

# 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

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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.

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## 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].  $\beta$ -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- $\beta$ -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

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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://gcu.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 (24–134aa) was PCR amplified with forward and reverse primers 5' ATTGATCCGGGGCGTTTGATCATAC3' and 5' ATTCTTGAGTCCGCTCCTTC 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 Quick-Change 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) co-ordinates 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/](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.caspar.it/PMDB/>) respectively [28].

### 2.4. Heterologous expression and purification of OtLOV1

BL21 ( $\lambda$  DE3) *E. coli* cells harboring hexa histidine tagged OtLOV1 construct were grown in TB medium at 37 °C to OD<sub>600</sub> = 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<sup>++</sup>-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 III 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<sub>2</sub>PO<sub>4</sub>. 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  $\mu$ l of 2.5% (V/V) freshly prepared glutaraldehyde was added to 85  $\mu$ l of the reaction mixture containing 25  $\mu$ M 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  $\mu$ l 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

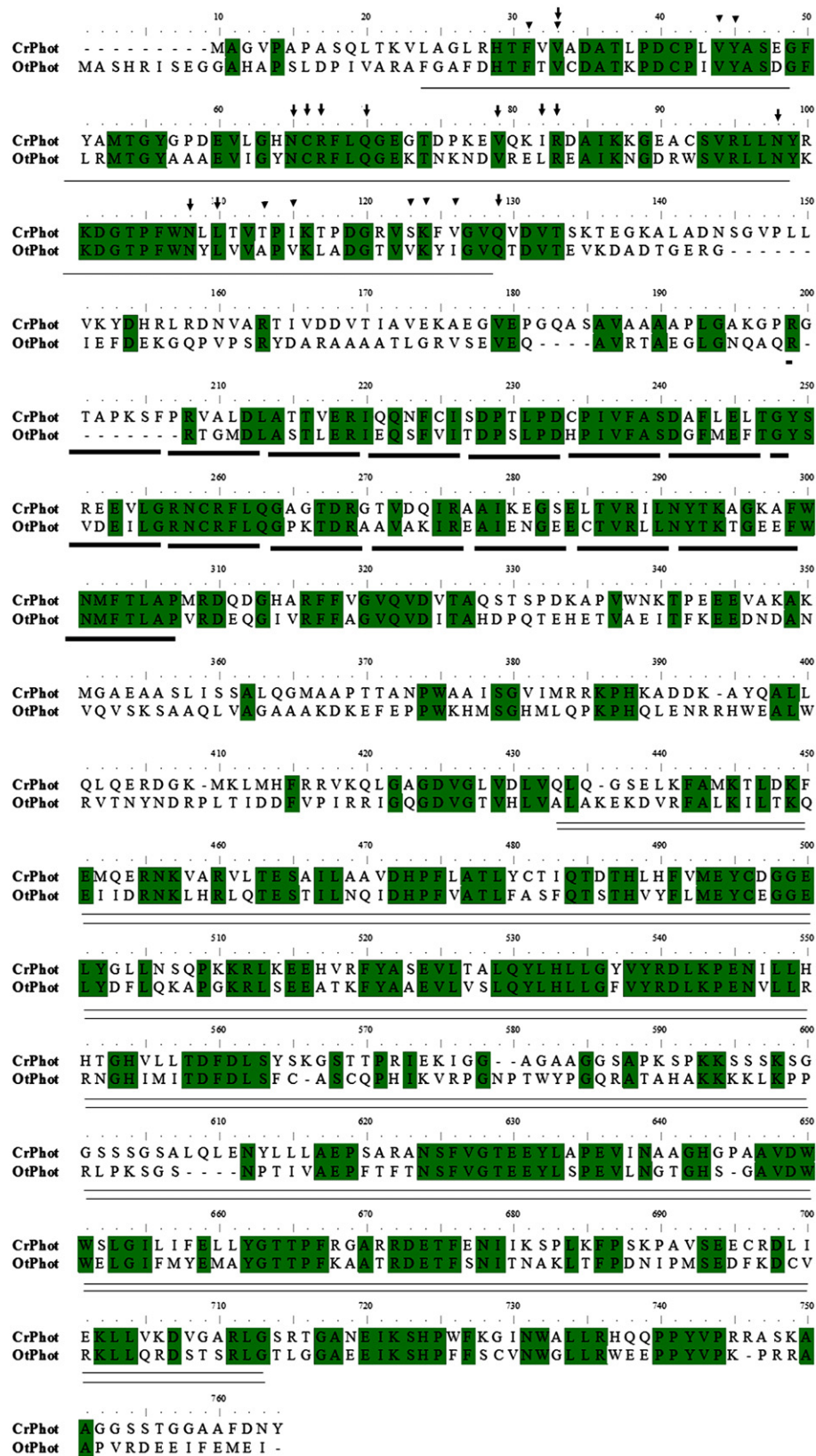
Protein model database of monomer accession ID: PM0075834

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 intronless phototropin gene (otphot). The CDART [25]



**Fig. 1.** Homology analysis of OtPhot and CrPhot. Identical amino acid residues are shown in black on green background. Residues which interact directly with FMN are shown with arrowheads. Amino acids important for dimerization interface are indicated here with solid triangles. LOV1 and LOV2 domains at the N-terminus are indicated by solid and dashed lines respectively. Ser/Thr kinase domain at the C-terminus is indicated by double lines.



