Proteins

Light-Regulated Tetracycline Binding to the Tet Repressor
Jayoung Moon,[a] Jongsik Gam,[b] Seung-Goo Lee,[b] Young-Ger Suh,[a] and Jeeyeon Lee*[a]

Abstract: Elucidation of the signal-transmission pathways between distant sites within proteins is of great importance in medical and bioengineering sciences. The use of optical methods to redesign protein functions is emerging as a general approach for the control of biological systems with high spatiotemporal precision. Here we report the detailed thermodynamic and kinetic characterization of novel chimeric LOV proteins and engineered light-sensing protein modules with TetR as a valuable tool that deepens our understanding of the mechanism of signal transmission within proteins. In addition, the light-regulated changes of drug binding that we describe here suggest that engineered light-sensitive proteins may be used for the development of novel therapeutic strategies.

Introduction

Living cells have evolved their own ways of responding to extracellular environmental changes.[1] A current focus of protein chemistry research is that protein functions are responsive to pH, light, ligand binding, and other environmental factors. In general, these adaptations are mediated by conformational changes in the protein structure.[2] In particular, the responses of sensory proteins to environmental signals play a vital role in cellular adaptations. Light–oxygen–voltage (LOV) domains are a subgroup of the Per-Arnt-Sim (PAS) superfamily, a sensory protein module present in organisms from all kingdoms of life.[3] Among LOV domains, the As-LOV domain from Avena sativa phototropin has been widely examined in biochemical, biophysical, and structural studies.[3b,4] This domain has a unique α-helical structural element, denoted as a Jα helix, that is located at the C terminus of the PAS core domain. The light-induced unfolding of the Jα helix is triggered by the formation of a reversible covalent adduct between flavin mononucleotide (FMN) and a conserved cysteine (Cys) residue in the chromophore binding site.[5] The transition from a light-activated covalent adduct state to a noncovalent cysteine-FMN complex state occurs on a time scale of tens of seconds in the As-LOV domain.[3a]

The LOV domain was initially discovered in plant phototropin, but subsequent studies identified LOV-domain proteins in bacterial genomes. These bacterial proteins regulate the general stress-response factor O6, virulence, and cell-envelope physiology.[3b,6] YtvA, the first identified prokaryotic photoreceptor-like protein from Bacillus subtilis, has an N-terminal light-sensing LOV domain and a C-terminal signaling sulfate transporter/anti-α-factor antagonist (STAS) domain.[4a,7] The LOV domain acts as a sensing module, and various effector domains are connected to the C terminus of this domain by an α-helix linker in bacteria. Structurally, the α-helix linker (residue 127–147) in YtvA corresponds to the Jα helix in the As-LOV domain, which is located at the C terminus of the PAS core (Figure 1A). However, the function of the Jα helix of YtvA appears to be different from that of As-LOV, and light-induced unfolding of the Jα helix of YtvA has not been reported. Most structural and biophysical studies of LOV proteins have included the Jα helix of the YtvA-LOV domain in their characterizations, and it is agreed that the YtvA-LOV domain exists as a dimer in solution.[4a,7a] Additional research indicated that the full length of YtvA adopts a dumbbell-like shape, in which LOV–LOV and STAS–STAS domains interact on either side.[7a] Blue-light absorption activates the YtvA-LOV as in other FMN binding LOV domains; absorption of blue light leads to the formation of a covalent protein-FMN adduct and the appearance of an absorption band at 390 nm. The YtvA-LOV domain differs from other LOV domains in that it has a much slower dark regeneration time (t1/2 ≈ 45 min).[7b,8] In the present study, we took advantage of the slow dark regeneration rate of this protein to develop a new light-controlled switch module by use of biomolecular engineering.

