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A conserved isoleucine in the LOV1 domain of a novel phototropin from the marine alga *Ostreococcus tauri* modulates the dark state recovery of the domain

Sindhu Kandoth Veetil, Chitvan Mittal, Peeyush Ranjan, Suneel Kateriya *

Department of Biochemistry, University of Delhi South Campus, Benito Juarez Road, New Delhi-110021, India

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ABSTRACT

Background: Phototropins are UV-A/blue light receptor proteins with two LOV (Light-Oxygen-Voltage) sensor domains at their N terminus and a kinase domain at the C-terminus in photoautotrophic organisms. This is the first research report of a canonical phototropin from marine algae *Ostreococcus tauri*.
Methods: We synthesized core LOV1 (OtLOV1) domain-encoding portion of the phototropin gene of *O. tauri*, the domain was heterologously expressed, purified and assessed for its spectral properties and dark recovery kinetics by UV–Visible, fluorescence spectroscopy and mutational studies. Quaternary structure characteristics were studied by SEC and glutaraldehyde crosslinking.
Results: The absorption spectrum of OtLOV1 lacks the characteristic 361 nm peak shown by other LOV1 domains. It undergoes a photocycle with a dark state recovery time of approximately 30 min ($\tau = 300.35$ s). Native OtLOV1 stayed as dimer in aqueous solution and the dimer formation was light and concentration independent. Mutating isoleucine at 43rd position to valine accelerated the dark recovery time by more than 10-fold. Mutating it to serine reduced sensitivity to blue light, but the dark recovery time remained unaltered. I43S mutation also destabilized the FMN binding to a great extent.
Conclusion: The OtLOV1 domain of the newly identified OtPhot is functional and the isoleucine at position 43 of OtLOV1 is the key residue responsible for fine-tuning the domain properties.
General significance: This is the first characterized LOV1 domain of a canonical phototropin from a marine alga and spectral properties of the domain are similar to that of the LOV1 domain of higher plants.

1. Introduction

Phototropins belong to the family of UV-A/blue light receptors that are present in both higher plants and lower organisms and are responsible for a variety of photoresponses [1,2]. Phototropins consist of two flavin mononucleotide (FMN) binding Light-Oxygen-Voltage (LOV1 and LOV2) domains at the N-terminus as the sensor domains and a kinase domain at the C-terminus [3] as the effector domain. In higher plants, phototropins are responsible for optimizing photosynthetic efficiency and avoiding photo-oxidative damage by chloroplast relocation [4], stomatal opening [5], leaf movements [6], leaf curvature [7] and solar tracking [8,9]. Interestingly, phototropin (CrPhot) from the fresh water alga *Chlamydomonas reinhardtii* is shown to be involved in regulating its sexual life cycle [10].

LOV domains bind FMN noncovalently to form a chemical species having an absorption maximum at 450 nm in darkness. Upon illumination with blue light, the chromophore of LOV domains bleaches at 450 nm and forms an intermediate covalent adduct between a conserved cysteine in the domain and the bound chromophore [11] that absorbs maximally at 390 nm. This leads to the activation of the proximal kinase domain and subsequent autophosphorylation of the photoreceptor, which in turn triggers photoresponses. The LOV domains of phototropins undergo a photocycle with the LOV390 adduct spontaneously breaking down to its dark-adapted state. LOV1 and LOV2 domains of phototropins are structurally similar, yet the LOV2 domain has a higher quantum yield and undergoes significant blue-light induced conformational changes [12]. Due to this photophysical nature, LOV2 plays the major role in mediating phototropin activity [13–16]. Unlike LOV2, photoexcitation with blue light does not change the conformation of the LOV1 domain greatly [17]. The LOV1 domain is known to function as the dimerization site and is responsible for controlling activation sensitivity of the whole photoreceptor to the incident blue light [18]. β-scaffold of the molecule mainly manifests the dimer interface of the quaternary structure of LOV domain [19,20].

