 min in positive sensitivity mode. The MS^E raw data were directly submitted to the ProteinLynx Global Server (V2.5, Waters) to search against the NCBI and UniProt database. The raw data from LTQ-Orbitrap was converted into MGF format by MM file conversion (from MassMatrix software package). The MGF file was searched against NCBI database by Mascot.

Amino acid sequences of LHCs were aligned by ClustalW in MEGA 6. The maximum likelihood phylogenetic tree was generated using MEGA 6 with 1000 bootstrap replications.

3. Results

3.1. Protein purification and identification

Thylakoid membranes of *Symbiodinium* sp. CS-156 were solubilized using DDM, and acpCP was purified by ion exchange and gel filtration chromatography. Spectroscopic properties of *Symbiodinium* acpPC are very similar to those of the counterpart complex from *Amphidinium* [24,26–28]. The absorption spectra of the acpPC at room temperature (294 K) and 77 K are shown in Fig. 1 and Figure S1, respectively. At RT, the Q_y band of Chl *a* is centered at 672 nm. The 440 and 459 nm peaks are assigned to Chl *a* and Chl c_2 Soret bands, overlapping with carotenoid absorption which further extends to the blue-green region. The 77 K spectrum is also dominated by three major peaks at 438, 462 and 671 nm. The emission spectra of acpPC excited at 460, 495 and 535 nm at room temperature (not shown) and 77 K (Figure S2, Figure S3) all display a single characteristic Chl *a* fluorescence peak at 679 nm and a broad band around 750 nm, consistent with other reports [27,28]. The UV/Vis circular dichroism spectrum (Fig. 2) shows that carotenoids and chlorophylls within the complex are highly dichroic, indicating a well-defined pigment arrangement in acpPC, as the ellipticity completely disappeared when the complex was thermally denatured (data not shown), although its CD exhibits no similarity to those of other LHCs [49–52].

3.2. Oligomeric state of acpPC

To analyze the oligomeric state of acpPC, we estimated the protein size via SDS-PAGE, size exclusion chromatography and non-denaturing electrophoresis, and then obtained the accurate size by mass spectrometry (LC/MS and native MS).

The size of acpPC polypeptide was examined by SDS-PAGE and LC/MS (Fig. 3). SDS-PAGE revealed a single band of a mass between 15 and 20 kDa, which was determined to be 18.3 kDa by LC/MS.

A Superdex 75 10/300 GL column (GE Healthcare) was calibrated with bovine serum albumin (BSA, 67 kDa), β-lactoglobulin (BLG, 36.6 kDa), ribonuclease A (RNase A, 13.7 kDa), vitamin B_{12} (1355 Da) and blue dextran (~2 MDa) (Fig. 4A). The calibration curve (plot of K_{av} versus the logarithm of MW) is linear with an R^2 of 0.999 (Fig. 4C). The elution profile of acpPC revealed a single peak at 9.4 mL, which corresponds to a MW of 64 kDa (Fig. 4). The non-denaturing gel revealed a single band slightly smaller than 67 kDa (Fig. 5). The unstained gel shows no significant loss of pigments, suggesting that acpPC kept its native state under the running conditions.

To measure the accurate mass, acpPC stabilized with DDM was characterized by native MS. In studies of protein assembly by mass spectrometry, intact proteins are introduced into the gas phase by gentle nanoESI. The folded intact protein complex only has a limited amount of positive charged side chains on the surface, and thus usually carries fewer charges in a mass spectrum than the denatured protein under regular ESI conditions. In the case of acpPC, a membrane-embedded protein, which was protected by the detergent micelle before introduction to the gas phase, a broad peak was observed in the initial experiments. The collision energy is required to remove those bound

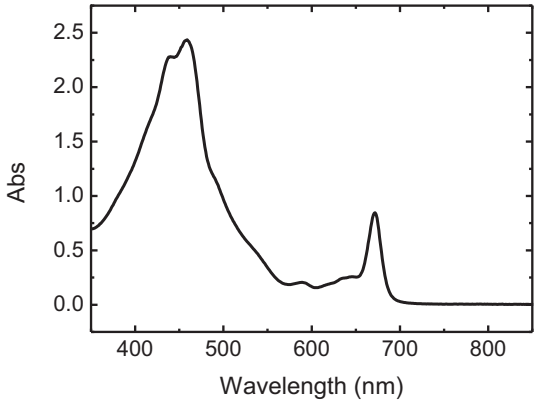

Fig. 1. The steady-state absorption spectrum of *Symbiodinium* acpPC at room temperature. Three peaks at 440 nm, 459 nm and 672 nm are identified.