The goal of the present research was to provide a better understanding of the mechanism of light-signal transmission within LOV proteins. Thus, we replaced output domains (STAS...
in YtvA-LOV and the kinase domain in As-LOV) with the TetR protein, a DNA binding protein that regulates the transcription of genes that code for resistance to tetracycline (Tc). TetR is a homodimeric α-helical protein, the monomers of which are 24 kD, and is composed of an N-terminal DNA binding domain and a C-terminal tetracycline binding domain (TBD). Binding of Tc to TetR triggers conformational changes that abrogate TetR binding to DNA. In the absence of Tc, the TetR dimer binds to its target DNA sequences, tetO1 and tetO2, which are operators of the tetR and tetA genes. The binding of TetR to tetO has a reported binding affinity ($K_d$) of $10^{-11}$ M, but this affinity decreases by nine orders of magnitude when the protein binds Tc. This leads to increased expression of TetA and TetR proteins, thereby resulting in rapid reduction of the cytoplasmic concentration of Tc. Previous studies have taken advantage of the tight control of the TetR–tetO system with Tc to regulate gene expression in many prokaryotic and eukaryotic systems.

In addition, structural and biochemical studies have revealed the presence of tetracycline-induced allostery in the TetR protein. Owing to the large distance of approximately 33 Å between the Tc binding site and the DNA binding region of TetR, previous researchers have used this protein as a model for mechanistic studies of allosteric regulation. Signal transmission from one site to another distant site within a protein has great significance for biomedical engineering. The engineering of allosteric TetR proteins with light-sensing domains, as described in the present study, provides a versatile tool for study of the interdomain signal-transmission pathways of proteins. In particular, operations of light switches on different timescales will provide valuable information on the engineering of light-signal transmission in LOV domains.

**Results**

**Protein design**

We constructed PAS-TetR chimeras by use of the YtvA-LOV and As-LOV light-sensing domains (Figure 1). These LOV domains that exist in a "light state" or a "dark state", depending on light intensity and wavelength, were connected to TetR in a head-to-tail manner (Figure 1B). The Jα helix of the As-LOV domain, which consists of approximately 20 amino acids at the C terminus of the protein, undergoes a conformational change upon irradiation. Many biochemical assays have confirmed the significance of the Jα helix in light-signal transmission in the As-LOV domain. Thus, we included the Jα helix in the design of the As-TetR construct (Figure 1B) so that the Jα helix of the As-LOV domain could be connected to the N terminus of the TetR domain.

The YtvA domain also has an α helix at the C-terminal region, but this α helix appears to have a different function. Our initial studies demonstrated that the Jα helix of the YtvA domain is not the main signaling element for signal transmission (unpublished data). Therefore, we did not include the Jα helix of YtvA in the design of the Ytv-TetR chimeric protein. The N-cap of 24 amino acids in the YtvA domain, the function of which has not yet been investigated, was included in the chimera (Figure 1B).

Fusion proteins (Ytv-TetR and TetR-Ytv) were made at each terminus (N or C) of YtvA-LOV, because the exact signaling pathway for light-signal transmission in the YtvA domain has not yet been determined. A cysteine-to-serine mutation in the FMN binding site of each LOV-TetR chimeric construct was generated to lock the proteins in the dark state, thereby preventing light-induced formation of the covalent adduct.

For the TetR domain as an output module, TetRs from type-B and type-D sources were utilized to construct PAS-TetRs. TetR(B) has been used in most biochemical characterizations, but its structure has not been solved. TetR(D) has been used in X-ray crystallography, which as a complex with the DNA binding tetO sequence has been solved by X-ray crystallography. TetR(D) was also used for chimeric protein design during our initial work. However, we found that TetR(D) fusion proteins had poor solubility, and the expression and folding of the chimeras were not suitable for the production of a large quantity of TetR(D) fusion proteins for biochemical assays (data not shown). Therefore, all fusion protein assays reported here were performed using the TetR(B) fusion proteins.
Fast-switch and slow-switch chimeric proteins

