

Unusual photodynamic characteristics of light-oxygen-voltage domain of phototropin linked to terrestrial adaptation of *Klebsormidium nitens*

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Abstract

Phototropin (Phot), a blue light sensing LOV domain protein, mediates blue light responses and is evolutionary conserved across the green lineage. *Klebsormidium nitens*, a green terrestrial alga, presents a valuable opportunity in studying adaptive responses from aquatic to land habitat transitions. We determined the crystal structure of *Klebsormidium nitens* Phot LOV1 domain (KnLOV1) in the dark and engineered different mutations (R60K, Q122N, D33N) to modulate the lifetime of photo recovery cycle. We observed an unusual slow recovery in native KnLOV1 domain ($\tau = 2467$ seconds) compared to different mutants (R60K: $\tau = 122$ seconds, Q122N: $\tau = 101$ seconds, D33N: $\tau = 580$ seconds). Crystal structures of native KnLOV1 and mutants revealed subtle but critical changes near the protein chromophore that is responsible for modulating protein recovery rate. Our findings shed light on the unique structural characteristics of the newly studied KnLOV1 and its evolutionary importance for phototropin mediated physiology.

Introduction

Phototropins are highly conserved blue light-sensing flavin-binding proteins found across the plant kingdom^{1–5}. It comprises two LOV domains (LOV1, LOV2) belonging to the PAS superfamily at the N-terminus, followed by a serine-threonine kinase (STK) domain at the C-terminus. The LOV protein has several α -helices (C, D, E, F, J) surrounding five β -sheets (A, B, G, H, I)^{6,7}, and both LOV domains (LOV1 and LOV2) bind non-covalently with flavin (FMN) chromophore in the dark inactive state (LOV447)⁸. Upon blue light illumination, a covalent-cysteinyl adduct (LOV390) is formed between the well-conserved cysteine of LOV domain and C(4) atom of an isoalloxazine ring in flavin. This causes conformational changes in the LOV domain, leading to other photo-transitions, oligomerization, and structural changes in phototropin^{9,10}. The active state (LOV390) slowly reverts to the inactive state (LOV447) during dark incubation^{11–13}.

The photophysical behavior and photocycle duration of LOV domain depends upon the residues present in the binding pocket and hydrogen bonding (HB) network around the chromophore^{6,7,14,15}. The highly conserved glutamine (Gln)^{15–17}, arginine (Arg)¹⁸, and aspartic acid (Asp)¹⁹ residue plays a critical role in eliciting photo-transitions in the LOV domain. The Gln residue located within the I β strand is responsible for transmitting photo-induced conformational changes to the FMN binding pocket of the LOV domain^{15,20,21}. To maintain a hydrogen bond with N(5) hydrogen, the Gln residue must flip its side chain^{15,22,23}. Upon covalent adduct formation, the FMN ring reduces and induces protonation of N(5) atom. The Arg residue, positioned centrally in the HB network of the LOV domain, interacts directly with the FMN phosphate group and makes a hydrogen bond with the Asp residue of the LOV protein. Thus, it regulates the lifetime of the covalent flavin-cysteinyl adduct and light-induced conformational changes in the LOV protein^{7,19}.

Klebsormidium nitens (*K. nitens*), a green terrestrial alga, is of significant importance from an evolutionary perspective as it belongs to the charophytes, which provide insight into the early transition from aquatic to land habitat. Consequently, it has proven helpful for studying algal adaptations in transitioning from aquatic to land habitat²⁴. Despite its significance, the photoreceptor machinery in this alga remains unexplored. A thorough understanding of this machinery could facilitate the study of the key characteristics of the newly discovered *K. nitens* phototropin LOV1 domain (KnLOV1), which plays a role in the transition from aquatic to land habitat. Additionally, the unique characteristics of the novel KnLOV1 domain including its photocycle modulation and oligomerization behavior could be useful for optogenetic applications in different biological systems. In the current study, we have characterized the novel LOV1 domain from the blue light-sensing photoreceptor, phototropin, in *K. nitens* (KnPhot), and compared its unique characteristics with those of the algal and higher plant phototropin LOV1 domains. We have also highlighted the key residues which influence the photochemical and photophysical properties of KnLOV1 protein which in turn affects its photoreaction kinetics.

Notably, LOV proteins have been extensively studied for their optogenetic potential due to their precise spatiotemporal activity in regulating physiological responses in plants^{15,18,19}. The photocycle modulation of the novel KnLOV1 domain could prove useful for optogenetic applications in different systems. Furthermore, we have studied the oligomerization behavior of the KnLOV1 domain to describe its role in light-mediated physiology in terrestrial algae.

Material and methods

Bioinformatic analysis

The phototropin sequence of *K. nitens* was retrieved from *K. nitens* database (http://www.plantmorphogenesis.bio.titech.ac.jp/~algae_genome_project/klebsormidium/) using BLAST server with *C. reinhardtii* phototropin LOV1 domain as query sequence. LOV1 domains from different algae and higher plants were retrieved from Phytozome v.12 genome database³⁸ (<https://phytozome.jgi.doe.gov/pz/portal.html>). Conserved domain analysis was performed using SMART³⁹ (<http://smart.embl-heidelberg.de/>), CD search^{40–43} (<https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>) and CDART tool⁴⁴ (<http://www.ncbi.nlm.nih.gov/Structure/lexington/lexington.cgi>). Sequence alignment and phylogenetic analysis was performed out using CLUSTALW⁴⁵ (<https://www.ebi.ac.uk/Tools/msa/clustalw2/>) and MEGA software^{46–48} (<https://www.megasoftware.net/>). The alignment was represented using BioEdit tool⁴⁹ (<https://bioedit.software.informer.com/7.2/>). To construct the phylogenetic tree, we utilized the JTT (Jones-Taylor-Thornton) model with 1000 bootstrap replicates to generate the Maximum-likelihood (ML) tree.

Cloning, expression and mutagenesis of KnLOV1 proteins

The gene sequence of *K. nitens* phototropin was optimized for codon usage and commercially synthesized. The sequence encoding the phototropin LOV1 domain (KnLOV1) was amplified using specific primers and cloned into the pET21a (+) expression vector containing a His-tag. Subsequently, the BL21 (λ DE3) expression strain was transformed with the KnLOV1 gene construct, and the cells were grown in TB media at 37 °C with 200 rpm speed until the optical density reached 0.4-0.6. Following induction with 0.5 mM IPTG, heterologous expression was carried out for approximately 48 hours at 16 °C. The protein was purified using ion metal affinity chromatography (IMAC) with cobalt resin (Takara) at 4 °C and eluted with an imidazole elution buffer (250 mM, pH ~8.5).

The Quick-Change site-directed mutagenesis kit (Agilent Technologies Inc., Santa Clara, USA) was utilized to produce different mutations of KnLOV1, including R60K, Q122N, and D33N, following the quick change protocol. The mutation-specific primers were designed using Primer-X software. After PCR amplification, the site-directed mutagenesis (SDM) product was treated with DpnI enzyme (Thermo scientific) and incubated at 37 °C for one hour. All constructs were verified by sequencing. A list of primers used is provided in Suppl. Table S5.