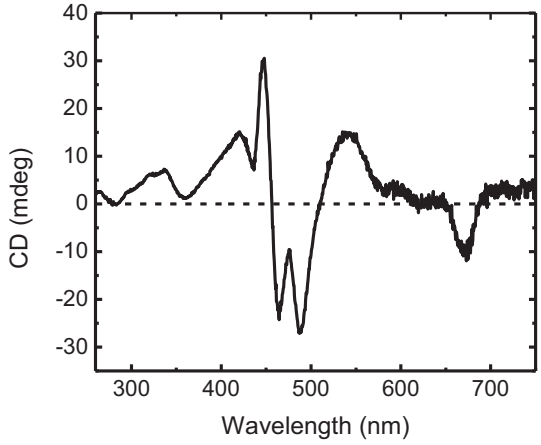

Fig. 2. UV/Vis CD spectrum of acpPC in 20 mM sodium phosphate pH = 7 and 0.02% DDM. A CD spectrum of the buffer was subtracted from the raw CD data of acpPC.

detergents and release the intact protein assembly. We observed two series of charged states by ramping the collisional energy, representing two species with a MW of 18.3 kDa (charge states 6+ to 8+) and 66.5 kDa (charge states 14+ to 16+), respectively (Fig. 6). The former was assigned to the apoprotein of acpPC, as the MW is consistent with previous measurements (Fig. 3), and the latter to the native acpPC. DDM was used to solubilize acpPC before its introduction to MS, as DDM has proved to be the most successful detergent in native MS, although other detergents can improve the spectra of certain membrane proteins [39].

In addition, acpPC in 200 mM ammonium acetate containing 0.02% DDM without adjusting pH was studied by native MS and SEC (data not shown). We also measured the size of acpPC in nonvolatile buffers (e.g. 20 mM sodium phosphate pH = 7, 20 mM bis-Tris pH = 6–6.5, 20 mM Piperazine-HCl pH = 5–5.8) with various concentrations of DDM (0.02–0.05%) via SEC, and found that different buffers or/and detergent concentrations did not affect the elution profile of the protein complex (data not shown).

To summarize, the size of native acpPC measured by native ESI-MS, SEC and BN-PAGE agrees with each other, indicating it is probably an oligomer since the size of the apoprotein is only 18.3 kDa, which is much smaller compared to the native size (66.5 kDa).

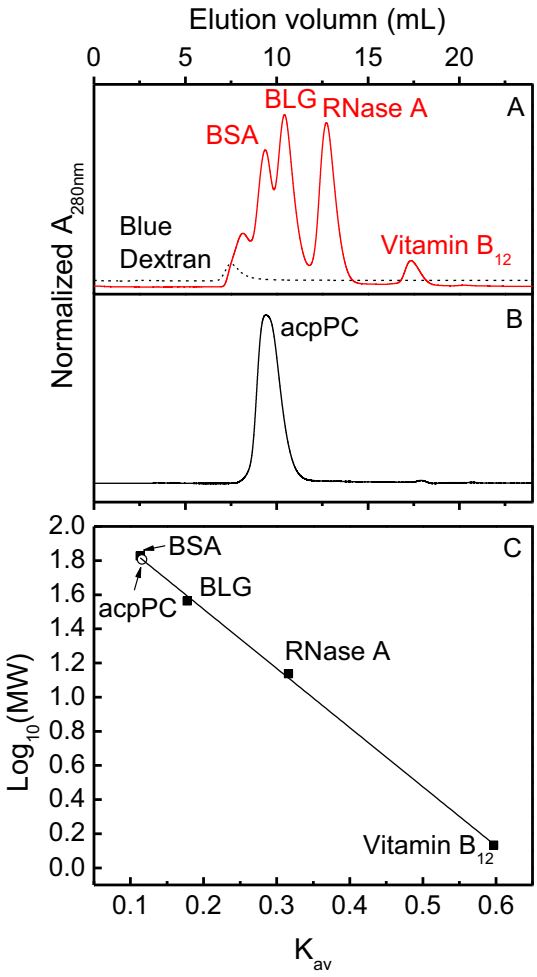

Fig. 4. SEC analysis of acpPC in 200 mM ammonium acetate pH = 5 supplemented with 0.02% DDM. A. Elution profile of standards (BSA, BLG, RNase A, vitamin B_{12}) and the void volume marker Blue Dextran. B. Elution profile of acpPC in the same buffer under the same running conditions for calibrants. C. Linear regression analysis determines the average molecular mass of acpPC. K_{av}, partition coefficient, equals to $\frac{V_e - V_o}{V_c - V_o}$, where V_e is the elution volume, V_o is the column void volume, which is the elution volume for Blue Dextran, and V_c is the geometric column volume.