analysis of the Otphot (762a.a) sequence suggested the presence of two sensory LOV domains at the N-terminus and a Serine/Threonine kinase domain at its C-terminus. Based on the homology analysis it is apparent that OtPhot shared an overall homology of 55% with the phototropin from fresh water alga *C. reinhardtii* (CrPhot), and the linker region between the two LOV domains is shorter in OtPhot (Fig. 1). Comparison of the amino acid residues of LOV1 domains of OtPhot and CrPhot revealed that all the FMN interacting residues are conserved in the putative OtLOV1 (Fig. 1). The conserved cysteine residue (C66) responsible for photo-adduct formation during photocycle of the LOV domain is embedded in NCRFLQ motif and glycine residue (G71), which serves as a ligand for the N (1) and C (2) of the isoalloxazine ring of the flavin [1,2] is also present in OtLOV1 (Fig. 1). The salt bridge [15] between E60 and K101 is conserved in OtLOV1 (Figs. 1 and 2A). The presence of amino acids K116 and D119 suggests the possibility of another salt bridge formation.

### 3.2. Conserved structural features of OtLOV1

To understand the structural organization of the OtLOV1 domain, we generated a homology based 3D model. The CrLOV1 domain of CrPhot (PDB ID 1N9L) [31] shared 65% amino acid identity with OtLOV1 and hence was selected as the structural template for building the OtLOV1 model. The predicted structure was validated by VERIFY3D [32] and ERRAT programs. It showed significant scores of 80.19% and 91.53% [33] respectively. Ramachandran plot analysis showed 93.4% of the residues in the most favorable region, 6.6% in the allowed region and none in the disallowed region. The predicted tertiary structure of OtLOV1 possessed a structural fold similar to that of CrLOV1, containing 4 helices, 7 strands and 7 turns (Fig. 2A). It also suggested that the FMN binding pocket has the conserved cysteine (C66) buried deep in it and the C66 is in proximity to the hydrophobic side chains of the V33, F50, F68 and V79. As reported in *A. sativa* the hydrophilic amino acid residues in close proximity to FMN binding pocket (N65, R67, G71, D102 and G127) are also conserved [34]. Comparative analysis of the conserved residues of FMN binding pocket of OtLOV1 with CrLOV1, especially residues below the isoalloxazine ring [31], showed one key replacement (L to I) at position 43 in OtLOV1 [35].

Recently, many experimental evidences have pointed towards the role of LOV1 as a dimerization site in the protein [19,36,37]. Nakasako et al. [36] have found that LOV1 domains of both Phot1 and Phot2

from *Arabidopsis* form dimer upon crystallization and their monomers interact using  $\beta$ -scaffold in an anti-parallel manner [36]. However, there are reports of existence of monomer interactions using parallel arrangements in proteins with similar characteristics [37]. Protein–protein interface analysis of the OtLOV1 model by PPI-Pred revealed that the residues 31F, 33V, 44V, 45Y, 113A, 115V, 123V, 124K and 126I (corresponding residues in the predicted OtLOV1 structure are 7F, 9V, 20V, 21Y, 89A, 91V, 99V, 100K and 102I) are involved in hydrophobic interactions at the dimer interface (Fig. 2B) in the  $\beta$ -scaffold. Positions of the residues important for the dimerization of the OtLOV1 domain are comparable to that of the LOV domain of YtvA and the LOV1 domain of *Arabidopsis* phot1 and phot2 [19,36].

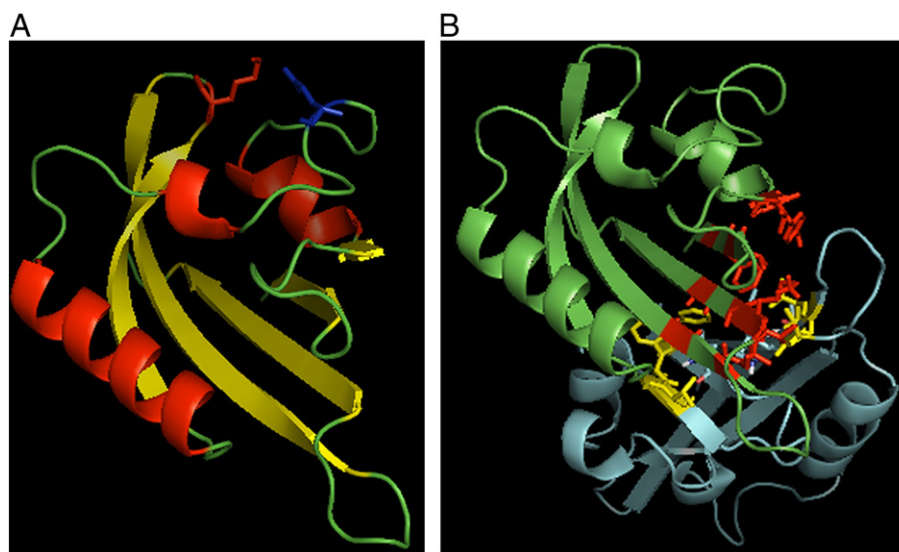
### 3.3. Expression and purification of recombinant OtLOV1

The OtLOV1 construct used for this study included amino acid residues from 24 to 134. 24 amino acids in the N terminus and the C terminal linker region between LOV1 and LOV2 region were not included in the OtLOV1 construct. The OtLOV1 domain boundary was decided based on CDART analysis, comparative structural studies and homology analysis with known CrLOV1 crystal structure.

