RESEARCH

Reversible photocontrol of oxidase activity by inserting a photosensitive domain into the oxidase

Tongjing Sun, Baoqi Zhang, Jinping Lin and Yuhong Ren[*]

Abstract

Background: Photocontrol of protein activity has become a helpful strategy for regulating biological pathways. Herein, a method for the precise and reversible photocontrol of oxidase activity was developed by using the conformational change of the AsLOV2 domain.

Results: The AsLOV2 domain was inserted into the nonconserved sites exposed on the surface of the AdhP protein, and the alov9 fusion was successfully screened for subsequent optical experiments under the assumption that neither of these actions affected the original activity of AdhP protein. The activity of alov9 was noticeably inhibited when the fusion was exposed to 470 nm blue light and recovered within 30 min. As a result, we could precisely and reversibly photocontrol alov9 activity through the optimization of several parameters, including cofactor concentration, light intensity, and illumination time.

Conclusions: An efficient method was developed for the photoinhibition of enzymatic activity based on the insertion of the light-sensitive AsLOV2 domain, providing new ideas for photocontrolling metabolic pathways without carriers in the future.

Keywords: AsLOV2, Enzyme activity, Insertion, Oxidase, Photocontrol

Background

Photocontrol of protein dynamics is a powerful tool for the precise spatial and temporal control of signal transduction (Pathak et al. 2014; Zhang and Cui 2015). The light-sensitive LOV2 domain of *Avena sativa* phototropin 1 (AsLOV2) is part of the PAS superfamily of domains (Crosson and Moffat 2001), each of which consists of a flavin nucleotide and a C-terminal Jα helix (Harper et al. 2003; Halavaty and Moffat 2007). During the conformational change of the AsLOV2 domain, the Jα helix will unfold under illumination (Harper et al. 2004; Swartz et al. 2002), and thus it has been utilized to construct engineered optical switches. As a photosensitive element, the AsLOV2 domain has many advantages, including high spatial and temporal resolution, fast response to

blue light, and good reversibility (Lee et al. 2014). Therefore, the AsLOV2 domain has been widely used as an engineered light switch in recent years.

There are two general approaches to achieve the photocontrol of proteins based on the AsLOV2 domain. One is based on the peptide-tagged LOV2 domain (Kawano et al. 2015). Devin et al. developed tunable light-inducible dimerization tags (TULIPs) to control protein localization (Strickland et al. 2012). The other approach is based on peptide-binding (Lungu et al. 2012). Hui et al. constructed the LOVTRAP system for reversible light-induced protein dissociation (Wang et al. 2016). Most studies have focused on interfering protein–protein interactions (PPIs) using the photosensitive domain, but studies have only rarely concentrated on the kinetics of individual proteins. Here, a method was introduced for achieving reversible photocontrol of oxidase AdhP in time and space based on insertion of the AsLOV2 domain.

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Sun *et al. Bioresour. Bioprocess.* (2019) 6:28

Page 2 of 6

In our work, the LOV2 domain was inserted into surface-exposed and evolutionarily nonconserved sites of the AdhP protein to prevent possible structural perturbations. The AdhP was cloned from *E. coli* MG1655 and catalyzes the oxidation–reduction reactions of short-chain alcohols and aldehydes (Thomas et al. 2013). The 10 Å spacing between its N and C termini prevented structural interference of the original protein (Dagliyan et al. 2016). Therefore, we assumed that when the LOV2 domain underwent a conformational change upon illumination, its disorder can be efficiently transmitted to the active center of the AdhP protein to produce a structural disturbance, which would inhibit the activity of the AdhP protein.

Materials and methods
Cloning, expression, and purification of enzymes
The gene for AdhP was subcloned into pET-28a(+) via *Bam*HI/*Xho*I (Thermo Fisher Scientific, Rockford, IL, USA) restriction sites to create an in-frame N-terminal 6*histidine tag. The LOV2 and adhP genes were spliced together using the splicing by overlap extension polymerase chain reaction method (SOE-PCR) (the primer information is listed in Additional file 1: Table S1) (Chen et al. 2016). The plasmids were then transformed into *Escherichia coli* BL21 (DE3)-competent cells.