Crystallization of KnLOV1 native and mutant proteins

The proteins were crystallized at 25 mg/ml protein concentration by hanging drop vapour diffusion method in light protected area from Mosquito robot. Several commercial crystallization screens were set up, and after about a week, the crystals were obtained in quite a few condition of Natrix (Hampton Research) at 16 °C temperature. The best crystals were replicated in the condition containing 0.05 M magnesium sulfate hydrate (salt), 0.05 M HEPES sodium pH 7.0 (buffer) and 1.6 M lithium sulfate monohydrate (precipitant). The crystals were small yellow/green in colour and diamond shape. A native and three mutant proteins were subjected to the same process for crystallization. First, D33N mutant crystal was obtained in 0.056 M sodium phosphate monobasic monohydrate and 1.344 M potassium phosphate dibasic, pH 8.2, from the Index (Hampton Research). Second, R60K mutant was formed in 0.49 M sodium phosphate monobasic monohydrate and 0.14 M potassium phosphate dibasic, pH 6.9, from the Index (Hampton Research). Third, Q122N mutant crystal was formed in 0.01 M nickel chloride hexahydrate, 0.1 M Tris, pH 8.5, and 1.0 M lithium sulfate monohydrate from the Crystal Screen 2 (Hampton Research).

X-ray data collection, and structure determination

Protein crystals were subjected to X-ray diffraction, and data was collected at the ID30B beamline at ESRF synchrotron, Grenoble, France. 30% Glycerol in mother liquor was used as cryo-protectant. Crystal was picked in small nylon loop and dipped into cryo-protectant then flash froze in liquid Nitrogen. The diffracted data was collected and processed with HKL-2000 software. Structure solution was performed through a molecular replacement (MR) approach with the Phaser MR program⁵⁰ of the CCP4 program suite⁵¹. The crystal structure of the KnLOV1 protein was solved using AtPhot2-LOV1 as a template (PDB ID: 2Z6C), which showed the highest sequence and structural similarity (~66%). After numerous cycle of

manual building in the COOT software, the structure was refined with the REFMAC5 program⁵². For the final model of native KnLOV1 protein, R_{work} and R_{free} values were 19% and 21%, respectively. The native protein structure was used as a template to solve the structure of all the mutants, which were determined through the same process as the native protein. The final data collection and refinement statistics can be found in Suppl. Table S1.

Spectroscopic analysis

The study investigated the dark recovery phenomenon in both native KnLOV1 and its mutant proteins. UV-visible spectra of wild-type and mutants were obtained using a Shimadzu UV-2600 spectrophotometer with a peltier attachment. The spectra were recorded in the dark and at various time intervals after 30 seconds of blue light exposure from a blue LED (Conrad, Luxeon III Emitter LXHL-PB09, Germany). Steady-state fluorescence spectra of proteins were acquired on a Perkin Elmer Lambda 645 spectrophotometer with a peltier setup (at 16 °C) by exciting the samples at 450 nm and recording the fluorescence emission from 460 to 600 nm. All the graphs were normalized and plotted using OriginLabs software Origin v8.5.

CD spectra of both the wild type and mutant KnLOV1 proteins were obtained using a JASCO J-815 CD Spectropolarimeter (Jasco, Japan) equipped with a peltier. To investigate the light-induced changes in protein secondary structure, the spectra were recorded in the far-UV range (200-250 nm) in a quartz cuvette with a pathlength of 1 mm at a protein concentration of approximately 8 μM (100 $\mu\text{g/mL}$) in 100 mM PBS buffer (pH ~7.1). The spectra were recorded in the dark and immediately after 30 seconds of blue light exposure. The spectra were collected at 4 °C with a scanning speed of 100 nm/second and a bandwidth of 1 nm. A total of five accumulations were taken and averaged. Data analysis was performed using OriginLabs software Origin v8.5.

The fluorescence decay time of both the native KnLOV1 and mutant proteins was measured using time resolved fluorescence spectroscopy (TRFS) on an FLS1000 fluorescence spectrometer (Edinburgh Instruments, UK). To measure the fluorescence decay curve using time-correlated single photon counting (TCSPC), a 467 nm pulse laser diode (PLD) with a pulse width of 50 picoseconds was used for excitation, and the emission was collected at 495 nm. The fluorescence decay curves were plotted with the instrument response function (IRF), and the graph fitting was performed by iterative re-convolution of IRF. The FAST software package was used for all decay curve fittings. The decay curve for all proteins was fitted using equation (1) below:

$$f(t) = \sum_{n=1}^{\infty} \left(\alpha_n \exp \frac{-t}{\tau_n} \right) ; n = 1, 2 \text{ or } 3 \quad (1)$$

Where, α_n is the relative amplitude and τ_n is the lifetime of n^{th} component. The average lifetime of the fluorescence decay curves for all proteins was calculated using equation (2) below:

$$\langle \tau \rangle = \sum_{i=1}^m (f_i \tau_i) ; m = 1, 2 \text{ or } 3 \quad (2)$$

Where, τ_i is the lifetime, and f_i is the fraction of the i^{th} component.

Glutaraldehyde crosslinking assay

The glutaraldehyde crosslinking assay was employed to investigate protein crosslinking. To initiate the assay, 50 μ L of freshly prepared 2.5% glutaraldehyde (GA) was added to 450 μ L of proteins (~165 mM conc.) in 100 mM PBS buffer (~pH 7.1) (as described in reference^{35,53}). The study was conducted under three different conditions: dark, light, and light-dark, to evaluate the impact of light on protein crosslinking. In the dark condition, GA (Sigma Aldrich) was added to the protein and the sample was incubated in the dark for ~1 hour at room temperature. In the light condition, the protein was illuminated with blue light for 1 minute, followed by the addition of GA, and then incubated in blue light for 1 hour at room temperature. In the light-dark condition, the protein was illuminated with blue light for 1 minute, followed by a 15 minutes dark incubation, and then GA was added. After GA addition, the sample was incubated in the dark for ~1 hour at room temperature. Crosslinking was halted by adding 20 μ L of 1M Tris, pH 8.0, and samples were kept at 4 °C. The crosslinked protein samples were subsequently separated on 12.5% SDS-PAGE gel, and the protein oligomers were visualized by staining with Coomassie brilliant blue dye. Protein oligomers concentration in all the light conditions was quantified using Fiji (ImageJ)⁵⁴ software.

Atomic force microscopy (AFM)

In order to characterize the morphology of protein oligomers and obtain visual representations, AFM imaging was performed using crosslinked protein samples in intermittent mode. The purified protein samples that had been crosslinked under different light conditions were applied onto a freshly cleaved mica sheet and air-dried in the dark at room temperature for approximately 1 hour. The dried protein samples were gently washed with 2 mL of freshly filtered double distilled water and air-dried overnight before scanning with an AFM machine. The WITech alpha 300 AFM machine was used with a Nano NSG30 probe under intermittent contact mode, with a resonate frequency of 320 kHz and driving amplitude of 0.4 (Vpp). The initial scanning size was set to 10 μ m x 10 μ m with 1290 x 1290 pixel resolution and scanning speed of 0.500. Data analysis was carried out using Project 4 software with a

line subtraction of order 4 used for background subtraction. The height of the molecules was measured after background subtraction and image cross-section was used for topological view.

