The plant blue light receptor, phot1, a member of the phototropin family (1), is a plasma membrane-associated flavoprotein that contains two (~110 amino acids) flavin-binding domains, LOV1 and LOV2, within its N terminus and a typical serine-threonine protein kinase domain at its C terminus. The LOV (light, oxygen, and voltage) domains belong to the PAS domain superfamily of sensor proteins. In response to blue light, phototropins undergo autophosphorylation. E. coli-expressed LOV domains bind riboflavin-5′-monophosphate, are photochemically active, and have major absorption peaks at 360 and 450 nm, with the 450 nm peak having vibronic structure at 425 and 475 nm. These spectral features correspond to the action spectrum for phototropism in higher plants. Blue light excitation of the LOV2 domain generates, in less than 30 ns, a transient ~660 nm-absorbing species that spectroscopically resembles a flavin triplet state. This putative triplet state subsequently decays with a ~4-μs time constant into a 390 nm-absorbing metastable form. The LOV2 domain (450 nm) recovers spontaneously with half-times of ~50 s. It has been shown that the metastable species is likely a flavin-cysteine (Cys39 thiol) adduct at the flavin C(4a) position. A LOV2C39A mutant generates the early photoproduct but not the adduct. Titrations of LOV2 using chromophore fluorescence as an indicator suggest that Cys39 exists as a thiolate.

Near-UV blue light regulates a variety of different responses in higher plants. These include phototropism, the inhibition of hypocotyl elongation, the expression of various genes, and stomatal opening. Phot1 (nph1), the recently discovered blue light receptor, is a member of the phototropin receptor family (1). Phot1 is a plasma membrane-associated flavoprotein that functions as the primary photoreceptor mediating phototropic plant movement (2–4). Phot1 has two 12.1-kDa flavin-binding domains, LOV1 and LOV2, within its N-terminal region and a typical serine-threonine protein kinase domain at the C-terminal region. Heterologous expression studies have shown that phot1 binds FMN1 as a chromophore and undergoes autophosphorylation in response to light treatment. It has therefore been proposed that this receptor functions as a light-activated serine/threonine kinase (4). The isolated LOV domains from oat phot1 (5) have been shown to undergo a cyclic photoreaction upon the absorption of light; LOV1 recovers with a half-time of 11.5 s, whereas LOV2 recovers with a half-time of 27 s (5). In addition, the quantum efficiencies for photoproduct (adduct) formation for LOV1 and LOV2 are ~0.045 and 0.44, respectively (5). The ground forms of the LOV domains have major absorption peaks at 360 and 450 nm with the 450 peak having vibronic structure at 425 and 475 nm. Upon absorption of light, the chromophore bleaches in the 450 nm region generating a species that absorbs maximally at 390 nm. This intermediate has been assigned as a flavin-cysteinyl adduct between the protein and the C(4a) carbon of the FMN chromophore. This adduct breaks down spontaneously, returning the protein to its ground form. A LOV2 mutant (LOV2C39A) in which the cysteine that forms the adduct has been mutated to alanine does not undergo this photoreaction (5).

Recently the crystal structure of the LOV2 domain from the fern Adiantum capillus-veneris phy3 (6) was solved to 2.7 Å resolution (7). Phy3 is a chimeric photoreceptor with homology to phytochrome at its N-terminal end and an almost complete phototropin at its C-terminal end. Its LOV2 domain shares a 70% sequence homology to the oat phot1 LOV2 (6). The structure indicates that the FMN molecule is held noncovalently within a chromophore-binding pocket. It places the sulfur of cysteine 39 at ~4.2 Å from the C(4a) carbon of the FMN chromophore. These observations are consistent with the light-induced formation of an FMN-cysteinyl adduct.

Here, we characterize the photocycle of the LOV2 domain of Avena sativa (oat), phot1. We have identified a new intermediate state in this photocycle and present evidence that Cys39 exists as a thiolate in the ground-state chromoprotein. We propose a photocycle scheme for this domain of the photoreceptor consistent with these observations. In addition we show that the LOV2C39A mutant undergoes a truncated photocycle in which this early intermediate reverts to the pigment’s ground form.

