Characterization of a Flavin Radical Product in a C57M Mutant of a LOV1 Domain by Electron Paramagnetic Resonance†

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ABSTRACT: In the flavin mononucletide-binding LOV1 domain of the Phot1-receptor from *Chlamydomonas reinhardtii* the photoreactive cysteine C57 has been replaced by methionine. Photoexcitation of this C57M mutant yields a metastable photoproduct (C57M-415) that thermally decomposes into a stable paramagnetic species (C57M-675) with extremely red-shifted absorption in the visible range. In this contribution, we describe the characterization of this radical by multi-frequency electron paramagnetic resonance and electron−nuclear double resonance. The main features of the spectra identify the paramagnetic species as a flavin neutral radical. However, detailed analysis shows that the isoalloxazine moiety of the flavin is alkyl substituted at N(5), rather than protonated as is usually the case. The implication of these observations on the likely mechanism of photoproduct generation in wild-type LOV domains is discussed.

Phot proteins comprise a family of blue-light photoreceptors (phototropins and homologues) in plants (*1*, *2*). These blue-light receptors regulate biological responses such as plant movement (phototropism) (*3*, *4*), chloroplast relocation (*5*−*7*), and stomatal opening (*8*) as well as gametogenesis in green algae (*9*). Phot receptors contain two photoactive LOV (light, oxygen, and voltage change sensitive) domains and a kinase domain. Each of the two LOV domains, LOV1 and LOV2, contains a single flavin mononucleotide (FMN)[1] chromophore. In the ground state (LOV-447), these are noncovalently bound to the protein. An X-ray structure of the LOV2 domain from the fern *Adiantum capillus veneris* has been solved at 2.7 Å resolution (*10*) and of the LOV1 domain from *Chlamydomonas (C.) reinhardtii* at 1.9 Å (*11*).

After light excitation, in probably all LOV domains, the FMN cofactor undergoes intersystem crossing (ISC) into the excited triplet state on a nanosecond time scale (*12*, *13*). The flavin triplet then decays on a microsecond time scale, concomitant with the appearance of a meta-stable photoproduct with an absorption maximum at 390 nm, LOV-390 (*14*). By NMR spectroscopy, this species has been shown to be an FMN-C(4a)−cysteinyl adduct (*15*), a result later confirmed by X-ray crystallography (*11*, *16*). The adduct formation is thought to trigger the kinase activity of the enzyme for downstream signaling. It decays within minutes back to the ground state, LOV-447. This reaction scheme is summarized in Figure 1.

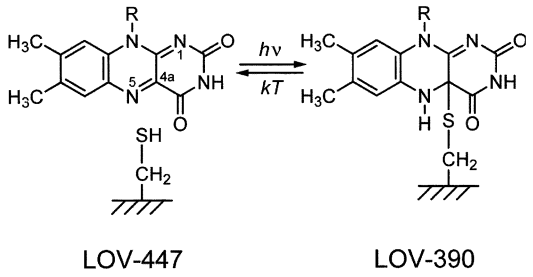

FIGURE 1: The formation of a cysteinyl-flavin-C(4a) covalent adduct (LOV-390) in a LOV domain following the absorption of blue light by the FMN cofactor bound to the ground-state species (LOV-447).

The molecular mechanism of the FMN-C(4a)−cysteinyl adduct formation is not yet unequivocally established. An ionic reaction mechanism has been suggested in which the cysteine is deprotonated and thus present as a thiolate (*12*). However, data from vibrational spectroscopy contradict this suggestion and imply that in the dark the cysteine is in its protonated thiol form (*17*, *18*). On the basis of these findings and on the observation that the flavin triplet is a potent oxidant capable of withdrawing an electron even from a remote redox-active amino acid, a radical-pair reaction mechanism should be considered (*19*, *20*).

To better characterize the mechanism of the photoinduced flavin-C(4a)−cysteinyl adduct formation, several mutations have been introduced into the LOV domains, e.g., replacement of the reactive cysteine residue by alanine or serine. These mutations preclude adduct formation. Instead, in some of them FMN neutral radicals can be generated upon photoexcitation (*19*, *20*). In a LOV1 C57M mutant in which the cysteine is replaced by methionine, photoactivity similar to the wild-type is observed (*21*). Upon blue-light irradiation, this mutant bleaches and forms a metastable photoproduct (C57M-415) with two absorption maxima at 350 and 415 nm. This photoproduct decomposes thermally with a time

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[1] Abbreviations: CPD: cyclobutane pyrimidine dimer; cw: continuous wave; DFT: density functional theory; EPR: electron paramagnetic resonance; ENDOR: electron−nuclear double resonance; FAD: flavin adenine dinucleotide; FMN: flavin mononucleotide; hfc: hyperfine coupling; ISC: intersystem crossing; RF: radio frequency.