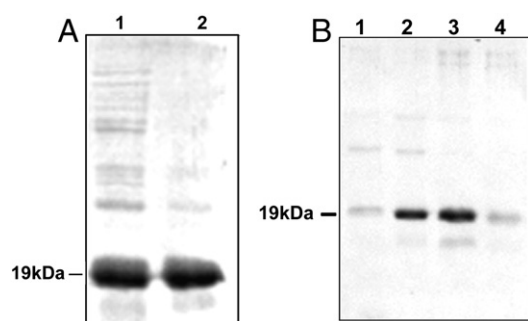
The 6 $\times$  histidine tagged OtLOV1 protein was expressed well in soluble form and purified to near homogeneity using affinity and gel filtration chromatography. The purified protein was resolved on SDS PAGE and visualized by Coomassie staining (Fig. 3).

### 3.4. OtLOV1 is a blue light sensor and uses FMN as chromophore

The absorption spectra of purified OtLOV1<sub>24-134a.a</sub> showed spectral characteristics typical of FMN bound LOV domain having peak maxima at 449 nm with vibronic band signatures (Fig. 4A) and another peak around 376 nm. The LOV1 domain spectra from organisms like *C. reinhardtii*, *A. sativa*, etc. show a vibronic structure with double peaks at 376 nm and 361 nm respectively [12,34] apart from the prominent peak around 450 nm. The OtLOV1 spectra differed from the canonical double-peaked LOV1 spectrum with the peak at 361 nm missing. This kind of spectral characteristics is a general property of the LOV2 domain and not for the LOV1 domain. Interestingly, OtLOV1 mutants C66A (Fig. 4C) and I43V (Fig. 5C) showed the appearance of a peak at 361 nm suggesting of an altered electronic environment around the chromophore which might be



**Fig. 2.** Tertiary structure and dimerization interface prediction of OtLOV1. (A) Cartoon representation of the 3D structure of the OtLOV1 domain of *O. tauri*, modeled using automated Swiss Model server. The structure has 4 helices, 7 strands and 7 turns. The salt bridge between E60 and K101 is shown in blue and red stick representation. (B) Predicted 3D model of the OtLOV1 dimer. The dimerization interface of the OtLOV1 lies in a deep hydrophobic pocket. Side chains of predicted interacting amino acid residues of monomers are depicted in yellow and red respectively.



**Fig. 3.** Purification of recombinant the OtLOV1 domain. (A) SDS PAGE analysis of the  $\text{Co}^{2+}$  affinity purified recombinant OtLOV1. Lanes 1 and 2 contain the eluents 1 and 2 respectively collected from the affinity column. (B) SDS PAGE analysis of the recombinant OtLOV1 purified by SEC. Lanes 1–4 contain SEC fractions showing OD at 449 nm in the order of elution.

modulated by more than one amino acid residue. To study the light response of the OtLOV1 domain we performed photobleaching experiments and observed that OtLOV1 exhibited photobleaching (Fig. 4A) upon irradiation with blue light. These blue-light induced spectral changes in OtLOV1 were completely reversible upon incubation (~30 min) in dark (Fig. 4A and B) and the lifetime of the recovery process was determined to be 300 s, suggesting that the OtLOV1 domain undergoes a photocycle and could function as a blue light sensor. Similar experiments were also performed using the full-length protein (OtPhot). The spectral and photochemical properties of OtPhot (full-length protein) were similar to that of OtLOV1 (data not shown). We performed mutational studies to assess the functionality of the purified OtLOV1 domain. The conserved C66A variant failed to form the flavin-cysteinyl adduct upon illumination with blue light (Fig. 4C). Disruption of the conserved salt bridge between E60 and K101 with an E60K variant showed spectral characteristics and dark recovery kinetics as that of wild type (Fig. 4D) this may be due to the