**EXPERIMENTAL PROCEDURES**

Light-induced Absorption Changes at Long Times—Difference spectra in the 1–100 s region were collected on a Hewlett Packard 8452A diode array spectrometer. The optical path length was 1 cm. The blue light pulse was provided by a white light camera strobe flash (1 ms, ~100 mJ pulse) filtered through Corning Glass filters 3-73 and 4-96, and a Corning 100-nm band pass filter with maximum transmission at 400 nm. Control of data acquisition and flash were automated with software written in LabVIEW (National Instruments, Austin, TX). Temperature was not controlled but was measured to be 20 ± 2 °C.

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§ This abbreviation used: FMN, riboflavin-5′-monophosphate; LOV, light, oxygen, and voltage.

† We define bleach as the amount of ground-state absorption missing at a given time in the photocycle.
Light-induced Absorption Changes at Short Times—Difference spectra in the 30-ns to 1-ms time window were collected on an instrument described previously (8). In brief, a dye laser pumped by the third harmonic of a Nd:Yag laser provided a 10-ns, 80-MW/cm² light pulse at 477 nm. The optical path length was 2 mm. A fresh sample was provided for each laser flash, allowing the averaging of absorbance data of several samples. The temperature for all measurements was 20°C. Light used to probe absorbance was polarized linearly to the magic angle (54.7°) relative to the laser polarization axis to prevent rotational diffusion artifacts (9).

Sample Preparation—Samples were prepared as outlined previously (5). In brief, the LOV domains were expressed in E. coli and purified by calmodulin affinity chromatography. The LOV2 domain used for these experiments was derived from oat phot1. The LOV2C39S mutant was made following the same procedure used for the LOV2C39A mutant (5). FMN solutions were made by dissolving FMN (Sigma) in the same buffer as that used for the protein preparation.

D₂O Exchange—A LOV2 sample was divided into two aliquots and lyophilized in the dark to a dry powder (20 h). One sample was then resuspended in D₂O (Aldrich); the second (control) sample of LOV2 was resuspended in H₂O.

Analysis—All data were transferred to a personal computer for analysis using programs written in a Matlab environment (The Mathworks, Natick, MA). Absorption difference spectra taken at different delays following light excitation pulse were arranged in the columns of a data matrix. The data matrix was then subjected to singular value decomposition (SVD) followed by global exponential fitting (10, 11). The global exponential fitting analysis assumes that the dark reactions following light excitation are first order processes. Kinetic changes are decomposed into a sum of exponential components. The exponents contain the apparent rate constants for the observed kinetic changes, and the amplitudes (pre-exponential factors) at different wavelengths represent the spectral changes associated with the exponential process and are called the b-spectra (10, 12).

Fluorescence Titrations—Concentrations of protein stock solutions were determined with a Hewlett Packard 8452A diode array spectrometer using εmax(LOV2) = 13,800 M⁻¹ cm⁻¹ (5). Stock protein was diluted with buffer (50 mM Tris, 10 mM NaCl, pH 8 for acid titrations, pH 6 for base titrations) to a concentration of about 1 μM. Corrected fluorescence excitation spectra were recorded between 300 and 515 nm with constant stirring on a Spex Fluorolog fluorometer, and fluorescence emission was monitored at 535 nm. The fluorometer was equipped with a lid containing holes fitted for a pH electrode and syringes so that pH adjustments could be made and monitored without opening the sample compartment. The pH was changed in a stepwise manner using 0.5–1 M HCl or NaOH and was monitored with a Corning Digital 110 meter and a Beckman Futura (model 511063, Fullerton, CA) semi-micro AgCl combination electrode. The total volume change over the course of the titrations was a maximum of 3%, precluding significant volume effects.