Previous research reported that the transition from the light-excited state to the dark-ground state was much slower in the YtvA-LOV domain than in the As-LOV domain. This enabled us to design a "slow-light-switch" protein, a critical requirement for characterization of the light-activated state of the light-sensing domain. The $t_{1/2}$ of the light-activated species in the As-LOV domain is approximately 30 s, thus making characterization of this state very difficult for most biochemical analyses. In the Ytv-TetR switch, the rate constant (measured as the increase in absorbance at 447 nm owing to dark-state recovery) was 0.01421 min$^{-1}$, which corresponds to a $t_{1/2}$ of 48.8 min (Figure 2). This is similar to that reported for the YtvA domain. This result suggests that engineering of YtvA with TetR did not affect the intrinsic nature of the photocycle of the YtvA domain, and indicates intact folding of YtvA in the chimera.

Electrophoretic mobility-shift assay

Figure 3 shows the results of our electrophoretic mobility-shift assays (EMSAs) of Ytv-TetR and As-TetR with a DNA oligomer that contained the tetO sequence. For the Ytv-TetR chimera (Figure 3A), the complex with tetO in the upper bands of the gel completely disappeared at 10 $\mu$M Tc in the light and at 50 $\mu$M Tc in the dark. These results indicate that Tc binding caused tetO DNA dissociation from the repressor protein and that the light state of the protein binds Tc at lower concentrations. The apparent Tc binding affinity ($K_a$) was calculated as 6.5 $\mu$M in light and 20.2 $\mu$M under dark conditions. For a chimera in which TetR was connected to the N terminus of the YtvA-LOV domain (TetR-Ytv), there was no consistent light dependence in Tc binding to the protein (data not shown). This implies that signal transmission through the N terminus of YtvA to TetR does not significantly contribute to the light-mediated regulation of TetR-Ytv.

Interestingly, light had the opposite effect on the As-TetR protein (Figure 3B). In particular, the dark state of As-TetR was more sensitive to Tc ($K_a$ less than approximately 8 $\mu$M) than the light state. The complete dissociation of the DNA–protein complex occurs at 50 $\mu$M Tc in the light. These results suggest the presence of different mechanisms in the light-induced changes in the DNA binding affinities of As-TetR and Ytv-TetR.

Fluorescence spectroscopy

Analysis of protein fluorescence can indicate changes in the environment of specific amino acids, and this method is widely used to monitor ligand binding of proteins. When Ytv-TetR and As-TetR fusion proteins were excited at 280 nm, maximum emission was at 340 nm, which is indicative of tryptophan fluorescence (Figure 4A). Upon Tc binding, fluorescence at 340 nm decreased and fluorescence at 514 nm increased owing to energy transfer to Tc. This fluorescence resonance energy transfer (FRET) has been widely used to monitor Tc binding to TetR. However, in the switches we designed, the fluorescence at 514 nm was complicated with another fluorescence emission signal from FMN. Although Tc binding had a large effect on emission at 340 nm (arrows in Figure 4A), illumination had a small effect on emission at 340 nm (black versus gray lines in Figure 4A). Thus, we used the fluorescence change at
340 nm to monitor Tc binding to LOV-TetR chimeric proteins in an equilibrium binding titration and stopped-flow measurements with a band-pass filter for 300–400 nm.

Equilibrium dissociation constant for Tc

Titration of Tc concentration indicated a \( K_d \) of \((0.265/0.1) \) \( \mu \)M in the light state and a \( K_d \) of \((0.537/0.1) \) \( \mu \)M in the dark state of Ytv-TetR (Figure 4B, C and Table 1). These results are consistent with the EMSA results. In other words, the YtvA domain in the light state has a more favorable orientation of the two TetR monomers, presumably owing to the release of structural constraints. In addition, Tc binds to the As-TetR protein more tightly in the dark state \((K_d = 0.41 \mu \text{M})\) than in the light state \((K_d = 0.78 \mu \text{M})\). Again, these results are consistent with the EMSA experiments.

