Hydration and Temperature Similarly Affect Light-Induced Protein Structural Changes in the Chromophoric Domain of Phototropin

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Received February 14, 2007; Revised Manuscript Received March 13, 2007

ABSTRACT: Phototropin is a blue-light sensor protein in plants, and LOV domain binds a flavin mononucleotide (FMN) as a chromophore. A photointermediate state, S390, is formed by light-induced adduct formation between FMN and an S−H group of nearby cysteine, which triggers protein structural changes for kinase activation in phototropin. We previously studied the low-temperature Fourier transform infrared (FTIR) spectra between the S390 and unphotolyzed states for a LOV2 domain of a phototropin from *Adiantum* (neo1-LOV2), and found that the protein structures of the S390 intermediate are highly temperature dependent (Iwata, T., Nozaki, D., Tokutomi, S., Kagawa, T., Wada, M., and Kandori, H. (2003) *Biochemistry 42*, 8183−8191). At physiological temperature, amide-I vibration at 1640−1620 cm^{-1} is significantly changed, implying structural alteration of β-sheet region. Such changes are largely suppressed at low temperatures, though S390 is formed. This observation suggested the presence of progressive protein structural changes in the unique active state (S390). Here we report that the hydration dependence of the amide-I vibrational bands in neo1-LOV2 is similar to the temperature dependence. As hydration of the sample is lowered, amide-I vibration at 1640−1620 cm^{-1} is significantly reduced. Instead, amide-I vibration at 1694 cm^{-1} newly emerged at low hydration as well as at low temperature, which shows a weakened hydrogen bond in the loop region. Spectral coincidence between low hydrations and temperatures strongly suggested that protein structural changes are similarly restricted under such conditions. It is likely that protein fluctuations are prerequisite for formation of the active state of neo1-LOV2.

Phototropin is a blue-light receptor in plants, which is involved in physiological responses such as phototropism (*1*), relocation of chloroplasts (*2*), and stomata opening (*3*) (also see ref *4* as a recent review). Phototropin possesses two flavin mononucleotide (FMN[1]) binding domains at the N-terminal side and Ser/Thr kinase motif at the C-terminal side. Blue-light absorption by the chromophore domain thereby leads to the regulation of the kinase activity, probably through the change in domain−domain interaction. The FMN binding chromophoric domain is highly homologous in primary (*5*) and tertiary (*6*) structures to bacterial light-sensor PYP (photoactive yellow protein), oxygen-sensor FixL, and voltage-sensor HERG of a channel protein, so that the domain is called LOV (light, oxygen, and voltage sensing) domain. The protein fold belongs to the PAS (Per−Arnt−Sim) superfamily.

It is intriguing that LOV domains activate diverse output domains despite unique protein architecture of LOV. In fact, LOV domains regulate several enzymatic activities such as Ser/Thr kinases, His kinases, phosphodiesterases, EAL domains, and phosphotransfer domains (*7*). In addition, there are zinc finger, response regulator, STAS domains, F-box and Kelch repeats in the downstream of LOV domains (*7*), suggesting physical interactions with these domains upon blue-light absorption of LOV domains. This fact demonstrates the importance of the protein structural changes in the LOV domains.

Activation of the LOV domains is initiated by adduct formation between FMN and a nearby cysteine (*8*−*12*). Following light absorption by FMN, intersystem crossing leads to formation of a triplet excited state absorbing at 660 nm (L660), appearing with a time constant of 3 ns in *Adiantum* neo1-LOV2 and oat phot1-LOV2 (*13*). Then, adduct formation accompanies appearance of the S390 intermediate with a time constant of 4 μs in oat phot1-LOV2 (*10*) and with 0.9 and 4 μs in *Chlamydomonas* phot-LOV1 (*14*). While various models have been proposed for the reactive cysteine, previous FTIR studies revealed that the cysteine is protonated in both the ground (*15*−*19*) and triplet excited (*20*) states of FMN. S390 is converted to the original state slowly (*8*, *19*, *21*, *22*), and hence the S390 state probably activates Ser/Thr kinase domain.