The recombinant *E. coli* BL21 (DE3) was incubated in lysogeny broth (LB) containing 50 mg/L kanamycin at 37 °C. When the OD_{600} reached approximately 0.6, enzyme expression was induced by the addition of 0.1 mM IPTG (final concentration). The culture was incubated at 18 °C for an additional 12 h at 200 rpm. After centrifugation (6000×*g*, 10 min, 4 °C) and washing with 0.9% NaCl, the cells were resuspended in 20 mM sodium phosphate buffer (pH 8.5) and disrupted by ultrasonication. After centrifugation (12,000×*g*, 30 min, 4 °C), the crude extract was used for protein purification using a 5-mL HisTrap FF crude column (GE Healthcare, Waukesha, WI, USA). The protein was eluted with an increasing gradient from 20 to 500 mM of imidazole in sodium phosphate buffer. Proteins were evaluated using SDS-PAGE, and the concentration was conducted using a Bradford assay kit (Beyotime, Shanghai, China). The purified enzyme was concentrated and stored at −20 °C for further use.

Enzyme assay
Enzyme activity was determined by measuring the rate of NAD^+ reduction at 340 nm on an ultraviolet (UV)/visible spectrophotometer (BioTek Instruments, Winooski, VT, US); a molar extinction coefficient of 6.22 mM^{-1} cm^{-1} for NADH was used for the calculation (Zhang et al. 2002). Standard reactions were performed in 200 μL of sodium phosphate buffer (100 mM, pH 8.5) at 30 °C containing 0.2 mM NAD^+, 5 mM substrate (in 5% v/v DMSO), and an appropriate amount of purified enzyme. One unit of enzyme activity was defined as the reduction of 1 μmol geraniol per minute. The light group activity was measured immediately after irradiation for a precise period. Specific wavelength 470 nm LED light sources was used for blue light illumination. All of the measurements were carried out over 2 min and performed at least three times.

Structure analysis and homology modeling
The structural model of alov9 was built using the modeler 9.20 program (Schwede et al. 2003). The crystal structure of AdhP (PDB ID: 4kgv) and AsLOV2 domain (PDB ID: 5hzj) were used as templates. Model evaluation was performed using the Ramachandran plot Web server (Betteridge et al. 2003). Ramachandran plot analysis results showed that 95.4% of the total number of residues was in the favored and allowed region for alov9. The model could be considered reliable only if the percentage of residues in the favored and allowed region was greater than 90%. Thus, the evaluation results indicated that our model of alov9 was reliable. Structural analysis was performed using the PyMOL 1.8.0 program.

Results and discussion
Strategies to inhibit AdhP activity
As described above, we hypothesized insertion of a light-sensitive domain at the active sites around catalytic center could lead to optogenetic control of AdhP. Our experiment is organized as follows: First, insertion sites were selected on the surface-exposed loops of AdhP by analyzing the crystal structure to avoid possible structural interference (Fig. 1a). Eighteen allosteric sites were selected in this round. Then non-conservative active sites were selected from those exposed sites by sequence alignment (Fig. 1b). Ten sites were finally identified as insertion sites. Finally, sensory domain was inserted into those insertion sites to form fusion Lov2-AdhP, here called alov1-10 (Additional file 1: Figure S1). All of the fusions can achieve soluble expression (Additional file 1: Figure S2).

