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Review

# Photo-induced inter-protein interaction changes in the time domain; a blue light sensor protein PixD

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**ABSTRACT**: For understanding molecular mechanisms of photochemical reactions, in particular reactions of proteins with biological functions, it is important to elucidate both the initial reactions from the photoexcited states and the series of subsequent chemical reactions, e.g., conformation, intermolecular interactions (hydrogen bonding, hydrophobic interactions), and inter-protein interactions (oligomer formation, dissociation reactions). Although time-resolved detection of such dynamics is essential, these dynamics have been very difficult to track by traditional spectroscopic techniques. Here, relatively new approaches for probing the dynamics of protein photochemical reactions using time-resolved transient grating (TG) are reviewed. By using this method, a variety of spectrally silent dynamics can be detected and such data provide a valuable description about the reaction scheme. Herein, a blue light sensor protein TePixD is the exemplar. The initial photochemistry for TePixD occurs around the chromophore and is detected readily by light absorption, but subsequent reactions are spectrally silent. The TG experiments revealed conformational changes and changes in inter-protein interactions, which are essential for TePixD function. The TG experiments also showed the importance of fluctuations of the intermediates as the driving force of the reaction. This technique is complementary to optical absorption detection methods. The TG signal contains a variety of unique information, which is difficult to obtain by other methods. The advantages and methods for signal analyses are described in detail in this review.

## 1. Introduction

The identification, characterization of intermediate species, and elucidation of the associated kinetics are fundamental and essential steps for research efforts aimed at understanding photochemical reactions in detail. Spectroscopy is a useful tool for this purpose and has been successfully applied to many photochemical reactions. In particular, light absorption or emission detection approaches have proven to be very sensitive and powerful techniques to detect intermediate species with high time resolution.[1] The monitoring of an absorption change after photoexcitation of molecules (known as flash photolysis or the transient absorption method) has been frequently used for studying photochemical reactions.

These techniques (sometimes combined with a stopped flow system) are very powerful to study reactions of biological molecules, including proteins.[2,3] One may detect the reaction intermediate after photoexcitation of the chromophore by monitoring an absorption change and the intermediate is characterized by the characteristic absorption spectrum. However, compared with photochemical reactions of relatively small molecules, one may encounter several difficulties when studying protein reaction dynamics. For example, characteristics of a protein reaction are such that the reaction of the chromophore is not final, rather, the chromophore reaction is usually just an initial triggering step of the subsequent reaction of the protein part. The reaction initiates a conformational change or a change in intermolecular interactions, and these changes are associated with biological function. Compared with tracing the reaction of the chromophore, the detection of such subsequent reactions in the protein is generally much more difficult to measure, because the absorption spectrum or emission are sensitive only to changes in the vicinity of the chromophore and are not perturbed by conformational or intermolecular interaction changes. Such dynamic events are called spectrally silent dynamics. The detection of such dynamics in the time domain has been very difficult. Recently, using Fourier transform infrared spectroscopy, Raman spectroscopy, and circular dichroism spectroscopy, it might be possible to trace the reaction dynamics in the time domain for a protein Although these techniques provide useful information on relatively local conformational changes, global reactions such as changes in intermolecular interactions cannot be detected. Furthermore, although infrared (IR) light absorption may detect conformation changes without detecting the absorption of the chromophore, application to protein dynamics remains limited because of the strong IR absorption by water. In this article, we refer to "the spectrally silent dynamics" as dynamics without spectral change in the ultraviolet/visible region.

An important, spectrally silent dynamic event involves inter-protein interactions that lead to oligomer formation or dissociation. Some monomers are bound by attractive interactions to form the oligomer. Oligomeric states and changes in these states are often key steps that facilitate biological reactions. Hence, it is essential to detect changes in the number of monomer units of an oligomer during a reaction. However, as stated above, such changes in the oligomeric state are often spectrally silent. Hence, it has been very difficult to trace the dynamics of oligomer formation or dissociation. For overcoming this difficulty, we have developed techniques for tracing such spectrally silent dynamics based on the pulse laser-induced transient grating (TG) method.[4-22] In this review, the power of the TG technique for studying the reaction of TePixD, which is a blue light sensor protein, will be demonstrated.[23-27] As described later, the UV/Vis absorption spectrum does not change after sub-nanoseconds of the photoexcitation, implying that spectrally silent dynamics are important for the biological function of TePixD, and this is a good example showing how the reaction dynamics of the oligomeric state of TePixD can be traced.

## 2. Principles of the TG technique

The principles of the TG method are briefly described.[28-31] For creating a grating, two coherent pulsed laser beams are crossed in a sample solution at an angle, which creates an interference (grating) pattern with a fringe length $\Lambda$ (Fig. 1).