We have studied protein structural changes in the S390 state of a LOV2 domain of neochrome1, a chimerical protein of the chromophoric domain of phytochrome and phototropin (recently renamed from phytochrome3 (*23*)), from *Adiantum* (neo1-LOV2) by means of low-temperature FTIR spectroscopy (*16*, *19*, *24*−*26*). The FTIR study showed highly temperature-dependent nature for amide-I vibrations of neo1-

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[1] Abbreviations: FMN, flavin mononucleotide; LOV, light−oxygen−voltage; PAS, Per−Arnt−Sim; FTIR, Fourier transform infrared; UV, ultraviolet.

10.1021/bi7003087 CCC: $37.00
Published on Web 05/16/2007

LOV2, indicating the presence of progressive protein structural changes in a single intermediate state (S390) (*16*, *26*). In particular, structural changes of the β-sheet (frequencies: 1640−1620 cm^{-1}) were observed only at room temperature, not at low temperature, which were confirmed by use of ^{13}C-labeling of protein (*26*). Such structural changes of the β-sheet were not observed for the Q1029L mutant, implying that Gln1029 has an important role in the intramolecular signaling of neo1-LOV2 (*24*). Recent NMR spectroscopy revealed deformation of an extra helix (called Jα) in the light activated state (*27*, *28*). Since Gln1029, involved in a β-sheet region, is located between FMN and the Jα-helix, light signal is presumably relayed from FMN to Jα through the β-sheet (Gln1029) in the case of LOV domains of phototropins.

Different protein structural changes at low temperatures possibly originate from limited protein motion under reduced fluctuation. This might be also the case in the protein crystal, where almost no surface changes were observed between the unphotolyzed and S390 states of neo1-LOV2 (*12*). Such restriction of protein motion could occur in other environments. In this article, we report hydration dependence of the structural changes in neo1-LOV2. FTIR spectroscopy was used for studying light-induced protein structural changes of the protein film of neo1-LOV2 under various hydration conditions. Consequently, we found that hydration dependence of the amide-I bands in neo1-LOV2 was similar to the temperature dependence, suggesting that protein fluctuation plays a crucial role in driving into the active state of S390 that accompanies structural alterations of β-sheets.

MATERIALS AND METHODS

Preparation of Adiantum Neo1-LOV2. The neo1-LOV2 construct in the present experiment contains N-terminal calmodulin-binding peptide and spanning amino acid residues Pro905−Pro1087 of neo1, which include Jα helix (Asp1050−Arg1072). The neo1-LOV2 domain was expressed in *E. coli* BL21 (DE3) and was purified by calmodulin affinity column as described previously (*16*, *19*, *24*). Purified protein was concentrated to 2 mg/mL and dialyzed against 1 mM K/phosphate buffer (pH 7). The protein solution was placed on a BaF_2 window, and then dried to a film under reduced pressure with an aspirator. The dry film was hydrated by putting 0.5 μL of pure water and 10%, 25%, and 50% glycerol/water (v/v) next to the film on the BaF_2 window. This window was covered by another BaF_2 window with a greased spacer and sealing film, and then left for 2 h at room temperature to stabilize the hydration.

Spectroscopy. UV−visible and infrared spectra of the samples were measured using V-550DS (JASCO) and FTS-7000 (Bio-Rad) spectrophotometers, respectively, as described previously (*16*, *19*, *24*). The films were illuminated by a >400 nm light, which was supplied with a combination of a halogen−tungsten lamp (1 kW) and a long-pass filter (L42, Asahi Techno Glass). Neo1-LOV2 molecules are randomly oriented in the film as confirmed by linear dichroism experiments.

RESULTS

Infrared Absorption Spectra of Neo1-LOV2 with Various Hydration Conditions. Figure 1e shows the infrared absorption spectrum of an unhydrated film of neo1-LOV2 at 295 K. The other spectra in Figure 1 correspond to those of the hydrated films with 0.5 μL of pure water (a) and 10% (b), 25% (c), and 50% (d) glycerol/water. Increased absorbances at 3600−3000 cm^{-1} and 1650 cm^{-1} at elevated hydration originate from stretching and bending vibrations of water. The absorption spectra did not change during the FTIR measurements.