Stopped-flow analysis

Next, we used stopped-flow analysis to elucidate the effect of light on the ligand-binding kinetics of fused chimeric TetRs. These experiments were performed under pseudo-first-order conditions (a large excess amount of Tc relative to LOV-TetR protein). The results suggest that Tc binding to LOV-TetR is a two-step processes, but only one of these steps is light-dependent. In the Ytv-TetR chima, the association rate constant \((k_{on})\) calculated from the slope of Tc versus k_{obs} was 50% greater in the light state (Figure 5). This result is consistent with the tighter binding to the light-state protein, as presented in the steady-state fluorescence titration (Figure 4B).

There was no reproducible light dependence of Tc binding in As-TetR (data not shown). This was not surprising, because the light-to-dark recovery of this chimera has a half-life of approximately 30 s, similar to the timescale of the data acquisition (100–200 s) in these experiments. This was not a limitation for the slower switch of Ytv-TetR (Figure 5).

Circular dichroism

Finally, we determined circular dichroism (CD) spectra of the Ytv-TetR light- and dark-state proteins in the 200–250 nm regions (Figure 6A). The CD signals of the light and dark states were significantly different, which suggests that light perturbed the protein structure. However, analysis of these results with the K2D3 web server (http://www.ogic.ca/projects/k2d3/index.html) suggested that the protein had similar \( \alpha \)-helical secondary structures in both states. The CD signal in the 250–320 nm region was also different for the light and dark states, thus indicating a non-symmetric environment with regard to the aromatic groups.

Table 1. Fluorescence \( K_d \) value.

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Figure 4. A) Fluorescence emission spectra of Ytv-TetR (left) and As-TetR (right) chimeric proteins in the presence (dashed line) and absence (solid line) of tetracycline in the dark state (black) and light state (gray). B) Titration of tetracycline binding to Ytv-TetR (left) and As-TetR (right) based on fluorescence. Gray circles and black squares represent the light state and dark state, respectively. C) \( K_d \) of Ytv-TetR and As-TetR in the light state and dark state (left), and ratios of the \( K_d \) in the light state to dark (right).
residues (tryptophan and tyrosine). The light state had a small positive signal (maxima at 290 nm) and the dark state had no signal in this region. We initially attributed this difference to the presence of the FMN chromophore in its binding site. However, CD signals of the YtvA-LOV domain with and without a Jα helix were also different (Figure 6B). Thus, the different CD spectra in the near-UV region in the light and dark states may also be explained by changes in the quaternary structure around CD-sensitive residues.

Discussion

Biological engineering of the signaling pathway from a light-sensitive module to an effector module of a protein has been used to understand the “switch” system of the light-sensing domains. This approach, which we used in the present study, has become a pivotal strategy for examination of the physiological effects of signaling proteins in which light is the stimulus. However, the fast dark recovery kinetics of the As-LOV domain made it difficult to study the light-activated state of engineered As-LOV chimeric proteins. Thus, we used the YtvA-LOV domain, which has a much slower dark recovery time (t1/2 ≈ 48 min), to study the light-activated state of a YtvA-LOV chimera in CD and stopped-flow experiments.

Our results demonstrated that optimal light-signal transmission requires proper alignment of the two modules of the chimeric protein. In particular, the Ytv-TetR chimera showed a light-dependent sensitivity to Tc binding, but the TetR-Ytv chimera did not. Our Tc titration data and stopped-flow measurements indicated that Ytv-TetR binds tightly to Tc in the light. However, TetR-Ytv (in which the C terminus of TetR is connected to the N terminus of YtvA) did not show reproducible light-dependent activity changes in most assays. This indicates that light-signal transmission occurs from the C terminus of the YtvA-LOV domain to the N terminus of TetR, even without the Jα helix in YtvA.

Addition of tetracycline leads to dissociation of tetO DNA from the TetR binding site. More specifically, the binding of Tc to TetR decreases the affinity of TetR for tetO DNA by nine orders of magnitude. The association constant (Ka) of the Tc–Mg complex to TetR was reported to be \(6.4 \times 10^6\) M\(^{-1}\), \(K_d = 0.16\) μM\(^{-1}\). The apparent dissociation constants of chimeras obtained from our fluorescence binding titration experiments were 0.2–0.8 μM, similar to the previous report. This suggests that the intrinsic function of TetR was retained in our chimeric proteins.