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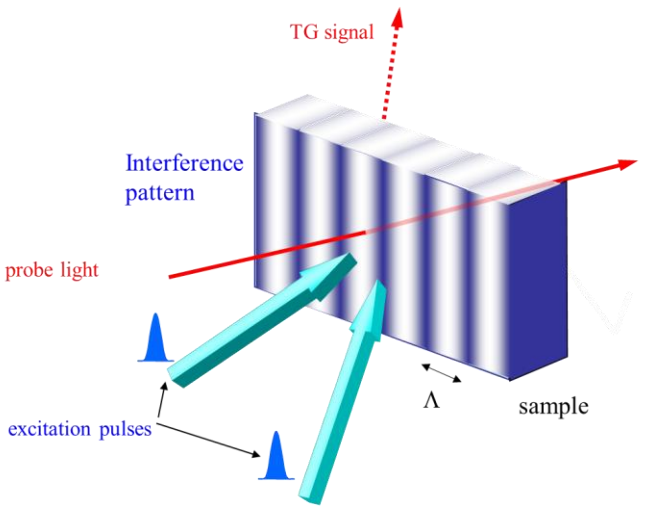


**Figure 1**. Illustration of the principle of the TG method.

The wavenumber $q$ of the grating is given by $2\pi/\Lambda$. A chemical reaction is initiated by this light and concentration modulations of the chemical species are created in the sample solution. When a probe beam is introduced to the photoexcited region at a Bragg angle, a part of the light is diffracted as the TG signal. When the absorption change at the probe wavelength is negligible, a sample thickness is longer than the fringe length, and the diffraction efficiency is small, the TG signal intensity ($I_{TG}$) is proportional to the square of the refractive index difference ($\delta n$) between the peak-null of the grating pattern. There are several origins of the grating.[31] One is the temperature change of the medium (thermal grating; $\delta n_{th}$), which is induced by the thermal energy released from the excited states and by the enthalpy change of the reaction. More importantly in this review, changes in the absorption spectrum (population grating), and in molecular volume (volume grating) also contribute to the signal. The sum of the population grating and volume grating terms is called the species grating. The species grating signal intensity is given as the difference between $\delta n$ due to the reactant ($\delta n_R$) and product ($\delta n_P$). Hence, the observed TG signal ($I_{TG}(t)$) is expressed as:

$$I_{TG}(t) = \alpha\{\delta n_{th}(t) + \delta n_P(t) - \delta n_R(t)\}^2, \quad (1)$$

where $\alpha$ is a constant representing the sensitivity of the experimental system.

The decay of the thermal grating signal is determined by the thermal diffusion; i.e., it decays with a rate constant of $D_{th}q^2$ ($D_{th}$: the thermal diffusivity). The time profile of the species-grating component is determined by the kinetics of the chemical reactions as well as the diffusion processes. When the reaction kinetics is negligible and the molecular diffusion coefficient ($D$) is time independent in the observation time range, the temporal profile of the species grating signal can be calculated by a simple molecular diffusion equation. Because the $q$-Fourier component of the concentration modulation decays with a rate constant $Dq^2$ for both the reactant and the product, the time development of the species grating signal can be expressed by,[4-17]

$$I_{TG}(t) = \alpha\{\delta n_P \exp(-D_P q^2 t) - \delta n_R \exp(-D_R q^2 t)\}^2, \quad (2)$$

where $D_R$ and $D_P$ are the diffusion coefficients of the reactant and the product, respectively. Furthermore, $\delta n_R$ (> 0) and $\delta n_P$ (> 0) are, respectively, the initial refractive index changes due to changes in the reactant and the product concentrations.

When $D$ does not change by the reaction, the first and the second terms of Eq. (2) are cancelled and give rise to a single exponential decay. If $D_R$ and $D_P$ are different, the both terms give rise to a characteristic rise-decay signal. The $D$ depends on various parameters of the solution (e.g., viscosity and temperature) and also the diffusing molecule.[32-34] When the size of a molecule changes because of an association or dissociation reaction, then clearly the value of $D$ changes. Generally, $D$ decreases with increasing molecular size. Besides the molecular size, $D$ also depends on the conformation of proteins. It was reported that $D$ of a protein in the native form is larger than that in the unfolded state.[18-20] Moreover, the TG signal associated with a chemical reaction can be detected as long as $D$ is different ($D_R \neq D_P$), even if there is no refractive index change by the reaction. Because $D$ depends on the conformation and the association/dissociation of the molecules, the time evolution of $D$ represents the dynamics of the changes in the interaction and conformation states. Hence, this TG technique is very suitable for detecting changes in the oligomeric state, which cannot be detected by UV/Vis spectroscopy (spectrally silent dynamics).

An advantage of the TG method is the high time resolution. This feature enables us to monitor the time dependence of $D$. The time resolution is determined by the magnitude of $q^2$ and $D$. For example, let us consider the following reaction scheme,

$$R \xrightarrow{h\nu} I \xrightarrow{k} P, \quad \text{(Scheme 1)}$$

where R, I, P, and $k$ represent the reactant, the intermediate species, the final product, and the rate constant of the change, respectively. In this case, $\delta n_R(t)$ and $\delta n_P(t)$ are given by:[21,22]

$$\delta n_R(t) = \delta n_R \exp(-D_R q^2 t)$$

$$\delta n_P(t) = [\delta n_I - \{k\delta n_P/((D_I - D_P)q^2 + k)\}]\exp(-(D_I q^2 + k)t) + \{k\delta n_P/((D_I - D_P)q^2 + k)\}\exp(-D_P q^2 t) \quad (3)$$

where $\delta n_I$ and $D_I$ are the initial refractive index change and $D$ of the intermediate species, respectively. By fitting the observed TG signal by this equation, one can determine various parameters in this equation.