RESULTS

Nanosecond Laser Flash Spectroscopy of LOV2—The long recovery times of the LOV2 photocycle (~200 s) precluded the use of simple signal averaging techniques to acquire transient laser-induced absorption changes over the full time range. We therefore obtained the data in two time scales using different instrumentation. For the short times (30 ns–100 μs), we used the gated diode array flowing a new 1-μl sample into the cuvette for every laser flash and recorded absorption data at about 720 wavelengths at selected delay times of 0.03, 0.13, 0.33, 1, 3, 10, and 100 μs. For extended time intervals (0.5–200 s) we used the Hewlett Packard diode array with blue-filtered narrow-band flash lamp excitation. The LOV2 absorption difference spectra at short times, averaged over eight laser flashes, are shown in Fig. 1. The spectra show a bleach of the 450 nm peak and a transient increase of absorption in the greenish and near-UV regions. This transient state relaxes within 10 μs into a metastable intermediate state that decays into the original ground state in tens of seconds (Fig. 2). LOV1 shows spectroscopically similar transitions with a much lower quantum efficiency (data not shown).

The above data show that the spectral features at 10 and 100 μs are nearly identical to those at 500 ms, indicating that the long-lived state decays in seconds. The recording time gap (100 μs–500 ms) does not therefore influence our kinetic analysis in this data set. The difference in pulse width of the excitation sources could have complicated the analysis of the data if the millisecond lamp flashes, which overlap in time with the metastable-state life span, caused second photon hits on this intermediate. Fortunately, the first absorption difference spectrum recorded in the long time window (500 ms) is nearly identical to
the spectrum recorded at 100 μs, indicating that there are no additional spectral transitions within the time gap, and second photon-induced photochemistry did not occur. This was apparent when we merged both data sets, adjusted for identical amounts of bleaching at 447 nm, as shown in Fig. 3.

The main feature of the nanosecond transient species is the absorption at longer wavelengths (500–700 nm), showing broad peaks at 510 and 660 nm. Various flavin photoproducts are known to show absorption in this wavelength range, including charge transfer states, triplet states, and neutral flavo-semiquinones (13, 14). To explore the nature of the early photoproduct, we generated the triplet and flavo-semiquinone states of FMN in aqueous solution by laser flash excitation (15, 16) and compared their spectral features with those of the intermediate states of LOV2. Although charge transfer bands of flavoenzymes also absorb maximally at wavelengths longer than that of the ground state (17, 18), they were excluded from consideration because the LOV2C39A mutant, which cannot form a charge transfer complex, formed an almost identical transient species (see below). Fig. 4 compares the LOV2 absorption difference spectra at 30 ns and 500 ms with the absorption difference spectra of the FMN triplet and semiquinone forms. Clearly, the early transient only fits well to the spectral features of well established triplet-state difference spectra of flavins in aqueous solution (15, 16).

Global Kinetic Analysis of LOV2—Transient absorption spectra are the algebraic sum of absorption spectra of all co-existing intermediate states and are not very informative. The spectra of the individual intermediate states can be obtained from the transient data by using global kinetic analysis, which involves singular value decomposition and exponential fitting. Global exponential fitting of the short time data gave a single decay time constant of 2 μs. Adding more exponential decay time constants did not improve the fit significantly. The residuals (difference between spectral data and calculated data from exponential fitting) using one exponential were already within the signal-to-noise ratio in the data. The b-spectra \( b_1 \) and \( b_0 \) from the exponential fit are shown in Fig. 1B. In general, the b-spectra reflect spectral changes associated with the exponential process and contain useful information about the kinetic scheme. If the decays of kinetic components are well separated in time, the b-spectra can be interpreted as difference spectra between decaying and forming intermediates. By assuming a given kinetic scheme, we calculated the spectra of the intermediates from the b-spectra. Because only one apparent rate was found, a linear scheme with two intermediates is sufficient to account for the data at early times. The observation of an isosbestic point around 420 nm for this transition is consistent with a two-state system. As expected, the calculated difference spectrum of the first light-activated state that we designate \( \text{LOV}^{447}_{2660, L} \) coincides with the earliest transient and has a bleach in the 450 nm region that is twice as large as that observed in the subsequent species, which we designate \( \text{LOV}^{390}_{2390, S} \). This can be explained by including a back-reaction from the \( \text{LOV}^{447}_{2660} \) species to the ground-state \( \text{LOV}^{2447} \). Because global exponential fitting produces only one rate constant, this assumption implies that \( \text{LOV}^{447}_{2660} \) decays to \( \text{LOV}^{390}_{2390} \) and back to the ground state simultaneously as follows.