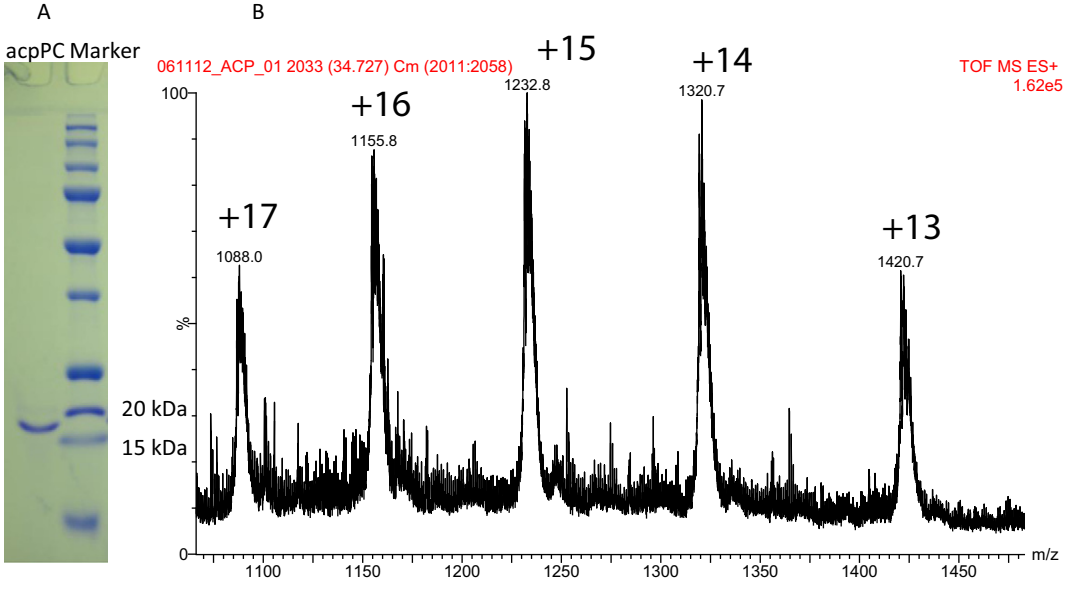

Fig. 3. A. SDS-PAGE analysis of acpPC, indicating the apoprotein size is ~18 kDa. B. LC/MS spectrum of denatured acpPC protein, showing that the apoprotein MW is 18.3 kDa.

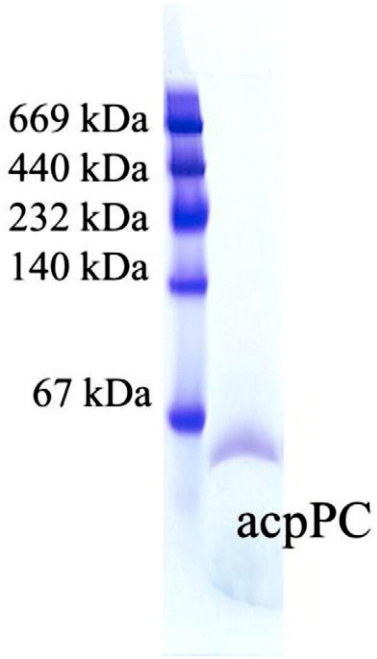

Fig. 5. Blue native-PAGE analysis, left lane: native protein marker (GE Healthcare), right lane: acpPC. The MW of native acpPC is very close to 67 kDa, suggesting that it associates to form a trimeric complex.

3.3. Sequencing of acpPC

Mass spectrometric sequencing results confirmed that acpPC belongs to the Chl c_2-containing proteins of *Symbiodinium*. The one with most hits is a fraction of a putative protein translated from an mRNA sequence (GenBank: FN646416.2) for acpPC in a clade C_3 strain of *Symbiodinium* [32] (Fig. 7). The maximum likelihood tree was generated using MEGA 6 with 1000 bootstrap replications.

4. Discussion

The coral endosymbionts *Symbiodinium* are essential constituents of coral reefs, the most productive and diverse ecosystems on Earth [4]. They give corals their coloration and provide corals photosynthetically-produced nutrients. Their photosynthetic activity is thought to be the key to maintain the symbiotic relationship between the algae and hosts [6–8]. However, their photosynthesis is largely unknown. In this study, we characterized the intrinsic light-harvesting complex (acpPC) from *Symbiodinium* and determined the oligomeric state of the complex, based on the accurate masses of the apoprotein and native protein, as well as the pigment content.