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constant of 2 h at 40 °C (*21*). The resulting blue product (C57M-675) shows absorption maxima at 320, 370, 450, 625, and 675 nm and is exceptionally stable under aerobic conditions for weeks. The UV/vis spectral features of C57M-675 are reminiscent of a flavin neutral radical; however, the long-wavelength absorption band is significantly (55 nm) red-shifted. Here, we show by multi-frequency electron paramagnetic resonance (EPR) that C57M-675 is indeed a flavin radical species, and by using a combination of EPR and electron−nuclear double resonance (ENDOR) techniques we have provided a detailed molecular characterization. In conjunction with the related study (*21*), we provide a convincing picture of the reaction products in the LOV1 C57M mutant protein.

MATERIALS AND METHODS

LOV1 Expression. Phot1 LOV1 was expressed with a His-Tag or as a fusion protein with maltose binding protein in *Escherichia coli* and purified as described previously (*13*). LOV1−C57M was generated by site-directed mutagenesis and purified as the wild type**.** Samples were transferred into the desired buffer (usually 10 mM sodium/potassium phosphate, pH 8, 10 mM NaCl) in H_2O or D_2O by dilution and ultrafiltration through microconcentrators (Amicon C10) at 4 °C. The cycle was repeated five times to give a final D_2O enrichment of 93−97%.

EPR Sample Preparation. The enzyme preparations were transferred into 3 mm (i.d.) quartz tubes for X-band (9−10 GHz) EPR and 0.7 mm (i.d.) quartz tubes for W-band (93−95 GHz) EPR. The LOV1 C57M mutant samples were then illuminated with light of 420−480 nm from a filtered Xe-lamp (ILC PS800SW-1) and afterward warmed to 40 °C for 4 h. LOV1 C57S mutant samples were illuminated under anaerobic conditions in the presence of EDTA. All samples were then frozen in liquid nitrogen.

EPR Instrumentation. Continuous-wave (cw)-EPR spectra at X-band frequencies (9−10 GHz) were obtained using a laboratory-built spectrometer. It consists of a X-band microwave (mw) bridge (Bruker ER041MR) and an AEG-20 electromagnet. Samples were placed in a dielectric resonator (Bruker ER4118X-MD-5W1), which was immersed in a laboratory-built helium-gas flow cryostat regulated by a temperature controller (LakeShore 321).

cw-EPR spectra at W-band frequencies (93−95 GHz) were obtained with one of two spectrometers: (a) A laboratory-built high-frequency/high-field EPR spectrometer equipped with a cylindrical TE_{011} cavity. The six-line EPR signal of a Mn(II)/MgO standard, placed near the sample in the cavity, was recorded simultaneously for *g*-factor calibration. (b) A commercial W-band EPR spectrometer (Bruker Elexsys E680). A Li/LiF sample was used for subsequent *g*-factor calibration.

ENDOR Instrumentation. cw-ENDOR spectra were recorded using a laboratory-built spectrometer consisting of an AEG-20 electromagnet and a X-band mw bridge (Bruker ER041MR). A radio frequency (RF) synthesizer (Hewlett-Packard 8647A) in conjunction with a high-power RF amplifier (ENI A-300) was used to generate the cw RF field in the laboratory-built TM_{110} ENDOR resonator ($Q \approx 1800$, 1 turn per mm NMR coil). The temperature was adjusted using a nitrogen-gas flow regulated by a temperature controller (Bruker ER4111VT).

Pulsed ENDOR spectra were recorded using a Fourier transform EPR spectrometer (Bruker ESP 380E) equipped with a pulsed-ENDOR accessory (Bruker ESP 360D−P) including an RF amplifier (ENI A-500) and a dielectric-ring ENDOR resonator (Bruker ER4118-MD-5W1-EN), which was immersed in a helium-gas flow cryostat (Oxford CF935) regulated by a temperature controller (Oxford ITC-4). For Davies-type ENDOR a pulse-sequence using 64 and 128 ns microwave $\pi/2$- and π-pulses, respectively, and an 8-μs RF pulse were used.

Calculations. To assist in the assignment of experimentally determined hyperfine couplings (hfcs) to individual nuclei in the FMN radicals, density functional theory (DFT) calculations were performed using the program package Gaussian 98 (*22*). The geometries of the FMN radicals were optimized at the unrestricted B3LYP/EPR−II level of theory and single-point calculations of hfcs were performed at the same level.