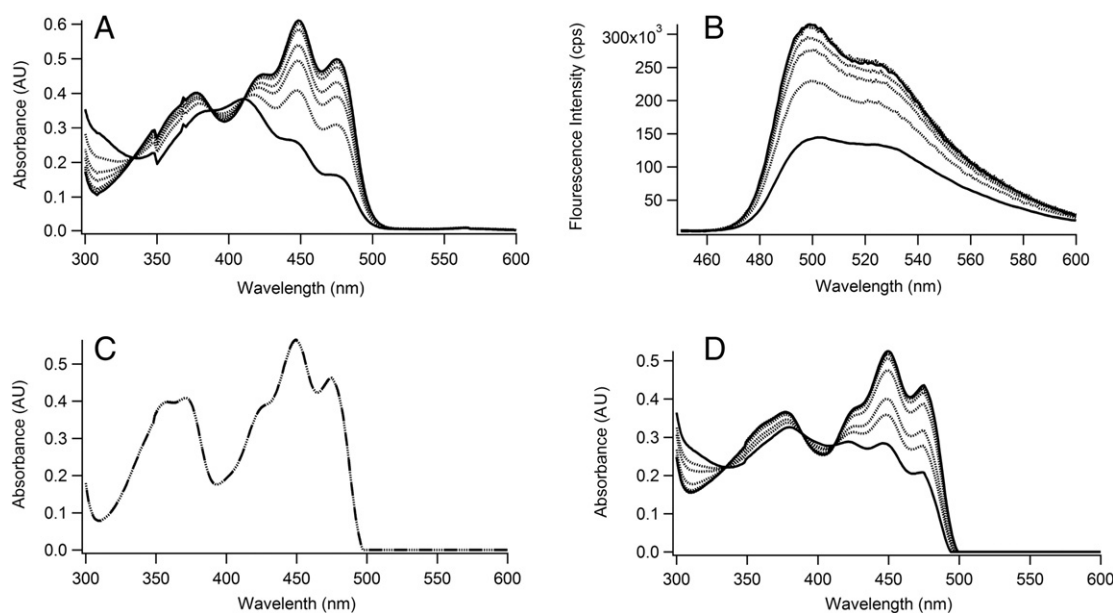
formation of another competing salt bridge between K116 and D119. These studies corroborate with previously reported studies [15,34].

Steady state fluorescence spectroscopy showed maximum fluorescence emission at 499 nm with a shoulder at 521 nm, which is the typical feature of a functional LOV domain (Fig. 4B). The fluorescence emission maximum (499 nm) of OtLOV1 was red shifted compared to the YtvA LOV1 (emission  $\lambda_{\text{max}}$  496 nm) [38] and CrLOV1 (emission  $\lambda_{\text{max}}$  495 nm) domains [39]. Photobleaching and recovery to dark state were observed from the fluorescence spectra of OtLOV1 (Fig. 4B) and the time taken for recovery to dark state was as noted in the UV-vis spectra.

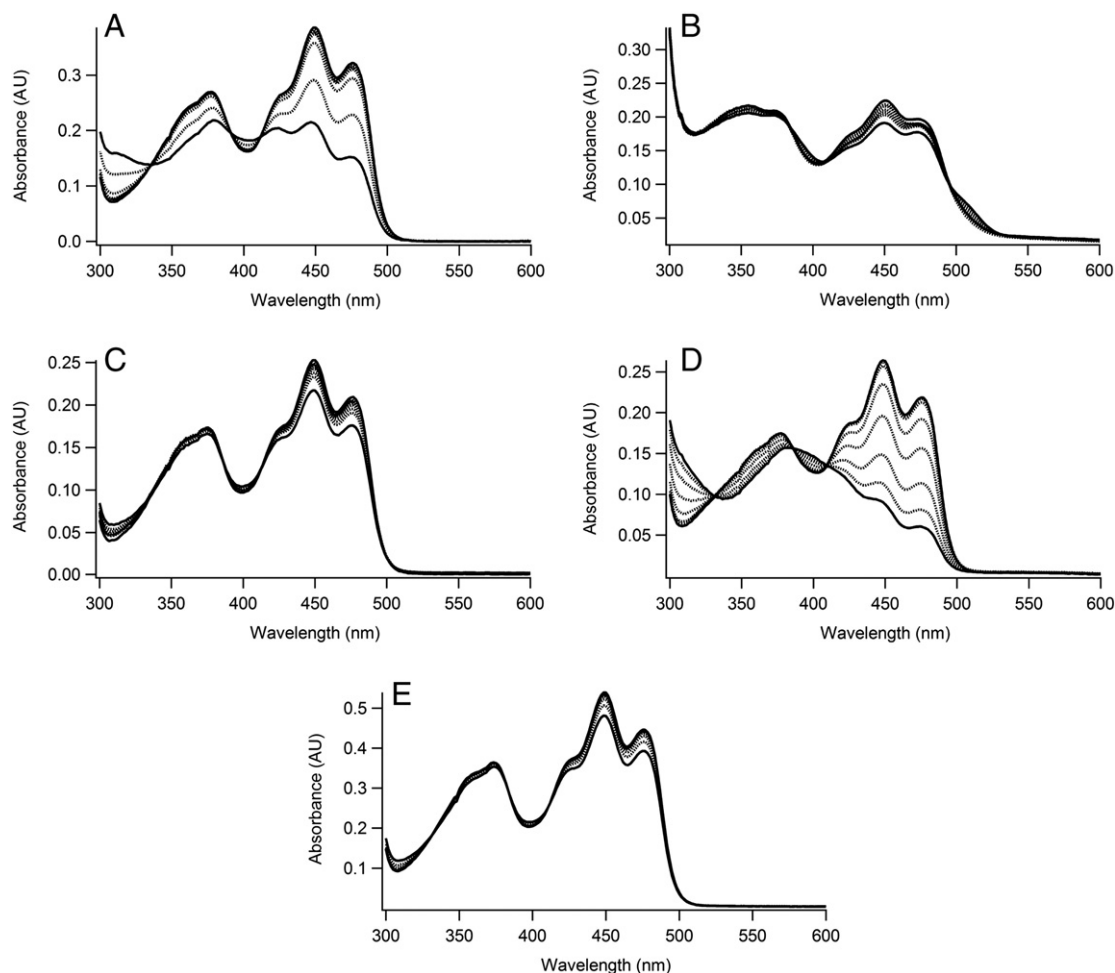
Homology analysis and spectral characteristics of OtLOV1 had indicated that the protein moiety would use FMN as chromophore. To establish the identity of the bound chromophore of OtLOV1, we spectrally characterized the extracted chromophore, and it showed spectral characteristics like FMN in aqueous solution (data not shown). Comparative UPLC analysis showed that the extracted chromophore(s) and commercially available FMN treated with TCA eluted with identical retention time (1.513 min) under isocratic condition (Fig. S1) confirming the chromophore identity.

