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Tetramer Formation Kinetics in the Signaling State of AppA Monitored by Time-Resolved Diffusion

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ABSTRACT The photoreaction kinetics of the BLUF domain of AppA was studied by monitoring time-dependence of an apparent diffusion coefficient (D) using the pulsed laser-induced transient grating technique. It was found that D of the photoproduce is time-dependent. From the concentration dependence of the reaction rate, it was concluded that the BLUF domain of AppA forms a dimer upon the photoexcitation. Since AppA exists as a dimeric form in the ground state, this dimerization reaction indicates the tetramer formation in the signaling state. From the slope of the plot of observed rate constants (kobs) against the AppA concentration, the second order rate constant is determined to be ~2.5 × 10^6 M⁻¹ s⁻¹, which is ~4 orders in magnitude lower than the diffusion controlled reaction. It indicates that a relative orientation of the protein molecules during the dimerization process causes additional constraint, which slow down the reaction rate.

INTRODUCTION

Recently, a novel family of blue-light photoreceptors, the BLUF domains (for sensors of blue light using FAD (1)) emerged burgeoning interest. These BLUF domains have been identified by sequence homology in purple photosynthetic bacteria, in cyanobacteria, and in the unicellular eukaryote, Euglena gracilis. They are involved in photophobic responses in E. gracilis (2), in transcriptional regulation in Rhodobacter sphaeroides (3), and in phototaxis in Synecochysis (4). Until now, five members of this family have been spectroscopically characterized: AppA from Rb. sphaeroides (3,5,6), YcgF from Escherichia coli (7), Slr1694 from Synecochysis PCC6803 (8), TII0078 from Thermosynechococcus elongatus BP-1 (9), and the photoactivated adenylyl cyclase (PAC) from E. gracilis (2). Among them the BLUF domain of AppA from Rb. sphaeroides (3,5,6,10–16) has been extensively investigated with respect to both function and photochemistry. AppA is a light and redox-responding regulator of photosynthesis gene transcription in Rb. sphaeroides, where it can be found in two different functional forms (3). Under anaerobic low-light growth conditions, AppA is in a “dark-adapted” form, which is able to bind and inactivate the repressor PpsR, thus allowing the RNA polymerase to maximally transcribe photosynthesis genes. Under aerobic highlight conditions or under strong blue light illumination, FAD in AppA is photoexcited and AppA is transformed into a signaling state (“light-adapted” form) that is incapable of interacting with the photosynthesis repressor PpsR. Under these conditions, there is a maximal repression of the photosynthesis gene expression (3). However, despite of the efforts, the photochemistry and structural dynamics underlying the signaling state formation in the BLUF domain of AppA are still unclear.

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blue light illumination (11,12,14). A size-exclusion chromatography study of full-length AppA suggested that the signaling state is accompanied by a conformational change that increases the Stokes radius and/or the aggregation state of the protein (3). Recently, it was established that the ground state of the BLUF domain of AppA exists as a dimer even in a very dilute solution (16). However, it was not clear whether the BLUF domain of AppA aggregated or not in the signaling state. There has been no report on dynamical behavior of AppA after the photoexcitation.

The kinetics of chemical reactions may be monitored by the transient absorption or time-resolved fluorescence techniques. Indeed, the transient absorption method was applied to the photoreaction of AppA, but a detailed study showed that the absorption detected signal indicated only the decay of the excited triplet state in a microsecond time range, not the other process, which may be expected for creating the signaling state. An inherent limitation of these techniques is that the signal appears only if a reaction induces some structural changes near the chromophore. If there is a very little change of the environment around the chromophore, these two techniques cannot detect the protein dynamics.

It was recently reported that the diffusion coefficient is a useful physical property to monitor the spectrally silent dynamics of structural changes or conformational changes (17–22). For monitoring the diffusion in time-domain, it was reported that the pulsed laser induced transient grating (TG) method is a powerful and suitable technique (17–22). In this study, we have investigated conformational changes or aggregation kinetics of this protein in the signaling state by the time-resolved TG technique. We found clear evidence for the tetramer formation of the photoexcited AppA for the first time. Not only that, the kinetics of the protein association was measured and the reaction mechanism is discussed.