We relied on native mass spectrometry (native MS) to determine the size of native acpPC, supplemented with traditional biochemical methods, namely size exclusion chromatography (SEC) and blue native PAGE (BN-PAGE). Recent developments in MS have overcome the difficulties of maintaining membrane protein stabilities in the gas phase, allowing this technique to provide insights into subunit stoichiometries and specific phospholipid interactions [36–40,53]. Compared to traditional biophysical methods such as X-ray crystallography and NMR spectroscopy, which are hindered due to the hydrophobicity, flexibility and instability of membrane proteins, native MS is very sensitive and much less time-consuming, while requiring relatively small amount of protein. The first membrane protein complex directly observed in the gas phase was a rat microsomal glutathione transferase. The noncovalently-associated homotrimer (53 kDa), the proposed active form of the enzyme, was identified by electrospray mass spectrometry analysis [53]. With the advances of this approach, several other membrane proteins have been successfully characterized, including the *E. coli* ammonium channel [39], a vitamin B_{12} importer [36], several ATP-binding cassette transporters [36], a diacylglycerol kinase [40], a sensory rhodopsin II [40]. This technique has also been extended to very large complexes: V-type ATPases from *Thermus thermophilus* and *Enterococcus hirae*, ~700 kDa protein complexes containing 26 subunits, where both soluble and membrane subunits were preserved in the gas phase [38]. Recently, our group has applied native MS to photosynthetic protein complexes. The MW of the intact FMO trimer was measured by native MS, indicating the existence of an eighth pigment (a bacteriochlorophyll *a*) and its partial occupancy in the complex [41], which is consistent with the previous finding in newly resolved FMO crystal structures [54,55]. We also successfully demonstrated the dimer-to-monomer transition of the orange carotenoid protein (OCP) upon illumination and the reverse process in the dark [42]. Native MS has been proven to be a reliable technique to accurately measure the sizes of various types of proteins, and can be applied to proteins lack of structural information. In this study, we observed two series of charged states representing the acpPC apoprotein (18.3 kDa, charge states 6+ to 8+) and native acpPC (66.5 kDa, charge states 14+ to 16+), respectively (Fig. 6). The mass determined by native MS is consistent with the size estimated by SEC and BN-PAGE. Compared to native MS, which gives m/z values for direct mass calculation, these two traditional

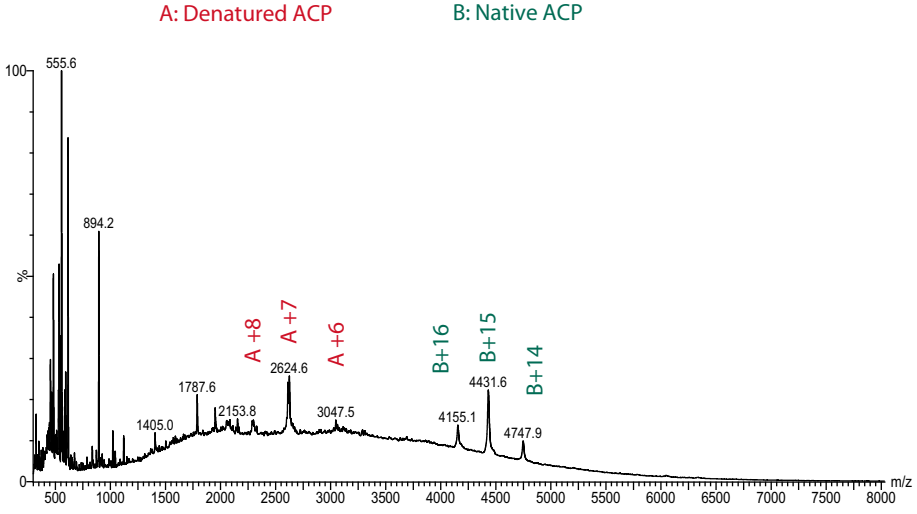

Fig. 6. Native-ESI mass spectrometry detected both denatured and native acpPC. Native acpPC has a mass of 66.5 kDa.