To establish whether fusion enzyme activity remains stable after insertion into the sensitive domain, activity assays were performed in a catalytic system using a microplate reader. The results showed that the activity of these 10 fusions had varying degrees of reduction except the insertion of ninth point (Thr215) (Fig. 1c), which might be caused by the insertion of AsLOV2 at the ninth site on the surface of the AdhP protein and prevents influencing the correct folding of the AdhP protein. The results confirmed that insertion of LOV2 domain causes
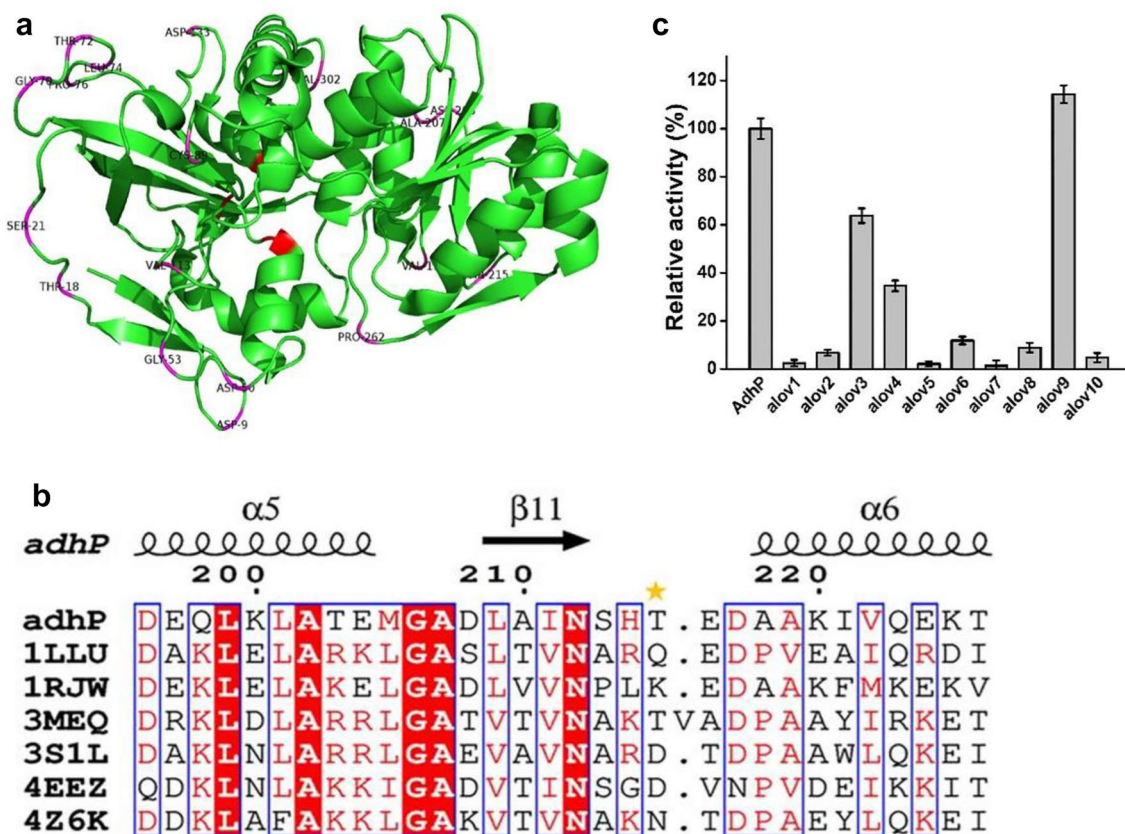
Fig. 1 Designing alovX (X present different insertion sites) photoswitches. **a** Crystal structure analysis of wild-type AdhP protein to select surface-exposed loop (red represents active center of AdhP. Magenta represents exposed sits in surface loop). **b** Screening unconservative sites from surface-exposed loops by sequence alignment (yellow star represents the selected site. The information of ten sites at Additional file 1: Figure S3). **c** Comparing catalytic activity between WT AdhP and alov1-10 fusions in the dark state. Error bars show SEM (*n* = 3)

different structural interferences to the AdhP protein. From the perspective of maintaining stable protein activity, alov9 fusion was selected for a subsequent study.