**EXPERIMENTAL PROCEDURES**

The experimental setup and the principle of the TG measurement were similar to that reported previously (17–26). Briefly, a laser pulse from a dye laser (Lumonics, Ontario, Canada, HyperDye 300; wavelength = 465 nm) pumped by an excimer laser (Lambda Physik, Göttingen, Germany, XeCl operation; 308 nm) was used as an excitation beam and a diode laser (780 nm) was used as a probe beam. The excitation beam was split into two by a beam splitter and crossed inside a sample cell. The sample was photoexcited by the created interference pattern to induce the refractive index modulation in the sample. A part of the probe beam was diffracted by the modulation (TG signal). The signal was isolated from the excitation laser beam with a glass filter and a pinhole, as detected by a photomultiplier tube (Hamamatsu, R1477, Hamamatsu, Japan), and recorded by a digital oscilloscope. The spacing of the fringe was measured by the decay rate constant of the thermal grating signal from a calorimetric standard sample (bromocresol purple in water), which releases all the photon energy of the excitation as the thermal energy within a time response of our system. All measurements were carried out at room temperature.

Wild-type AppA(5-125) was expressed and purified essentially as described previously (13). Heterologous protein (over)production was performed in E. coli/ M15 (pREP4), grown in production broth (PB), which contains 20 g L−1 tryptone, 10 g L−1 yeast extract, 5 g L−1 dextrose, 5 g L−1 NaCl and 8.7 g L−1 K2HPO4, pH 7.0). Ampicillin and kanamycin were used at 100 and 50 µg mL−1, respectively. Before proceeding with purification, using nickel-affinity resin, cell-free extract was incubated for 1 h on ice, with a large molar excess of FAD. Purified protein was dialyzed to 10 mM Tris-HCl, pH 8.0, and stored at −20°C. Purity of the samples was checked by SDS-PAGE, using the PHAST system (Amersham Biosciences, Piscataway, NJ) and with UV/Vis spectroscopy. The flavin composition of the purified protein was determined by thin-layer chromatography (TLC) as described in (13).

**PRINCIPLE AND THEORETICAL**

Under weak diffraction conditions, the TG signal intensity (ITG) is proportional to the square of variations in the refractive index (δn) and in the absorbance. We can neglect the absorption term by selecting a probe wavelength at which the absorption change is sufficiently small. Then, the refractive index change consists of the following three components: temperature rise due to the released thermal energy (δnth, thermal grating), the molecular refractive index difference between the reactant and products due to the change of the absorption spectrum (δnspec, population grating), and the density change caused by the reaction volume (δnv, volume grating). We call the sum of δnth, pop and δnv the species grating (δnspec), because the time profiles of δnth, pop and δnv are identical for most cases. The TG signal intensity (ITG) is given by

\[ I_{TG} = \alpha (\delta n_{th}(t) + \delta n_{pop}(t)) \]

(1)

where α is a constant representing the sensitivity of the system. If the thermal energy is released promptly, the thermal grating signal may be expressed by

\[ \delta n_{th}(t) = \delta n_{th} \exp(-q^2 D_{th} t) \]

(2)

where Dth is the thermal diffusivity and q is the grating wavenumber. We can separate the thermal contribution from the other two components by the time-resolved method (23–25). The key point of the separation is based on the fact that the thermal grating signal decays with a rate constant given by the thermal diffusivity (Dth) but the time development of the other signal is determined by the kinetics of the reaction and the molecular diffusion. Since the thermal diffusivity is usually 2 or 3 orders of magnitude larger than usual molecular diffusion coefficients in solution, the thermal component can be easily separated from the species grating signal. If we can neglect the reaction kinetics in the molecular diffusion kinetics, the time-dependence of δnspec is given by (17–22)

\[ \delta n_{spec}(t) = -\delta n_{rg} \exp(-D_{rg} q^2 t) + \delta n_{tp} \exp(-D_{tp} q^2 t) \]

(3)

where δnrg(>0) and δntp(>0) represent the refractive index changes by the reactant and product, respectively, Drg and Dtp are the molecular diffusion coefficients of the reactant and product, respectively. The reaction kinetics can be separated from the diffusion process by measuring the TG signal at different q^2, because the decay due to the diffusion process depends on q^2, whereas the reaction kinetics should not.