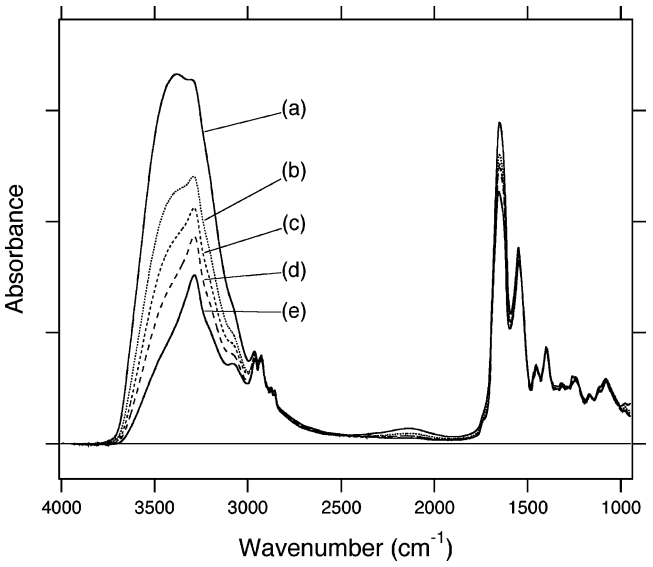

FIGURE 1: Infrared absorption spectra of the neo1-LOV2 films hydrated with 0.5 μL of pure water (a) and 10% (b), 25% (c), and 50% glycerol/water (d) at 295 K. The spectrum of unhydrated film is also shown (e). One division of the *y*-axis corresponds to 0.5 absorbance unit.

Molar ratio of protein and water molecules in the neo1-LOV2 films was estimated by using molar extinction coefficients of visible absorption of neo1-LOV2 (11,200 M^{-1} cm^{-1} at 450 nm (*29*)) and infrared absorption of water O−H stretch (100 M^{-1} cm^{-1} at 3500 cm^{-1} (*30*)). From the UV−visible and FTIR spectra of the unhydrated and fully hydrated films, the molecular extinction coefficient of the amide-II (at 1550 cm^{-1}) was estimated as 70,000 M^{-1} cm^{-1} for neo1-LOV2. It is now possible to estimate how many water molecules are contained for one protein molecule from absorbances at 3500 and 1550 cm^{-1}. The values were calculated to be 1100, 730, 560, 440, and 300 molecules per one protein molecule of neo1-LOV2 in the hydrated films with pure water (Figure 1a) and 10% (Figure 1b), 25% (Figure 1c), and 50% (Figure 1d) glycerol/water and in an unhydrated film (Figure 1e), respectively.

Difference UV−Visible Spectra between the S390 and Unphotolyzed State. In all hydration conditions, formation of S390 was observed after illumination. Figure 2a shows typical UV−visible spectra for the hydrated film with pure water (solid lines) and unhydrated film (dotted lines). The UV−visible spectra of the unphotolyzed state were almost identical between the fully hydrated and unhydrated films, possessing peaks at 448 and 474 nm. After illumination, both films showed photoconversion to S390 with a slight difference with the conversion yield. Formation of S390 was reduced (~10%) for the unhydrated film as judged from the absorbance change at 420−480 nm even after prolonged illumination. Similar reduced formation of S390 was observed at low temperature, where the S390 formation was reduced by 64% and 36% at 77 and 100 K, respectively (*16*).

