A way to sense light intensity: Multiple-excitation of the BLUF photoreceptor TePixD suppresses conformational change

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Abbreviations: BLUF, sensors of blue light using FAD; FAD, flavin adenine dinucleotide; D, diffusion coefficient; DSCC, diffusion-sensitive conformational change; TC, transient grating; TA, transient absorption

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A B S T R A C T
TePixD, a cyanobacterial sensor of blue light using flavin adenine dinucleotide (FAD) (BLUF) which exists in a decamer form, was found to exhibit photoreaction sensitive to light intensity. While the number of excited molecules increased monotonically as the laser power increased, the number of decamers exhibiting a global conformational change initially increased, and then decreased with the increase of excitation intensity. This unusual power dependence was analyzed based on a Poisson distribution equation, demonstrating that decamers containing more than one excited monomer subunit do not undergo conformational change. Our results suggest that TePixD functions not only as a photosensor, but also by sensing light intensity.

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1. Introduction

Most organisms possess photoreceptors that adequately sense and adapt to light environments to allow their survival. The sensors of blue light using flavin adenine dinucleotide (FAD) (BLUF) are a photoreceptor domain found in many species [1,2]. PixD proteins are ones of photosensors containing the BLUF domain, and identified in cyanobacteria. They include Slr1694 of the mesophilic Synechocystis sp. PCC6803 (SyPixD) and Tll0078 of the thermophilic Thermosynechococcus elongatus BP-1 (TePixD) [3]. SyPixD regulates phototaxis of cyanobacteria [3–5]. Crystallographic analyses showed that these homologous PixD proteins have a unique oligomeric structure; a decamer comprised of two stacked pentameric rings [6,7]. Because oligomer formation of PixD proteins is also observed in solution, PixD may function as an oligomer in cyanobacterial cells [3,8,9]. The importance of decamer formation for signal transduction in SyPixD has been demonstrated [9]. In the presence of the response regulator PixE, a stable PixD10-PixE5 complex is formed in solution in the dark, and this complex dissociates into a PixD dimer and PixE monomer upon blue light irradiation [5,9]. The initial photochemical reaction of the PixD proteins was investigated by using the absorption detection, and it was shown that a hydrogen bond switching around the chromophore results in a reversible red shift of ~10 nm in their absorption spectrum. This primary red-shifting reaction proceeds on picosecond time scale [10]. Following the spectral change, we found that conformational change associated with a volume expansion and a significant diffusion coefficient (D)-change during the photocycle of TePixD by the transient grating (TG) method. From the concentration dependence of the TG signal and of molecular mass in solution determined by the gel filtration chromatography, we revealed a pentamer–decamer equilibrium in the dark state and showed that only the decamer form of TePixD undergoes the conformational change [11]. However, the reason why PixD proteins exist in the decamer form is unknown. We discovered a peculiar dependence of the conformational change of the TePixD decamer on the intensity of the excitation light, which could be related to the importance of the oligomeric form. The Poisson equation was used to analyze the dependence of the conformational change of the TePixD...
decamer on the light intensity, and we concluded that multiple-excitation of the TePixD subunits suppresses the global conformational change.

2. Materials and methods

2.1. Sample preparation

TePixD was expressed with pET28a vector (Novagen) in Escherichia coli BL21 (DE3) as described elsewhere [3]. The His-tagged protein was purified by nickel affinity column chromatography (GE Healthcare, HisTrap FF column). The protein concentration was determined from the absorbance at 450 nm using the molar extinction coefficient of FAD (11 300 M⁻¹ cm⁻¹).

2.2. TG and transient absorption (TA)

TG measurements were performed with a similar setup to that reported previously [11,12]. Two excitation pulses with a wavelength of 465 nm [a dye laser (Lumomics, HyperDye 300) pumped by a XeCl excimer laser (Lambda Physik, Complex 102); duration of ~20 ns] and a continuous wave probe light of 830 nm (Crysta Laser) were arranged to satisfy the phase-matching condition. The TG signal was detected by a photomultiplier tube (Hamamatsu, R1477), and averaged by a digital oscilloscope (Tektronix, TDS-7104). The repetition rate of the photoexcitation was 1012 Hz (tsu, R1477), and averaged by a digital oscilloscope (Tektronix, TDS-7104).

The TG signal was detected by a photomultiplier tube (Hamamatsu, R928), and accumulated by the digital oscilloscope. The diameter of the illuminated area was estimated to be 0.8 mm from the intensity of light transmitted through a pinhole with a diameter of 20 μm, which was positioned vertically to the beam axis in 20 μm steps around the excitation position using a micrometer.

3. Results

Fig. 1 shows a typical TG signals after the photoexcitation of TePixD in buffer solution at q² = 4.5 × 10¹⁷ m⁻² and 170 μM at different laser intensity. The red-shifted state is formed within our experiment. The temporal change in the intensity of the probe beam after photoexcitation was detected by a photomultiplier tube (Hamamatsu, R928), and accumulated by the digital oscilloscope.

