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Photoreaction of the Cysteine S−H Group in the LOV2 Domain of *Adiantum* Phytochrome3

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So far, the biological light-signal transduction has been known to be initiated by cis−trans photoisomerization of chromophore molecules. In fact, photoisomerization of retinal, phytochromobilin, and *p*-coumalic acid takes place in rhodopsins, phytochromes, and photoactive yellow protein (PYP), respectively.[1] In these proteins, altered chromophore structure after isomerization enforces protein structural changes, followed by specific protein−protein interaction to activate transducer protein. However, such a general scheme now needs to be reconsidered by recent discoveries of sensor proteins with flavins as the chromophore.[2,3a] How is the light energy converted into the protein structural changes for flavin-binding proteins so as to activate transducer?

Phototropin (phot) is a blue-light photoreceptor for tropic responses,[2b] reorientation of chloroplast[3b] and stomata opening[3c] in plants, which are deeply concerned in the regulation of photosynthetic activities. Phot binds two flavins (FMN) noncovalently in its N-terminal half, and the remaining half of phot is a serine/threonine kinase. Light absorption of the FMN induces autophosphorylation of phot molecules.[4] The crystal structure of the second flavin-binding domain (LOV2) of *Adiantum* phytochrome3 (phy3), a fusion protein of phot with phytochrome chromophoric domain,[4b] clearly demonstrated that the flavin-binding domain is characteristic of the LOV (light-oxygen-voltage) domain, which also belongs to the PAS (Per-Arnt-Sim) superfamily.[5] Two photoactive LOV domains (LOV1 and 2) are obtainable by expression systems.[4b] Light illumination of the phot-LOV domain causes loss of yellow colors, forming the product absorbing at ∼390 nm (S390).[6] Because the spectrum of S390 resembles that of a covalent C(4a) flavin-cysteinyl adduct,[7] formation of a flavin-cysteinyl adduct in the LOV domain has been suggested. Several experiments such as mutation of the cysteine,[6,8] NMR spectroscopy,[9] and X-ray crystallography of S390[10] have provided experimental evidence in favor of the formation of the C(4a)flavin-cysteinyl adduct in S390 (scheme 1, Figure 1a).

By using time-resolved absorption spectroscopy of oat phot1-LOV2, Swartz et al. have shown that there are only two photocycle intermediates, L660 and S390, and L660 is likely to be an excited-triplet state.[8] Therefore, S390 is the only intermediate in the electronic ground state which is formed from L660 with a time constant of 4 μs. This is in contrast to those of other sensor proteins mediated by photoisomerization. In visual rhodopsin, for instance, the primary intermediate is formed in femtosecond regime, followed by the appearance of other intermediates in pico-, nano-, micro-, and milliseconds sequentially.[1a]

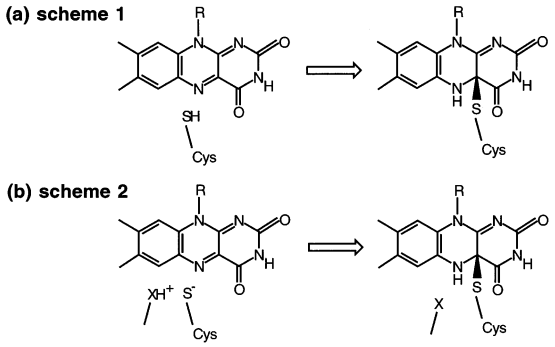

Figure 1. Two possible photoreaction schema for the plant LOV domain. (a) Cysteine is protonated in the initial state (scheme 1). (b) Cysteine is deprotonated, which is stabilized by the counterion (scheme 2).[8]

Swartz et al. have also proposed that the cysteinyl group is deprotonated in the phot1-LOV2 on the basis of the pH titration of fluorescence from FMN for the wild-type and cysteine-mutant proteins. To stabilize the thiolate, they postulated the presence of the counter charge, the XH^+ group (scheme 2, Figure 1b).[8] However, the crystal structure of LOV2 of phy3 showed no suitable amino acids, as the XH^+ group is nearby the only cysteine (Cys966) in phy3-LOV2.[5] Cys966 is surrounded by hydrophobic amino acids. How is the thiolate stabilized in such a hydrophobic pocket? Does the cysteinyl group of the phy3-LOV2 domain exist in thiolate? X-ray crystal structure with 2.7 Å resolution could not distinguish between SH and S^-.[5]

Vibrational spectroscopy is one of the potential methods in determining if the cysteine is protonated or not, because the stretching frequency of cysteine S−H is in the 2580−2525 cm^{-1} region, where the other vibrations are absent.[11] On the basis of this understanding, we developed a new approach that utilizes the cysteine S−H as a hydrogen-bonding probe inside a protein.[12] In the present study, we have measured the change in the infrared spectra upon illumination of LOV2 at 295 and 150 K. The FMN-bound phy3-LOV2 domain was expressed in *E. coli* and was purified by calmodulin affinity column as described elsewhere.[5] The sample in 1 mM potassium phosphate buffer (pH 7.0) was dried on a BaF_2 window and rehydrated by placing 1 μL of H_2O aside before mounting into a cryostat. FTIR spectroscopy was applied to the hydrated film as described previously.[12,13]

Before the FTIR measurements, UV−visible absorption spectra of the films in a cryostat were measured both before and after the illumination with a >400 nm light for 1 min. The spectra look similar between 295 and 150 K, exhibiting negative peaks at 449 and 475 nm (Figure 2). Besides, two peaks appeared at 304 and 400 nm. They are characteristic spectra of the S390 minus original state,[6,8] indicating that S390 is formed in the hydrated film sample.