\[
\text{LOV}^{2447} \rightarrow \text{LOV}^{447}_{2660} \rightarrow \text{LOV}^{390}_{2390} \]

**Scheme 1**

\(^3\) We use the following nomenclature for intermediate spectra. Each intermediate contains a base name with a superscript and subscript. The superscripts we chose are D for dark, L for light-activated, and S for signaling (we have kept them in alphabetical order). The subscripts denote the absorption maximum of the intermediate in its longest wavelength absorption band. For this paper we chose LOV2 as a base name (e.g., LOV2447). We suggest that as new intermediates are assigned they be named following this convention.
This scheme shows LOV2\textsubscript{660} decaying exponentially with an apparent single rate constant \( k_{\text{app}} = 1/\tau_{\text{app}} = 1/\tau_1 + 1/\tau_2 \). The amplitude of the bleach at 447 nm in the LOV2\textsubscript{660} species is 50% of that observed at early times for the LOV2\textsubscript{447} intermediate. This result indicates that about 50% of LOV2\textsubscript{660} returns to the ground-state LOV2\textsubscript{447} and the other 50% decays into LOV2\textsubscript{290}, forcing the time constants \( \tau_1 \) and \( \tau_2 \) to be equal and twice the observed 2-\( \mu \)s apparent time constant. Therefore \( \tau_1 = \tau_2 = 4 \mu \)s. Analysis of the slow back-reaction decay shown in Fig. 2 reveals only one time constant of 70 s, where half-life \( t_{1/2} = \ln(2)/r \).

The calculated spectra of the photointermediates are shown in Fig. 4B. The spectrum of LOV2\textsubscript{290} was obtained by adding enough ground-state spectrum to the second intermediate spectrum to eliminate negative absorption. To calculate the spectrum of LOV2\textsubscript{290} we added twice the amount of the ground-state spectrum to the first intermediate because the ground-state depletion is twice as large, with one half of it being recovered via the parallel pathway \( \tau_2 \). The spectrum calculated for LOV2\textsubscript{290} (Fig. 4B), presumably the FMN triplet state, fits well with the triplet state spectrum measured for lumiflavin in solution (19). Because the ground-state spectrum added was obtained in the Hewlett Packard spectrophotometer at much higher spectral resolution than that attainable in our flash spectrometer, we applied a 12-point Savitsky-Golay smoothing to the spectrum to have comparable spectral resolution. The molar extinction coefficients are calculated relative to the published value for LOV2, \( \varepsilon \) for LOV2 = 13,800 M\(^{-1}\) cm\(^{-1}\) (5).

LOV2C39A Forms LOV2\textsubscript{660}, Which Decays Back to the Ground State—Because it was shown earlier that the site-specific mutant LOV2C39A is apparently photochemically inactive (5), thus implicating Cys\textsuperscript{39} as the reactive protein side chain, we carried out laser flash photolysis studies on this mutant. Absorption spectra were collected in the 10 ns to 1 ms time window and are the average of 6 laser flashes. The unexpected result is that LOV2C39A shows absorbance changes at early times nearly identical to those observed for LOV2, but at late times the system returns to the original ground state and does not form the LOV2\textsubscript{390} metastable species as shown in Fig. 5. Global exponential fitting gives satisfactory residuals (data not shown) with a fit to one time constant of 72 \( \mu \)s. The quality of the analysis and the amplitude of residuals for the LOV2C39A data set were comparable with those of the wild-type pigment. We see formation of only one species, which is the spectrum of the product formed.

