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

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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.

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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

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