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When Is the Helix Conformation Restored after the Reverse Reaction of Phototropin?

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Phototropin (Phot1, Phot2) is a well-known blue-light receptor in plants.[1] Phototropins consist of two photoreceptive light-oxygen-voltage (LOV) domains, LOV1 and LOV2, at their N terminus, a Ser/Thr kinase domain at their C terminus, and a linker region that connects the LOV2 domain and the kinase domain.[2] The two LOV domains noncovalently bind a flavin mononucleotide (FMN) as a chromophore.[3] When the dark-adapted state of this protein (the D state) is exposed to blue light, a photochemical reaction that forms an adduct between a cysteine and FMN occurs, and the absorption spectrum shifts to blue with a time constant of a few microseconds (formation of the S state).[4,5] After this step, some spectrally silent reactions have been reported for Phot1LOV2 combined with a linker (Phot1LOV2−linker) and for Phot2LOV2−linker by NMR[6] and FT-IR[7] spectroscopy, volume change detection,[8] and diffusion coefficient (D) measurements.[8,9] The reaction scheme is as follows. Following the adduct formation, the linker region dissociates from the LOV2 domain with a time constant of 300 μs. After the dissociation of the linker, the α-helix of the linker region unfolds with a time constant of 1 ms (the T state). This biologically active state remains for ∼29 s for *Arabidopsis* Phot1LOV2−linker.[10,11] In the dark, the covalent bond between the cysteine and flavin is disrupted by a thermal reaction, and the linker refolds to the original inactive helical conformation. This refold or back-reaction is important for inactivation of Phot. However, when is the conformation of the linker region restored?

Previous research has shown that the formation of α-helices of synthesized polypeptides is quite fast in solution (within μs).[12] These results suggest that formation of the α-helix of the linker is likely to be fast after the disruption of the adduct. However, these studies were performed using artificial polypeptides with stable helices. On the other hand, a helical wheel analysis of the Jα-helix showed an amphipathic character, and its hydrophobic region may bind to the hydrophobic surface of the LOV2 core region.[13] NMR studies reported that the construct consisting of only the Jα-helix exists as a random coil in solution.[14] These observations indicate that the linker region cannot form helical structures without the LOV2 core. Therefore, the folding process of the linker domain may reflect the interdomain interaction, and the recovery rate should give us a deeper insight to the deactivation mechanism of the native protein.

Previous circular dichroism measurements have shown that the lifetime of the unfolded conformation of the linker region is almost identical to that of the absorption change.[8,15] Furthermore, the lifetime of the T state measured by the absorption change is almost independent of the existence of the linker region.[11] These observations indicate that the disruption of the cysteine−FMN bond is the rate-determining step for the folding of the linker domain. To overcome this limitation, we disrupted the covalent bond of the T state by light irradiation to initiate the folding process. Recently, Kennis et al.[16] reported the direct observation of the photochemical reverse reaction using the transient absorption technique and found that the D state is regenerated on an ultrafast time scale (100 ps) upon photoexcitation of the T state. Using this method combined with a two-step transient grating (TG) method,[17] we measured the refolding dynamics of the linker by initially accumulating the T state by preirradiation with blue light and subsequently photoexciting the T state using a pulsed UV laser (308 nm) to measure the TG signal.

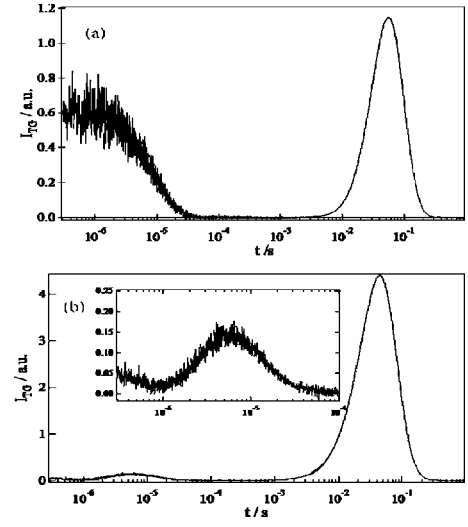

Figure 1. Observed TG signals of the Phot1LOV2−linker in the buffer for (a) the reverse reaction and (b) the forward reaction at $q^2 = 4.1 \times 10^{11}$ m^{-2}. A magnified signal in the adduct formation process is shown in the inset of (b).

The experimental setup and the principle are described in the Supporting Information (SI). Phot1LOV2−linker in the T state was accumulated by blue-light preirradiation at 449 nm. Since the absorbance of the T state at 449 nm is small, most of the D state was converted to the T state by the preirradiation. Furthermore, since the absorbance of the T state at 308 nm is larger than that of the D state,[5] the T state was predominantly photoexcited by the excimer laser. These two conditions made it possible to selectively measure the TG signal of the reverse reaction.