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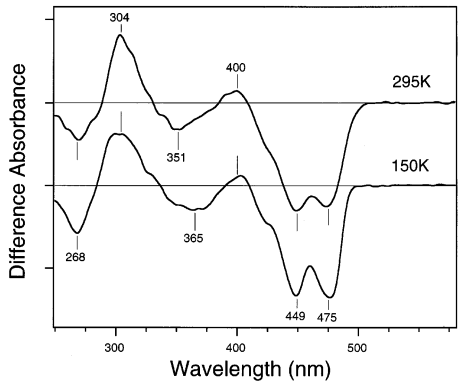

Figure 2. Light minus dark difference spectra for phy3-LOV2 (pH 7.0) in the UV−visible region. The spectra are measured at 295 K (upper) and 150 K (lower). One division of the *y*-axis corresponds to 0.03 absorbance unit.

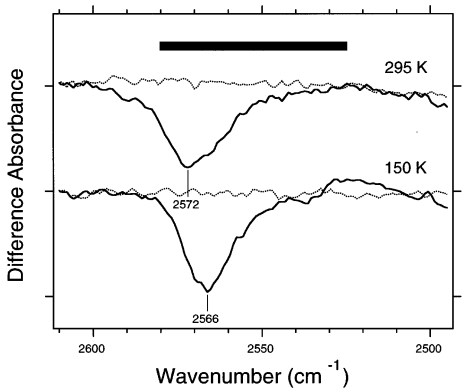

Figure 3. Light minus dark (solid line) and dark minus dark (dotted line) infrared difference spectra for phy3-LOV2 (pH 7.0) in the 2620−2470 cm^{-1} region. The spectra are measured at 295 K (upper) and 150 K (lower), being the average of nine and eight recordings, respectively, each of which was calculated from 128 interferograms with 2 cm^{-1} resolution. The thick horizontal bar (2580−2525 cm^{-1}) shows the frequency region of S−H stretch in the literature.[11b] One division of the *y*-axis corresponds to 0.00008 absorbance unit.

Interestingly, observation of S390 at as low as 150 K suggests that the formation of S390 does not accompany global protein structural changes. This is consistent with the time-resolved spectroscopy that detected formation of S390 within a short time scale of 4 μs.[8] The spectral difference in the 320−380 nm region (Figure 2) may suggest different protein structures between 295 and 150 K.

Next, we have measured the IR difference spectra under the same illumination conditions as those for UV−visible spectroscopy. Figure 3 shows the presence of the negative band at 2572 cm^{-1} (295 K) and 2566 cm^{-1} (150 K) in the light minus dark spectrum (solid line), which is absent in the baseline (dotted line). The result clearly demonstrates the presence of S−H groups in the initial state and the disappearance of them upon S390 formation. Because the phy3-LOV2 has only one cysteine, the S−H stretching vibration comes from Cys966. No positive band at 295 K suggests either adduct formation or deprotonation of the S−H group upon photoconversion to S390. The former is consistent with the adduct formation between FMN and Cys966 in S390.[6−10] Accordingly, our data support scheme 1 in Figure 1, as the molecular mechanism of S390 formation in phy3-LOV2.

S−H stretching vibrations appear in the 2580−2525 cm^{-1} region (thick bar in Figure 3), and their frequencies are reduced as the hydrogen-bonding of the S−H groups becomes stronger.[11b] The frequency of 2572 cm^{-1} at 295 K, therefore, indicates that the S−H group is located under little hydrogen-bonded condition. This is consistent with the local structure around Cys966, where there is no hydrogen-bonding acceptor site for Cys966.[5] The hydrogen-bonding character is slightly strengthened at 150 K, as shown by the low-frequency shift by 6 cm^{-1} (compare Figure 3 150 K with 295 K). Peak amplitude of the S−H stretch in phy3-LOV2 at 150 K is 1.2 times that at 295 K, while full-width at half-maximum (fwhm) is 20 and 16 cm^{-1} at 295 and 150 K, respectively. It is noted that the S−H stretch is much broader than that in the M intermediate of T89C mutant bacteriorhodopsin (frequency, 2576 cm^{-1}; fwhm, 9 cm^{-1} at 230 K),[12] suggesting that the S−H group of Cys966 in the LOV2 has a large degree of freedom in motion. Such multiple vibrational structures of Cys966 may be advantageous in interaction with FMN in the excited-triplet state.

The low-temperature spectroscopic measurements revealed the formation of S390 even at 150 K (Figure 2), suggesting that the process takes place without global structural rearrangement in the protein moiety. The S390 minus phy3-LOV2 spectrum in the UV−visible region was obtained at any temperatures between 150 and 295 K (not shown). This aspect of LOV2 is remarkable in contrast to the other light sensor proteins. In rhodopsins, phytochromes, and PYP, photoisomerization takes place in femtosecond and early picosecond time scales, while the intermediates activating transducer appear in milliseconds and seconds.[1] There are time differences with more than 10 orders of magnitude, during which proteins alter their structures through several intermediates.

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