### 3.5. Molecular determinants of dark state recovery and light sensitivity of OtLOV1

To identify the key amino acid residues responsible for modulating the photocycle of the OtLOV1 domain, we identified a number of amino acid residues from our homology model and made mutants with amino acid replacements at those positions in the FMN binding pocket. We then analyzed these mutant proteins for its characteristic spectrum, photobleaching (illumination for 30 s or 120 s with blue light) and time taken for recovery to dark state. It was evident from our alignments that the LOV1 domain of *C. reinhardtii* harbors a leucine at position 43 (number corresponds to OtLOV1), whereas OtLOV1 has an isoleucine at the corresponding position. An I43L mutation did not show any alteration in the recovery kinetics as well



**Fig. 4.** Spectral analysis and blue light sensitivity of the OtLOV1 domain. (A) The protein sample was irradiated with high intensity blue light for 30 s. The uppermost and lowermost traces (solid traces) represent dark state spectrum and spectrum immediately after illumination with blue light respectively. Dotted traces represent spectra at intervals of 2, 7, 12, 22 and 27 min of dark incubation (from bottom to top) respectively. (B) The protein sample (OtLOV1) was excited at 390 nm and the fluorescence emission spectrum was monitored in the range of 400 nm–600 nm. Uppermost and lowermost solid traces represent dark adapted and spectra immediately after illumination respectively. Dotted traces (from bottom to top) are spectra obtained at 2, 7, 12, 22 and 27 min of recovery in the dark. (C) Absorption spectra of C66A mutant. Dotted trace represents dark state spectrum and dashed trace represents spectrum after the protein sample was illuminated with high intensity blue light for 2 min. (D) Absorption spectra of E60K mutant. Uppermost and lowermost solid traces represent dark adapted and spectra immediately after illumination respectively. Dotted traces (from bottom to top) are spectra obtained at 2, 7, 12, 22 and 27 min of recovery in the dark.



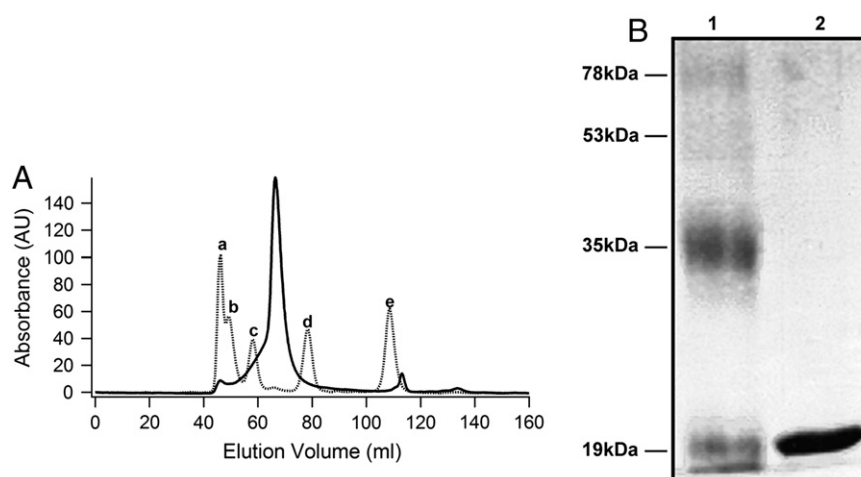
**Fig. 5.** Absorption spectra of OtLOV1 mutants. (A) Absorption spectra of I43L mutant, uppermost and lowermost solid traces represent dark state and bleached state spectrum. Dotted traces represent recovery at 30 s, 2 min, 5 min, 10 min, 20 min, 30 min respectively (from bottom to top). (B) Absorption and recovery spectra of I43S mutant, traces labeled as in (A). (C) Absorption and recovery spectra of I43V mutant. Recovery traces recorded at intervals of 30 s till 3.5 min (bottom to top) respectively. (D) Absorption and recovery spectra of T32V mutant, traces labeled as in (A). (E) Absorption and recovery spectra of double mutant I43V T32V, traces labeled as in (C).