### 3. TePixD reaction induces a change in diffusion

PixD is a relatively short protein (17 kDa), which consists of the BLUF (blue light sensors using a flavin chromophore) domain and additional short helices.[35-37] PixD possesses FAD as the chromophore. PixD from the thermophilic cyanobacterium *Thermosynechococcus elongatus BP-1* (Tll0078) is called TePixD. The homologous PixD protein from a mesophilic cyanobacterium *Synechocystis* (SyPixD, Slr1694) was reported to be involved in phototactic movement.[36] A crystal structure of TePixD has been reported.[37] The structure revealed an interesting and characteristic feature of this protein; the structure is composed of two pentamer rings that form a decamer (Fig. 2). The decameric structure seems to reflect the native form, as size-exclusion chromatography (SEC) showed that TePixD maintains oligomeric structures even in solution.[35-37] Because no signal output domain was identified in the amino acid sequences, signal transduction was proposed to be mediated by direct protein–protein interactions of monomeric units. Hence, the oligomeric form should play an important role in the signal transduction of PixD proteins.

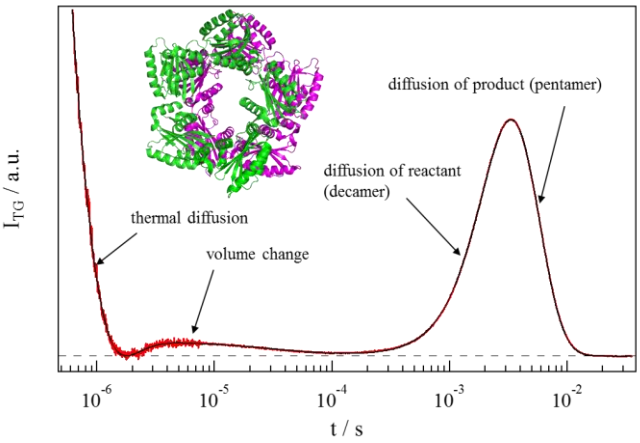


**Figure 2**. A typical TG signal (red line) after photoexcitation of TePixD at a concentration of 300 μM and $q^2 = 1.0 \times 10^{13}$ m$^{-2}$. The best fitted curve based on the time-dependent $D$ model (Eq. 3) is shown by the black line. (Inlet) Structure of TePixD. The green part indicates one pentamer ring and the purple represents another ring. Each ring is composed of five monomers.

The photochemical reaction of TePixD was initially studied by absorption spectroscopy and the spectral data revealed that this protein exhibits the typical photochemistry of a BLUF protein.[38,39] Upon blue light irradiation, the ultraviolet-visible absorption band of FAD is red-shifted by ~10 nm.[38] The kinetics of the red-shift was determined by the flash photolysis method to be sub-nanoseconds. The red-shifted intermediate is generated with a quantum yield of 0.29.[40-42] The spectrum does not change after this initial reaction, and it returns back to the dark state with a time constant of 12 s at room temperature.[40-42]

The spectrally silent reaction after the formation of the red-shifted species was investigated by the TG method.[23] After photoexcitation of TePixD, the TG signal was observed over a wide time range. A typical signal in a buffer at a concentration of 300 μM and at a grating wavenumber $q^2 = 1.0 \times 10^{13}$ m$^{-2}$ is shown in Fig. 2. The observed temporal profile was analyzed and assigned as follows. Because the initial decay rate agreed well with that of the signal from a calorimetric reference sample, which converts all the absorbed photon energy to the thermal energy, this component was attributable to the thermal grating caused by the thermal energy released from the excited molecule and the enthalpy change of the reaction. The other phases should be due to the species grating components. The second weak decay component (around 10 μs region in Fig. 2) was expressed well by a single exponential function with a time constant of 40 μs. The rate constant was independent of the $q^2$ value, so the kinetics should represent intrinsic reaction kinetics of TePixD. Because the transient absorption change has not been detected on this time scale, there is no population grating component, and hence, this component should be attributed to the volume grating. The dip between the thermal grating and the species grating components (around 2 μs in Fig. 2) indicates that the sign of the refractive index change due to the volume change is positive. Therefore, this 40-μs process represents the density decrease because of the increase of the partial molar volume of TePixD.

The rate constant of the rise-decay curve on the millisecond time scale was dependent on $q^2$, indicating that this peak represents the molecular diffusion process. If $D$ did not change during the reaction, the species grating signal would decay single-exponentially ($D_P = D_R$ in Eq. (2)). The rise-decay profile certainly indicates that $D$ is changed by the photoreaction of TePixD. From the signal profile, it was found that the signs of the refractive index changes of the rise and the decay parts were negative and positive, respectively. Thus, the rise and the decay parts, respectively, can be assigned to the diffusion of the reactant and a product; ($D_R > D_P$). The assignments of these components are shown in Fig. 2.

## 4. Kinetics

The change in $D$ suggests significant conformational changes or a change in the oligomeric state. The kinetics of this change were measured from the diffusion signals at various $q^2$.[23] Figure 3 depicts the $q^2$-dependence of the diffusion signal, indicating that the peak intensity was weak on a fast time scale and increased with increasing the observation time by decreasing $q^2$.