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When proteins are dimerized during the diffusion process, the apparent $D$ is time dependent, and hence, the observed TG signal should be different from that predicted by Eq. 3. For analyzing the observed TG signal, we may use the following model:

$$A \rightarrow A^* + A \rightarrow (A^* : A),$$  
(Scheme 1)

where $A^*$ indicates an intermediate created by the photoexcitation and the dimer is formed between this intermediate ($A^*$) and the ground state protein ($A$) with a rate constant $k$. The time dependence of the spatial modulation of these species is given by the following rate equations:

$$\frac{\partial[A]}{\partial t} = D_k \frac{\partial^2[A(x,t)]}{\partial x^2}$$  
(4)

$$\frac{\partial[A^*]}{\partial t} = D_I \frac{\partial^2[A^*(x,t)]}{\partial x^2} - k[A(x,t)][A^*(x,t)]$$  
(5)

$$\frac{\partial[A^*:A]}{\partial t} = D_p \frac{\partial^2[A^*(x,t):A]}{\partial x^2} + k[A(x,t)][A^*(x,t)],$$  
(6)

where $x$ is the coordinate along the grating wavevector, $k$ is the reaction rate constant, and $D_k$, $D_I$, and $D_p$ denote the diffusion coefficients of the reactant, intermediate, and the product, respectively. Solving these equations under a condition that the concentration of $A$ is sufficiently large so that it can be treated as a constant, we may find the time dependence of the TG signal as

$$I_{TG} = \alpha \left[ \delta n_1 \exp \left( -D_k q^2 t \right) + \left\{ \delta n_2 + \frac{\delta n_3 k[A]}{(D_p - D_k)q^2 - k[A]} \right\} \exp \left( -D_q q^2 t \right) \right],$$

(7)

where $\delta n_1$, $\delta n_3$ and $\delta n_3$ are the refractive index of the ground state protein, the intermediate, and the product, respectively.

In this study, we found that $D_I$ is nearly equal to $D_k$ from a fact described below. By replacing $D_I$ by $D_k$ and $k[A]$ by $k_{obs}$, the above equation is expressed as

$$I_{TG} = \alpha \left[ \delta n_1 \exp \left( -D_k q^2 t \right) + \left\{ \delta n_2 + \frac{\delta n_3 k_{obs}}{(D_p - D_k)q^2 - k_{obs}} \right\} \exp \left( -D_q q^2 t \right) \right],$$

(8)

This functional form is the same as that calculated based on the two-state model used for detecting conformational changes of proteins (17,18,22).

RESULTS AND DISCUSSION

Time-dependent diffusion

Fig. 1 represents the TG signal of AppA after the photoexcitation. The signal rose quickly with the time-response of our system (∼20 ns) and there appeared a slow weak rising component with a time constant of ∼3.4 μs. Measuring the TG signal at different $q^2$, we found that this time constant of the rise component did not depend on $q^2$. Hence the rising part of the TG signal represents the species grating signal, which reflects an intrinsic dynamics of the protein, not the diffusion. This species grating signal may be due to the volume change of the protein or absorption change of FAD. Recently, Gauden et al. (11) investigated the photocycle of AppA by the transient absorption and time-resolved fluorescence methods and found that FAD triplet state is formed at a low yield of ∼9% and decays to the ground state with a lifetime of 3 μs. Hence, we attributed the ∼3.4 μs-dynamics in the TG signal to the decay rate of the triplet state of FAD. After this species grating signal, the TG signal decayed to zero with a time constant of several microseconds, which depended on the grating conditions. This decay rate constant agreed well with $D_{obs} q^2$ at the experimental $q^2$ determined by the thermal grating signal from the calorimetric reference sample. Hence, we was the thermal grating component created by the thermal energy due to the nonradiative transition from the excited state of FAD.