Optical inhibition enzyme activity

To evaluate the inhibitory activity of the alov9 in response to specific wavelength (470 nm) of illumination, the activity of AdhP and alov9 was measured under dark and light conditions, respectively. In vitro activity assays revealed that the alov9 fusion was indeed strongly inhibited upon exposure to blue light (Fig. 2a), whereas the control group AdhP, in both the presence and absence of irradiation, displayed similar activities (Fig. 2a). These data indicated that the Jα helix unfolding of the AsLOV2 protein upon illumination caused a structural disturbance in the AdhP protein, which caused a change in the substrate channel and a decrease in enzyme activity at last. The conformational change of enzyme influenced by illumination can be further confirmed by an experiment based on the Circular Dichroism date. As shown in Additional file 1: Figure S4, the

alov9 without illumination showed an abundant α-helix at 208 nm and 222 nm negative peak and β-sheet at the 195–198 nm positive peak. After illumination, the circular dichroism value of α-helix and β-sheet of alov9 increased significantly. This indicated that the alov9 undergoes a conformational change upon illumination, which led to a decrease in oxidase activity. All of the results confirmed that LOV2 insertion could markedly inhibit the activity of AdhP under illumination.

The FMN cofactor is necessary for the unfolding of the AsLOV2 protein in the blue state (Christie et al. 1998). It has been confirmed that the incorporation of cofactor FMN is required for the light sensitivity of alov9 (Yu et al. 2016). As expected, the catalytic activity of alov9 in the absence of cofactor FMN did not decrease under irradiation (Fig. 2b). The data indicated that the participation of cofactor FMN is required in the next optical experiment.

Exploration of factors affecting optical suppression

To precisely photocontrol alov9 in time and space, several parameters were optimized that affect optical

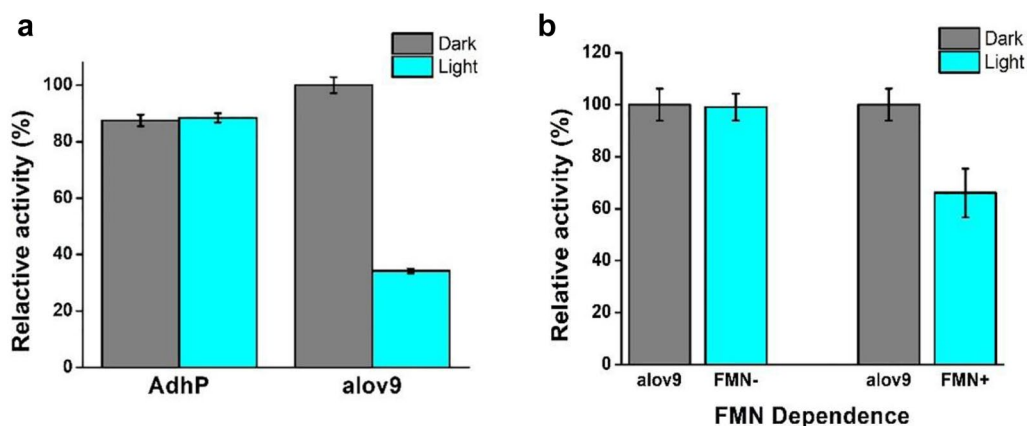Sun *et al. Bioresour. Bioprocess.*   (2019) 6:28