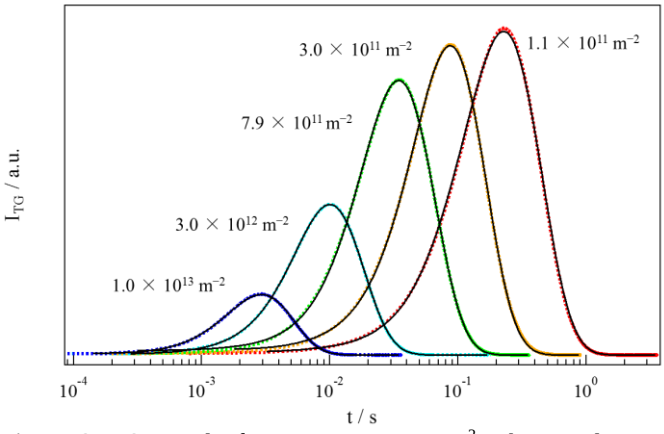


**Figure 3**. TG signal of TePixD at various $q^2$. The signals were normalized by the thermal grating intensity measured under the same condition. The $q^2$ values are shown in the figure. The black lines are the best fitted curve based on the time-dependent $D$ model (Eq. 3).

This dependence was explained qualitatively as follows. When $D_P$ is similar to $D_R$, the intensity of the peak should be weak because of the cancellation of the two terms in Eq. (2).

As the difference between $D_R$ and $D_P$ increases, the diffusion peak intensity becomes stronger. Thus, the $q^2$-dependence of the peak intensity indicates that the difference in $D$ increased with time. The TG signal due to the time-dependent $D$ was analyzed on the basis of Eq. (3). The observed signals at different $q^2$ were consistently reproduced with parameters of $D_R = 4.6 \times 10^{-11}$ m$^2$ s$^{-1}$, $D_I = 4.3 \times 10^{-11}$ m$^2$ s$^{-1}$, $D_P = 3.0 \times 10^{-11}$ m$^2$ s$^{-1}$, and $k^{-1} = 4$ ms.[23]

## 5. Reaction depends on the oligomeric state

The determined $D$ of the reactant ($D_R$) of $4.6 \times 10^{-11}$ m$^2$ s$^{-1}$ is much smaller than that expected for water-soluble globular proteins with a molecular size similar to that of TePixD, but is typical for proteins with a size of 160 kDa.[43,44] This observation suggests that TePixD exists as a decamer in the dark state. The reactivity of the oligomeric state was investigated by the concentration dependence of the signal. Interestingly, from curve fitting of the signals after normalization by the thermal grating signal, which means that the signal was normalized by the number of photoexcited molecules, it was found that the amplitude of $\delta n_R$ significantly increased with increasing concentration (Fig. 4).[23]

Because the refractive index change of the reactant ($\delta n_R$) corresponds to the number of reactive species, this concentration dependence indicates that the fraction of the reactant drastically increases with increasing concentration. On the contrary, the transient absorption signal intensity representing the concentration of the initial red-shift species was independent of the concentration after the correction of

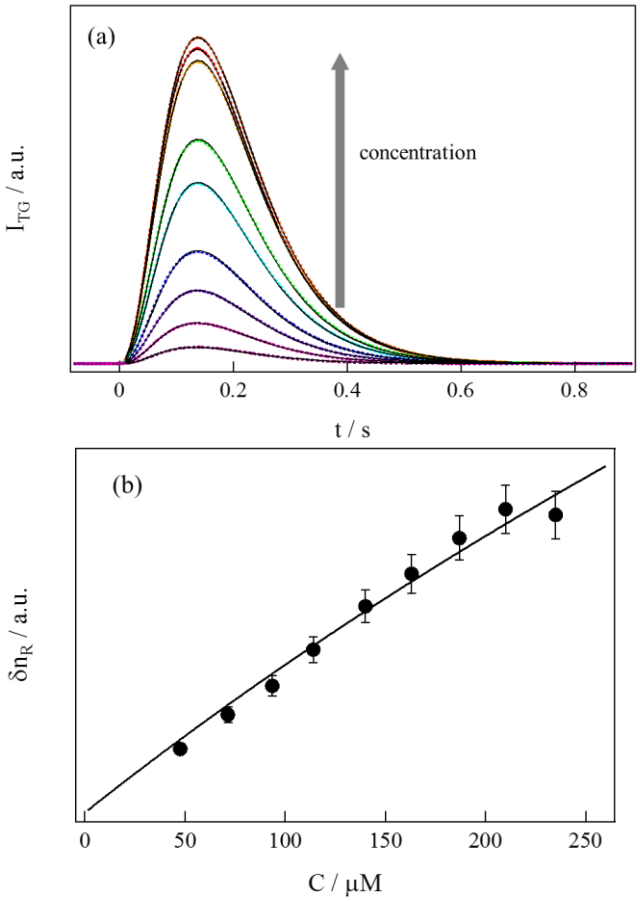


**Figure 4**. (a) The diffusion signal of TePixD measured at various concentrations at $q^2 = 1.0 \times 10^{13}$ m$^{-2}$. The signal intensity increased as the concentration increased, although these signals were normalized by the thermal grating intensity (number of the photoexcited molecules). The concentrations were 300, 270, 240, 210, 180, 150, and 120 μM. (b) Amplitude of the reactant (closed circles) determined from the TG signals of (a). The best fitted curve based on the equilibrium model between the pentamer and the decamer is shown by the solid line.

the absorbance at the excitation wavelength. Hence, the creation efficiency of the red-shifted species does not depend on the concentration. Furthermore, the amplitude of the second volume change dynamics (40-μs phase), representing the initial conformational change, did not depend on the concentration after correction by the thermal grating signal intensity. Thus, the decrease of the diffusion signal intensity is explained by an increase in the formation efficiency of the final species; with $D_P$ increasing as a function of protein concentration, but the formation efficiency of the first red-shifted species does not depend on the concentration. This observation cannot be explained without considering the existence of two species (reactive and unreactive species), which cannot be distinguished from the absorption spectrum, and the fraction of the reactive species increased as the protein concentration increased.