There are reports of blue light mediated photobehavioral response only from a single marine alga, *Fucus vesiculosus*. In this alga the blue-light triggered responses are mediated by a LOV domain coupled

Abbreviations: IPTG, isopropyl-β-d-thiogalactopyranoside; SDS, sodium dodecyl sulfate; FMN, flavin mononucleotide; TCA, Trichloro Acetic Acid; SEC, size exclusion chromatography; FPLC, Fast Performance Liquid Chromatography; UPLC, Ultra Performance Liquid Chromatography; LOV1, Light Oxygen Voltage 1; Co++-IMAC, Cobalt Immobilized Metal Affinity Chromatography; BLAST, Basic Local Alignment Search Tool; CDART, Conserved Domain Architecture Retrieval Tool; PCR, Polymerase Chain Reaction; cps, counts per second

* Corresponding author. Tel.: +91 11 24111955; fax: +91 11 24110283.
E-mail address: skateriya@south.du.ac.in (S. Kateriya).

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transcription factor called aureochrome, which regulates photomorphogenesis [21]. However, there is no report of the characterization of a canonical phototropin from any other marine algae. The recent availability of the completely sequenced genome of *Ostreococcus tauri* [22] has generated considerable interest in studying this smallest free-living eukaryote to decipher its photobiological adaptation and other physiological responses especially because of the evolutionarily significant position occupied by this organism [22–24]. Here we report for the first time the photophysical properties, thermal recovery kinetics and quaternary structure characterization of the LOV1 domain (OtLOV1) of a novel phototropin (OtPhot) from the marine alga *O. tauri*. Detailed mutational studies show that replacement of isoleucine at position 43 of the OtLOV1 domain with other amino acids can modulate the photobleaching efficiency as well as thermal recovery kinetics of the domain.

2. Materials and methods

2.1. Identification and cloning of novel phototropin

Putative phototropin sequence of marine algae *O. tauri* was identified using BLAST from its genome database (http://genome.jgi-psf.org/Ostta4/Ostta4.home.html) with the LOV1 domain of *C. reinhardtii* as query sequence. All relevant protein sequences retrieved were further analyzed for their domain architecture using CDART (http://www.ncbi.nlm.nih.gov/Structure/lexington/lexington.cgi) [25]. Sequence alignment between CrPhot (CAC94940) and OtPhot (Protein ID 29659; *O. tauri* v.2.0 JGI) was carried out using ClustalW (http://www.ebi.ac.uk/Tools/clustalw2/index.html) [26].

Codon-usage analysis was carried out by GCUA program (http://gcua.schoedl.de/). The Otphot gene was codon optimized for expression in *E. coli* and the gene fragment encoding the core OtLOV1 domain was synthesized from Bioserve, India. The synthesized gene fragment (24-134a.a) was PCR amplified with forward and reverse primers 5′ ATTGGATCCGGGGCGTTTGATCATAC3′and 5′ATTCTTGAGTCCGCGTCCTTC ACCTCG 3′ respectively and cloned into pET21a (+) vector (Novagen, USA) after digesting both vector and insert with the appropriate restriction enzymes. The sequence of OtLOV1 was submitted to NCBI (EU153548.1) database.

2.2. Site directed mutagenesis of OtLOV1

Single amino acid replacements were carried out using QuickChange site directed mutagenesis kit (Stratagene) as per manufacturer's instruction and verified by sequencing.

2.3. Prediction of the 3D structure and analysis of dimerization interface of OtLOV1

Tertiary structure of OtLOV1 was generated by homology modeling using the program http://swissmodel.expasy.org/. The model was refined by energy minimization steps in the Swiss-pdbViewer 4.0.1 and validated using programs Verify3D, Procheck, and ERRAT (http://nihserver.mbi.ucla.edu/SAVES/). The validated PDB (PM0075834) coordinates of OtLOV1 were submitted to ClusPro [27] (http://cluspro.bu.edu/home.php) for protein–protein docking analysis using DOT 1.0 as default program. The model with lowest free energy was selected for 3D structure validation (as described earlier) and submitted to protein model database [28] depository (PM0075844). Residues which were potential binding sites for dimer formation were identified from the model PM0075834 using PPI-Pred program (http://bmbpcu36.leeds.ac.uk/ppi_pred/) [29]. These residues were located and highlighted in the dimer model (PM0075844) using PyMol program. Atom coordinate files of monomer (PM0075834) and dimer (PM0075844) were deposited in protein model database (http://mi.caspur.it/PMDB/) respectively [28].