Dynamic light scattering (DLS)

To determine the size distribution of protein molecules in the oligomerized sample, the Zetasizer Nano ZS DLS instrument (Malvern Instruments, UK) was employed. A native purified sample was used as a control. The protein samples were diluted to a concentration of 1 mg/mL in freshly filtered 100 mM PBS buffer. The scans were conducted in the dark at room temperature, and a total of 3 scans were taken. The Zetasizer Nano software was used for analysis, and size distribution fitting was performed using a Gaussian function in the OriginLabs software Origin v8.5.

Results

KnLOV1 more closely related with higher plants phototropin LOV1 than algal phototropin LOV1

To perform evolutionary and homology analysis, well-characterized LOV domain sequences from several algae and plants were selected, including OlPhot LOV1 from *Ostreococcus lucimarinus*, DsPhot LOV1 from *Dunaliella salina*, CsPhot LOV1 from *Coccomyxa subellipsoidea*, CrPhot LOV1 from *Chlamydomonas reinhardtii*, VcPhot LOV1 from *Volvox carteri*, MpPhot LOV1 from *Marchantia polymorpha*, SmPhot from *Sellaginella moellendorffii*, Ac-vPhot LOV1 from *Adiantum capillus-veneris*, GmPhot LOV1 from *Gnetum montanum*, and AtPhot LOV1 from *Arabidopsis thaliana*. The results indicated that KnPhot sequence shares an overall sequence homology of ~42% with algal phototropin and ~57% with higher plant phototropin. However, KnPhot LOV1 domain exhibits ~65% similarity to algal LOV1 domain and ~70% similarity to higher plant LOV1 domain, as depicted in Fig. 1b. Interestingly, the linker region between LOV1 and LOV2 domain was longer than the algal Phot(s) and shorter than the higher plant Phot(s), indicating the evolutionary changes in *K. nitens* phototropin to adapt from aquatic to land habitat. Moreover, homology analysis showed the presence of a conserved NCRFLQ motif and other key residues regulating photocycle time in *K. nitens* phototropin LOV1 domain, but differ in some binding pocket residues from other LOV domains. The residues involved in protein oligomerization were also conserved in KnLOV1 domain similar to algal and higher plant LOV1 domain.

For phylogenetic analysis, we utilized phototropin gene sequences from different algae and plants like *Ostreococcus lucimarinus* (OlPhot), *Micromonas* sps. (MspPhot), *Dunaliella salina* (DsPhot), *Coccomyxa subellipsoidea* (CsPhot), *Chlamydomonas reinhardtii* (CrPhot), *Volvox carteri* (VcPhot), *Marchantia polymorpha* (MpPhot), *Anthoceros bharadwajii* (AbPhot), *Sellaginella moellendorffii* (SmPhot), *Adiantum capillus-veneris* (Ac-vPhot), *Gnetum montanum* (GmPhot), and *Arabidopsis thaliana* (AtPhot). The phylogenetic analysis revealed that KnPhot LOV1 domain is closer to higher plant Phot LOV1 domain than the algal Phot LOV1 domain, as illustrated in Fig. 1a. These findings provide valuable insights into the evolutionary history and functional characteristics of phototropin genes in *K. nitens* and other related organisms. The blue and green color of node branches represent the algal and higher plants similarity.

KnLOV1 protein is structurally more similar to higher plant phototropin LOV1 domain

The KnLOV1 protein possesses a structure comprising of five β -sheets bordered by five α -helices, as depicted in Fig. 2a. The C-terminal α -helix of the protein is rotatable and essential for its dimerization, and it contains both polar and non-polar residues in its flavin binding pocket. Six active amino acid residues interact with the FMN ring at six distinct positions: (i) FMN-O4-NE2-Gln122, 2.89 Å; (ii) FMN-N5-NE2-Gln122, 3.33 Å; (iii) FMN-O4-ND2-Asn101, 2.99 Å; (iv) FMN-N3-ND2-Asn91, 2.92 Å, (v) FMN-O2-NE2-Gln63, 2.94 Å; and (vi) FMN-O4-NE2-Gln63, 2.78 Å. The side chains of Cys59(S) interact with FMN ring C4A and N5 atom at distances of 3.8 Å and 3.5 Å, respectively. Additionally, the phosphate of FMN interacts with the guanidinium chain of Arg60 (Arg60-NE-O3P-FMN, 2.9 Å; Arg60-NH2-O2P-FMN, 2.88 Å) and Arg76 (Arg-76-NE-O1P-FMN, 3.21 Å; Arg76-NH2-O1P-FMN, 2.82 Å) (as shown in Fig. 2c and 3a).

In terms of structural similarity, the KnLOV1 protein is most closely related to the AtPhot2-LOV1, with a similarity of ~64%, as indicated by a Z score of 20.2 and an RMSD value of 1.5 in the DALI server. The *C. reinhardtii*, a freshwater alga, phototropin LOV1 domain (CrPhotLOV1) has a similarity of ~66%, with a Z score of 19.7 and an RMSD value of 1.1. One notable difference between KnLOV1 and AtPhot-2LOV1 is the presence of an α -helical structure in the latter, while CrPhotLOV1 has a similar loop structure to KnLOV1. The first and second β -sheets in KnLOV1 are shorter than the third, fourth, and fifth β -sheets in AtPhot2-LOV1, and all of the α -helices in KnLOV1 are shorter than those in AtPhot2-LOV1. The length of the loop between the first and second β -sheets is more variable and has a more hanging structural orientation in AtPhot-LOV1A than in KnLOV1 (as seen in Fig. 2a). The FMN binding pocket in KnLOV1 is larger than that in AtPhot-LOV1, and the isoalloxazine ring of FMN is surrounded by Cys59 (in the third α -helix) and Leu103 (in the third β -sheet) residues, with an average distance between them of 7.1 Å. This distance is 7.0 Å (Cys170 and Leu214) and 6.55 Å (Cys57 and Leu101) in AtPhot2-LOV1A and CrPhotLOV1, respectively (Suppl. Fig. S1a-d).