After the thermal grating signal decayed to zero, the signal rose again and finally it decayed to the baseline. This rise-decay component depended on $q^2$ as shown in Fig. 2. This $q^2$-dependence indicates that these components represent the diffusion processes. The sign of the preexponential factor can be determined without any ambiguity by using the fact that the sign of the thermal grating signal is negative ($\delta n_{th}<0$). As the thermal grating signal reaches zero, the rise and decay parts of the diffusion signal represent the reactant and product diffusions, respectively. This rise-decay feature of the diffusion signal indicates that $D$ of the parent and the product are different each other, and the product diffuses slower than reactant. We will explain the origin of the slower diffusion of the product in a latter section.

It is more interesting to note that not only the rate but also the temporal profile of the signal depends on $q^2$. If $D$ of the parent and the product are constants in time, and the product is created from the parent promptly, the profile should be...
Tetramer Formation of AppA

FIGURE 2 Normalized TG signals of concentrated solution of BLUF domain of AppA (0.95 mM) at various $q^2$ conditions. The $q^2$ are i), 4.5 $\times$ 10^{-10} m^{-2}, ii), 1.3 $\times$ 10^{-11} m^{-2}, iii), 5.6 $\times$ 10^{-11} m^{-2}, and iv), 1.3 $\times$ 10^{-11} m^{-2}. The best fitted curve based on Eq. 3 is shown by the solid line.

expressed by Eq. 3. However, we found that this profile could not be fitted by the biexponential function. If the profile represents only the diffusion process, the time-dependence should be expressed by a combination of terms of exp($-D_2 q_2 t$) (e.g., Eq. 3). In this case, if the signal measured at various $q^2$ is plotted against $q^2 t$, the shape of the signals should be identical. However, the signals are totally different depending on the $q^2$-value (Fig. 3). Therefore, the failure of the biexponential function cannot be explained by simply adding more diffusion terms. One explanation we can think from this fact is that the diffusion process is time-dependent (17,18,22). For analyzing the observed TG signal, a model that represents the time-dependent $D$ (e.g., Eq. 8) should be used.

Origin of diffusion change

Since the theoretical equation based on the two-state model presented in the Principle and theoretical section contains many parameters, we have to know some parameters independently for a reliable determination of parameters from the fitting. For that purpose, we determined $D_R$ and $D_P$ without using the two-state model as follows. It should be mentioned that after the reaction (conformational change or aggregation) completes, $D$ should be time-independent. Therefore, the temporal profile of the TG signal after this time should be expressed by a biexponential function (Eq. 3) and, from the rate constants, $D_R$ and $D_P$ can be determined.

To perform this analysis, we measured the diffusion signal at a low $q^2$ condition (3.9 $\times$ 10^{-10} m^{-2}, Fig. 4), and fitted the signal from 80 ms after the photoexcitation. Later we will see that this time is sufficiently slower that the time constant of the kinetics. We found that the temporal profile after this time can actually be fitted well by the biexponential function, and this fact ensures that $D$ does not depend on time after 80 ms. From the biexponential fitting, $D_R$ and $D_P$ are determined to be (8.8 $\pm$ 0.4) $\times$ 10^{-11} m^2 s^{-1} and (7.2 $\pm$ 0.4) $\times$ 10^{-11} m^2 s^{-1}, respectively. Therefore, we found that the product diffuses 1.22 times slower than the reactant.

First, we compare $D_R$ with $D$ of other proteins. The molecular mass of the BLUF domain of AppA is $\sim$15.5 kDa. $D$ of a protein with a similar size, e.g., myoglobin (18 kDa) measured by the TG method is 10 $\times$ 10^{-11} m^2 s^{-1}, which is larger than $D_R$ of AppA (27). We think that this difference in $D$ reflects the dimeric form of AppA in solution (16). Indeed, $D$ of Green fluorescent protein having a molecular mass of $\sim$30 kDa (about the same size as the dimer of AppA) was reported to be 8.7 $\times$ 10^{-11} m^2 s^{-1} in water (28). The similar $D$ to $D_R$ of AppA ensures the dimeric form of AppA. This is the first reported $D$ of the ground and signaling states of the BLUF domain of AppA.