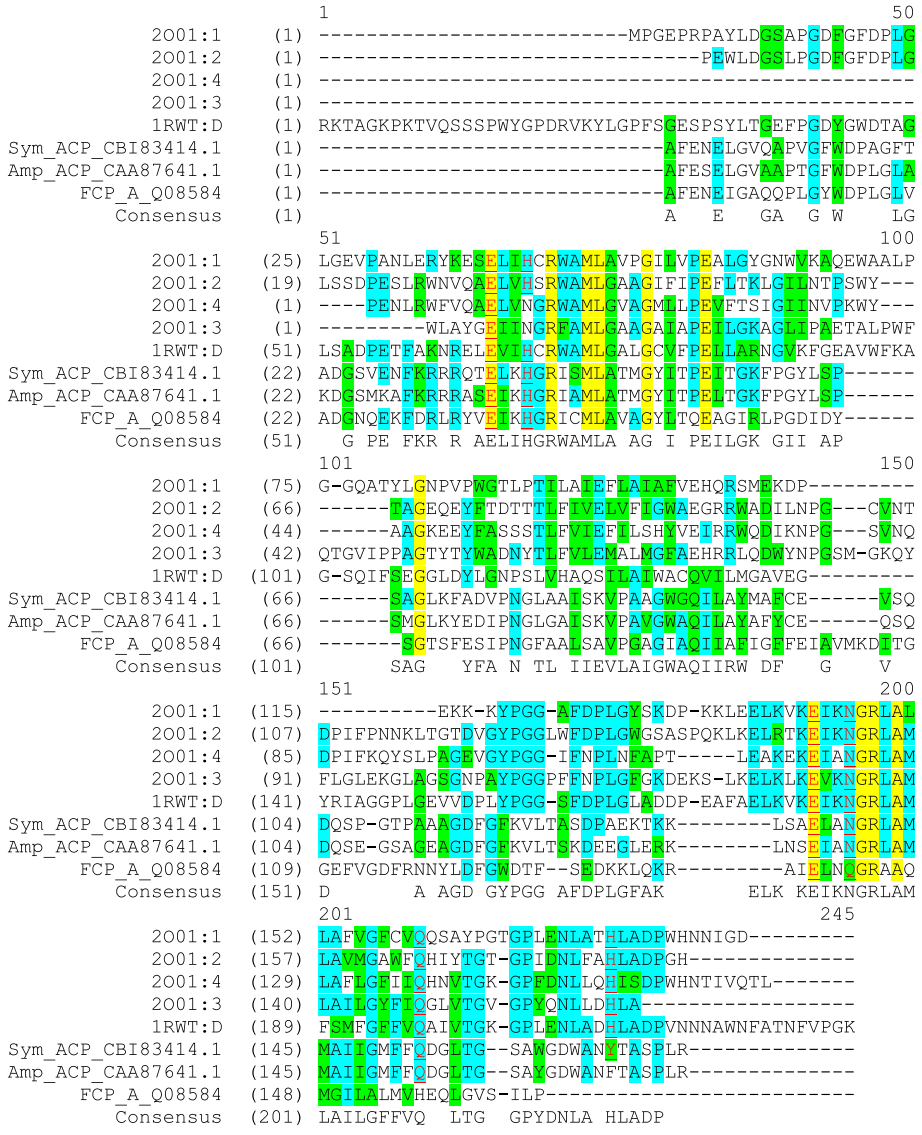

Fig. 7. Comparison of LHCs of different organisms. 2O01:1–4 are Lhca 1–4 from *Pisum sativum* [60]; 1RWT:D is LHCII from *Spinacia oleracea* [61] (2O01 and 1RWT are PDB IDs); Sym_ACP_CBI83414.1 is acpPC of *Symbiodinium* sp. [32]; Amp_ACP_CAA87641.1 is acpPC of *Amphidinium carterae* CS-21 [25] (CBI83414.1 and CAA87641.1 are Genbank IDs); FCP_A (Uniprot Q08584) is FCPA (fucoxanthin-chlorophyll *a-c* binding protein A) from *Phaeodactylum tricornutum* [62,63]. (Color codes: conservative block of similar identical Chl *a* central ligands in 1RWT).

techniques more depend on the shape of the protein rather than the actual size of the protein. However, they are still widely used to approximate the sizes of membrane proteins, although standards are usually water-soluble proteins [56–59].