The Hydrogen-bonding (HB) network residues in KnLOV1 are critical for protein dark recovery. The residues involved in HB network in KnLOV1, namely Gln122, Asn101, Asn91, Gln63, Arg60 and Arg76, are similar to those found in higher plant (AtPhot2-LOV1) and algal phototropin LOV1 (CrPhotLOV1). Additionally, Ile36 and Ile119 in KnLOV1 are structurally more similar to Ile147 and Ile230 in AtPhot2-LOV1, but differ from Leu34 and Val117 in CrPhotLOV1. However, KnLOV1 is structurally more similar to CrPhotLOV1 (Asp31 and Ile77) than to AtPhot2-LOV1 (His144, Val190), due to the presence of Asp33 and Ile79 in the ligand binding pocket (Suppl. Fig. S1a-d). These residues in KnLOV1 are located farther from FMN compared to CrPhotLOV1. The flanking residue Asp33 in KnLOV1 is comparable to Asp31 in CrPhotLOV1, which forms a salt bridge connection with Arg58 to cover FMN ring and stabilize the structure. Therefore, mutation at the similar site in KnLOV1 at Asp33 could lead to structural deformity. In AtPhot2LOV1, His143 residue is present and is located slightly further from Arg171 compared to CrPhotLOV1. However, the comparable distance is higher in KnLOV1 between Asp33 and Arg60 (Suppl. Fig. S1e). A significant difference between these three proteins is the presence of a proline residue before Asp31 (CrPhotLOV1) and His143 (AtPhot2LOV1), which restricts the flexibility of loop. Nevertheless, in KnLOV1, a lysine residue is present instead of proline residue, which maintains slight flexibility (Fig. 3c).

Alteration in protein chromophore environment affects photorecovery kinetics of KnLOV1 proteins

The native KnLOV1 protein is a monomeric structure and all the three mutant named D33N, R60K, and Q122N, are dimer in an asymmetric unit of the crystal structure. The KnLOV1 mutants D33N, R60K and Q122N crystals were diffracted up to 2.0 Å, 2.1 Å and 1.9 Å respectively and were processed in the $P4_32_12$ space group. The R_{work} and R_{free} values of D33N, R60K and Q122N mutants are 16/21, 17/22 and 18/22 respectively. The summary of molecular refinement statistics has been mentioned in Suppl. Table S1. The structural comparison between native and mutants shows high similarity with the following RMSD values of D33N (0.170), R60K (0.352) and Q122N (0.132).

The primary difference in all mutants is interaction of Gln122 residue with N5 atom of FMN which introduces change in structure; culminating ultimately to the loose binding and faster recovery rate (Fig. 3). The first promising position that is Asp33 do not interact directly with FMN ligand but affecting the stability of the protein. The mutation D33N then causes a steep decrease in the recovery time which is 580 seconds. D33N mutation causes an increase in the interaction distance 3.3 Å to 3.5 Å between Gln122 to N5-FMN atom compared to native KnLOV1. The distance of all H-bond increases that are interacting with FMN ring along and decreases in those which interacts with phosphate group (Fig. 3b, Table 1). D33N mutation make FMN more dynamic compare to native because it destabilizes it which results to reduction in recovery time. The second mutant R60K structure shows that the distance between N5-FMN and Gln122 increased from 3.3 Å to 3.4 Å compared to native. An increase in the H-bond distances (0.1 Å) with active site residue and decreasing bond distance towards Arg76 (Fig. 3d, Table 1). All these interactions then make FMN unstable compare to native KnLOV1 leading to drastic fall in recovery time from 2647 seconds to 122 seconds. In Q122N mutation structure, there is shorting of first and third beta sheet compared to native structure. This mutation also causes the loss of complete interaction with N5 atom of FMN and only weak interactions of FMN is leftover with active site residue compare to native KnLOV1. Due to main interacting residue (Gln), the structural alteration collectively responsible for the destabilization of cofactor FMN leading to remarkable reduction in recovery time from 2647 seconds to 101 seconds. The mutated residue Asn have no interaction with N5-FMN ring and distance increased from 3.3 to 3.8 Å and distance between ND2 group of O4-FMN decreased from 3.0 to 2.7 Å (Fig. 3c, Table 1).

The comparison between all the three mutant shows that differential recovery time ranging from highest 580 seconds (D33N), 122 seconds (R60K) to lowest 101 seconds (Q122N) is depends on the interaction of Gln122 with N5-FMN. As Q122N mutation lost direct interaction with N5-FMN but R60K and D33N persists weak interaction and correlates with the time of recovery rate fastest in Q122N and lowest in D33N. The effect of all the mutations also increases the area of binding pocket size of ligand with protein might also contributing the effect on recovery rate (Fig. 3b).

Hydrogen-bonding network residues attribute to unusual long dark recovery of KnLOV1 protein

The duration of dark recovery in the LOV domain is influenced by the photophysical behavior of the chromophore and its environment in the binding pocket. Alterations in the HB network and the chromophore binding pocket can affect the photophysical behavior and lifetime of the photocycle^{13,14,22,23}. In this study, we investigated the changes in the photophysical characteristics and recovery kinetics of KnLOV1 by measuring the absorption spectra in the dark and at various time points after blue light illumination (see Fig. 4a). Our results showed that the protein exhibits maximum absorbance (λ max) at 448 nm for the S0 $\rightarrow$ S1 transition bands, with shoulder peaks at 426 and 475 nm, which differs from the algal (λ max = 447 nm) and higher plant LOV1 (λ max = 449 nm). Additionally, we observed prominent S0 $\rightarrow$ S2 bands with minor peak at 351 nm and major peak at 369 nm, differing from algal LOV1 at 360 nm and higher plant LOV1 at 361 nm. Upon blue light illumination, protein absorbance decreased, and in the dark, the protein exhibited dark reversion after some time. The KnPhot LOV1 protein showed a dark recovery time of approximately 2.5 hours, which was much longer than that of algal and higher plants LOV1 protein^{16,17} (as shown in Fig. 4a).

To explore the role of different amino acids in dark recovery, we introduced various mutations in the protein. We selected mutations by substituting binding pocket residues and part of HB network involved in the photo-recovery kinetics of the protein, such as R60K, Q122N, and D33N. We expressed and purified mutants such as LOV1 R60K, LOV1 Q122N, LOV1 D33N, and LOV1 R60K D33N, LOV1 Q122N D33N, with an intact chromophore. The absorption and fluorescence spectra revealed that the mutations R60K and R60K D33N caused a slight blue shift of approximately 1 nm, while Q122N D33N caused a shift of approximately 2 nm in the transition I peaks. Wild-type KnLOV1 protein and different mutants exhibited different recovery time constants (τ), representing the recovery rate of the protein from the signaling state to the ground state. Wild-type KnLOV1 protein exhibited a slower recovery rate (τ = 2467 seconds) compared to different mutants, i.e., R60K (τ = 122 seconds), Q122N (τ = 101 seconds), D33N (τ = 580 seconds), R60K D33N (τ = 31.8 seconds), and Q122N D33N (τ = 36.5 seconds), which accelerated the recovery kinetics of the protein (as shown in Fig. 4b-f).

The Q122N mutation in the LOV1 domain of KnPhot accelerated the photocycle of the protein, and the recovery was about 80 times faster in the Q122N mutant compared to the KnLOV1 wild-type protein. The faster recovery rate indicates the destabilization of the covalent adduct due to the mutation.