The significant difference in $D$ between the reactant and product is very interesting. According to the Stokes-Einstein relationship, $D$ is given by (29,30)

$$D = \frac{k_B T}{6\pi a r}$$

where $k_B$, $T$, $\eta$, $a$, and $r$ are Boltzmann constant, temperature, viscosity, a constant representing the boundary condition between the diffusing molecule and the solvent, and radius of the molecule, respectively. If the difference in $D$ between the reactant and product is interpreted in terms of the difference

FIGURE 3 TG signals of the BLUF domain of AppA (0.95 mM) at various $q^2$ values are i), 1.3 $\times$ 10^{-11} m^{-2}, ii), 5.6 $\times$ 10^{-11} m^{-2}, and iii), 4.5 $\times$ 10^{-12} m^{-2}.

FIGURE 4 TG signals of the BLUF domain of AppA at various concentrations at low $q^2$ (3.9 $\times$ 10^{-10} m^{-2}) condition. The concentrations are i), 0.95 mM, ii), 0.27 mM, and iii), 0.17 mM.

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in the molecular radius, the molecular volume of the product should be 1.9 times larger than that of the reactant and it is unreasonable to consider that the intrinsic molecular volume increases 1.9 times by a simple chemical reaction.

One possible explanation is the conformational change of the protein, which leads to increase the interaction between the solvent and the protein. For example, it was found that $D$ of the LOV2 domain with the liker part of phototropin decreased 0.77 times by the product formation compared with $D$ of the reactant, and this decrease in $D$ was attributed to a strong interaction between the protein and solvent due to unfolding of the $\alpha$-helix in the linker part in the product state (17). The BLUF domain of AppA consists of a five-stranded $\beta$-sheet, two $\alpha$-helices on top of this, and the FAD chromophore anchored between these two $\alpha$-helices (15). Some rearrangements of the hydrogen bonding between flavin to the conserved residues were observed after the photoexcitation (12,14).

Another possible explanation for the large reduction in $D$ is the dimerization of the BLUF domain after the photoexcitation. (Since the BLUF domain of AppA already exists as a dimer in the ground state even in a very diluted solution (15, 16), which was confirmed by the TG measurement described above, the formation of the dimer in this case means the tetramer formation in the signaling state. Hereafter, we refer this process “dimerization”, because this process is a bimolecular reaction.) The 1.9 times increase of the molecular volume is a very reasonable value for this dimerization reaction.

To examine these possibilities, we measured the TG signal at various AppA concentrations. If the dimerization is the main cause of the difference in $D$, this reaction rate should be slower at a lower concentration. On the other hand, if a conformational change is responsible for the 1.22 times reduction in $D$ in the signaling state, the temporal profile of the TG signal should not depend on concentration, besides the absolute intensity.

Under a low $q^2$ condition ($q^2 = 3.9 \times 10^{10}$ m$^{-2}$), the temporal profile of the diffusion signal is relatively similar at any concentrations we used; i.e., all TG profiles in this timescale consist of the rise-decay component (Fig. 4). At this low $q^2$, the diffusion peak can be reproduced by a biexponential function with $D_R = (8.8 \pm 0.4) \times 10^{-11}$ m$^2$s$^{-1}$ and $D_P = (7.2 \pm 0.4) \times 10^{-11}$ m$^2$s$^{-1}$ after 80 ms at any concentration. Therefore the final product should be the same at all concentrations after a sufficiently long time.

On the other hand, in a middle $q^2$ condition ($5.6 \times 10^{11}$ m$^{-2}$), the temporal profile of diffusion signal depended on the concentration rather significantly (Fig. 5). In a fast timescale, the temporal profile of the TG signals changes much drastically with the concentration (Fig. 6). The signal became an approximately single exponential decay as the concentration decreased (Fig. 6). Considering that the diffusion peak arises due to the difference between $D_R$ and $D_P$, one may understand that the nearly single exponential behavior indicates a small change in $D$ in this time range. As $D_R$ and the final $D_P$ are always constant as shown above at low $q^2$ experiment, the small change in $D$ should be interpreted in terms of the slower rate of change in $D_P$ with decreasing the concentration. This concentration dependence of the TG profile and the 1.22 times decrease in $D$ (i.e., ~2 times increase in molecular volume) in the product state support the dimerization mechanism in the excited state of this protein.

