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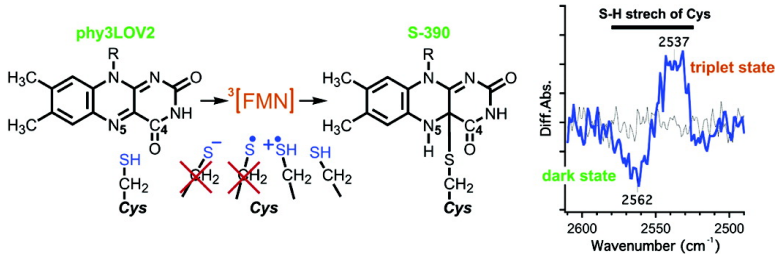

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Reactive Cysteine Is Protonated in the Triplet Excited State of the LOV2 Domain in *Adiantum* Phytochrome3

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Phototropin is a plant blue-light sensor protein that possesses a flavin mononucleotide (FMN) as the chromophore.[1] Upon light absorption, structural changes of the FMN-binding LOV domain activate serine/threonine kinase domain, leading eventually to various functions such as tropic responses, relocation of chloroplast, and stomata opening in plants.[1] The primary reaction is an adduct formation between FMN and a nearby cysteine (Cys; Figure 1), which takes place in a few microseconds through a triplet excited state of FMN.[2,3] In the case of the LOV2 domain of *Adiantum* phytochrome3 (phy3LOV2), the distance between the C4a position of FMN and the sulfur atom of cysteine (Cys966) is 4.2 Å.[4] Thus, molecular mechanism of the adduct formation through the triplet excited state of FMN has been of interest.

Swartz et al. proposed that Cys is deprotonated in the LOV2 domain of oat phototropin1 (phot1LOV2) on the basis of the pH titration of fluorescence from FMN for the wild-type and cysteine-mutant proteins.[2] However, Fourier transform infrared (FTIR) spectroscopy directly detected S−H stretching vibrations in the unphotolyzed state of phy3LOV2[5] and phot1LOV1 of *Chlamydomonas reinhardtii*.[6] The next question is the chemical structure of Cys in the triplet excited state, from which an adduct is formed. From the ultrafast spectroscopic results, Kennis et al. suggested a possibility that proton-transfer reaction takes place from Cys to FMN, so that Cys has an S^- form.[7] In contrast, Kay et al. proposed a radical-pair mechanism based on the EPR study on mutations, where a hydrogen atom is transferred from Cys to FMN so that Cys has a neutral radical form (S).[8] The same group also suggested another possibility that Cys is an SH^+ form.[9] This model argues an electron-transfer reaction from Cys to FMN. Thus, there are several possible structures for Cys in the triplet excited state (Figure 1), which is essential for the reaction mechanism of adduct formation.

Vibrational spectroscopy is a potential method in determining if Cys 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.[10] This led to finding of the protonation state of Cys in LOV domains.[5,6] In contrast to the unphotolyzed state, it is not easy to apply FTIR spectroscopy to the triplet excited state because of its short lifetime (a few microseconds). For step-scan time-resolved FTIR spectroscopy, highly repeated photoexcitation is required, whereas LOV domains have a long photocycle period such as minutes. In this communication, we determine the protonation state of Cys in the triplet excited state of phy3LOV2 by means of low-temperature FTIR spectroscopy.

Illumination of phy3LOV2 causes formation of the S390 intermediate (Figure 1, right). S390 is the sole intermediate of LOV domains, and we found that S390 is formed at any temperatures between 77 and 295 K for phy3LOV2.[11] Nevertheless, interestingly, formation yield of S390 was reduced at low temperatures. We reported the presence of unreactive fractions to be 64 and 36% at 77 and 100 K, respectively.[11] This observation can be interpreted in terms of structural heterogeneity that prohibits molecular motion for adduct formation at low temperatures. This also suggests that prolonged illumination accumulates the triplet excited states, though they rapidly decay to the original state at 77 K. Consequently, both UV−visible and IR difference spectra after and before illumination were identical to the baseline. However, if the triplet excited state is populated enough during illumination at 77 K, we expected that the difference during and before illumination would possibly provide a meaningful spectrum, and this was indeed the case.