**Fig. 5.** A, difference absorption spectra of LOV2C39A after excitation with a 477-nm laser pulse. Spectra collected at 0.03, 0.13, 0.33, 1.0, 30, 100, and 1000 \( \mu \)s. Arrows indicate spectral changes with time. B, results of global multi-exponential fitting of difference absorption spectra from LOV2C39A. \( b_1 \) is the b-spectrum with an apparent rate constant of 70 \( \mu \)s, and \( b_0 \) is the spectrum of the product formed.

**Does LOV2C39A Exist As a Thiol (-SH) or a Thiolate(-S\textsuperscript{2-})?**—The typical pK for free thiol is around 8.5, but the values observed in proteins depend on local interactions such as ion pair formation or polarity of the environment. For example, the reaction mechanism of some cysteine-containing flavoenzymes active in the oxidoreduction of disulfide bonds involves the formation of flavin C(4a) thiol adducts as enzymatic intermediates, and the reactive species in those systems appears to be the thiolate ion with pK values well below 8.5 (17). Flavin fluorescence yield is also strongly affected by electrostatic environment and can be fully quenched in the presence of a neighboring thiolate, as was observed in the study of a mercuric ion reductase mutant (17). The close proximity of Cys\textsuperscript{39} to FMN in LOV2 (7) allowed us to probe the ionization state of Cys\textsuperscript{39} by monitoring pH-dependent perturbations in fluorescence yield. We monitored the pH-dependent fluorescence yield of aqueous FMN, LOV2, LOV2C39A, and LOV2C39S.

The titration of an aqueous solution of FMN (inset, Fig. 6) shows the typical fluorescence decrease that accompanies the deprotonation of N(3) with a pK of about 10 and a similar decrease in fluorescence in the acid region with an apparent pK around 1.7. Flavin fluorescence and absorption spectra are known to be affected by the ionization state of its N(3) atom. Deprotonation of N(3)H is accompanied by a significant decrease in fluorescence and a shift of the near-UV absorption band to shorter wavelengths. The pK of N(3) can be modulated by hydrogen bonding and the surrounding electrostatic environment. The mechanism of fluorescence quenching of flavins in solution at low pH is attributed to collisional quenching by protons (20). Riboflavin, which does not contain a phosphate...
group bound to its ribityl chain, shows the same fluorescence titration profile as FMN, indicating that fluorescence yield is not significantly affected by phosphate ionization state. Similar high and low pH fluorescence titration curves have been reported for flavins and flavinyl peptides (21). The other “titratable” groups on the chromophore, such as N(1), N(5), C(2)O, and C(4)O, have pKₐ values of less than or equal to zero and therefore do not contribute to the observed effect (14).

Fluorescence base titration of LOV2 and the two mutants LOV2C39A and LOV2C39S are similar to that of FMN, all showing the decrease in fluorescence associated with N(3) ionization. The reversibility of these titrations in the basic region shows that the N(3) group is readily accessible to bulk protons, presumably via a proton pathway provided by protein residues or structural water, which is present in the three-dimensional crystal structure of Adiantum Phyš LOV2. In addition, the reversible nature of the titrations shows that the flavin chromophore is stable in the binding pocket at alkaline pH.

Neither FMN nor the two thiol-free mutants showed fluorescence perturbations in the 4–9 pH range (Fig. 6). A fluorescence change would have been expected for LOV2 in that range indicating deprotonation of the Cys³⁹ thiol group. Strikingly, the titration of LOV2 does not show any changes in fluorescence in the 4.5–9.5 pH range, indicating that no change in the Cys³⁹ ionization state is taking place within this pH range.