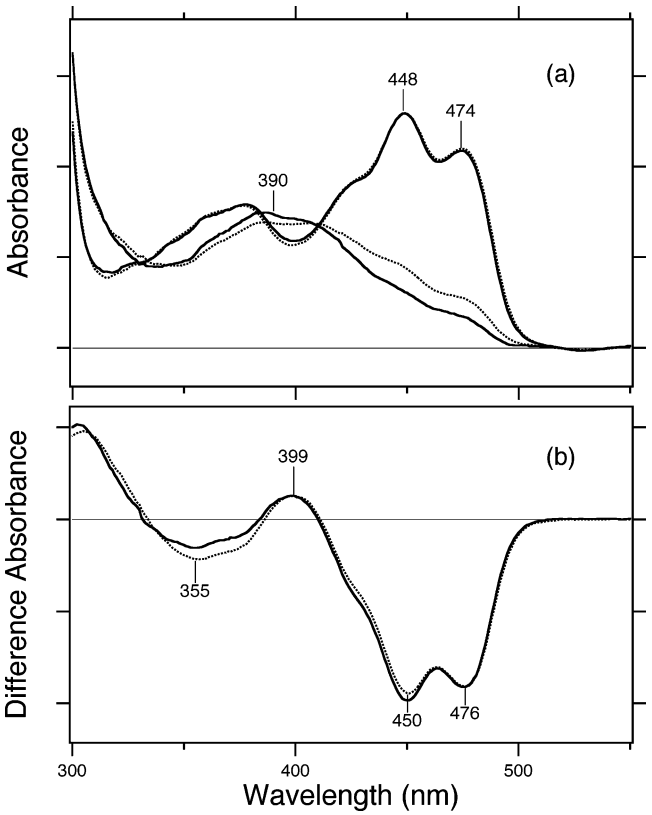

FIGURE 2: (a) UV−visible spectra of the unhydrated film (dotted line) and the hydrated film with 0.5 μL of pure water (solid line) of neo1-LOV2 in the dark, which possesses peaks at 448 and 474 nm. After light illumination, these peaks decrease and broad peaks appear at 386 nm (S390 intermediate). Decrease of the peaks at 448 and 474 nm is less for the unhydrated film (dotted line) than for the hydrated film with 0.5 μL of pure water (solid line). (b) Light-minus-dark difference UV−visible spectra of the unhydrated film (dotted line) and the hydrated film with 0.5 μL of pure water (solid line) of neo1-LOV2. Difference spectrum of the unhydrated film is multiplied by 1.1 so that the amount of the unphotolyzed state converted to S390 becomes the same. Spectra are measured at 295 K, and one division of the *y*-axis in panels a and b corresponds to 0.5 absorbance unit.

We inferred that structural heterogeneity of neo1-LOV2 at low temperatures prevents adduct formation between FMN and the reactive cysteine. The present observation suggests that some population of neo1-LOV2 molecules in the unhydrated film possesses similar structural heterogeneity that prevents adduct formation. Despite the presence of such an unreactive portion, light-induced difference spectra look similar between hydrated and unhydrated films (Figure 2b).

Difference FTIR Spectra between the S390 and Unphotolyzed State. We then measured difference infrared spectra of neo1-LOV2 under various hydration conditions. The FTIR difference spectra between the S390 and unphotolyzed states were very similar for hydrated and unhydrated films in the 1600−950 cm^{-1} region (data not shown). Since the structural changes of FMN mainly appear in this frequency region, this result indicates similar changes of the FMN chromophore between the films with different hydration conditions. Such observation was also the case for different temperatures (*16*).

On the other hand, highly different spectral features were gained for the amide-I region. Figure 3a shows the FTIR difference spectra between the S390 and unphotolyzed states in the 1750−1600 cm^{-1} region. For the unhydrated film, peaks were observed at 1724 (+), 1710 (−), 1694 (+), 1687 (+), 1675 (−), and 1644 (−) cm^{-1} (thick solid line at the top of Figure 3a). The spectrum of the unhydrated film has little absorbance at 1630−1620 cm^{-1}, which increases upon hydration together with the bands at 1655 (+) and 1643 (−) cm^{-1} (Figure 3a). In contrast, positive bands at 1694 and 1687 cm^{-1} are reduced upon hydration. Interestingly, the spectrum for the unhydrated film (thick solid line at the top of Figure 3a) is similar to that of the fully hydrated film at 100 K (thick solid line at the top of Figure 3b), and the effect of hydration is also similar to that of increasing temperature. In fact, positive band at 1694 cm^{-1} only appears at low hydration or low temperature, which lacks for the fully hydrated film at room temperature. Positive band at 1630−1620 cm^{-1} is much reduced at low hydration or low temperature.