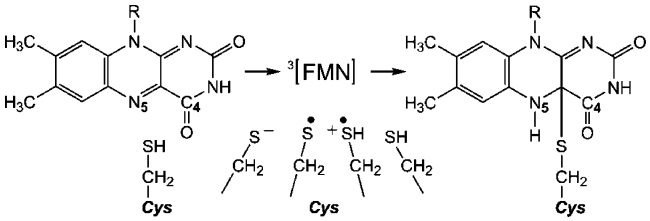

Figure 1. Possible structures of Cys in the triplet excited state of LOV domains (middle). The structures of FMN and Cys in the unphotolyzed (left) and S390 (right) states are also shown.

Figure 2a shows the difference FTIR spectrum of phy3LOV2 after and before illumination at 77 K. The spectrum reproduced the reported one between S390 and phy3LOV2,[11] and about 30% was photoconverted to S390 in this study. Although no further changes were observed for the difference spectra after and before illumination, the difference FTIR spectrum during and before illumination (Figure 2b) was similar to neither the baseline nor the difference spectrum of S390 and phy3LOV2 (Figure 2a). This observation suggests that Figure 2b originates from the spectrum of the triplet excited minus original state in phy3LOV2. Amplitude of vibrational bands was proportional to the light intensity (data not shown), indicating that the product is formed as a single photon event. Figure 2c shows the difference FTIR spectrum of the C966A mutant during and before illumination at 77 K. The difference spectrum after and before illumination was identical to the baseline (data not shown). It is known that the triplet excited state is directly reverted to the original state in this mutant because of the lack of adduct formation.[2] Similar spectra in Figure 2b and c strongly suggest that both products originate from the triplet excited state.

Figure 2b shows negative bands at 1681 and 1546 cm^{-1}, which are assignable for the vibrations of FMN.[6,11] On the positive side, strong peaks were observed at 1493, 1439, 1156, and 1140 cm^{-1} for the wild type (Figure 2b), while similar bands were present at slightly modified frequencies for C966A (Figure 2c). Previous time-resolved resonance Raman spectroscopy of the triplet excited state

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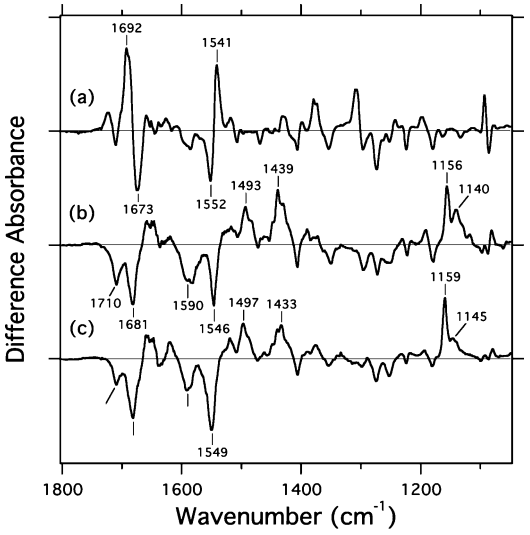

Figure 2. Difference FTIR spectra of the wild-type (a, b) and C966A mutant (c) proteins of phy3LOV2 in the 1800−1050 cm^{-1} region at 77 K. Illumination of the wild type causes formation of the stable S390 intermediate with the yield of 30 %. (a) The difference spectrum after and before illumination corresponds to that of the S390 and unphotolyzed state. (b) The difference spectrum during and before illumination for the remaining unreactive fraction (70%). (c) The difference spectrum during and before illumination of C966A, which forms only the triplet excited state. One division of the *y*-axis corresponds to 0.001 absorbance units.