The apoprotein and the native complex has a MW of 18.3 kDa and 66.5 kDa, respectively, both of which were determined in this study, and the 4/6/6/1 (Chl *a*/Chl c_2/peridinin/diadinoxanthin) pigment ratio was reported by the same authors elsewhere [29]. Pigments of pure acpPC were extracted by adding HPLC grade methanol, followed by centrifugation. The absorption spectrum of the supernatant (methanol extract) was used for pigment ratio determination. Because each pigment present in the acpPC has known molar extinction coefficient(s) in methanol at certain wavelength(s), the reconstruction of the pigment extract spectrum using absorption spectra of individual pigments in methanol was performed (Fig. S2 and Table S1 of [29]). The molar ratio of the pigments in the acpPC was determined to be 4/6/6/1 (Chl *a*/Chl c_2/peridinin/diadinoxanthin). Then we reconstructed the 77 K absorption spectrum of native acpPC (not methanol extract) with spectra of individual pigments taken in 2-MTHF (2-methyl tetrahydrofuran) or diethyl ether/isopentane/ethanol, 5/5/2 (v/v/v), in which pigments have similar spectral properties compared to those in native protein environments. The same ratio was obtained. If we consider that acpPC forms a trimeric complex and contains 4 Chl *a* ($MW_{Chl\ a}$ = 893.49 Da), 6 Chl c_2 ($MW_{Chl\ c2}$ = 608.93 Da), 6 peridinins ($MW_{peridinin}$ = 630.81 Da) and 1 diadinoxanthin ($MW_{diadinoxanthin}$ = 582.85 Da) per complex, the MW of native protein would be 66.5 kDa ($18{,}300 \times 3 + 893.49 \times 4 + 608.93 \times 6 + 630.81 \times 6 + 582.85 = 66{,}495$ Da), which is consistent with our observations. Some reports showed different pigment ratios in acpPC from different dinoflagellates: the acpPC of *Amphidinium carterae* binds 7:4:12 (or 10):2 of Chl *a*, Chl c_2, peridinin and diadinoxanthin depending on the detergent used to solubilize thylakoid membranes [24], and *Prorocentrum minimum* has a ratio of 4:7:8:2 or 4:7:6:4 depending on growth conditions [30]. Although these values are quite different from the 4:6:6:1 ratio we established [29], native acpPCs isolated from different organisms may not have the same pigment composition and in any case would have MWs ranging from 68 to 72 kDa, which are close to the size of the complex in this study, if those acpPCs can form trimers. It would be very interesting to examine the sizes of acpPCs isolated from various sources to see if they fit the trimer assumption.

To further characterize acpPC, we conducted phylogenetic analysis of various LHCs. Fig. 7 compares amino acid sequences of LHCs from different organisms, including LHCI from *Pisum sativum* (PBD ID: 2O01:1–4 [60]), LHCII from *Spinacia oleracea* (PBD ID: 1RWT:D [61]), *Amphidinium* acpPC (Genbank CAA87641.1 [25]), FCPA (fucoxanthin-chlorophyll *a-c* binding protein A, encoded by *Lhcf*, see discussion below) from *Phaeodactylum tricornutum* (Uniprot Q08584 [62,63]), as well as *Symbiodinium* acpPC (Genbank CBI83414.1 [32]) in this study. We found that acpPCs from two genera share ~80% similarity, while they have about 55% and 40% similarities with diatom FCP and plant LHCs, respectively. Phylogenetic analysis (Fig. 8) indicates that acpPCs are most closely related to FCP from diatoms, and distantly related to LHCs from higher plants, with LHCII a closer relative compared to LHCI. This agrees with other studies [25,32,64]. FCPs, the closest relative to acpPC among all known groups of LHCs, serve as intrinsic LHCs in diatoms, which belong to the heterokonts, a eukaryotic group of algae. FCP and acpPC are similar in size and share some photosynthetic pigments, Chl *a*, Chl *c* and diadinoxanthin. Although containing distinct primary carotenoids (fucoxanthin versus peridinin), they belong to the intrinsic Chl *a*/*c* LHC family, which consists of various LHCs from Chl *c*-containing algae in four major lineages, heterokonts, haptophytes, dinoflagellates and cryptophytes. These lineages are thought to share a common ancestor that acquired a plastid by engulfing a red alga (secondary endosymbiosis) and are thus designated as chromalveolates [65,66]. Another piece of evidence is the fluorescence emission spectrum of *Symbiodinium* whole cells at 77 K, revealing a single peak at 687 nm, which is generally thought to be PSII fluorescence [5,67], indicating that most LHCs transfer excitations to PSII under normal conditions (Figure S4). This suggests that acpPCs in dinoflagellates have similar functions to the *Lhcf*-encoded FCPs [49,68]. The fact that FCPs can assemble into trimers and higher oligomers [49] supports our finding that acpPC exists as trimers, as the two closely related LHCs have originated from the rhodophyte ancestry, and both can feed energy to PSII.

