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Photoprotective sites in the violaxanthin–chlorophyll *a* binding Protein (VCP) from *Nannochloropsis gaditana*

Donatella Carbonera [a,*], Alessandro Agostini [a], Marilena Di Valentin [a], Caterina Gerotto [b], Stefania Basso [b], Giorgio Mario Giacometti [b], Tomas Morosinotto [b]

[a] Department of Chemical Sciences, University of Padova, Via Marzolo 1, 35131 Padova, Italy
[b] Department of Biology, University of Padova, via U. Bassi 58/B, 35121 Padova, Italy

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ABSTRACT

Violaxanthin–chlorophyll *a* binding protein (VCP) is the major light harvesting complex (LHC) of the Heterokonta *Nannochloropsis gaditana*. It binds chlorophyll *a*, violaxanthin and vaucheriaxanthin, the last in the form of 19′ deca/octanoate esters. Photosynthetic apparatus of algae belonging to this group have been poorly characterized in the past, but they are now receiving an increasing interest also because of their possible biotechnological application in biofuel production. In this work, isolated VCP proteins have been studied by means of advanced EPR techniques in order to prove the presence of the photoprotective mechanism based on the triplet–triplet energy transfer (TTET), occurring between chlorophyll and carotenoid molecules. This process has been observed before in several light harvesting complexes belonging to various photosynthetic organisms. We used Optically Detected Magnetic Resonance (ODMR) to identify the triplet states populated by photoexcitation, and describe the optical properties of the chromophores carrying the triplet states. In parallel, time-resolved EPR (TR-EPR) and pulse EPR has been employed to get insight into the TTET mechanism and reveal the structural features of the pigment sites involved in photoprotection. The analysis of the spectroscopic data shows a strong similarity among VCP, FCP from diatoms and LHC-II from higher plants. Although these antenna proteins have differentiated sequences and bind different pigments, results suggest that in all members of the LHC superfamily there is a protein core with a conserved structural organization, represented by two central carotenoids surrounded by five chlorophyll *a* molecules, which plays a fundamental photoprotective role in Chl triplet quenching through carotenoid triplet formation.

1. Introduction

Photosynthetic organisms harvest sunlight thanks to the chlorophyll (Chl) and carotenoid (Car) molecules bound to the protein supercomplexes, embedded in the thylakoid membranes, called Photosystems I and II. Each photosystem is composed of two moieties, (i) the core complex, responsible for charge separation and first steps of electron transport, and (ii) the peripheral antenna system, with a role in light harvesting, transfer of excitation energy to the reaction centers, and photoprotective reactions, like quenching of Chl triplet and singlet excited states, and Reactive Oxygen Species (ROS) scavenging. Reaction centers are widely conserved among all organisms performing oxygenic photosynthesis, going from cyanobacteria to higher plants [1]. Antenna systems are instead far more diversified. In all eukaryotes, the antenna system is composed of members of a multigenic family of proteins called light harvesting complexes (LHC) proteins. All proteins belonging to this family have a common evolutionary origin [2–4] and share a conserved structural organization characterized by three membrane-spanning α-helixes connected by stroma and lumen-exposed loops. Two of these helixes are homologous and present a "generic LHC motif" consisting of a highly hydrophobic sequence containing glutamic acids involved in the Chl binding and in the stabilization of the folding through salt bridges with arginines in the other helix [5].

Despite this common origin, LHC proteins diversified in different groups of photosynthetic eukaryotes, such as Chl *a*/*b* binding proteins found in *Viridiplantae* (LHCA/LHCB), fucoxanthin Chl *a*/*c* binding protein (FCP, or LHCF) in diatoms, LHCR, in red algae and diatoms, and LHCSR/LHCX, with a role in photo-protection and found in all the above-mentioned groups [2,4,6,7]. Pigment binding properties of LHCs are thus diversified due to adaptation to the light availability in the specific habitat. Different LHCs can bind not only different Chl and Car species