The EMSA results indicated the presence of a band that had a higher molecular mass than the Ytv-TetR/tetO complex in the dark state (lanes 5 and 6 in Figure 3A). This might be a tetramer of Ytv-TetR bound to tetO sequences in the presence of Tc. The higher-molecular-size band at approximately 20–50 μM Tc indicates a higher-order quaternary complex (i.e., a tetramer of YtvA-LOV), and this might be an intrinsic property of YtvA-LOV. The CD spectra also supported...
a significant structural perturbation of Ytv-TetR under light and dark conditions. The Jα helix unfolding in the As-LOV domain appears to affect Tc binding to the TetR module by disruption of the dimerization orientation of TetR. The Jα helix is thought to be docked on the surface of the PAS β fold in the dark state, and unfolding of this helix uncovers a hydrophobic region on the β sheet in the light state.\cite{11a} The exposure of this hydrophobic region might lead to structural constraints in TetR function, and this might explain the decreased binding affinity of Tc of As-TetR in the light state. One of the complications of studying engineered light-sensing domains during biochemical assays is that there might be heterogeneous populations of light- and dark-state samples. This might explain the small magnitude of light-induced changes in our PAS-TetR proteins.

Conclusion

We have demonstrated that light stimulates communication between two unrelated protein domains, LOV and TetR. Our EMSA and Tc titration experiments clearly indicated that light absorbed by the chromophore was transmitted through the N-terminal DNA binding domain to the C-terminal tetracycline binding domain in TetR, thus leading to altered Tc binding. Therefore our experiments confirm the alternative direction of signal transmission in TetR suggested by Reichheld et al. (N-terminal DNA binding domain to C-terminal Tc binding domain).\cite{11a}

The purpose of this report was to identify the major signaling elements of two LOV domains, YtvA and As-LOV, on the basis of bioengineering. We constructed "light switches" with different dark regeneration kinetics, in which light altered the binding of TetR to Tc, thereby controlling the tetO DNA binding affinity. Functional characterization indicated that the YtvA-LOV and As-LOV domains had different light effects in the engineered protein. In addition, it was found that the PAS β fold composed of five strands of β sheets in YtvA-LOV could contribute to the light-signal propagation even when the C-terminal Jα helix was eliminated. The successful modulation of TetR activity by light clearly demonstrates the presence of interdomain communication in both directions: from the DNA binding domain to the Tc binding site and from the Tc binding site to the DNA binding domain. Further studies of the optogenetic control of cellular function will improve our understanding of light-signal-transmission mechanisms in biological systems.\cite{11a}

In addition, the engineering of proteins in which light can regulate the binding to drugs provides a basis for the development of novel therapeutic strategies.

Experimental Section

Cloning

The DNA fragments of each module shown in Figure 1 were polymerase chain reaction (PCR)-amplified using primers listed in Table 2 with HF Phusion polymerase (NEB). These short PCR fragments were used as templates for the second-round overlap PCR to generate the AsLOV-TetR, YtvA-TetR, and TetR-YtvA chimeric fusion proteins. Each primer contained a Nhel or BamH restriction site, and the PCR products of the second step were treated with both restriction enzymes and subsequently ligased with a pET28a(+) vector cut that contained the same restriction enzyme cleavage using T4 ligase (NEB) at room temperature for 10 min. The clones with desired chimera sequences were sequence-verified and transformed to the BL21(DE3) strain.