The photodynamic properties of the KnLOV1 examined by measuring the fluorescence decay time in wild type and mutant proteins and was found 4.1 nanoseconds (wild type), 3.5 nanoseconds (R60K mutant), 3.9 nanoseconds (Q122N mutant), and 3.7 nanoseconds (D33N mutant), as mentioned in Suppl. Table S2.

We found that the mutations primarily modulating the photocycle lifetime significantly did not alter the triplet state decay or adduct formation in KnLOV1 protein. Steady state fluorescence for KnLOV1 wild type and different mutants revealed similar emission maxima (495 nm) and shoulder peaks (518 nm) across all proteins (Suppl. Fig. S4), which is consistent with the algal LOV1 domain.

Residues for dark recovery affect the light induced conformational changes in KnLOV1

The light-induced changes in secondary structure of the LOV1 protein investigated using circular dichroism (CD) spectroscopy in the far-UV region of wild type LOV1 protein displayed a characteristic α -helical conformation with distinct peaks at 208 and 222 nm, and a prominent peak at 218 nm indicating the presence of β -sheet structure (Suppl. Fig. S5a). Upon exposure to blue light, there was a gradual reduction in both α -helical and β -sheet content. Similar results were obtained for the Q122N and D33N mutants (Suppl. Fig. S5c and S5d). However, the R60K mutant showed an increase in both α -helical and β -sheet content (Suppl. Fig. S5b). Double mutations, such as R60K D33N, led to an increase in α -helix and a decrease in β -sheet content, whereas Q122N D33N resulted in a decrease in α -helix and an increase in β -sheet content (Suppl. Fig. S5e and S5f). The mutants used in this study are listed in Suppl. Table S3. These findings suggest that blue light induces secondary structural changes in the LOV1 protein and that different mutations can produce contrasting effects on the protein's conformation.

Photoinduced oligomerization of KnLOV1 protein

Effect of light on protein crosslinking suggested that in native conditions, KnLOV1 protein primarily exists in dimer form with trace amounts of tetramer, trimer, and monomer. SDS-PAGE gel analysis of crosslinking samples revealed the presence of protein monomers, suggesting non-covalent interactions among monomer molecules in protein oligomers (Suppl. Fig. S6a and S7). Glutaraldehyde crosslinking experiments confirm that the majority of the protein is in dimer form, with a small number of tetramers, trimers, and monomers (Suppl. Fig. S6a and b). We conducted crosslinking experiments under different light conditions to study the effect of light on protein crosslinking. The dark incubated crosslinked sample showed a higher concentration of dimer than the light and light-dark crosslinked samples (lane 2 and 3 dark (D), 4 and 5 light (L), 6 and 7 light-dark (LD) in Suppl. Fig. S6a). We used protein without GA addition as a control sample for oligomerization study in gel (lane 8 in Suppl. Fig. S6a). Dark incubated protein samples showed a higher number of oligomer species formation compared to light incubated samples (Suppl. Table S4). However, blue light illumination decreased the oligomer concentration and increased the monomer concentration in protein samples. These results coincide with AFM studies.

To visualize and characterize the morphology and structure of LOV1 oligomers, we studied crosslinked protein samples in various light conditions using AFM. We used native purified protein as a control sample in AFM for size comparison. The oligomer size was measured by examining the cross-section of different particles seen in AFM images. Protein size was also confirmed using DLS of crosslinked protein particles in different light conditions and control samples (Suppl. Fig. S8). The results indicate that the oligomers vary in size from approximately 1.2-5 nm for monomers, 6-12 nm for dimers, and 14-22 nm for trimer and tetramer molecules depending on the measuring axis for the cross-section of oligomeric molecules (Fig. 5). In AFM results, protein oligomers come in contact and form higher oligomeric species, resulting in an increase in the size of the oligomer depending on the surface and process of molecular oligomerization. In control samples (with native purified protein), monomeric size molecules were

predominant, with a trace amount of dimer molecule, which may have been formed due to storage or higher protein concentration in AFM samples.

Discussion

Green algae have been important in ecosystems for millions of years and are believed to have given rise to terrestrial plants^{55–57}. Recent studies showed that charophyte algae *Klebsormidium nitens* share features with land plants such as plant hormones and cell wall structure^{37,55}. Our study explores the evolutionary relationship across the green lineage with emphasis on *K. nitens* LOV1 protein. The present study provides an insight into the possible adaptations at structural and functional level in *K. nitens* phototropin during the evolution from aquatic to land habitat. Structure, phylogenetic and homology analysis suggested that the KnLOV1 domain exhibits closer relationship to higher plant LOV1 domain. Interestingly, KnLOV1 shows a very high dark recovery time compared to algal and higher plants LOV domains.. As a proof of concept, we characterized and studied the function of amino acids involved in HB network regulating protein recovery time. The key residues important for ligand binding, protein recovery, and oligomerization were found to be conserved in the KnLOV1, similar to higher plant and algal phototropin LOV1.

The conserved glutamine at position 122 (Gln122) in the LOV domain plays a crucial role in light-regulated physiology^{18,23,58}. Spectroscopy, simulation, and structural studies have shown that Gln122 is involved in the photo-transitions of the LOV domain²³. Despite some LOV proteins exhibit blue light (BL) responses without Gln122^{12,59,60}. The recent research has shown that water molecules can compensate for its absence by relaying hydrogen bond signals from the flavin N5 atom to the LOV domain beta-sheet¹². Interestingly, in our study water molecules are not there in the structure and protein with Gln122 mutation is functional. Only, duration of photocycle is affected due to mutation (Fig. 3c). Other amino acid mutations, such as R60K and D33, have been found to accelerate the photocycle and recovery time^{25,26,61,62}. In particular, the Q122N mutation in the LOV1 domain of phototropin (KnLOV1) accelerates recovery time by 25 times compared to the wild type protein. Although the role of glutamine has been studied in other LOV domains, but the acceleration in recovery time is not very prominent as observed in KnPhot LOV1 domain^{27,63}. The crystal structure of the native protein reveals that the amide group of Gln122 interacts with the O-4 and N5 of flavin in the H-bonding network of the flavin binding pocket. It is established that the lateral chain flips after the blue light illumination during the covalent adduct formation and forms interactive H-bond with the N(5) of flavin⁶⁴. In our study, the N5-Asn122 hydrogen bond is lost in the Q122N mutant structure due to which the stability of the FMN must be less and recovery rate is faster. The acceleration of photocycle duration in the mutant protein is due to increase in distance between FMN and Asn122 causing destabilization of the covalent adduct (Fig. 3c). The increased energy content and decreased in the activation energy might accelerate the photocycle in the mutant protein²³.