The diameter of the illuminated area was estimated to be 0.8 mm from the intensity of light transmitted through a pinhole with a diameter of 20 μm, which was positioned vertically to the beam axis in 20 μm steps around the excitation position using a micrometer.

Fig. 1. The typical TG signals after the photoexcitation of TePixD in buffer solution at q² = 4.5 × 10¹⁷ m⁻² and 170 μM. The signals measured at various laser intensities 0.80 (purple), 5.6 (aqua), and 29 mJ cm⁻² (red) are shown.
The dependence of the TG signal representing the D-change on laser power. The laser powers measured were (A) 0.80 (purple), 1.7 (blue), 3.4 (light blue), and 5.6 mJ cm$^{-2}$ (aqua) from top to bottom. The best fitting curves obtained using Eq. (2) are shown as black dotted lines; these completely reproduce the observed signals. The signal at 5.6 mJ cm$^{-2}$, where the signal intensity was saturated, is shown in both panels. (C) Signals in (A) and (B) normalized by peak intensity. Temporal profiles at any laser power were almost identical.

Fig. 4. The dependence of the reaction that induces a spectral red shift on laser power. The change in TA was observed at 488 nm at laser powers of 2.4, 5.2, 10, 14, 22, and 32 mJ cm$^{-2}$, and fitted by a single exponential function with a dark recovery rate constant of $k$. The amplitude of the TA signal ($\Delta A(0)$; red filled circles) was plotted against the laser intensity. The blue solid curve represents the fitting obtained using Eq. (4) with the same $b$, for the laser power dependence of $\delta n_{\text{m}}$ (Fig. 2B).

The rise and decay components of the TG signal shown in Fig. 3 represent the diffusion rates of the reactant and product, respectively. As the excitation laser power is increased, the intensity of the diffusion peak initially increased, reflecting an increase in the number of reactive molecules (Fig. 3A). However, surprisingly the signal intensity decreased beyond a certain laser power (5.6 mJ cm$^{-2}$ under these experimental conditions) (Fig. 3B). This decrease was not due to any possible photodamage effect, because the signal intensity returned to the original one by decreasing the light intensity, i.e., this light intensity effect was reproducible for the same sample many times. This intensity dependence cannot be explained neither by the saturation effect of excitation that is shown in Fig. 2B and Fig. 4.

There are several factors that determine the intensity of the diffusion signal; i.e., the difference in $D$ of the reactant and product, the rate of conformational change, and the number of protein molecules that exhibit a conformation change (DSCC). Considering these factors, three possible reasons could explain this strange phenomenon; a light effect causing a decrease in the difference of $D$ for unknown reasons, a decrease in the rate of the change in $D$ with increasing the light intensity, or a decrease in the quantum yield of the conformational change reflecting the change in $D$. For examining these possibilities, the signals were normalized to the peak intensity. We found that there was no dependence on laser power in the temporal profile, as shown in Fig. 3C. Because the profile is sensitive to the values of $D$ or the kinetics of the change in $D$ ((4 ms)$^{-1}$), this result indicates that both $D$ and the reaction rate are independent of the laser power. Hence, the dependence of the signal on light intensity should be caused by a change in the quantum yield of the TePtxD decamers undergoing a conformational change; i.e., the number of species showing DSCC decreases with increasing light intensity when the light intensity is strong.

To analyze these signals quantitatively, the signal was analyzed based on the following reaction scheme [11]

$$R \xrightarrow{hv} I \xrightarrow{k} P$$

where $R$, $I$, $P$, and $k$ represent the reactive decamer, the volume-expanded intermediate, the final product, and the rate constant of the DSCC, respectively. The time profile of the TG signal for this scheme can be expressed as

$$I_{\text{TG}}(t) = \frac{\delta n (\Delta n_r) \exp(-Dq^2t) + \delta n_i \exp(-Dq^2 + k)t}{(\Delta n_r - \Delta n_i)q^2 + k \exp(-Dq^2 + k)t - \exp(-Dq^2t)}$$

where $\delta n$ and $D$ are the initial change in refractive index and $D$ of each species indicated by subscript letters, respectively. We analyzed the signals by fixing the values of $D_r$, $D_i$, $D_p$, and $k$ as those determined in our previous paper (D$_r$ = 4.9 × 10$^{-11}$ m$^2$ s$^{-1}$, D$_i$ = 4.4 × 10$^{-11}$ m$^2$ s$^{-1}$, D$_p$ = 3.2 × 10$^{-11}$ m$^2$ s$^{-1}$, and $k$ = 250 s$^{-1}$) [11], and thus the change in refractive index of the reactive species was an only adjustable parameter. The fittings obtained using this
equation were almost perfect. In particular, $\delta n_0$, which is a value proportional to the number of reactive decamers responsible for DSCC, is plotted in Fig. 5. The obtained $\delta n_0$ showed an increase-decrease trend as the excitation intensity was increased, as observed in the intensity of the DSCC signal. Because we have demonstrated that only the decamer of TePixD undergoes the DSCC, the decrease of $\delta n_0$ at stronger excitation intensities implies some kind of suppression of the conformational change in the TePixD decamer.