There are three possibilities for the dimer formation mechanism. One possibility is that the photoexcited AppA (AppA*) is associated with the ground state AppA to yield the dimer (Scheme 2): $\text{AppA}^* + \text{AppA} \rightarrow \text{AppA} : \text{AppA}$. (Scheme 2)

In this case, if the concentration of AppA* is low enough compared to AppA, the rate equation is the same as that of the pseudo-first order rate equation and eventually, the temporal profile should be independent of the laser power.
Secondly, if a photoexcited AppA is associated with another photoexcited AppA (AppA*),

\[ \text{AppA}^* + \text{AppA} \rightarrow k_{\text{obs}}(\text{AppA}^*)_2, \]  

(Scheme 3)  

the rate equation becomes the second-order rate equation. In this case, the reaction rate depends on the concentration of the photoexcited protein; hence, the TG profile depends on the laser power.

We observed that the temporal profile of the TG signal is independent of the laser power; only the signal intensity increases with the increase in the laser power, but not the temporal shape. Therefore, we concluded that Scheme 2 is appropriate to describe the reaction. According to this scheme, the observed feature of the TG signal can be explained qualitatively as follows. Upon photoirradiation, this protein changes its conformation, and the dimerization reaction takes place. Since this dimerization occurs faster at a higher concentration, the biexponential feature is observed even at an early time range (i.e., under a high q^2 condition). In a diluted solution, the dimerization process and the D change become slower, so that a single exponential like feature is observed in the fast timescale. This single exponential behavior provides us another important information; i.e., D of the initially created product is similar to \( D_R \) (\( D_1 = D_R \) in Eq. 7).

**Kinetics of dimer formation**

On the basis of the above observations, we should use Eq. 8 for the fitting of the TG signal. The adjustable parameter is the rate constant \( k_{\text{obs}} \) and the refractive index change. The fitting is generally satisfactory (e.g., Figs. 5 and 6), and we determine \( k_{\text{obs}} \) at all concentrations. For example, the time constant was 4.5 ms at 0.95 mM. (This time is sufficiently shorter than the time range we used for the biexponential fitting in the previous section (>80 ms).) The rate constant \( k_{\text{obs}} \) decreased as the concentration decreased. From the slope of the plot of \( k_{\text{obs}} \) versus concentration (Fig. 7) and a relation of \( k_{\text{obs}} = k[\text{AppA}] \), we determined the second order rate constant, \( k \), to be \( \approx 2.5 \times 10^5 \text{M}^{-1}\text{s}^{-1} \). Interestingly, this value is much smaller than that of a diffusion controlled reaction \((\approx 10^9 \text{M}^{-1}\text{s}^{-1})\) calculated by the Smolochowski-Einstein equation for a bimolecular reaction in solution (31). This difference indicates that the collision between two protein molecules is not the sole criterion for the aggregation process; i.e., their relative orientations dictate additional constraints, which slow down the rate of the reaction by four orders of magnitude.

We think that this photoinduced dimer finally dissociates to the original species, because the TG signal is reproducible when the repetition rate of the excitation is slow enough. This leads to the conclusion that there is no covalent bond formation in the aggregated state.

Previously, the kinetics of the photoreaction of AppA was studied by the transient absorption method (11). The results showed that, as long as the reaction was monitored by the absorption change, the signaling state was formed directly from the singlet excited state of FAD on a fast (<1 ns) timescale after blue light excitation (11). The fast formation of the signaling state suggested that there was no large difference in the structure between the ground state and the signaling state. Parallel to the formation of the signaling state, the FAD triplet state is formed with a low quantum yield. The triplet state decays to the ground state with a 3-μs time constant and the signaling state returned to the ground state in ~30 min (11). In our TG signal, the signal rose quickly with the time response of our system (~20 ns) and then the weak slow rising component appeared, which was attributed to the decay of the triplet state of FAD. The time constant of this triplet state decay is very close to that measured by the absorption detection (11). After this dynamics, even though the absorption does not change, we observed a new kinetics with a few milliseconds time constant as the diffusion change dynamics, which should be attributed to the dimerization reaction. Therefore, this dimerization reaction is spectrally silent dynamics and arises due to the protein-protein interaction. We suggest that this state is the signaling state of AppA.