In the acid region the fluorescence intensities of both LOV2 mutants decrease as observed for free FMN. Proton collisional fluorescence quenching of protein-bound FMN and free FMN (that is slowly released from the protein) contribute to the observed decrease in fluorescence yield. In contrast, the LOV2 protein shows a marked fluorescence increase below pH 4. We interpret this fluorescence increase to reflect the protonation of the Cys³⁹ thiolate with an apparent pKₐ less than 4. This low pKₐ explains why no fluorescence perturbation was observed in the 4–9 pH range and suggests that Cys³⁹ is ionized (i.e., S⁻) in LOV2 under physiological conditions. The calculation of a precise value for the Cys³⁹ pKₐ is complicated by slow release of FMN from the protein at low pH. This release of chromophore also partially masks the maximally observable fluorescence increase of protein-bound FMN due to thiolate protonation. Maximal fluorescence levels as high as that observed for LOV2C39A were attained at low pH in some experiments. We conducted fast titrations, in which the pH was rapidly changed from 7 to 2.8 and returned to 7 within 60 s, to explore the observed low pH fluorescence changes in more detail. The pH-induced fluorescence changes of LOV2 were almost fully reversible, and chromophore loss was slow (data not shown). Chromophore loss was faster for LOV2C39A than for LOV2 and slower for LOV2C39S than for LOV2. This progression parallels the decrease in polarity and hydrogen bonding potential of the groups at low pH, e.g. -OH > -SH > methyl. Chromophore release in LOV2 can also be effected by the addition of iodoacetamide (known to react exclusively with thiolate (22)) at neutral pH, whereas there was no effect on the LOV2C39A mutant (data not shown).

The assignment of Cys³⁹ as a thiolate is also supported by the relative fluorescence levels observed for LOV2 and the two mutants in the 4–10 pH range. These fluorescence levels reflect the electrostatic environment of the chromophore. The LOV2C39A mutant (containing nonpolar alanine) shows the highest fluorescence yield followed by LOV2C39S (containing polar serine) and the LOV2 protein, which contains a negatively charged thiolate and shows the lowest fluorescence yield (Fig. 6). A similar effect was noted in thioredoxin reductase, in which a neighboring serine quenched flavin fluorescence to a greater extent than a neighboring protonated thiol (23).

The UV-Visible absorption spectra of LOV2 wild type and the two mutants, LOV2C39A and LOV2C39S, are shown in Fig. 7. The absorption spectra of the three proteins are nearly identical in the blue spectral region but show key differences in the near-UV region. These bands are strongly affected by electron redistribution in the chromophore, such as those associated with deprotonation of N(3) (24). These bands may also reflect changes in the electrostatic environment of the chromophore as well as hydrogen bonding (24, 25). The LOV2 protein spectrum shows a single maximum at about 375 nm with a slight shoulder at shorter wavelengths, whereas spectra for both the LOV2C39A and the LOV2C39S mutants have two nearly equal bands. This is consistent with Cys³⁹ perturbing the local chromophore electrostatic environment to a greater extent than the corresponding group in either LOV2C39S, which contains the polar -OH group or the nonpolar -CH₃ group in LOV2C39A. Because the -SH group is less polar than -OH, the Cys³⁹ would be expected to affect the electrostatic environment significantly in its negatively charged thiolate form. Although not specifically mentioned in the original cited publications, we have noticed that analogous effects occur in both lipoamide dehydro-
genase (18) and in a mutant of the flavoenzyme mercuric ion reductase. Mutating three of the four cysteine residues to alanine in mercuric ion reductase yielded an absorption spectrum with a single near-UV peak (17) similar to that observed for LOV2. The remaining cysteine, Cys\(^{140}\), of the mutated mercuric reductase was readily titrated and found to have a \(pK_a\) of about 6.7 (17). If the protein was taken to lower pH (such that the thiol was fully protonated), or if Cys\(^{140}\) was mutated to serine, the near-UV peak became a double band, confirming the effect of a thiolate negative charge on this near-UV flavin band.

\[ \text{D}_2\text{O Exchange} - \text{To investigate the possible role of a proton transfer reaction in the rate-limiting step of the back-reaction, we measured the back-reaction rate after exchanging D}_2\text{O for H}_2\text{O. The back-reaction in D}_2\text{O is three times slower than in H}_2\text{O as shown in Fig. 8. The ground-state absorption spectrum of LOV2 in D}_2\text{O is unaltered; the light-induced spectral changes are also the same as shown in Fig. 2. Usually slowing of a thiolate negative charge on this near-UV flavin band.} \]