The mutations R60K and D33N may alter the volume of the LOV domain²⁶. The arginine residue interacts with the phosphate group of FMN and stabilizes the interaction between the conserved cysteine in the LOV domain and the C4 atom of the chromophore¹⁴. The R60K mutation does not affect the photochemistry of LOV1 protein, as indicated by the minor effects on fluorescence and absorption spectra observed in this study, suggesting that the FMN surrounding environment is not significantly altered. However, modulation of photocycle duration may occur due to alteration in the H-bonding network of the LOV domain in R60K and D33N mutants (Fig. 3b and d), this observation was also seen earlier²⁶. After blue light illumination followed by covalent adduct formation, the side chain of Arg58 shifts away from FMN phosphate¹⁴, leading to a change in the length of the H-bond between Arg58 and FMN phosphate group, and altering the interaction with Asp31 residue in LOV (CrPhot) domain. The R60K mutation accelerates the photocycle about 20 times faster, and in the case of D33N, about 5 times faster than the wild-type KnLOV1 domain, which is more prominent than other LOV domains. The replacement of Arg60 with Lys weakens the H-bond with the phosphate group of FMN because Lys has only a single terminal amino group that forms only one H-bond interactions with FMN and there is small movement of FMN towards Lys, that changes the FMN interacting distances (Fig. 3d), whereas Lys barely forms an H-bond interaction with Asp33 residue. Therefore, the photoproduct might get destabilized due to this mutation, leading to the fast photocycle on LOV1 domain. The double mutation R60K/D33N has a weaker H-bond of Arg60 with the FMN phosphate and Asp33 residue in comparison to the wild-type LOV domain, suggesting the role of Arg60 interaction with the LOV domain in the stability of the active state (LOV390) covalent adduct.

In various physiological processes, the activation and deactivation of photoreceptors depend on their photocycle. For signaling processes that require a longer signaling state, a native protein with a slower reversion rate would be useful. Therefore, the recovery kinetics affect the downstream signaling pathways through photoreceptor switching between active and inactive states, which might be important for adaptation in the terrestrial environment of *K. nitens*. The native KnLOV1 protein with slower dark recovery and a protein engineered for faster dark reversion could be potential candidates for optogenetic applications.

Previous studies have shown that the LOV1 domain acts as an attenuator of light sensitivity³⁶. In our current study, oligomer structural prediction suggested that the F-helix and several loops are involved in generating an oligomeric interface between LOV domains (Fig. 2b). Simulation experiments revealed that the A β -B β and G β -H β loops are present at the interacting interface of the LOV domain, and light-induced covalent bond formation may be responsible for decreased structural alterations of these loops, leading to protein oligomerization⁶⁵. KnLOV1 protein was observed as a monomer in the crystal structure, crosslinking experiments and atomic force microscopy experiments demonstrated that the protein predominantly forms dimers in solution, with a small amount of monomer and higher oligomeric populations. This suggests that LOV1 may act as a protein dimerization site in the intact phototropin molecule^{34,66}. Oligomerization in the LOV domain remains in a dynamic equilibrium between monomer and dimer forms, depending on a wide range of concentrations and light exposure. The light-dark

incubated samples showed transient tetramerization for the dimerization of dimeric molecules of the LOV1 domain, which may have a possible role in the functional regulation of phototropin³⁶. These findings suggest the role of the LOV1 domain in phototropin oligomerization.

Conclusion

Our findings offer insight into the evolution of the LOV domain and its ability to adapt to diverse environments. The amino acids within the flavin binding pocket and H-bonding network of KnLOV1 protein impact its photocycle recovery kinetics. The duration of photoreceptor active state plays a crucial role in regulating specific physiological responses and achieving precise physiological outputs. The modulation of the photocycle kinetics and oligomerization through light enables fine-tuning of physiological responses. However, the amino acids that affect the oligomerization of LOV proteins require further exploration. Investigating how the moderation of protein oligomerization affects the photocycle kinetics and physiological activation in the in-vivo system will be particularly interesting.

Declarations

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Author contributions

S.S. and A.K.G. contributed equally. S.K. conceived the project and S.S. performed sequence and phylogenetic analysis, protein expression and purification, spectroscopy experiments, crosslinking and microscopy experiments. A.K.G. and S.G. performed crystallography experiments and refinement of structures. S.S. wrote the manuscript with the help of S.K., S.G. and A.K.G. All authors read and approved the final manuscript.

Competing interests. The authors declare no competing interests.

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Data availability

Atom coordinates and structure-factor amplitudes have been deposited in the Protein Data Bank under accession codes 8I11 (KnLOV1 native, dark), 8IYN (D33N mutant, dark), 8J68 (R60K, dark), and 8IL9 (Q122N, dark). Electron density maps of native KnLOV1 and mutants is shown in supplementary information (see Suppl. Fig. S2).

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Table

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Interacting Residue with FMN	Interacting residue Atom	FMN ligand Atom	Native KnLOV1 Distance (Å)	D33N Mutant Distance (Å)	Q122N Mutant Distance (Å)	R60K Mutant Distance (Å)
Gln 122	NE2	N5	3.33	3.5	-	3.4
	NE2	O4	2.89	2.84	-	2.89
Asn122	ND2	N5	-	-	3.8	-
		O4	-	-	2.7	-
Asn101	ND2	O4	2.99	3.06	3.10	3.07
Asn91	ND2	N3	2.94	2.93	2.85	3.01
Gln63	NE3	O2	2.94	2.95	3.06	2.90
		O4'	2.78	2.65	2.80	2.93
Arg60	NE	O3P	2.90	2.66	2.85	-
	NH2	O2P	2.88	2.97	2.84	-
Lys60	NZ	O2P	-	-	-	2.76
Arg76	NH2	O1P	3.21	2.88	2.83	-
	NE	O1P	2.82	2.85	2.87	2.7
	NH2	O3P	-	-	-	2.99