as spectral properties from that of the wild type (Fig. 5A). However, I43S mutant showed reduced photobleaching upon illumination for 120 s and no photobleaching was observed upon illumination for 30 s (Fig. 5B), the dark recovery time of this mutant was found to be slightly slower than that of wild type ( $\tau=384$  s). Moreover, this variant incorporated approximately seven fold less FMN than wild type and was found to form oligomers upon storage (data not shown). Another variant I43V showed partial photobleaching upon illumination for 30 s (Fig. 5C) and was reverted to dark state with an accelerated recovery time of  $\sim 3.5$  min ( $\tau=64$  s). An increase in illumination time to 120 s did not affect the amplitude of photobleaching and recovery kinetics of the I43V variant (data not shown). A similar isoleucine to valine (I85V in VVD) replacement had shown to enhance adduct decay in the fungal LOV domain of VVD protein [40,41] and a second isoleucine at position 74 in VVD further enhanced the thermal recovery process. A threonine at position 32 in OtLOV1 corresponds to isoleucine 74 of VVD, mutating threonine to valine (T32V) in OtLOV1 showed spectral and dark recovery characteristics like wild type OtLOV1 (Fig. 5D). The double mutant of VVD (I74V I85V) showed synergetic effect on dark recovery kinetics (increases recovery by 600 fold). However, the OtLOV1 double mutant (T32V I43V) showed properties similar to the single mutant I43V (Fig. 5E). These observations could implicate that the isoleucine at 43rd position could be the key molecular determinant for photosen-

sitivity and the dark recovery kinetics of the OtLOV1 domain. Other OtLOV1 mutants (A113E, L117E, V123E, and I126S) generated either did not express functional protein or showed properties similar to that of wild type.

### 3.6. OtLOV1 exists as dimer in native state

LOV domain belongs to the PAS (Per-ARNT-Sim) domain super family of proteins. PAS domain containing proteins quite often regulate function of the molecule by switching between different oligomeric states [42]. Studies show that the LOV1 domain also functions as an oligomeric switch to regulate the activity of the LOV2 domain of phototropin. Characterization of the oligomeric nature of OtLOV1 by size exclusion chromatography (SEC) showed that it eluted as a dimer under native condition (Fig. 6A) and showed no major peaks corresponding to monomer, trimer or higher oligomers. Quaternary structural characteristics of OtLOV1 were further confirmed by chemical crosslinking using glutaraldehyde which showed that majority of the protein stayed as dimer and a very small proportion of the sample was present in trimeric and higher oligomeric states. The presence of higher oligomeric species could be due to some degree of nonspecific crosslinking (Fig. 6B). Cross-linking experiments at different concentrations of OtLOV1 viz 25  $\mu$ M, 5  $\mu$ M, and 2.5  $\mu$ M were also performed using glutaraldehyde and



**Fig. 6.** Oligomeric state characterization of OtLOV1. (A) SEC profile of the recombinant OtLOV1. The SEC profile of OtLOV1 is shown as solid trace. The peak positions of the standard proteins used for calibration (dotted trace) are indicated by arrows as follows: (a) thyroglobulin (670 kDa); (b) bovine gamma globulin (158 kDa); (c) chicken ovalbumin (44 kDa); (d) myoglobin (17 kDa); (e) vitamin B-12 (1.35 kDa). (B) SDS PAGE profile of cross-linked OtLOV1. Lane 1 shows untreated sample and lane 2 shows glutaraldehyde treated OtLOV1.

dimerization/oligomerization was found to be independent of protein concentration. SEC purification of OtPhot (full length) showed that OtPhot existed as dimer. However, the expression of the recombinant OtPhot was not optimal with many truncated products being formed. These experiments clearly show that the OtLOV1 domain could provide site for dimerization of the phototropin in native condition.

#### 4. Conclusions

This study suggests that the LOV1 domain of *O. tauri* possesses characteristics of a functional FMN bound blue light sensor and it would provide site for the dimerization of the receptor in vivo. Double peak (361 nm and 376 nm) structure of the dark state spectrum of the OtLOV1 domain is the manifestation of electronic environment around FMN binding pocket controlled by more than one residue. The molecular determinant (I43) plays a pivotal role in modulating the photophysical properties of the LOV1 domain of OtPhot by regulating the recovery to dark state and the photosensitivity of the domain. Characterization of OtLOV2 and its interaction with other domains of OtPhot will be required to decipher the mechanistic basis of regulation of photoreceptor activity and to understand the physiological role of blue light mediated signaling in this (*O. tauri*) non-flagellated, free-living smallest marine alga that does not undergo sexual life cycle.

Supplementary materials related to this article can be found online at [doi:10.1016/j.bbagen.2011.04.008](https://doi.org/10.1016/j.bbagen.2011.04.008).