\[ \text{The LOV2 Photocycle and Its Mechanism} - \text{We have identified only one transient species preceding the formation of the metastable state LOV2}^{390} \text{ in LOV2. We chose the superscript S to indicate a possible signaling state of the protein, as has been found for the longest lived intermediates of other sensory systems (36). Upon absorption of a photon, LOV2 presumably is raised to its singlet excited state, which decays in times shorter than our time resolution (\(-30 \text{ ns}\)) to form an intermediate that we call LOV2}^{460}. \text{As stated earlier, the spectral properties of ion pair formation with a proton donor/acceptor group (22, 26, 27), hydrogen bonding (28, 29), and charge transfer complexes (to flavin (17)) and/or by indirect stabilization such as helix dipole interactions (30). We find no evidence for the expected long wavelength absorption typical of flavin-sulfur charge transfer complexes. A charge pair formation by partial or total proton transfer, with or without involvement of hydrogen bonding interactions, from the thiol to a neighboring group(s) seems the most plausible. The crystal structure of LOV2 (phy3 LOV2) (7) shows no basic residues (arginine, lysine, etc.) in the immediate proximity of Cys\(^{39}\). At longer distances basic residues are plentiful, but it is not clear which one (if any) could be involved in the photocycle reaction. It has been shown in DsbA, a protein containing a reactive thiolate with a \(pK_a\) value around 3.5, that site-specific replacement of all nearby charged residues did not modify the low cysteine thiol \(pK_a\) (31). The mechanism for thiolate stabilization in DsbA remains unclear. We therefore cannot identify on a structural basis which amino acid side chain stabilizes the thiolate and acts as the proton donor/acceptor group in the photocycle scheme. However, the crystal structure shows a water molecule in the vicinity of the FMN chromophore (7). It has been demonstrated that in bacteriorhodopsin structural water is required to stabilize the protein and participates in intramolecular proton transfer reactions during the proton-pumping photocycle (32, 33). This water itself could be either the proton donor/acceptor group, or it could act as a bridge between the cysteine and another remote amino acid side chain that donates/accepts the proton. It might also be possible that no single amino acid side group acts as the proton donor/acceptor but rather that a group of side chains and structural water provides an electrostatic environment that is modified by protein conformational changes associated with the LOV2 photocycle. In bacteriorhodopsin several protein groups and structural water contribute to the protonated Schiff base counterion (94, 35) and to the proton transfer reaction associated with the ion pumping process (35).

\[ \text{DISCUSSION} \]

\[ \text{Protein-Chromophore Interactions in the LOV2 Ground State} - \text{The data presented here strongly suggest that the ground state of LOV2 contains the thiolate form of Cys\(^{39}\). This hypothesis is supported by the following observations: (a) the constancy of the fluorescence intensity over a wide pH range (4.5–9.5); (b) the lower fluorescence yield in the wild type versus either of the Cys\(^{39}\) mutants, suggesting the presence of a quenching charged species; (c) the reversible fluorescence increase for the wild type at low pH, as expected if protonation removes such a charged group, and the fact that neither of the cysteine-deficient mutants shows this low pH titration profile; (d) the near-UV spectral differences between LOV2 and both Cys\(^{39}\) mutants.} \]

\[ \text{We considered the possibility of hydrogen bonding between the Cys\(^{39}\) sulfur and flavin N(5) because the distance, measured from the crystal structure, is about 3.6 Å. This distance is within the range of typical hydrogen bonds. However, we cannot fully account for all the above observations with arguments based solely on such an interaction.} \]

\[ \text{A thiolate must be stabilized by direct interactions such as dipole interactions (30). We find no evidence for the expected long wavelength absorption typical of flavin-sulfur charge transfer complexes.} \]