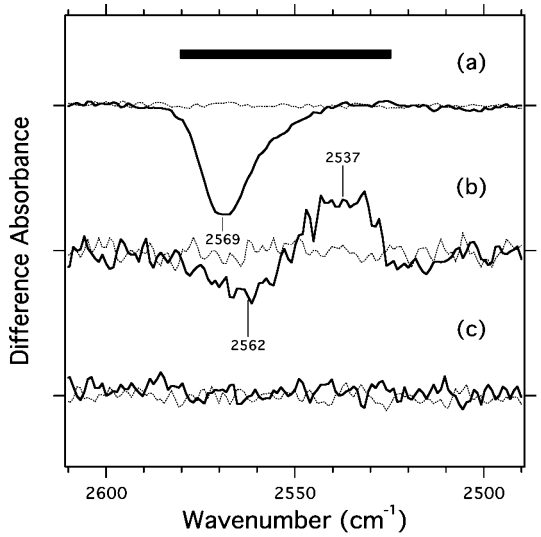

Figure 3. Difference FTIR spectra of the wild-type (a, b) and C966A mutant (c) proteins of phy3LOV2 in the 2610−2490 cm^{-1} region at 77 K. Experimental conditions for thick solid lines are the same as those in Figure 2, while dark minus dark spectra are shown by thin dotted lines (baseline). Thick horizontal bar (2580−2525 cm^{-1}) shows the frequency region of the S−H stretch known from the literature. One division of the *y*-axis corresponds to 0.000 03 absorbance units.

of FMN in solution did not show prominent vibrational bands in the 1500−1100 cm^{-1} region,[12] which may suggest the effect of the specific protein environment. FTIR studies by use of isotope-labeled FMN will reveal the structure of the triplet excited state of FMN in phy3LOV2, which is our future focus.

Spectra in Figure 3a−c correspond to those in Figure 2a−c, respectively, in the characteristic S−H stretching frequency region. Figure 3a shows the presence of a negative band at 2569 cm^{-1}, which reproduced the previous results.[5,11] It clearly demonstrates the presence of the S−H group for Cys966 in the initial state and the disappearance upon S390 formation, because phy3LOV2 has only one cysteine. In contrast to Figure 3a, the difference FTIR spectrum of the triplet excited and original state in the wild-type phy3LOV2 (Figure 3b) shows the presence of both positive and negative peaks at 2537 and 2562 cm^{-1}, respectively. The lower frequency in Figure 3b (2562 cm^{-1}) than that in Figure 3a (2569 cm^{-1}) may originate from structural heterogeneity of S−H groups. Such bands are not observed for the C966A mutant (Figure 3c). Thus, we concluded that both unphotolyzed and triplet excited states of phy3LOV2 possess S−H groups, whose stretching frequencies are 2569 and 2537 cm^{-1}, respectively.

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.[10] The frequency at 2569 cm^{-1} in phy3LOV2 indicates that the S−H group is located under weak hydrogen-bonded condition. This is consistent with the local structure around Cys966,[4] where there is no hydrogen-bonding acceptor site for Cys966. It is noted that upon formation of the triplet state, the frequency is remarkably downshifted to 2537 cm^{-1}. This indicates that the S−H group finds a hydrogen-bonding acceptor in the triplet excited state. Previously, we have not observed such low frequency as S−H stretches among bovine rhodopsin,[13] neurospora rhodopsin,[14] and quinol oxidases,[15,16] where S−H stretches are at >2540 cm^{-1}. The only exception is the T89C mutant bacteriorhodopsin, where the S−H stretch was observed at 2523 cm^{-1}.[10] In this case, the hydrogen-bonding acceptor is a negatively charged Asp85. Thus, the frequency at 2537 cm^{-1} might favor the SH^+ form in the triplet excited state. However, more experimental efforts are required to conclude whether Cys has an SH or SH^+ group. In any case, it is evident that the hydrogen-bonding interaction of the S−H group becomes very strong in the triplet excited state. We infer the hydrogen-bonding acceptor to be the N5 atom of FMN, and such strong interaction presumably drives adduct formation on a microsecond time scale.

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