Figures

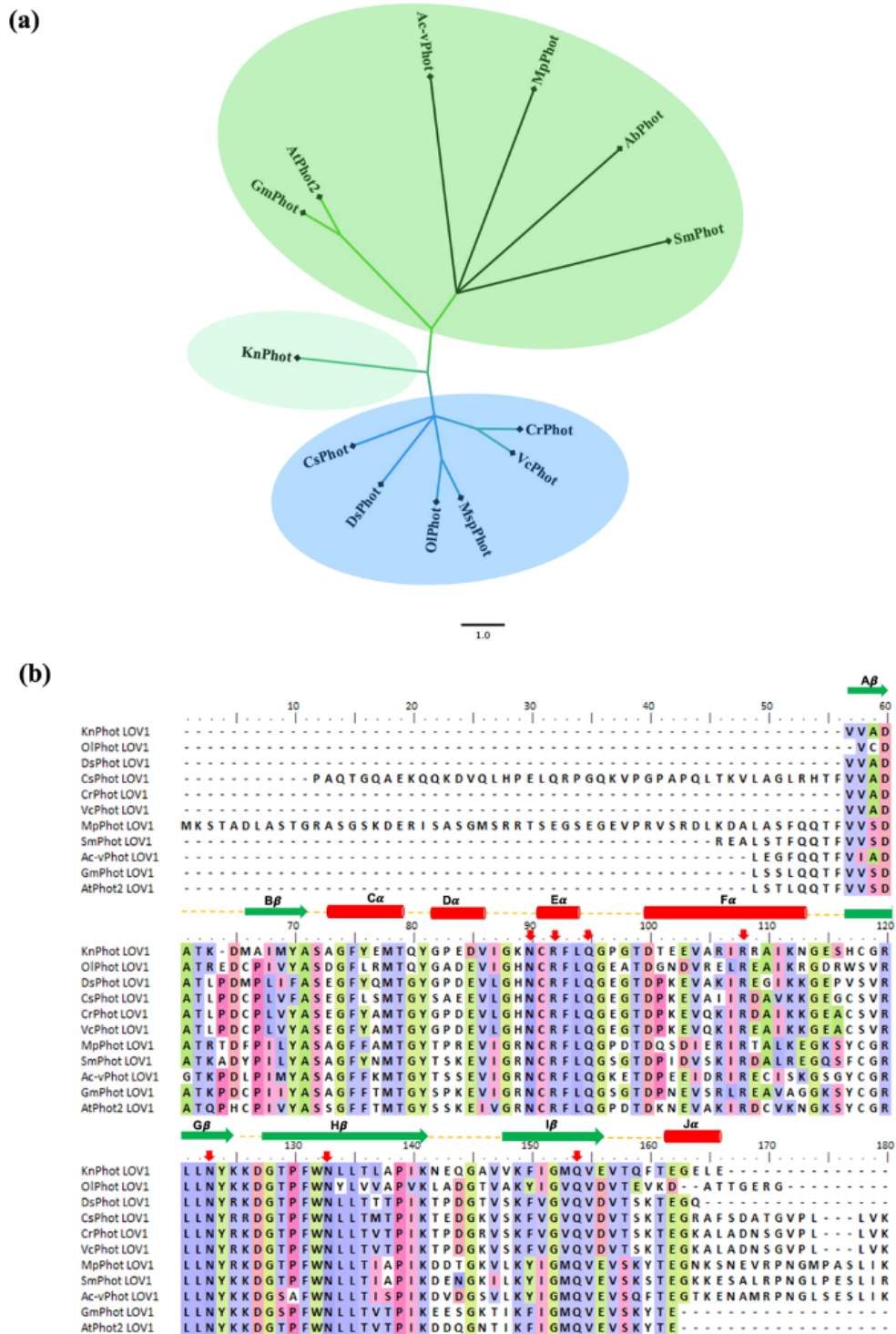


Figure 1

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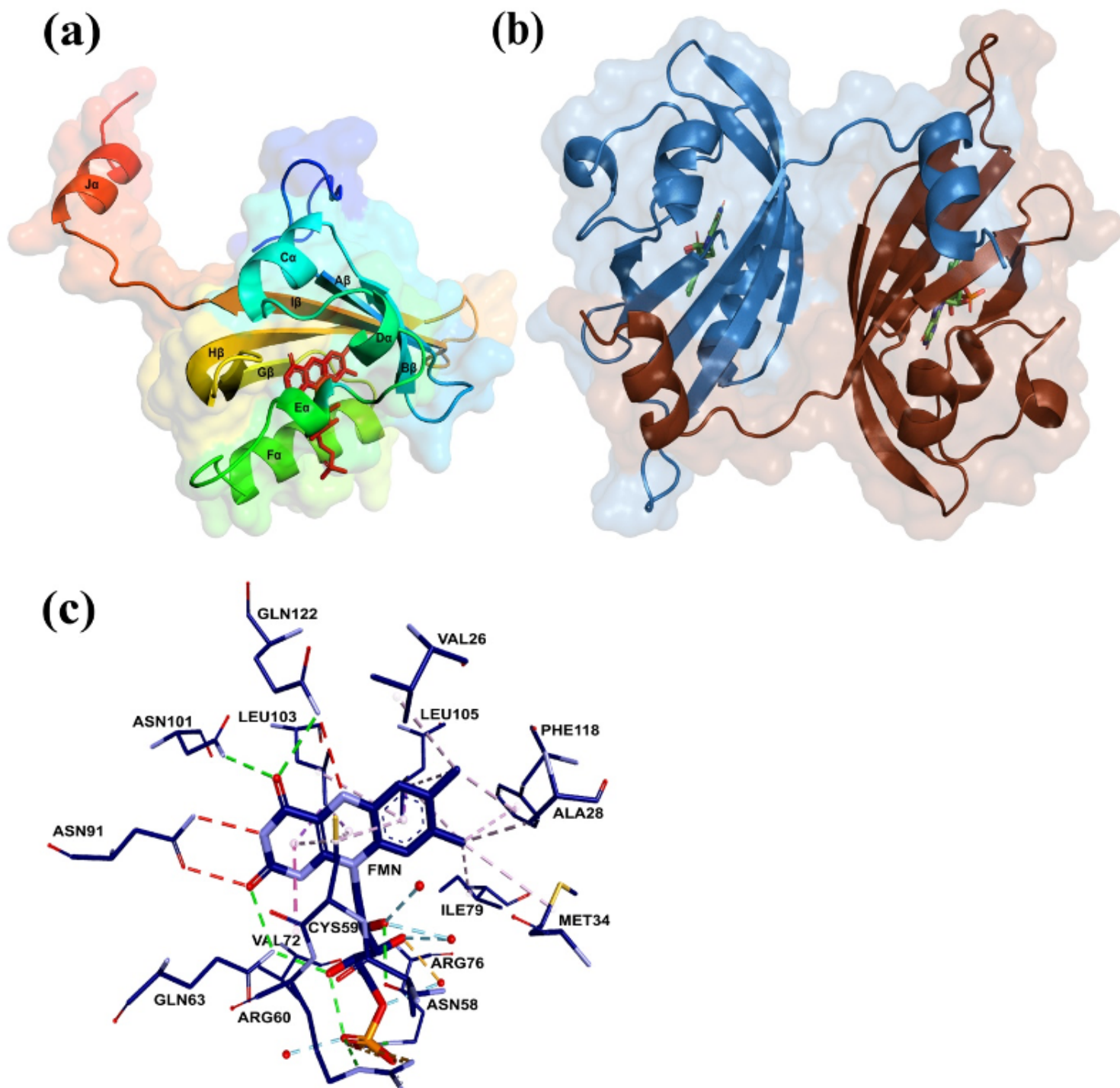
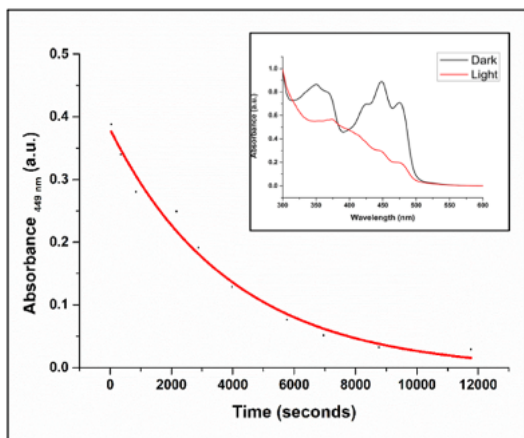
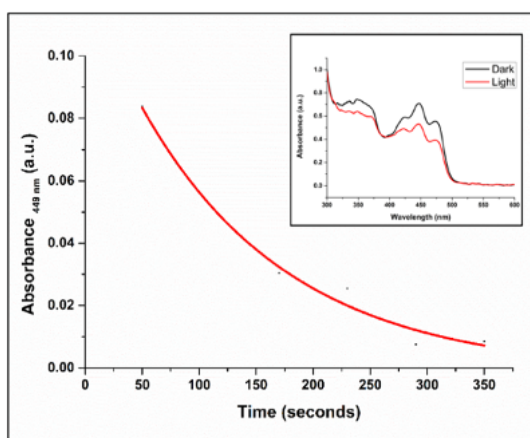