The fact that the fraction of the reactive species increased with increasing concentration suggests the involvement of oligomeric states to the reaction efficiency. From this observation, it was suggested an association equilibrium between the pentamer and the decamer forms in the dark state and that the decamer is only responsible for the *D*-change. This suggestion was examined by analysis of the observed concentration dependence of $\delta n_R$ (Fig. 4 (b)), based on an equilibrium model of the pentamer and decamer (Scheme 2),

$$2Pen \rightleftharpoons Dec, \qquad \text{(Scheme 2)}$$

where *Pen* and *Dec* represent the pentamer and decamer of TePixD, respectively. The equilibrium constant *K* is given by

$$K = \frac{[Dec]}{[Pen]^2}$$

The concentration dependence of $\delta n_R$ was fitted by $[Dec]/C$, where *C* is the total concentration, representing the correction of the absorbance. The concentration dependence of $\delta n_R$ was explained by this equilibrium model with *K* of 27 M$^{-1}$.

The equilibrium of the pentamer and the decamer of TePixD was also supported by the molecular size detection from the SEC measurements.[23] The elution profile showed essentially a single peak and the peak position was dependent on the concentration. When the concentration was increased from 10 to 230 μM (an initial concentration), the peak shifted to an elution volume that indicated a higher molecular mass species. By plotting the peak positions to the calibration curve, an apparent molecular mass at 230 μM was determined to be 160 kDa. The molecular mass of 160 kDa is in accordance with the molecular mass of the decamer. When the initial concentration was 10 μM, the elution volume of the peak shifted to represent a lower molecular weight species and the apparent molecular mass from the calibration curve was 110 kDa. The observed peak shift was interpreted as a shift in the equilibrium from the decamer to that of the pentamer, and was dependent on protein concentration.

It should be noted that the elution profile consisted of a single peak. If the pentamer and decamer existed as stable isomers without equilibrium in the elution time, one should observe two peaks corresponding to the pentamer and the decamer. The single peak that was dependent on concentration indicated that the pentamer and decamer are under equilibrium at least within the elution period (~15 min). On the basis of the SEC and TG measurements, it was concluded that the decamer and the pentamer are in equilibrium in the dark state and only the decamer undergoes the main reaction to change *D*.

Because the reaction is sensitive to the oligomer state, it was expected that the reaction is also sensitive to crowded conditions such as those found *in vivo*. This expectation was examined using a crowding molecule, Ficoll-70.[25] Although the crowding agent did not affect the quantum yield of the spectral red shift reaction, the recovery rate of the product, rate constant of the volume change reaction, and the magnitude of the volume change, the magnitude of the TG signal representing the diffusion change significantly increased on addition of Ficoll-70. This observation indicates that the decamer-pentamer equilibrium shifts to the decamer under the crowded condition used and the reaction becomes more efficient.

### 6. Reaction scheme of TePixD

The reaction product of TePixD was identified from the SEC method and the *D*-value.[26] The elution profiles of the SEC for the 1.1 mM TePixD solution were measured under illuminated conditions and it was found that the peak position shifted toward the smaller size, which was almost the same as that observed in the dilute (dark) condition. However, the elution profile of a diluted solution (40 μM), in which the pentamer is dominant, did not change by light illumination. These observations indicated that the photoreaction is the conversion from the decamer to the pentamer; i.e., the TePixD decamer undergoes light-induced dissociation.

The dissociation reaction was further confirmed by the diffusion measurement using dynamic light scattering (DLS).[26] At a very dilute concentration, the equilibrium between pentamers and decamers shifts almost completely towards the pentamer state. From the rate constant of the DLS profile at this concentration, $D = 3.7\times10^{-11}$ m$^2$ s$^{-1}$ was determined. This $D$ value should be for the pentamer ($D_{pen}$). This value of $D_{pen}$ is very close to the $D$ of the photoreaction product ($3.8 \times 10^{-11}$ m$^2$ s$^{-1}$), which was obtained after viscosity correction. The result that the $D$ value of the pentamer measured in the dark is almost the same as that of the photoproduct of the decamer confirms that the photoproduct of the decamer is the pentamer. Furthermore, this observation is very surprising; i.e., despite its smaller size relative to that of the decamer, the pentamer has a smaller $D$.

On the basis of above experimental measurements, the main reactions of TePixD are the creation of the red-shifted species (reaction around the chromophore), conformational change of the monomer unit with a time constant of 40 μs, and the dissociation reaction of the decamer to the pentamer. The reaction scheme of TePixD is depicted in Fig. 5.