Figure 1a depicts the TG signal for the reverse reaction of Phot1LOV2−linker. For comparison, we have also shown the TG signal for the forward reaction measured under the same conditions but without the blue-light preillumination (Figure 1b). These signals consisted of several phases. The phases for the forward reaction have been assigned previously.[8] The initial decay-rise component

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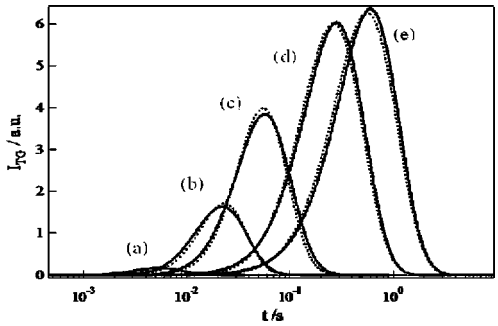

Figure 2. The q^2 dependence of the TG signals (dotted lines) after photoexcitation of the Phot1LOV2−linker in the buffer. The q^2 values are (a) 6.25×10^{12}, (b) 1.20×10^{12}, (c) 3.98×10^{11}, (d) 6.55×10^{10}, and (e) 3.01×10^{10} m^{-2}. The best-fit curves obtained using a two-state model are shown as solid lines.

in the <10 μs range in Figure 1b represents the adduct formation process. The second decay in the 10−50 range is attributable to the thermal grating. The rise-decay peak in the 10−100 ms range is due to the protein diffusion process and represents a change in diffusion coefficient (D) from D_R to D_P, where R and P signify the reactant and product, respectively.[8] The change in D ($D_R > D_P$) is a clear indication of the unfolding of the α-helix of the linker.[8]

The overall features of the TG signal for the reverse reaction appeared to be similar to those observed for the forward reaction. However, two different features were noted. First, the thermal grating signal for the reverse reaction rose quickly (within our instrumental response, ∼20 ns), and the decay-rise component observed for the forward reaction was absent. This absence of the adduct formation process is reasonable because the rate of the disruption of the covalent bond for the reverse reaction (∼100 ps[16]) is much faster than our instrumental time response. Since this adduct formation component has overlapped destructively with the thermal grating signal for the forward reaction, the thermal grating intensity of Figure 1b is weak. The lack of this phase is a clear confirmation that the observed signal is not contaminated by the forward reaction. To estimate a possible contribution of the forward reaction in the signal, we fitted the thermal grating signal of the reverse reaction by a superposition of the thermal grating signal and the adduct formation phase that were observed for the forward reaction (Figure S1 in the SI). The contribution of the forward reaction was found to be less than 7%. Second, the sign of the refractive index change (δn) for the diffusion peak is opposite to that of the forward reaction. Since the TG signal intensity is proportional to the square of δn, this difference is not apparent from just observing the signal. However, we confirmed in two ways that this sign is opposite (section S-3 in the SI). This observation ensures that D_P is larger than D_R, i.e., the refolding process of the α-helix occurs upon UV irradiation.

Next, to determine the kinetics of the folding process, we measured the TG signal in various time windows by changing q^2 (q is the grating wavenumber) (Figure 2). The diffusion peak intensity decreased in the faster time region. If both D_P and D_R are time-independent, the diffusion peak intensity should not depend on the observation time scale. Contrary to this prediction, the TG signal in a fast time scale (i.e., with a large q^2) was weak, and the intensity increased with increasing observation time (Figure 2). This time dependence of the signal intensity can be explained in terms of the time-dependent apparent D. More clear evidence for the time-dependent D was obtained from the q^2t plot of the signals (Figure S2).

On the basis of these results, the temporal profile of the TG signal was analyzed using the time-dependent D (eq 3 in the SI). With only a single adjustable parameter, the rate constant of the diffusion change, k, all of the TG signals were reasonably well reproduced, and the time constant of the change was 13 ± 3 ms. We have previously shown that the tertiary structural change of the phot1LOV2−linker sample does not change D and that the unfolding is the sole origin of the change.[8,9] Since D is recovered completely by this reverse reaction, this rate is determined by the refolding of the linker region and not by a tertiary structural change. This α-helix formation process in this reverse reaction should be studied by the other techniques (e.g., IR or NMR spectroscopy) in the future.

The α-helix formation rates of several polypeptides have been measured and reported.[12] In many cases, the rates are fast (100 ns to a few μs). However, the folding rate of the linker region is much slower than those for typical α-helices of polypeptides. This slower rate confirms that the LOV2 domain acts as a template for the formation of the α-helix of the linker region, and the formation of the α-helix is described by the induced fit model. The observed slow rate could reflect the probability of a perfect matching of an interdomain hydrophobic interaction. This study supports and further highlights the importance of interdomain interactions in deactivation of this protein.

The biological functions of phototropins are deactivated by three steps: recovery of the chromophore reaction (dissociation of the covalent bond), recovery of the conformation of the linker region, and return of the protein to its nonphosphorylated state. This is the first report describing the conformational recovery rate.

Supporting Information Available: Experimental procedures, principle of the TG experiment, and additional results as described in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

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