Abbreviations: VCP, violaxanthin–chlorophyll *a* binding protein; Car, carotenoid; Chl, chlorophyll; LHC, light harvesting complex; FCP, fucoxanthin chlorophyll *a*/*c* binding protein; LHC-II, light harvesting complex of Photosystem II; ZFS, zero-field splitting; TR-EPR, Time-Resolved Electron Paramagnetic Resonance; TTET, triplet–triplet energy transfer; ODMR, optically detected magnetic resonance; FDMR, fluorescence detected magnetic resonance; T − S, triplet-minus-singlet

* Corresponding author. Tel.: +39 498275144; fax: +39 498275161.
E-mail address: donatella.carbonera@unipd.it (D. Carbonera).

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but also pigments in different relative amounts, with the Chl/Car ratio that can change between the value of 3.5, observed in plants and green algae, and 0.9, characteristic of the fucoxanthin chlorophyll proteins of diatoms [8].

Nannochloropsis gaditana is a eukaryotic alga belonging to *Eustigmatophyceae*, a group of organisms wich originated from a secondary endosymbiosis between an ancestor of red algae and a eukaryotic host cell [9]. The cells contain a single, big, chloroplast, which is surrounded by four membranes and occupies most of the cell volume. The *N. gaditana* photosynthetic apparatus is not well characterized although interest in this organism is increasing owing to its high rate of lipid productivity, which suggests that it could be a valuable candidate for biofuel production [10–12].

Photosystem II light harvesting proteins of *N. gaditana* are characterized by a particular pigment composition, different from the one of diatoms and any other known alga. In fact, they bind only Chl *a*, lack accessory Chls *b* or *c*, and have violaxanthin as the main accessory light harvesting pigment. Vaucheriaxanthin is present in minor amount, in the form of 19′ deca/octanoate esters [13–15]. For this reason, these antenna complexes have been defined violaxanthin–chlorophyll *a* binding proteins (VCP). The VCP fractions, purified from thylakoids solubilized in glycosidic detergents, have as a major component a 22-kDa polypeptide which, according to sequence analysis, shows similarity with LHCF from diatoms [16,17].

The number of chromophores bound *per* apoprotein is still unknown, however the ratio Chl/Car has been determined to be 1.7–1.8 and the ratio violaxanthin/vaucheriaxanthin is 1.5–1.6, depending on the oligomeric state of VCP [17].

It is well known that in light-stress conditions the formation of Chl triplet states (^{3}Chl) and singlet oxygen ($^1O_2$) in the photosynthetic apparatus may be particularly severe. In this scenario the constitutive mechanism of triplet–triplet energy transfer (TTET), played by carotenoids to quench ^{3}Chl *via* their triplet states, ($Car + {}^3Chl \rightarrow {}^3Car + Chl$), represents the fastest way of response before further photoprotective mechanisms have the time to take place. Once populated, ^{3}Car, lying at a lower energy compared to $^1O_2$, relaxes harmlessly to the ground state in the microsecond time scale [18]. TTET has been shown to occur in all the antennas of the LHC superfamily studied until now, in particular in FCP from *Cyclotella meneghiniana* [19,20], LHC from *Amphidinium carterae* [21] and LHC-II from *Spinacia oleracea* [22].

In this work, isolated VCP proteins from *N. gaditana* in different oligomeric states have been studied by means of advanced EPR techniques in order to investigate the presence of the photoprotective mechanism based on TTET. Optically Detected Magnetic Resonance (ODMR), time-resolved EPR (TR-EPR) and pulse EPR have been successfully employed in the past to get insights into the TTET mechanism in several light harvesting complexes [19–24]. The comparison of results obtained for VCPs with those previously published for other light harvesting complexes belonging to the LHC superfamily pointed out that, despite the divergence in sequence and pigment binding properties, they share a protein core, composed of five Chl *a* and two Car molecules, highly conserved also in the structural organization. This core has a major role in ^{3}Chl quenching and its photoprotective function is likely fundamental in all antenna systems of the LHC superfamily, as its conservation suggests.