Previous FTIR study of ^{13}C-labeled FMN and apoprotein revealed that the bands at 1727 (+)/1710 (−) cm^{-1} and at 1687 (+)/1678 (−) cm^{-1} at room temperature (thick solid lines at the bottom of Figure 3a,b) originate from the C4═O and C2═O stretching vibrations of the FMN chromophore, respectively (*26*). In addition, the positive bands at 1692 and 1687 cm^{-1} at 150 K (thick solid line at the top of Figure 3b) were assigned as the amide-I vibration and C2═O stretch of FMN, respectively (*26*). A similar spectral feature suggests that the positive bands at 1694 and 1687 cm^{-1} in the unhydrated sample (thick solid line at the top of Figure 3a) originate from the amide-I vibration and C2═O stretch of FMN, respectively. Indeed, only the positive shoulder at 1694 cm^{-1} exhibits spectral downshift by ^{13}C-labeling of apoprotein (data not shown), indicating the same origin as the case at 150 K. Regarding the C4═O stretch in FMN, the frequency is independent of hydration and temperature for the unphotolyzed state, appearing at 1710 (−) cm^{-1} (Figure 3). In contrast, the frequency of the S390 state (1727 cm^{-1}) is lowered upon dehydration (1724 cm^{-1} in Figure 3a) or upon lowering temperature (1721 cm^{-1} in Figure 3b). Thus, the hydrogen bond of the C4═O group is dependent on hydration, as well as amide-I vibrations of peptide backbone.

Dark Reversion of S390 to the Unphotolyzed State. Figure 4 shows the dark reversion processes from the S390 to unphotolyzed state by monitoring the absorption at 450 nm with various hydration conditions. Lower hydrated samples have longer lifetime of S390. The recovery was fitted by double exponentials for the hydrated film with pure water (Figure 4a), whereas others were fitted by double exponentials and slower components (Table 1). In fact, less hydrated samples did not return to the original state even after 3−4 h, and we had to hydrate the samples for the complete recovery. Between the two kinetic components, the major one was affected by hydration, while the minor (faster) one was almost insensitive to hydration. Presence of slower components suggests that there are multiple S390 states under less hydrated conditions. Slowed kinetic feature at low hydration is also similar to the case at low temperature, where photocycle kinetics are generally slowed down at low temperature. The overall half-life time of the recovery ($\tau_{1/2}$) was obtained to be 300, 390, 750, 2500, and 5400 s for hydration with pure water and 10%, 25%, and 50% glycerol/water and the unhydrated film, respectively.