Figure 2

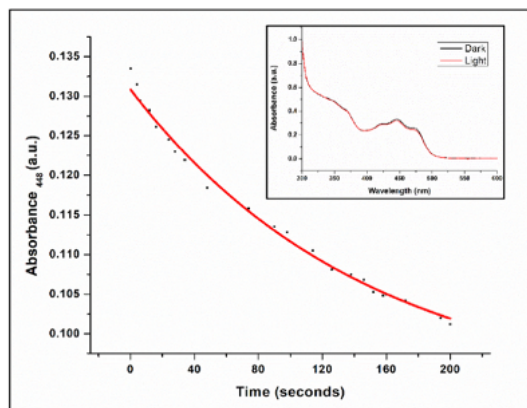
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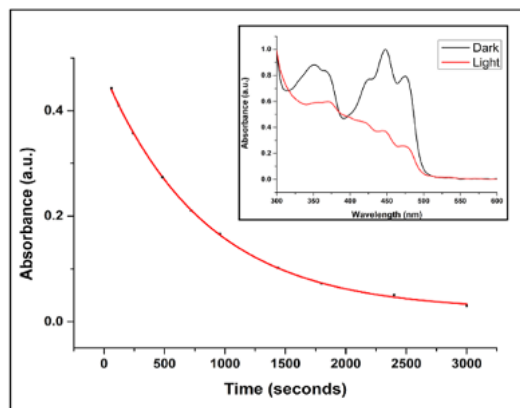
(a)



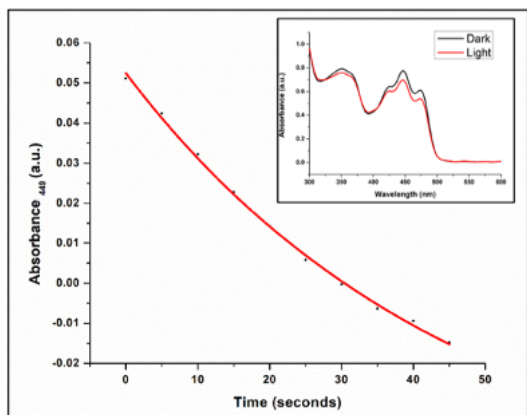
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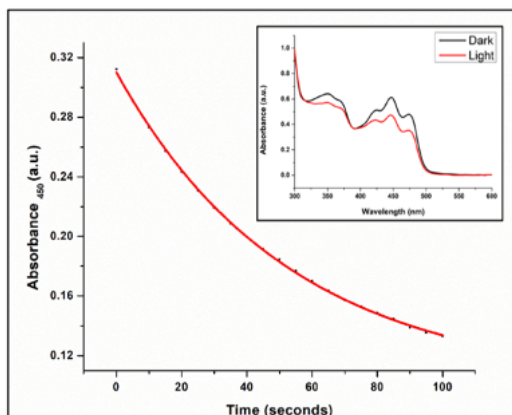
(c)



(d)



(e)



(f)

Figure 4

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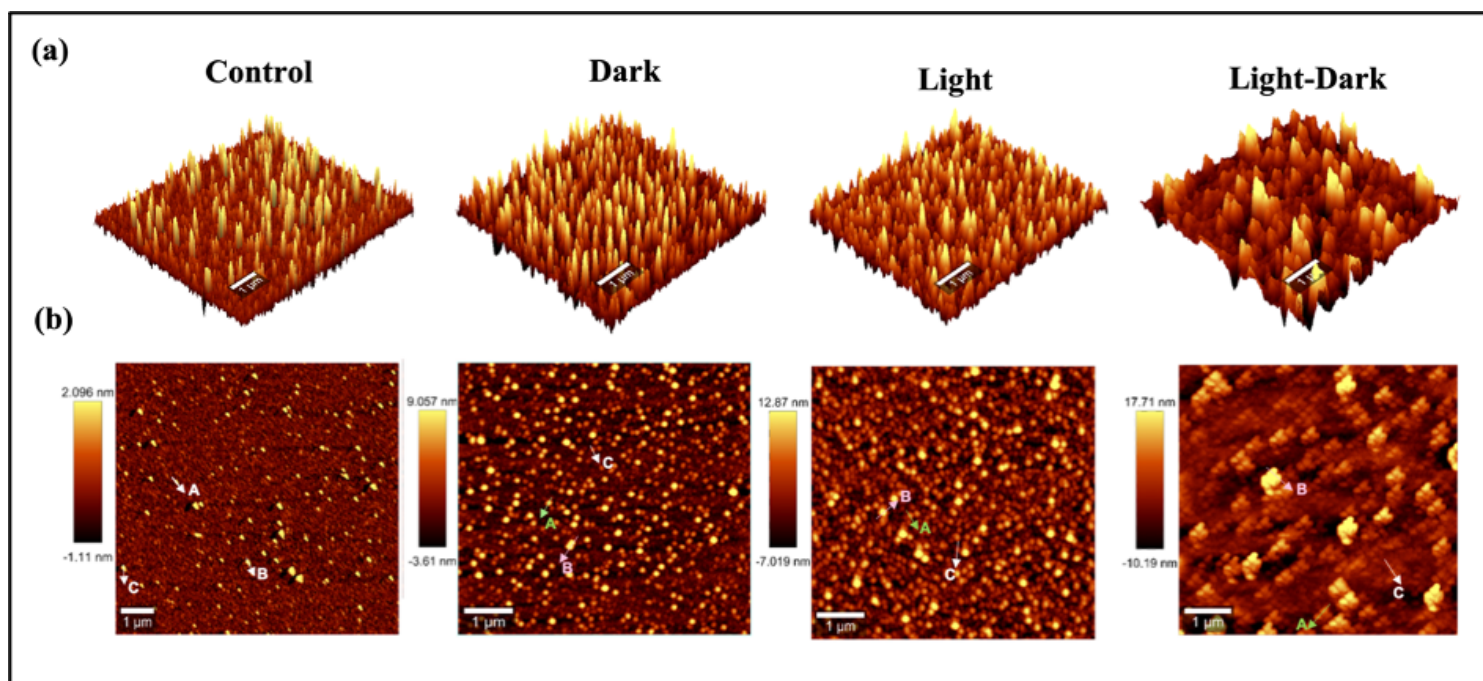


Figure 5

Legend not included with this version

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