Sequence analysis of FCPs revealed the existence of another type of FCP, LI818- or LHCSR-like FCP (encoded by *Lhcx*) which is involved in photoprotection [69]. However, only one type of acpPC has been discovered so far ([25] and this study), and genomic analysis using the gene encoding LHCSR-like FCP as the bait yields poor matches, which only cover less than 6% of the query sequence, indicating no sign of this type of LHCs in *Symbiodinium* (see supplementary information S1). Other studies also show the lack of the protective LHCs in other dinoflagellates [64]. The fact that acpPC contains the carotenoids diadinoxanthins (Ddx), which are equivalent to xanthophyll pigments central to plant photoprotection seems to suggest that it may have a similar role in dinoflagellates, but no acpPC bound with diatoxanthin (Dtx) has been isolated yet, despite the fact that the conversion from Ddx to Dtx was detected in cells under high light conditions [70]. Moreover, the Chl *a* triplet quenching is inefficient in acpPC [28,29]. This could mean that there may exist another type of acpPC/LHC regulating photoprotection in dinoflagellates, as *Lhcx*-encoded FCPs do in diatoms [64,68]. Some evidence indicates that acpPC may have a state transition response with redistribution from PSII to PSI under high light conditions [71]. Another way to protect cells from overexcitation is to disconnect LHCs (primarily PCPs and acpPCs) from PSII under intense illumination [5,71]. Alternatively, the lack of photoprotection mechanism could explain why some dinoflagellates (especially *Symbiodinium*) are so fragile and readily damaged upon increased irradiation.

Another interesting fact is that we did not observe acpPC monomers in this study (data not shown). After membrane solubilization, the supernatant obtained by ultra-centrifugation was applied to a gel filtration column. SEC analysis revealed it contained mostly acpPC trimers with very small amount of PCP but no indication of the existence of acpPC monomers (confirmed by LC-MS/MS, data not shown). This is different from FCP, which can also exist as monomers, although monomeric FCPs had lost most of the energy coupling between carotenoids and chlorophylls [49].

In this study, we utilized LC-MS/MS to sequence acpPC (sequence shown in Fig. 7). The theoretical MW is 18357.90 Da, which is very close to the MW measured by LC/MS. We further analyzed MS data with the *de novo* sequencing module in PEAKS (Bioinformatics Solutions). This module can derive a peptide sequence from mass spectrum without the need of a sequence database, capable of identifying novel peptides and studying unsequenced organisms. Ambiguities of some residues (data not shown) were found, indicating that *Symbiodinium* may be able to encode acpPCs with slightly different sequences. This is consistent with previous work done in *Amphidinium*: persistent ambiguities were observed at residue 4 and in the C-terminal region [24]. A recent study also confirmed the hyperdiversity of genes encoding this protein by screening EST libraries in *Symbiodinium* [32]. It is not surprising that acpPC has multiple gene copies in the genome as does PCP [10,14–16,19–21,23], since genes are often organized in tandem arrays in dinoflagellates [31]. A draft assembly of the nuclear genome suggests that in *Symbiodinium minutum*, genes representing 42.4% of the proteome may have originated by gene duplication, including 62 genes encoding Chl *a*/*b* binding proteins [31]. This does not necessarily mean that our strain also has an equal amount of this group of genes, because *Symbiodinium* of different clades can vary in the aspect of gene copy numbers [23]. A BLAST search was conducted by using the acpPC gene sequence in this study against the whole-genome shotgun contigs of *Symbiodinium minutum* [31]. We found 56 hits with the highest identity of ~80% (see supplementary information S2), indicating acpPC is not fully conserved even in the same genus. It is not clear why *Symbiodinium* can produce LHCs with sequence variations in different species or even in the same strain. It might be related to their habitats, where only blue green light of low intensity is available [33]. The diversity of LHCs ensures their efficient light capture and thus survival. Another fact is that *Symbiodinium* encodes transcripts comprised of multiple acpPC mRNAs [32]. It is likely that these LHCs are initially expressed in the cytosol as polyproteins and then transported into the chloroplast where the polypeptide is cleaved into individual mature acpPC [32]. For example, the mRNA sequence (GenBank: FN646416.2), to which our protein has the most hits, consists of two identical LHC transcripts. Further northern blot analysis suggests that it can have up to ten repeats [32]. A similar phenomenon was found in *Amphidinium carterae,* which possesses acpPC polyproteins with up to 10 different but closely related polypeptides [25].