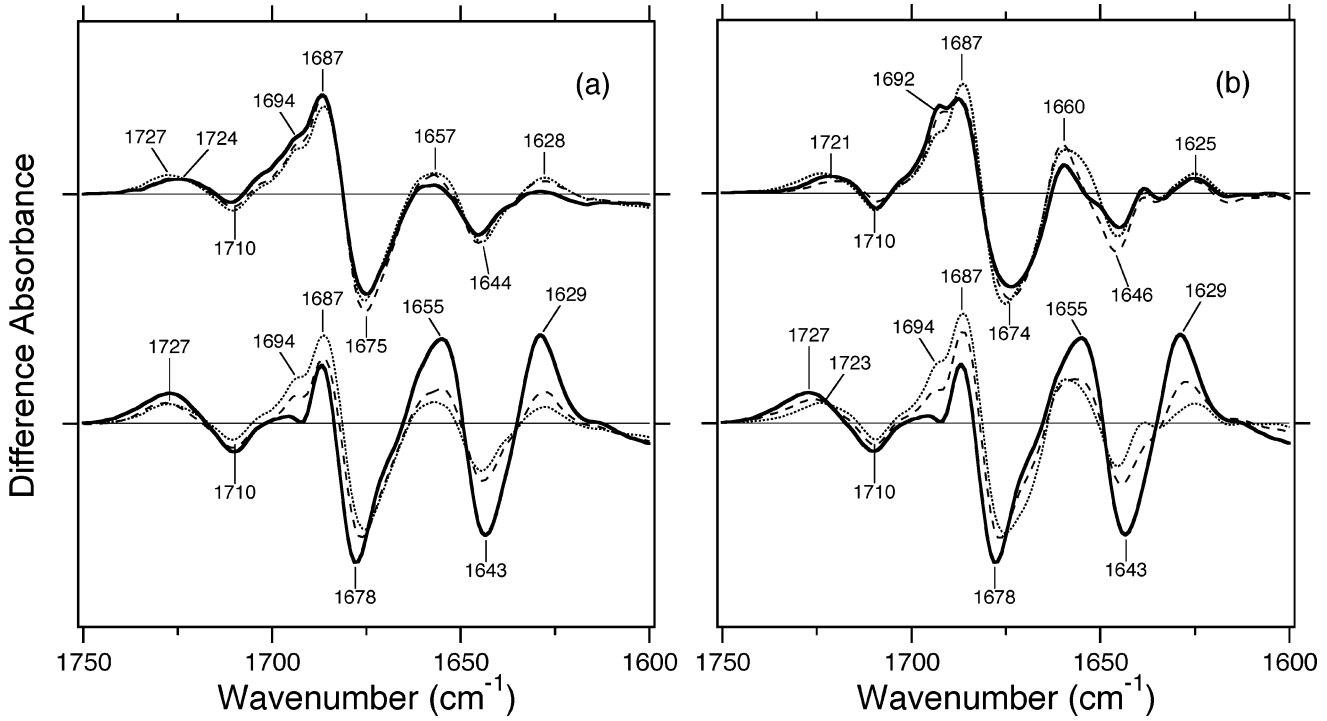

FIGURE 3: Light-minus-dark difference FTIR spectra of neo1-LOV2 in the 1750−1600 cm^{-1} region for various hydrations (a) and temperatures (b). (a) The sample was hydrated with no (thick solid line at the top), 50% (broken line at the top), 25% (dotted lines at the top and bottom), or 10% glycerol/water (broken line at the bottom) or pure water (thick solid line at the bottom) at 295 K. (b) The spectra were recorded at 100 K (thick solid line at the top), 150 K (broken line at the top), 200 K (dotted lines at the top and bottom), 250 K (broken line at the bottom), and 295 K (thick solid line at the bottom) with hydration of pure water. One division of the *y*-axis corresponds to 0.015 absorbance unit. The data in Figure 3b as well as the thick solid line at the bottom of Figure 3a are reproduced from ref *21*.

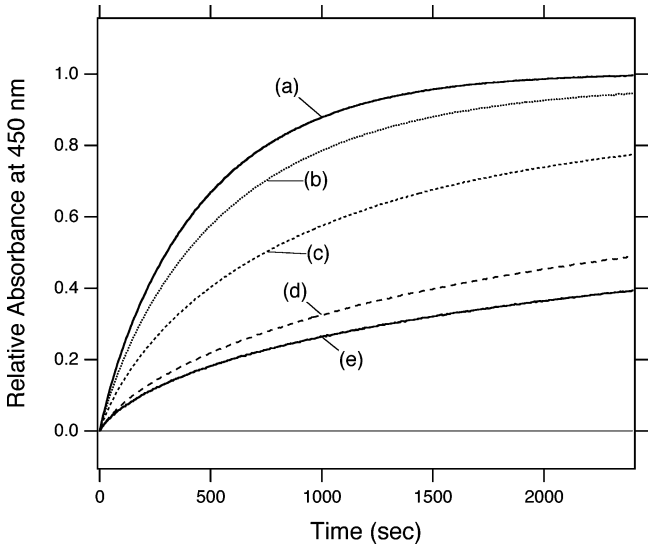

FIGURE 4: Time course of the dark recoveries of the neo1-LOV2 films hydrated with 0.5 μL of pure water (a) and 10% (b), 25% (c), and 50% glycerol/water (d), while that of unhydrated film is also shown (e). Absorption difference at 450 nm before and after illumination is normalized as 1.