Previously, it was reported that the dark-adapted wild-type AppA156 exhibited an elution profile of 35 kDa and light-adapted state exhibited an elution profile of 37 kDa by a gel chromatographic technique. It indicated that the light excitation of the chromophore caused a conformational change in AppA156, so that the Stokes radius increased, but any elution profile that corresponds to the dimer formation was not reported. It is difficult to specify the exact cause of the difference between this chromatographic result and this observation, because the experimental conditions were different. However, it should be noted that our TG technique monitors sensitively the refractive index change caused only by the creation of the photoexcited state, whereas the gel chromatography monitors all AppA proteins in the solution. It might be difficult to detect the dimer contribution among the whole proteins, unless the population of the dimer is dominant. This different sensitivity could be one possible
The kinetics of photoreaction of the BLUF domain of AppA5–125 is studied from a viewpoint of diffusion co-efficient (D) using the pulsed laser induced transient grating (TG) method. The temporal profile of the TG signals changed depending on the observation time, indicating that D of the product is time-dependent after the photoexcitation. We measured D of the ground and signaling states of AppA from the TG signal under a low q2 condition. The observed diffusion coefficients for the ground state and signaling state of AppA are $(8.8 \pm 0.4) \times 10^{-11} \text{m}^2\text{s}^{-1}$ and $(7.2 \pm 0.4) \times 10^{-11} \text{m}^2\text{s}^{-1}$, respectively. Comparing D of AppA with that of other proteins, we suggest that AppA form a dimer in the ground state. Moreover, the TG signal profile depended on the concentration drastically in particular on an early time-scale. The observed TG signal could be well fitted by the dimerization model. The dimerization rate constant ($k_{\text{obs}}$) decreases as the concentration decreases. From the plot of $k_{\text{obs}}$ against the concentration, the second order rate constant was determined to be $\approx 2.5 \times 10^5 \text{M}^{-1}\text{s}^{-1}$. This value is lower than the diffusion controlled rate expected for a bimolecular association reaction with a steric factor of unity. The slower rate of aggregation is attributed to the orientational constraint of AppA during the formation of the tetramer. This study can be a demonstration showing that the diffusion measurement is a powerful way to monitor spectrally silent dynamics including the dimerization reaction.

CONCLUSION

The kinetics of photoreaction of the BLUF domain of AppA5–125 is studied from a viewpoint of diffusion coefficient (D) using the pulsed laser induced transient grating (TG) method. The temporal profile of the TG signals changed depending on the observation time, indicating that D of the product is time-dependent after the photoexcitation. We measured D of the ground and signaling states of AppA from the TG signal under a low q2 condition. The observed diffusion coefficients for the ground state and signaling state of AppA are $(8.8 \pm 0.4) \times 10^{-11} \text{m}^2\text{s}^{-1}$ and $(7.2 \pm 0.4) \times 10^{-11} \text{m}^2\text{s}^{-1}$, respectively. Comparing D of AppA with that of other proteins, we suggest that AppA form a dimer in the ground state. Moreover, the TG signal profile depended on the concentration drastically in particular on an early time-scale. The observed TG signal could be well fitted by the dimerization model. The dimerization rate constant ($k_{\text{obs}}$) decreases as the concentration decreases. From the plot of $k_{\text{obs}}$ against the concentration, the second order rate constant was determined to be $\approx 2.5 \times 10^5 \text{M}^{-1}\text{s}^{-1}$. This value is lower than the diffusion controlled rate expected for a bimolecular association reaction with a steric factor of unity. The slower rate of aggregation is attributed to the orientational constraint of AppA during the formation of the tetramer. This study can be a demonstration showing that the diffusion measurement is a powerful way to monitor spectrally silent dynamics including the dimerization reaction.

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