\[ \text{We measured the back-reaction rate after exchanging D}_2\text{O for H}_2\text{O. The back-reaction in D}_2\text{O is three times slower than in H}_2\text{O as shown in Fig. 8. The ground-state absorption spectrum of LOV2 in D}_2\text{O is unaltered; the light-induced spectral changes are also the same as shown in Fig. 2. Usually slowing of a thiolate negative charge on this near-UV flavin band.} \]
this intermediate strongly resemble those of well characterized triplet states of flavins and are distinctly different from that of the flavin-neutral semiquinone. The data analysis indicates that only 50% of LOV2 excited to the adduct LOV2\textsuperscript{390} and the other 50% returns back to the ground-state LOV2\textsuperscript{390}. Because the measured quantum efficiency for LOV2\textsuperscript{390} formation is around 0.44 (5), the singlet excited state must undergo singlet/triplet intersystem crossing with an efficiency as high as 88%. The LOV2\textsuperscript{390} intermediate spectrally resembles the mercuric ion reductase thiol-C(4a) adduct (17). Based on this similarity it was previously proposed that the LOV2\textsuperscript{390} intermediate involves formation of a covalent bond between the LOV2 protein and the FMN chromophore (5). Fig. 9 shows the kinetic scheme for the LOV2 photocycle and the details of the proposed mechanism. Because the ground state of the pigment is thought to contain an ionized Cys\textsuperscript{39} we have to postulate the existence of a proton donor/acceptor group in the protein that actively participates in the reaction mechanism. The proton held by this group is donated to the flavin N(5) during the formation of the postulated C(4a)-thiol adduct. This latter bond is presumably formed by nucleophilic attack of the sulfide of Cys\textsuperscript{39} on the C(4a) carbon of FMN. This reaction should be strongly favored because the well known charge redistribution occurring in the triplet state polarizes the N(5)–C(4a) double bond (37, 38). The carbon acquires in this process a fractional positive charge and the nitrogen a fractional negative charge increasing significantly the N(5) pK\textsubscript{a} (38).

We propose the following mechanism. The increase in basicity causes the N(5) to become protonated by a proton-donating group in the protein. Upon protonation of N(5), the N(5)–C(4a) double bond becomes a single bond leaving a very reactive C(4a) carbo-cation that undergoes attack by the cysteine sulfide, resulting in formation of the long lived intermediate LOV2\textsuperscript{390}. In addition, the distance between the C(4a) carbon and the Cys\textsuperscript{39} thiolate is expected to change upon creation of a tetrahedral sp\textsuperscript{3} carbon from a planar sp\textsuperscript{2}. A decrease in distance could be important in the progress of this reaction. The C(4a) adduct contains an asymmetric chiral C(4a) center that may be responsible for the large light-induced chromatophore circular dichroism changes observed previously (5). The proton transfer reaction therefore initiates the process. In the absence of the large pK\textsubscript{a} shift induced by light activation this reaction cannot occur. In the model the sharp increase in the pK\textsubscript{a} of N(5) is the “molecular switch” that drives the photoreaction.

Our mechanism for the formation of the flavin-thiol adduct is based largely on that proposed by Miller et al. (17) for mercuric ion reductase in which a thiolate (generated by reduction of a cystine S–S bond) binds spontaneously to the flavin C(4a). The main difference is that in LOV2 the thiolate is the stable chromophore form, and modulation of the N(5) pK\textsubscript{a} by light activation promotes the formation of the flavin-cysteine adduct. A mechanism using similar arguments but not involving a stable Cys\textsuperscript{39} thiolate has been recently proposed for LOV2 (7).

The back-reaction must involve a reversal of this scheme. The ionized group that donated the proton in the forward reaction is now a base. In a base-catalyzed reaction, a proton from N(5) is abstracted by this group, resulting in the formation of LOV2\textsuperscript{390}. In this configuration the flavin and the protein are presumably in strained conformations and return to a lower energy state by breaking the S–C(4a) bond and regenerating the C(4a)–N(5) double bond. The back-reaction is three times slower in D\textsubscript{2}O as in H\textsubscript{2}O, indicating that formation or breakage of bonds involving hydrogen atoms and/or proton transfers are rate-limiting steps during this back-reaction. Interestingly, the yield of LOV2\textsuperscript{390} in D\textsubscript{2}O is nearly identical to that in H\textsubscript{2}O, suggesting that such processes are not rate-lim-
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