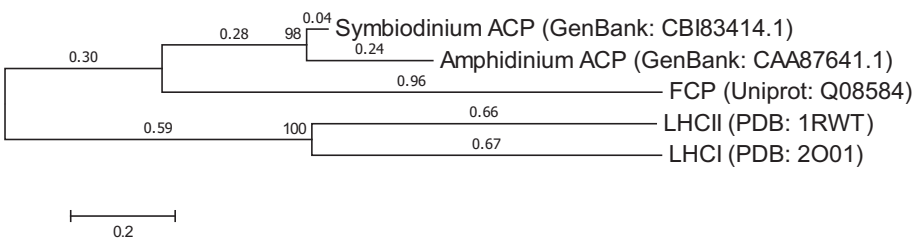

Fig. 8. Phylogenetic tree of LHCs. The maximum likelihood tree was generated using MEGA 6 with 1000 bootstrap replications. The numbers next to each node represent a measure of support for the node (bootstrap values) and the ones along the lines are branch lengths.

Unlike higher plants and diatoms, peridinin-containing dinoflagellates possess both membrane-intrinsic (acpPCs) and the water-soluble (PCPs) LHCs. Another algal group that shares this same feature are the cryptophytes, which also have additional unique LHCs, phycobiliprotein tetramers, in the thylakoid lumen [72].

PCP shares no sequence similarity to any other LHCs. It is not clear whether it can interact with acpPC or not. Energetically, PCP should be able to transfer excitations to acpPC, as the former emits at 674 nm (77 K, [14]) while the latter (77 K, this study) at 679 nm upon peridinin excitation. The energy gap makes the transfer possible. The situation also holds for PSII, which has fluorescence emission maximum at 687 nm (77 K, this study).

5. Conclusions and outlook

In this study, we isolated and characterized the major LHC chlorophyll *a*-chlorophyll c_2-peridinin-protein (acpPC). This complex is integral to the thylakoid membranes and contains Chl *a*, Chl c_2, peridinin and diadinoxanthin (Ddx), associated with a polypeptide of 18.3 kDa, the N-terminal region of which is related to the LHCII of higher plants. We utilized native electrospray ionization mass spectrometry, size exclusion chromatography and non-denaturing electrophoresis, and determined the size of the native complex to be 64–66 kDa. Along with the accurate MW of the apoprotein measured by LC/MS and the pigment stoichiometry, we conclude that the native acpPC is probably a trimer.

In this study, we identified acpPC at the protein level in *Symbiodinium* and showed direct evidence revealing the possible quaternary structure of the intrinsic LHC in dinoflagellates. It could provide some insights into the phylogeny of Chl *c*-containing algae. Although several genes encoding Chl *a*/*c* proteins have been established in different genera of dinoflagellates [64], only in *Amphidinium* the LHC was detected at the protein level [25]. Isolating representative LHCs and understanding the functions of them is essential to correctly interpret relationships among different linages in phylogenetic analysis, which is mainly based on cDNA or putative proteins, and may contribute to resolving many unknowns in this area in the future.

As the endosymbionts of coral reef builders, *Symbiodinium* have received increasing attention in recent years. The photosynthesis of the algae is critical to themselves and their hosts, but the details of the photosynthetic mechanism are still unclear. How do the LHCs (namely PCP and acpPC) interact with each other as well as photosystems? How do the two photosystems communicate? How do stressed conditions affect the behavior of photosynthetic machineries? As an important representative of Chl *c* LHC family, acpPC is distinct compared to other related proteins from chromists, e.g. ineffectiveness in photoprotection. Are there any other functions besides light-harvesting or any unidentified protoprotective proteins? Why have Chl *a*/*c* LHCs preserved the chlorophylls they bind while adapting to various primary carotenoids? What is the origin of Chl *c*? These are key questions to be addressed.

Acknowledgements

The authors would like to thank Drs. David Kramer and Atsuko Kanazawa of Michigan State University for providing the *Symbiodinium* culture. We would like to acknowledge the NIH NCRR Biomedical Mass Spectrometry Resource at Washington University supported by NIH NCRR Grant P41RR000954, for help with the Orbitrap LC-MS/MS experiments. We would also like to thank Dr. Dariusz Niedzwiedzki for discussions, and Ms. Mindy Prado for helping with cell culturing. This research is from the Photosynthetic Antenna Research Center (PARC), an Energy Frontier Research Center funded by the DOE, Office of Science, Office of Basic Energy Sciences under Award Number DE-SC 0001035.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbabio.2014.07.023.

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