Page 4 of 6

Fig. 2 Optical suppression of alov9 protein. **a** Enzyme activity assay shows that alov9's catalytic activity is inhibited upon irradiation. **b** Dependence of alov9 protein on cofactor FMN. Under the experimental conditions, the relative activity was expressed as a percentage of the maximum activity (100% activity = 7.19 U/mg). All error bars show SEM (n = 3)
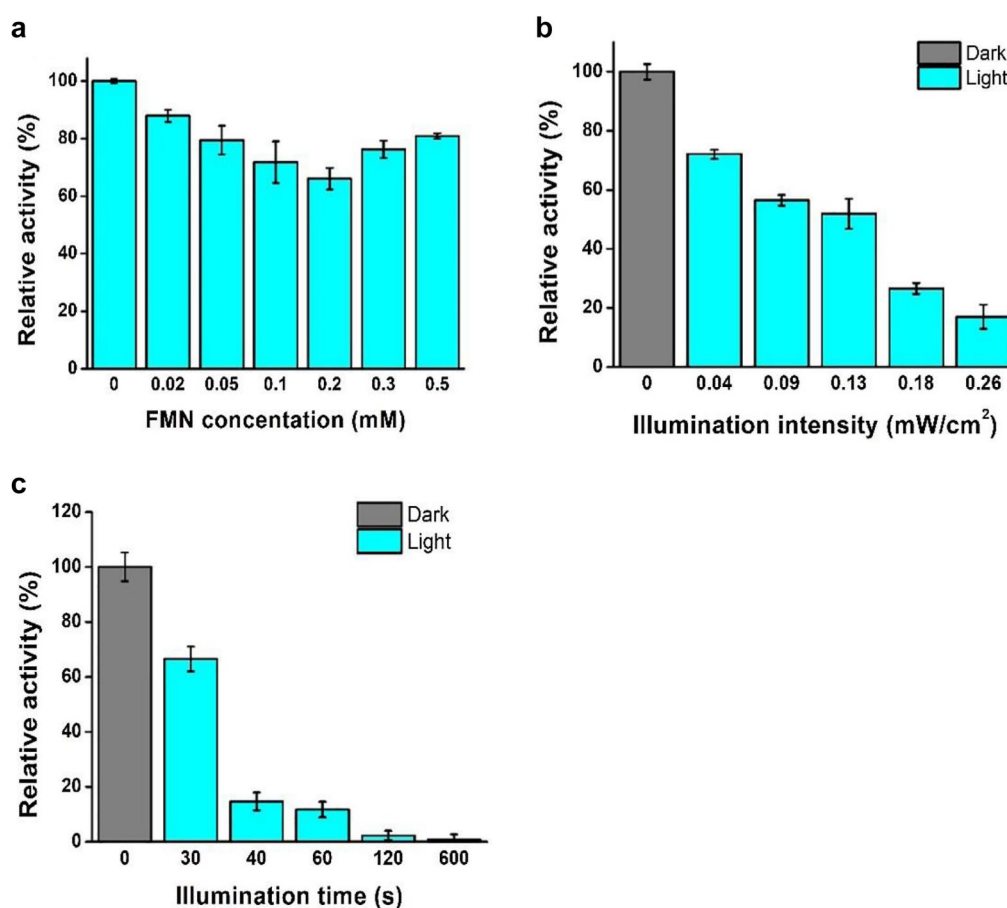

Fig. 3 Optical suppression factors. **a** Relationship between FMN concentration and the enzyme inhibition under irradiation at 470 nm for the same time. **b** Power dependency of alov9's inhibition under illumination at 470 nm for the same time. **c** Relationship between illumination time and alov9's inhibition under irradiation at 470 nm. All error bars show SEM (n = 3)