Table 1: Time Constants in the Recovery Process from the S390 to Unphotolyzed State[a]

hydration	t_1 (s)	t_2 (s)	slower components (%)	overall $\tau_{1/2}$ (s)
pure water	360 (89%)	88 (11%)	0	300
10% glycerol/water	500 (75%)	150 (21%)	4	390
25% glycerol/water	890 (65%)	180 (21%)	14	750
50% glycerol/water	1480 (54%)	180 (11%)	35	2500
no	1630 (43%)	180 (10%)	47	5400

[a] t_1 and t_2 correspond to the time constants of double exponentials, while $\tau_{1/2}$ represents the half-time of the overall recovery (relative absorbance at 450 nm is 0.5 in Figure 4).

DISCUSSION

The present results showed similar effects of hydration and temperature on the protein structural changes of neo1-LOV2. At physiological temperature, amide-I vibration at 1640−1620 cm^{-1} is significantly changed, implying structural alteration of β-sheet. These vibrations were identified by use of ^{13}C-labeling of apoprotein (*26*). Such changes are largely suppressed at low hydration (Figure 3a) and low temperature (Figure 3b). While amide-I vibration of the β-sheet (1629 cm^{-1}) is reduced, amide-I vibration of turn structure (1694 cm^{-1}) newly emerges at low hydration and temperature. This feature is well visualized in Figure 5, where positive absorbances at 1629 and 1694 cm^{-1} are plotted versus numbers of water molecules per protein (Figure 5a) and temperature (Figure 5b). These observations strongly suggested that protein structural changes are similarly restricted under such conditions. The lifetime of S390 and the structural changes of the β-sheet region are inversely correlated (Figure 5a). Namely, the β-sheet structure is not perturbed, and the photocycle of neo1-LOV2 became slowed down. This observation is consistent with our previous report for the Q1029L mutant of neo1-LOV2 and the wild-type neo1-LOV1 study, both of which showed longer lifetime of S390 and limited structural changes in the β-sheet region than the wild-type neo1-LOV2 (*19*).

It is generally accepted that a fast event corresponds to the local structural changes around the chromophore in photoreceptive proteins, while a slow event corresponds to