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#### References

- [1] J.M. Christie, M. Salomon, K. Nozue, M. Wada, W.R. Briggs, LOV (light, oxygen, or voltage) domains of the blue-light photoreceptor phototropin (nph1): binding sites for the chromophore flavin mononucleotide, *Proc. Natl. Acad. Sci. U. S. A.* 96 (1999) 8779–8783.
- [2] P. Hegemann, Algal sensory photoreceptors, *Annu. Rev. Plant Biol.* 59 (2008) 167–189.
- [3] W.R. Briggs, J.M. Christie, M. Salomon, Phototropins: a new family of flavin-binding blue light receptors in plants, *Antioxid. Redox Signal.* 3 (2001) 775–788.
- [4] J.A. Jarillo, H. Gabrys, J. Capel, J.M. Alonso, J.R. Ecker, A.R. Cashmore, Phototropin-related NPL1 controls chloroplast relocation induced by blue light, *Nature* 410 (2001) 952–954.
- [5] T. Kinoshita, M. Doi, N. Suetsugu, T. Kagawa, M. Wada, K. Shimazaki, Phot1 and phot2 mediate blue light regulation of stomatal opening, *Nature* 414 (2001) 656–660.
- [6] J.M. Christie, P. Reymond, G.K. Powell, P. Bernasconi, A.A. Raibekas, E. Liscum, W.R. Briggs, Arabidopsis NPH1: a flavoprotein with the properties of a photoreceptor for phototropism, *Science* 282 (1998) 1698–1701.
- [7] K. Sakamoto, W.R. Briggs, Cellular and subcellular localization of phototropin 1, *Plant Cell* 14 (2002) 1723–1735.
- [8] J. Ehleringer, I. Forseth, Solar tracking by plants, *Science* 210 (1980) 1094–1098.
- [9] A. Schwartz, D. Koller, Diurnal phototropism in solar tracking leaves of *Lavatera cretica*, *Plant Physiol.* 80 (1986) 778–781.
- [10] K. Huang, C.F. Beck, Phototropin is the blue-light receptor that controls multiple steps in the sexual life cycle of the green alga *Chlamydomonas reinhardtii*, *Proc. Natl. Acad. Sci. U. S. A.* 100 (2003) 6269–6274.
- [11] S.H. Song, B. Dick, P. Zarak, A. Penzkofer, T. Schiereis, P. Hegemann, Absorption and emission spectroscopic characterisation of combined wildtype LOV1–LOV2 domain of phot from *Chlamydomonas reinhardtii*, *J. Photochem. Photobiol. B* 81 (2005) 55–65.
- [12] M. Kasahara, T.E. Swartz, M.A. Olney, A. Onodera, N. Mochizuki, H. Fukuzawa, E. Asamizu, S. Tabata, H. Kanegae, M. Takano, J.M. Christie, A. Nagatani, W.R. Briggs, Photochemical properties of the flavin mononucleotide-binding domains of the phototropins from Arabidopsis, rice, and *Chlamydomonas reinhardtii*, *Plant Physiol.* 129 (2002) 762–773.
- [13] A.S. Halavaty, K. Moffat, N- and C-terminal flanking regions modulate light-induced signal transduction in the LOV2 domain of the blue light sensor phototropin 1 from *Avena sativa*, *Biochemistry* 46 (2007) 14001–14009.
- [14] S.M. Harper, L.C. Neil, K.H. Gardner, Structural basis of a phototropin light switch, *Science* 301 (2003) 1541–1544.
- [15] M.A. Jones, K.A. Feeney, S.M. Kelly, J.M. Christie, Mutational analysis of phototropin 1 provides insights into the mechanism underlying LOV2 signal transmission, *J. Biol. Chem.* 282 (2007) 6405–6414.
- [16] Y. Nakasone, T. Eitoku, D. Matsuoka, S. Tokutomi, M. Terazima, Dynamics of conformational changes of Arabidopsis phototropin 1 LOV2 with the linker domain, *J. Mol. Biol.* 367 (2007) 432–442.
- [17] T. Iwata, D. Nozaki, S. Tokutomi, H. Kandori, Comparative investigation of the LOV1 and LOV2 domains in *Adiantum phytochrome3*, *Biochemistry* 44 (2005) 7427–7434.
- [18] Y. Nakasone, T. Eitoku, D. Matsuoka, S. Tokutomi, M. Terazima, Kinetic measurement of transient dimerization and dissociation reactions of Arabidopsis phototropin 1 LOV2 domain, *Biophys. J.* 91 (2006) 645–653.
- [19] V. Buttani, W. Gartner, A. Losi, NTP-binding properties of the blue-light receptor YtvA and effects of the E105L mutation, *Eur. Biophys. J.* 36 (2007) 831–839.
- [20] A. Moglich, K. Moffat, Structural basis for light-dependent signaling in the dimeric LOV domain of the photosensor YtvA, *J. Mol. Biol.* 373 (2007) 112–126.
- [21] F. Takahashi, D. Yamagata, M. Ishikawa, Y. Fukamatsu, Y. Ogura, M. Kasahara, T. Kiyosue, M. Kikuyama, M. Wada, H. Kataoka, AUREOCHROME, a photoreceptor required for photomorphogenesis in stramenopiles, *Proc. Natl. Acad. Sci. U. S. A.* 104 (2007) 19625–19630.
- [22] E. Derelle, C. Ferraz, S. Rombauts, P. Rouze, A.Z. Worden, S. Robbens, F. Partensky, S. Degroove, S. Echeynie, R. Cooke, Y. Saeys, J. Wuyts, K. Jabbari, C. Bowler, O. Panaud, B. Piegau, S.G. Ball, J.P. Ral, F.Y. Bouget, G. Piganeau, B. De Baets, A. Picard, M. Delseny, J. Demailly, Y. Van de Peer, H. Moreau, Genome analysis of the smallest free-living eukaryote *Ostreococcus tauri* unveils many unique features, *Proc. Natl. Acad. Sci. U. S. A.* 103 (2006) 11647–11652.
- [23] P.J. Keeling, *Ostreococcus tauri*: seeing through the genes to the genome, *Trends Genet.* 23 (2007) 151–154.