2. Materials and methods

2.1. Cell growth

N. gaditana from CCAP, strain 849/5, was grown in sterile filtered F/2 medium [25], using sea salts 32 g/l from Sigma Aldrich, 40 mM TRIS/HCl pH 8, Sigma Aldrich Guillard's (F/2) marine water enrichment solution 1×. Cells were grown under 100 μE m^{-2} s^{-1} of illumination and mixed with air enriched with 5% CO_2. Temperature was set at 22 ± 1 °C.

2.2. VCP purification

Isolation of thylakoid membranes from *N. gaditana* was performed according to [17]. Thylakoid membranes were then solubilized with final 0.4% α-DM, 10 mM HEPES pH 7.5 and loaded in a 0.1–1 M sucrose gradient. The bands corresponding to monomeric and trimeric VCP of the sucrose gradient were then harvested with a syringe. All the manipulations performed to obtain final sampling for the ODMR and EPR experiments have been done in dim green light at 4 °C.

2.3. Sequence analysis

Alignment analysis was performed using T-Coffee [26,27] and manually modified with Bioedit 7.1.3.0. Chl binding sites were identified according to Liu et al. [5], α-helixes were named according to Dittami et al. [7]. *Nannochloropsis* sequences Naga 2 (Naga_100027g19), Naga3 (Naga_100012g50) Naga4 (Naga_100004g86) Naga9 (Naga_100017g59) and Naga17 (Naga_100013g28) were retrieved from nannochloropsis.org [28], while sequences from *Spinacia oleracea*, Lhcb1_So, (P12333.1), and *C. meneghiniana*, Fcp1_Cmen (AJ000670.1), were retrieved from NCBI.

2.4. ODMR measurements

The principle of the ODMR technique, reviewed in [29], will be briefly summarized in the following. ODMR is a double resonance technique based on the principle that, when a triplet steady state population is generated under continuous illumination, the application of a resonant microwave electromagnetic field between a couple of spin sublevels of the triplet state, generally induces a change of the steady state population of the triplet state itself, due to the anisotropy of the decay and population rates of the spin sublevels. The induced change of the triplet population may be detected as a corresponding change of the emission and/or absorption of the system. In particular, absorbance detected magnetic resonance (ADMR), detects the change in the steady state absorption of the chromophore carrying the triplet state, whereas the changes in the emission are detected by means of fluorescence detected magnetic resonance (FDMR).

FDMR and ADMR experiments were performed in the laboratory built apparatus, described in detail in [30,31]. Amplitude modulation of the applied microwave field is used to greatly increase the signal to

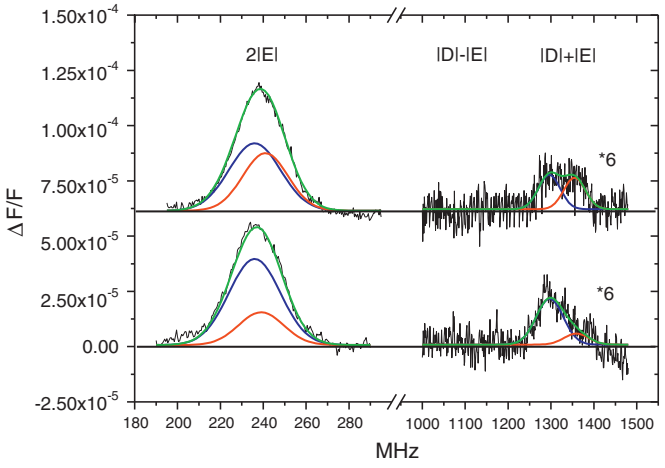

Fig. 1. ^{3}Car FDMR spectra of monomeric (top) and trimeric (bottom) VCPs detected at 690 nm. Amplitude modulation frequency: 333 Hz, time constant: 600 ms, temperature: 1.8 K. Reconstruction (green) of the experimental spectra (black) using two Gaussian components (blue and red). Spectra vertically shifted for comparison.