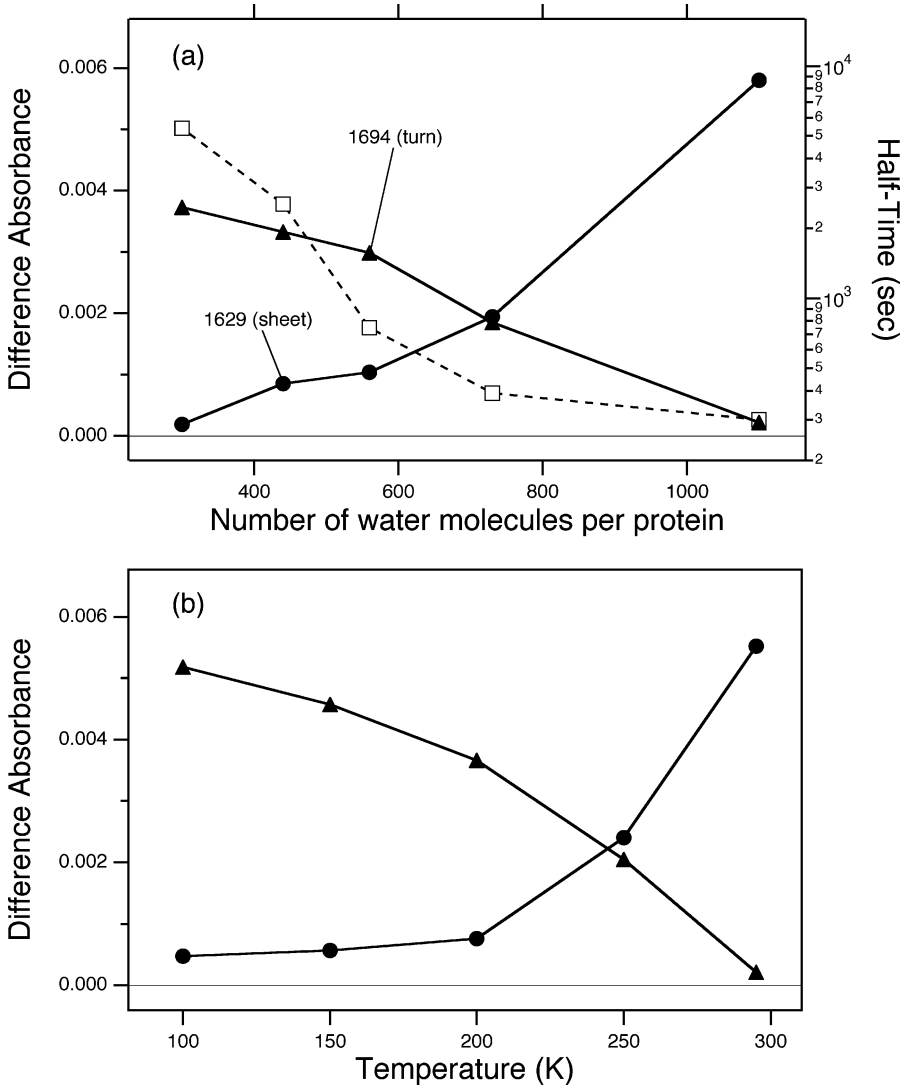

FIGURE 5: (a) Difference absorbances at 1694 cm^{-1} (filled triangles), and 1629 cm^{-1} (filled circles) in Figure 3a are plotted versus the numbers of water molecules per one protein molecule in the sample. Open squares show the half-time of the decay of S390. (b) Absorbances at 1694 cm^{-1} (filled triangles), and 1629 cm^{-1} (filled circles) in Figure 3b are plotted versus temperature.

global protein changes. That is why earlier intermediates are trapped at low temperature, while late intermediates are trapped at higher temperature. In these processes, protein fluctuation plays an important role in driving into large-scale motion of protein (*16*, *31*). Similar hydration dependence further emphasizes the importance of such protein fluctuation in forming the active state of S390, which is suppressed under low hydration or temperature. In the case of LOV domains, we are able to distinguish two states of S390, $S390_I$, and $S390_{II}$. $S390_I$ exhibits structural changes of turn structure, not β-sheet, which is stabilized at low temperature or low hydration. On the other hand, $S390_{II}$ exhibits structural changes of β-sheet largely, which is observed under physiological conditions (fully hydrated at room temperature). $S390_{II}$ is presumably the active state in LOV domains of phototropin. Although there have been no experimental observations of $S390_I$ by time-resolved measurements, we infer that it is the precursor of the active $S390_{II}$ state.

The crystal structure of the S390 intermediate reported very limited protein structural changes (*12*). In particular, there are almost no changes at the surface of protein, which raised a question on the mechanism how to output the light signal. On the other hand, global protein structural changes were suggested in hydrated films and solution by means of FTIR (*16*), NMR (*27*), and transient grating (*32*) techniques. There has been a suggestion that protein structural changes are restricted in crystals, possibly because of low hydration and/or protein−protein contact. While the latter may be important, Figure 5a shows that structural changes of the β-sheet region are small under low hydration conditions. Little change of the β-sheet region is consistent with the crystal structure of the S390 state that shows no surface changes (*12*). The analysis of protein structural changes in crystals has to be thus carefully interpreted. In the case of LOV domains, more than 1000 water molecules are necessary for the structural changes of one protein molecule under physiological conditions.

ACKNOWLEDGMENT

We thank M. Nabeno and Prof. M. Sakurai for valuable discussions.

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BI7003087