- [24] B. Palenik, J. Grimwood, A. Aerts, P. Rouze, A. Salamov, N. Putnam, C. Dupont, R. Jorgensen, E. Derelle, S. Rombauts, K. Zhou, R. Otillar, S.S. Merchant, S. Podell, T. Gaasterland, C. Napoli, K. Gendler, A. Manuell, V. Tai, O. Vallon, G. Piganeau, S. Jancek, M. Heijde, K. Jabbari, C. Bowler, M. Lohr, S. Robbens, G. Werner, I. Dubchak, G.J. Pazour, Q. Ren, I. Paulsen, C. Delwiche, J. Schmutz, D. Rokhsar, Y. Van de Peer, H. Moreau, I.V. Grigoriev, The tiny eukaryote *Ostreococcus* provides genomic insights into the paradox of plankton speciation, *Proc. Natl. Acad. Sci. U. S. A.* 104 (2007) 7705–7710.
- [25] L.Y. Geer, M. Domrachev, D.J. Lipman, S.H. Bryant, CDART: protein homology by domain architecture, *Genome Res.* 12 (2002) 1619–1623.
- [26] M.A. Larkin, G. Blackshields, N.P. Brown, R. Chenna, P.A. McGettigan, H. McWilliam, F. Valentin, I.M. Wallace, A. Wilm, R. Lopez, J.D. Thompson, T.J. Gibson, D.G. Higgins, Clustal W and Clustal X version 2.0, *Bioinformatics* 23 (2007) 2947–2948.
- [27] S.R. Comeau, D.W. Gatchell, S. Vajda, C.J. Camacho, ClusPro: a fully automated algorithm for protein–protein docking, *Nucleic Acids Res.* 32 (2004) W96–W99.
- [28] T. Castrignano, P.D. De Meo, D. Cozzetto, I.G. Talamo, A. Tramontano, The PMDB protein model database, *Nucleic Acids Res.* 34 (2006) D306–D309.
- [29] J.R. Bradford, D.R. Westhead, Improved prediction of protein–protein binding sites using a support vector machines approach, *Bioinformatics* 21 (2005) 1487–1494.
- [30] H. Katsura, K. Zikihara, K. Okajima, S. Yoshihara, S. Tokutomi, Oligomeric structure of LOV domains in *Arabidopsis* phototropin, *FEBS Lett.* 583 (2009) 526–530.
- [31] R. Fedorov, I. Schlichting, E. Hartmann, T. Domratcheva, M. Fuhrmann, P. Hegemann, Crystal structures and molecular mechanism of a light-induced signaling switch: the Phot-LOV1 domain from *Chlamydomonas reinhardtii*, *Biophys. J.* 84 (2003) 2474–2482.
- [32] J.U. Bowie, R. Luthy, D. Eisenberg, A method to identify protein sequences that fold into a known three-dimensional structure, *Science* 253 (1991) 164–170.
- [33] C. Colovos, T.O. Yeates, Verification of protein structures: patterns of nonbonded atomic interactions, *Protein Sci.* 2 (1993) 1511–1519.
- [34] M. Salomon, J.M. Christie, E. Knieb, U. Lempert, W.R. Briggs, Photochemical and mutational analysis of the FMN-binding domains of the plant blue light receptor, phototropin, *Biochemistry* 39 (2000) 9401–9410.
- [35] S. Crosson, K. Moffat, Structure of a flavin-binding plant photoreceptor domain: insights into light-mediated signal transduction, *Proc. Natl. Acad. Sci. U. S. A.* 98 (2001) 2995–3000.
- [36] M. Nakasako, K. Zikihara, D. Matsuoka, H. Katsura, S. Tokutomi, Structural basis of the LOV1 dimerization of *Arabidopsis* phototropins 1 and 2, *J. Mol. Biol.* 381 (2008) 718–733.
- [37] J.S. Lamb, B.D. Zoltowski, S.A. Pabit, L. Li, B.R. Crane, L. Pollack, Illuminating solution responses of a LOV domain protein with photocoupled small-angle X-ray scattering, *J. Mol. Biol.* 393 (2009) 909–919.
- [38] A. Losi, E. Polverini, B. Quest, W. Gartner, First evidence for phototropin-related blue-light receptors in prokaryotes, *Biophys. J.* 82 (2002) 2627–2634.
- [39] W. Holzer, A. Penzkofer, M. Fuhrmann, P. Hegemann, Spectroscopic characterization of flavin mononucleotide bound to the LOV1 domain of Phot1 from *Chlamydomonas reinhardtii*, *Photochem. Photobiol.* 75 (2002) 479–487.
- [40] J.M. Christie, S.B. Corchnoy, T.E. Swartz, M. Hokenson, I.S. Han, W.R. Briggs, R.A. Bogomolni, Steric interactions stabilize the signaling state of the LOV2 domain of phototropin 1, *Biochemistry* 46 (2007) 9310–9319.
- [41] B.D. Zoltowski, B. Vaccaro, B.R. Crane, Mechanism-based tuning of a LOV domain photoreceptor, *Nat. Chem. Biol.* 5 (2009) 827–834.
- [42] B.L. Taylor, I.B. Zhulin, PAS domains: internal sensors of oxygen, redox potential, and light, *Microbiol. Mol. Biol. Rev.* 63 (1999) 479–506.