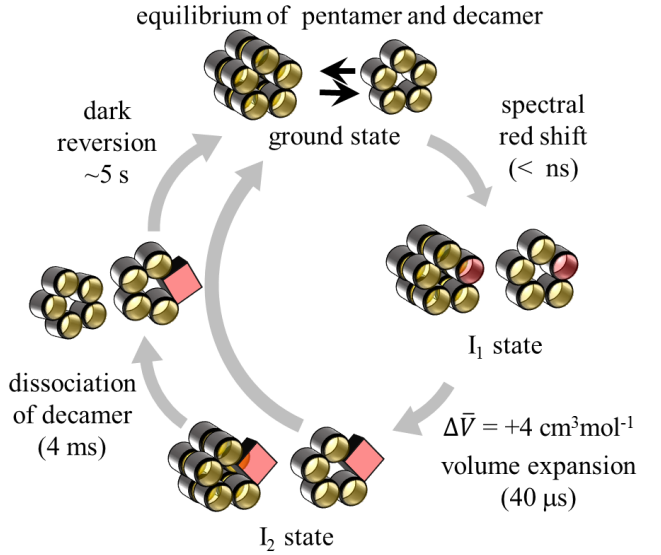


**Figure 5**. The reaction scheme of TePixD determined from the kinetics of $D$ change, concentration dependence, and laser power dependence. The intermediates are termed the $I_1$ and $I_2$ states.

**7. Intense light suppresses the dissociation**

A very surprising observation was reported on the dissociation reaction of TePixD; the dissociation reaction is suppressed by strong light illumination.[24] In general, stronger excitation light intensity leads to an increase in the number of molecules in the excited state and the reaction product. Indeed, the transient absorption signal at 488 nm (amount of the red-shifted species) increased with increasing light intensity. When the excitation light intensity increased more, the power dependence became weaker, which is a result of the saturation effect. Similarly, the signal intensity representing the volume expansion process increased monotonically and the power dependence became weaker at stronger excitation intensities (Fig. 6(a)); similar to that of the red-shifted species. The dependence of the volume change process on laser power can be understood as an effect of saturation of photoexcitation. This result implies that the conformational change represented by the volume change is localized in the monomer subunits of the oligomeric form.

However, as the excitation laser power is increased, the intensity of the diffusion peak increased only in a weak intensity region (Fig. 6(b)). The increase of the signal in a weak laser power region clearly reflects an increase in the number of reactive molecules. Surprisingly, however, the signal intensity decreased beyond a certain laser power (Fig. 6(c)). This intensity dependence cannot be explained by a simple saturation effect.

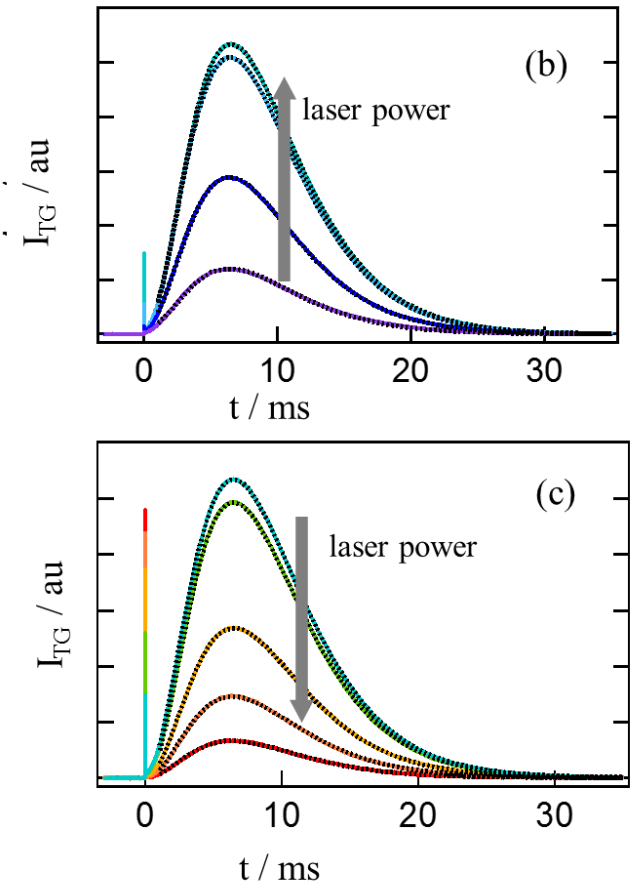


**Figure 6**. (a) The dependence of the volume change phase on laser power. The laser powers measured were 0.80, 1.7, 3.4, 5.6, 8.8, 15, 21, and 29 mJ cm$^{-2}$ from bottom to top. The decay and rise part observed in 0–10 μs is the thermal grating component. The best fitting curves by a single exponential function with a constant are shown as black dotted lines. (b) and (c): The dependence of the TG signal representing the $D$-change on laser power. The laser powers measured were (b) 0.80, 1.7, 3.4, and 5.6 mJ cm$^{-2}$ from bottom to top, and (c) 5.6, 8.8, 15, 21, and 29 mJ cm$^{-2}$ from top to bottom.

The dependence of the diffusion signal on light intensity is explained in terms of a change in the quantum yield of the TePixD decamers. Because only the decamer of TePixD undergoes the dissociation reaction, the decrease of the signal intensity at stronger excitation intensities implies some type of suppression of the conformational change in the TePixD decamer. The number of photoexcited monomer units, which are necessary for suppression, was determined from the number of photons per unit volume. It was found that this photon number was approximately one-tenth of the protein concentration. This estimation suggested that one monomer unit excitation is necessary for the reaction.