2.4. Heterologous expression and purification of OtLOV1

BL21 (λ DE3) *E. coli* cells harboring hexa histidine tagged OtLOV1 construct were grown in TB medium at 37 °C to $OD_{600} = 0.6$. Protein expression was carried out at 16 °C in darkness in the presence of 0.5 mM IPTG for 48 h. The recombinant protein was purified from the soluble fraction with Co^{++}-IMAC resin (Clontech, Laboratories Inc. USA) according to manufacturer's protocol. The eluted samples were further purified by gel filtration chromatography using AKTA explorer FPLC system equipped with Superdex 75pg16/60 column (GE Healthcare, Sweden) pre equilibrated with 1× PBS.

2.5. UV–visible and fluorescence spectroscopy of recombinant OtLOV1

The absorption spectra for OtLOV1 were recorded with Cary300® UV–visible spectrophotometer (Varian, Inc., USA) using a scan speed setting of 2500 nm/min at 16 °C. The fluorescence emission spectra were recorded with fluorolog-3 spectrofluorometer (FL3-22, Horiba, Jobin Yvon, USA) at 16 °C. Fluorescence emission spectra were recorded by using an excitation wavelength of 390 nm. All data obtained were plotted using IGOR software. Dark state spectrum was obtained from protein that was dark adapted for more than 1 h, while bleached state spectrum was obtained immediately after exposing the protein for 30 s or 120 s to high intensity blue light from an LED (Conrad, luxeon lll Emitter LXHL-PB09, Germany) with a power of 1 W at 460 nm. For recovery to dark state studies, the protein was bleached as before for 30 s or 120 s and spectra recorded at intermittent time intervals after dark incubation. Similar experiments were performed to probe the changes in the fluorescence emission.

2.6. Chromophore extraction and characterization

Purified OtLOV1 was treated with 10% (V/V) TCA, vortexed and incubated on ice for 5 min followed by centrifugation at 13,000 *g* for 2 min at 4 °C. The supernatant was collected and neutralized with equal volume of 1 M NaH_2PO_4. The extracted chromophore was analyzed by reversed phase HPLC method using ACQUITY BEH C18 column with ACQUITY UPLC system (Waters Corp; USA). The mobile phase consisted of (A) 0.1% v/v formic acid in water and (B) acetonitrile containing 0.1% formic acid.

2.7. Gel filtration and protein cross-linking of OtLOV1

All gel filtration experiments were carried out in 1× PBS buffer at 4 °C on a sephadex-75 column using AKTA FPLC system (GE Healthcare, USA). Gel filtration protein standards (Bio-Rad, USA) were eluted under similar conditions. Elution profiles were monitored at three different wavelengths (280 nm, 449 nm and 370 nm).

Protein cross-linking was carried out using glutaraldehyde as described elsewhere [30]. Briefly, 5 μl of 2.5% (V/V) freshly prepared glutaraldehyde was added to 85 μl of the reaction mixture containing 25 μM purified OtLOV1 in phosphate buffer (10 mM sodium phosphate, pH 8.0, 10 mM NaCl) and incubated at 37 °C for 15 min. The reaction was terminated by the addition of 5 μl of 1 M Tris–Cl, pH 8.0. Crosslinked protein samples were resolved on 12.5% SDS-PAGE.

Accession numbers:

Nucleotide sequence accession ID: EU153548.1
Protein model database of monomer accession ID: PM0075834
Protein model database of dimer accession ID: PM0075844

3. Results and discussion

3.1. Identification of a novel phototropin from O. tauri

Mining of the *O. tauri* genome database led to the identification of a single putative intronlesss phototropin gene (otphot). The CDART [25]