Sun *et al. Bioresour. Bioprocess.* (2019) 6:28

Page 5 of 6
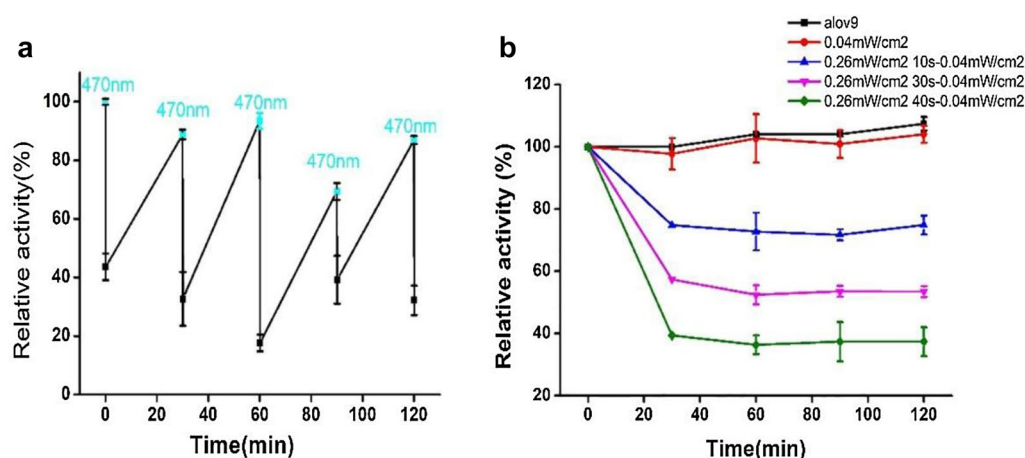
Fig. 4 Reversible photocontrol of alov9 activity. **a** The activity of alov9 will decrease rapidly in a short time under the irradiation of 470 nm blue light. Then the enzyme activity will slowly recover in the dark state. **b** The activity of alov9 will rapidly decrease with a strong irradiation. We keep lighting with weak intensity, and alov9 activity does not recover but remains at a certain level. All error bars show SEM ($n = 3$)

suppression, including cofactor concentration, illumination intensity, and duration of illumination. Further analysis concerning the correlation between FMN concentration and light-induced inhibition level suggested that 0.2 mM is preferable under the conditions used in our experiments (Fig. 3a). In the following experiments, alov9 activity was monitored while varying the light intensity. When alov9 was exposed to illumination of increasing intensity for 40 s, higher intensities were found to induce a progressively decreasing rate of activity (Fig. 3b). The effects of irradiation time on alov9 excited at 470 nm exhibited a decrease similar to that of the light intensity (Fig. 3c). These results suggested that the inhibitory effect of blue light on alov9 underwent pronounced changes with varying illumination factors. Consequently, the enzyme activity of alov9 fusion can be controlled as desired by changing various factors under blue light excitation.

Reversible regulation of alov9

To verify the stable reversibility of alov9 catalytic activity, the alov9 activity were analyzed through five cycles of illumination at 470 nm followed by incubation in the dark. The results showed that 87% of alov9 activity could be maintained after four rounds of illumination (Fig. 4a). This suggested that irradiation shifts of alov9 from its physiological active state to a strongly inhibited state and that this process was reversible; alov9 recovered its native activity in the dark. In addition, the maintenance of alov9 activity was achieved by varying the light intensity (Fig. 4b). Based on all of the evaluated results, it is suggested that the precise and reversible regulation of the alov9 fusion can be achieved.

Conclusions

In summary, based on the conformational change of the light-sensitive AsLOV2 domain, a method was successfully developed to reversibly photocontrol oxidase activity. We successfully constructed the alov9 fusion, in which the AsLOV2 domain was inserted into the allosteric sites of the AdhP protein, without interfering in the original AdhP activity. This alov9 fusion was indeed strongly inhibited upon exposure to blue light. Our results showed that alov9 fusion activity can be reversibly photocontrolled and still retain 87% of its initial activity after four illumination cycles. Finally, oxidase activity can achieve spatial and temporal control in a precise time. The control of protein activity via LOV2 insertion may prove to be a valuable tool for other biological processes in the future.

Additional file

Abbreviations
alov9: AsLOV2-AdhP; AsLOV2: LOV2 domain isolated from *Avena sativa*; FMN: flavin mononucleotide; IPTG: isopropyl β-ᴅ-1-thiogalactopyranoside; LB: lysogeny broth; OD_{600}: optical density of bacteria at 600 nm; PAS: Per-Arnt-Sim.

Sun *et al. Bioresour. Bioprocess.*   (2019) 6:28

Page 6 of 6

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