Furthermore, the light intensity dependence of the number of the reaction product was analyzed more quantitatively as follows.[24] The number of TePixD decamers containing one red-shifted subunit at

various excitation intensities was calculated by a Poisson distribution. When $\lambda$ 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:[45]

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

At low photon density (smaller $\lambda$), $P_x$ increases as the photon number increases. However, $P_x$ begins to decrease gradually at higher photon densities (larger $\lambda$), because the contribution of two excited monomers in the decamer can no longer be neglected. Eqaution (4) was used to analyze the dependence of $\delta n_R$ on laser power. First, the number of photoexcited molecules ($\lambda$) was determined from the saturation effect of the volume change signal intensity. Next, the number of decamers containing one red-shifted species was calculated from the probability of the Poisson distribution at $x$ = 1 ($P_1$). Because the dependence of $P_1$ on light intensity reproduced the observed light intensity dependence of the signal intensity very well, the above reaction model was concluded to be correct, i.e., only a decamer containing one red-shifted monomer unit is responsible for the dissociation and the excitation of multiple subunits in the decamer suppresses the reaction.

**8. Driving force of the dissociation**

According to previous studies using circular dichroism spectra of TePixD[26] or NMR of AppA,[46,47] the conformational change was not observed upon illumination. These results imply that significant structural changes do not occur in the BLUF domain upon photoexcitation. What could be the driving force of the dissociation reaction? On this point, a molecular dynamics (MD) simulation on another light-sensing domain, the light-oxygen-voltage-sensing (LOV) domain, which also uses flavin as a chromophore, suggested that a fluctuation of the LOV core structure could be important for the reaction.[48-50] Recently, the driving force of the reaction was experimentally revealed as a fluctuation of the intermediate species of TePixD.[27]

For detecting the fluctuation, the partial molar isothermal compressibility ($\bar{K}_T = -(\partial\bar{V}/\partial P)_T$) is an essential quantity, because this quantity is directly linked to the mean-square fluctuations of the protein partial molar volume by $\langle(\bar{V} - \langle\bar{V}\rangle)^2\rangle \equiv \langle\delta\bar{V}^2\rangle = k_B T\bar{K}_T$.[51] (Here, <X> means the averaged value of a quantity of X.) To measure this compressibility change, it is necessary to measure the volume change at various pressures. Using traditional thermodynamic methods, however, a quantitative measurement of the volume change is very difficult even at one atmosphere and it has been almost impossible to detect the compressibility change in the time- domain. Nevertheless, the TG method can detect the time-dependent volume change, as shown in the above sections. If this measurement can be performed at various pressures then the detection of this fluctuation change is possible.

Recently, such a measurement was reported.[27] Initially, the reactions probed by the transient absorption signal were measured at various pressures and it was found that reactivity of the TePixD is not sensitive to pressure. Therefore, the TG signal representing the volume change directly reflects the volume change during the reaction at various pressures. Interestingly, the volume property was very sensitive to pressure.[27] Figure 7 shows the pressure dependence of the TG signal of the volume expansion process. It is clear that the TG signal of TePixD depended significantly on the pressure, in contrast to the results of ultraviolet-visible and transient absorptions. The pressure-dependent volume change implies a compressibility change during the reaction.

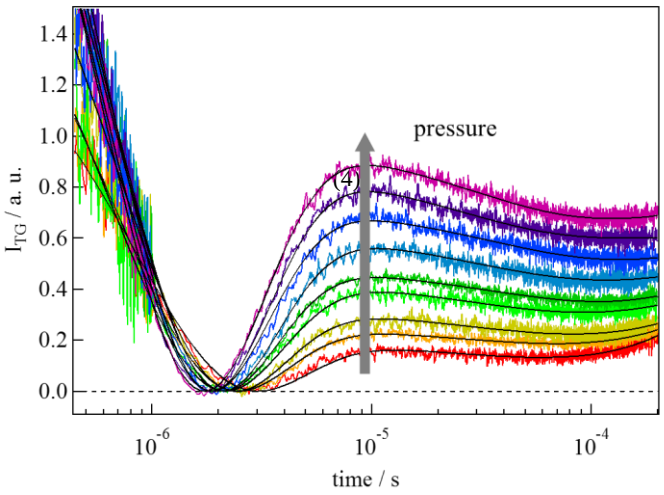


**Figure 7**. Typical TG signals of TePixD in the sub-millisecond time region, which represents the volume expansion process from the intermediate $I_1$ to $I_2$, recorded every 25 MPa from 0.1 MPa to 200 MPa (from bottom to top) with $q^2$ = 3.5×10$^{12}$ m$^{-2}$. Fitting curves using the thermal grating component, volume grating component, and diffusion signal are shown by black solid lines

From the pressure dependence of the amplitude of the volume grating of $I_1$ and $I_2$ states, the pressure dependences of the volume changes ($\Delta\bar{V}_{g\to s}$) for $I_1$ and $I_2$ states were determined. From the slope of the pressure-dependent volume change, compressibility of $I_1$ and $I_2$ were calculated to be: $\Delta\bar{K}_T$ = +(5.6 ± 0.6) × 10$^{-2}$ cm$^3$ mol$^{-1}$ MPa$^{-1}$ (for $I_1$) and $\Delta\bar{K}_T$ = +(6.6 ± 0.7) × 10$^{-2}$ cm$^3$ mol$^{-1}$ MPa$^{-1}$ (for $I_2$). Therefore, this result showed that the partial molar volume fluctuation of the short-lived intermediate states is indeed enhanced transiently compared with that of the ground state.