Protein expression and purification

AsLOV-TetR, YtvA-TetR, and TetR-YtvA chimeric constructs in pET28al(+) were overexpressed in the Escherichia coli BL21(DE3) strain at 18 °C overnight with 0.2 mM isopropyl β-D-thiogalactopyranoside (IPTG) as described previously.\cite{11a} The cell pellet was lysed with sonication and proteins were purified using Ni-NTA affinity chromatography under native conditions. After elution with buffers containing 250 mM imidazole, the proteins were dialyzed against a 25 mM tris(hydroxymethyl)amino- methane (Tris) buffer (pH 7.0, 10% glycerol, 1 mM DTT) overnight and concentrated with centrifugation.

EMSA

DNA oligomers (48 bp) that contain the tetO sequence were purchased from CosmoGenetech, and the sequences of the oligomers were as follows: tetO1 5’- CCAAATTGGGTGATACATCTACATGGATAGTATTTTTCACCCTC-3’ and tetO2 5’- GAGTGGTAAAAATACCTCTA-

Table 2. Primers used for cloning of LOV-TetR chimeric constructs.

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CAATGATAAGTGTTCAACAAAATTAGG-3’. Equal molar amounts of each oligomer were hybridized by mixing both oligomers in water at 96 °C for 5 min followed by cooling to room temperature within 2 h. The annealed DNA was incubated with the chimeric TetR proteins in a binding buffer composed of 20 mM Tris-HCl (pH 8.0), 50 mM NaCl, 1 mM MgCl₂, 0.1 mM DTT, 5% v/v glycerol, and 0.001 mg/mL BSA at 10 min at room temperature. For the preparation of light-activated proteins, samples were illuminated with a white light source at this step. The DNA was loaded onto an 8% polyacrylamide gel and run at 50 V for 1 h in a Tris-boric acid buffer that included MgCl₂ 5 mM. The gel was stained with Safe- Pinky DNA staining solution (Genedepot) and quantitatively analyzed using an ImageQuant LAS 4000.

Fluorescence measurements

Emission spectra of chimeric TetR proteins were measured from 295 to 550 nm with an excitation of proteins at 280 nm. Light-state samples were prepared by illuminating proteins with a white-light lamp for 1 min. Equilibrium binding titration experiments were carried out at 20 °C using a Jasco FP 6500 spectrophotometer. Proteins were dissolved in 100 mM Tris-HCl (pH 8.0), 100 mM NaCl, 1 mM MgCl₂, and 1 mM dithiothreitol, and the quenching of the intrinsic tryptophan fluorescence as a function of Tc concentration was measured to monitor the binding of Tc to proteins. The spectral bandwidths used for measurement were 1 nm for excitation and 5 nm for emission, varied depending on the strength of the fluorescence signal.

Stopped-flow measurement

Pre-steady-state binding kinetic data were obtained using a SX-20 stopped-flow spectrophotometer (Applied Photophysics Ltd., UK) at (20 ± 0.4) °C. Proteins were dissolved in a buffer that contained 10 mM Tris (pH 8.0), 150 mM NaCl, and 2 mM dithiothreitol (DTT) with 10 mM MgCl₂. A decrease in fluorescence emission owing to the Tc binding to TetR was detected using a UG-1 band-pass filter (Andover Corporation) with maximum transmittance at 300–400 nm and excitation at 280 nm. The experiments were performed using 0.1 μM proteins with excess amounts of Tc in varying concentrations from 2.5 to 20 μM to maintain pseudo-first-order kinetic conditions. Generally, eight thousand data points were obtained in each kinetic trace, and three to five traces were averaged to fit to double-exponential decay curves using PRO-Data software (Applied Photophysics Ltd., UK).

Circular dichroism

CD measurements were carried out using a Jasco J715 spectropolarimeter. Protein samples were prepared at a final concentration of 25 μM in 10 mM Tris (pH 8.0), 150 mM NaCl, and 2 mM DTT with 10 mM MgCl₂. The spectra were obtained in the far-UV spectral region from 190 to 250 nm and the near-UV spectral region from 250 to 350 nm at 25 °C. The buffers were also scanned under the same conditions, and the buffer background was subtracted. The optical pathlength was 0.1 cm.

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