4. Discussion

To obtain deeper insight into this peculiar phenomenon, we roughly estimated how many photoexcited molecules are necessary for suppression of the DSCC as follows. First, the number of photons per unit volume was calculated from the laser power and excitation volume, e.g., the laser power at 5.6 mJ cm$^{-2}$ (at the peak of the TG signal intensity (Fig. 3)) corresponds to a photon density of ca. 110 $\mu$M. By using the absorbance at the excitation wavelength (0.56) and the quantum yield of the red-shifted species formation (0.29) [13], this photon density can excite molecules to form the red-shifted species with a concentration of 18 $\mu$M. It is interesting to note that this concentration is approximately one-tenth of the protein concentration (170 $\mu$M). Considering this number, an intriguing hypothesis presents itself; a decamer that contains one red-shifted species can undergo a conformational change, but that containing more than one excited monomer cannot.

To quantitatively examine this hypothesis, we calculated the number of TePixD decamers containing one red-shifted subunit at various excitation intensities using a Poisson distribution. When $x$ subunits in the decamer are excited on average, the probability ($P_x$) of the excited decamer containing $x$ excited subunits may be described by a Poisson distribution [14]

$$P_x = \frac{\exp(-\lambda) \cdot \lambda^x}{x!}. \quad (3)$$

At low photon density (smaller $\lambda$), $P_x$ increases as the photon number increases. However, $P_x$ begins to decrease gradually at higher photon density (larger $\lambda$), because the contribution of two excited monomers in the decamer can no longer be neglected.

Eq. (3) was used to analyze the dependence of $\delta n_0$ on laser power, under the assumption that only decamers containing one red-shifted subunit exhibit the DSCC. First, we determined the dependence of the number of photoexcited molecules ($\lambda$) on the laser power from those of $\delta n_{\text{red}}$ and AA(0), which should be proportional to the number of red-shifted molecules. These dependences on laser power were analyzed in terms of absorption saturation. The saturation effect of light absorption is usually written as [15]

$$\alpha_a = \frac{\alpha_0}{1 + l/l_s}. \quad (4)$$

where $\alpha_0$ is the measured non-linear absorption coefficient, $\alpha_0$ is the absorption coefficient at low light intensity, $l$ is the laser intensity, and $l_s$ is the saturation intensity. As shown in Figs. 2B and 4, the numbers involved in the primary spectral red shift (AA(0)) and that in the subsequent volume expansion ($\delta n_{\text{red}}$) processes were well reproduced by Eq. (4) with the same $l_s$.

Next, we calculated the number of decamers containing one red-shifted species from the probability of the Poisson distribution at $x = 1$ ($P_1$) using a relation of $\lambda = c \cdot \alpha_a (c$: constant). Surprisingly, the dependence of $P_1$ on light intensity reproduced the observed light intensity dependence of $\delta n_0$ very well (Fig. 5). This close agreement strongly supports the above hypothesis; only decamers containing one red-shifted monomer are responsible for the DSCC, and the excitation of multiple subunits in the decamer suppresses the conformational change.

As yet, we do not have a clear molecular mechanism to explain the above observation. However, to our knowledge, such interesting effects of light intensity on the photoreaction dynamics have not been observed for other photoreceptor proteins. We believe that this dependence has some biological relevance, because it has been shown that DSCC is important in many photoreceptors [16–19]. In this experiment, we used pulsed laser light. It may be interesting to consider if the excitation of multiple subunits is possible in nature. According to available data, the average solar irradiance at the surface of the earth in the visible light region is around 1000 mW m$^{-2}$ nm$^{-1}$ [20]. If we assume that the width of the absorption band of flavin is 100 nm (centered at 400 nm) and the lifetime of the activated state is 10 s, the light density from the sun during this lifetime is approximately 100 mJ cm$^{-2}$, which is significantly stronger than that used in our TG experiments. Therefore, it is plausible that excitation of multiple subunits in the TePixD decamer suppresses the conformational change in natural environments.

Because blue light is generally harmful for living cells, the fact that photoactivation of multiple subunits in the decamer of TePixD suppresses the conformational change may have an important meaning for the physiological function of TePixD as a photoreceptor. In conclusion, we propose that the decamer form of TePixD plays a role not only in sensing the light, but also in sensing the intensity of light.

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