**9. Fluctuation of multi-monomer excited species**

In the above section, it was shown that the fluctuation is increased in the intermediate species and this result suggested that the fluctuation could be the driving force of the dissociation reaction. For further confirmation of this hypothesis, the correlation between the fluctuation and the reactivity was reported by using the light intensity dependence of the dissociation reaction.[27] As described in the above section, photo-excitation of a monomer of TePixD yields $I_1$ and $I_2$ intermediates at any laser power, but does not produce the final product when multiple monomers within one decamer were excited. Thus, the compressibility of the intermediates of reactive and non-reactive TePixD were measured from the laser power dependence of the compressibility changes. Interestingly, the pressure dependence gradually decreased with increasing laser power. This observation implies that the compressibility decreased monotonically as the excitation laser power was increased. Therefore, it is qualitatively apparent that a TePixD decamer (or pentamer) containing multiple excited monomers possesses smaller compressibilities than a decamer containing only one excited monomer.

The observed compressibility change is the sum of contributions from a decamer with different numbers of excited monomers at any laser power. By fitting the pressure dependence at different laser powers, the compressibility change of double-excited species ($\Delta\bar{K}_T^{(2)}$) was determined to be –(4.3 ± 1.5) × 10$^{-2}$ cm$^3$ mol$^{-1}$ MPa$^{-1}$

for $I_1$ and $-(6.7 \pm 2.4) \times 10^{-2}$ $10^{-2}$ $cm^3$ $mol^{-1}$ $MPa^{-1}$ for $I_2$ of Fig.5. The compressibility of both $I_1$ and $I_2$ of two excited decamers was much smaller than the compressibility of one excited species, and even smaller than the decamers ground state. In conclusion, the enhanced compressibility is important in facilitating the dissociation reaction of the TePixD decamer.

These results are schematically illustrated in Fig. 8.

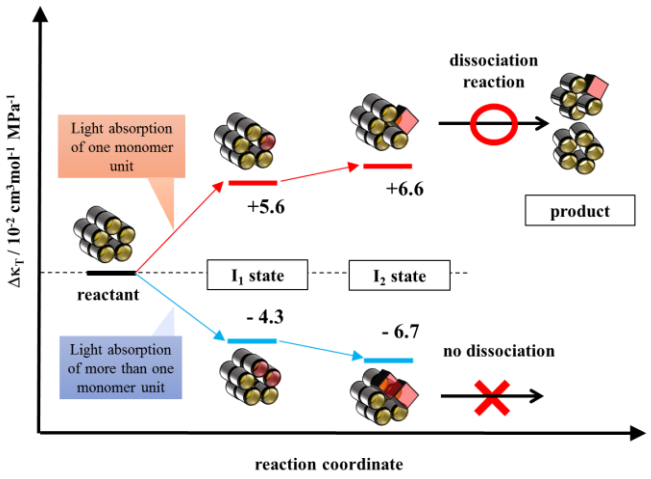


**Figure 8**. Schematic illustration of the volume fluctuation change from the ground state, depicted along the reaction coordinate of TePixD for both cases in which one monomer is excited (red lines) or multiple monomers are excited (blue lines). In the figure, volume fluctuation change is expressed per mol of TePixD monomers.

## 10. CONCLUSION

In this review, approaches to study spectrally silent dynamics in photochemical reactions were presented by examining, as an example, the photochemical reaction of TePixD. Although the absorption spectrum change completes within a nanosecond following photoexcitation, the TG studies revealed a variety of chemical reactions that lead to the final state. After changes associated with the chromophore, the conformational change measured by the TG signal occurs with a time constant of 40 μs. This reaction occurs to both the penatmer and decamer in the dark state. The dissociation reaction then takes place from the decamer to the pentamer with a time constant of 4 ms. This reaction is light intensity dependent, i.e., this reaction is induced upon photoexcitation of one monomer unit of the decamer. However, this reaction is suppressed by multi-monomer excitations. It was shown that this reaction is induced by the fluctuation of the enhanced intermediate species of the protein.

At this point, it is worthy to mention the photochemistry of another PixD protein, SyPixD. The structure of SyPixD is very similar to that of TePixD. Similar to TePixD, it forms a decamer in solution in the dark state.[52] The initial reaction characterized by the red-shifted species formation is also very similar. However, spectrally silent photochemical reactions of SyPixD are very different from that of TePixD. The TG technique revealed that the initial conformational change of SyPixD is much slower (45 ms) when compared with the conformational change of TePixD (40 μs).[53] Furthermore, the photochemical reaction after the conformational change is a dissociation from a decamer to a dimer, not the pentamer.[52,53] More significantly, the dissociation reaction is induced by the multi-monomer excitation of the decamer, which contrasts the TePixD case, the multi-monomer excitation suppresses the dissociation.[54] These different behaviors illustrate the diversity of protein reactions for biological functions of even similar proteins.

Besides PixD proteins, the present TG technique has revealed various changes in other oligomeric protein species.[9,10,13-16] For studying such reactions, information describing the spectrally silent dynamics is essential if a full understanding of the reaction is to be obtained. The TG approach is unique and no other method can extract such information. This method should be suitable for many other reaction systems and therefore reveal hidden reaction mechanisms of proteins.

**KEYWORDS**: protein reaction, diffusion, inter-protein interaction, dynamics, transient grating

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