RESULTS

EPR. Figure 2 shows the 9-GHz cw-EPR spectrum of C57M-675 recorded in derivative mode ($d\chi''/dB_0$). The spectra of C57M-675 in protiated and deuterated buffer are compared in traces a and b. Both EPR spectra closely resemble those of protein-bound neutral flavin radicals such as those observed in CPD photolyases (*23*), although it should be noted that the *g*-factor of C57M-675 is slightly higher, 2.0039 as opposed to 2.0034 of the neutral flavin radical in CPD photolyase (*23*, *24*), and the peak-to-peak EPR line width of 1.69 mT is slightly smaller than the 1.87 mT determined for $FADH^{\bullet}$ in CPD photolyase (*23*). To make a more direct comparison with a flavin radical in an identical environment shown in trace c and d of Figure 2 are EPR spectra of the photogenerated FMN radical in the LOV1 C57S mutant. The isotropic *g*-factor of this species is 2.0033 and the peak-to-peak EPR line width of 2.04 mT is typical of a neutral flavin radical and is also very similar to that observed in a LOV2 C450A mutant (*20*). Partially resolved doublet structures on the low- and high-field sides are visible in the two C57M-675 spectra (Figure 2a,b) and in the spectrum of C57S in protiated buffer (see Figure 2c). In a typical neutral flavin radical, these structures are attributed to the partially resolved hfc of the proton at N(5). This assignment is based on the fact that the doublet splitting vanishes upon deuteriation of the buffer (Figure 2d) by which the exchangeable proton is replaced by a deuteron with its 6.5-fold smaller magnetic moment and, therefore, significantly smaller hfc. This also results in a substantial reduction of the EPR line width from typically 1.8−2 to 1.5 mT in a protein-bound neutral flavin radical. The spectrum of C57S in deuterated buffer (see Figure 2c) has a peak-to-peak line width of 1.32 mT, which confirms its identity as an N(5)-protonated neutral flavin radical.

The remarkable observation at this point is that in contrast to the case of LOV1 C57S and other protein-bound flavin radicals, the doublet splitting on the low- and high-field sides of the spectrum of C57M-675 does not vanish upon buffer deuteriation. Rather, the two spectra are virtually identical, although the peak-to-peak line width of 1.64 mT in deuterated buffer is marginally smaller than that in protiated buffer. A possible explanation of this fact arises when considering

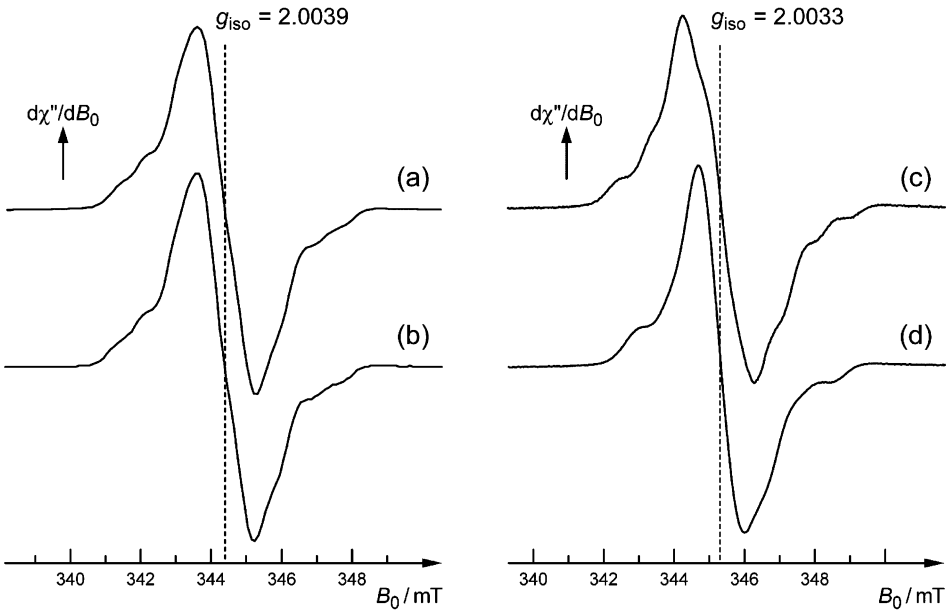

FIGURE 2: X-band frozen-solution cw-EPR spectra of C57M-675 in (a) H_2O and (b) D_2O buffer. The spectra were recorded at 150 K and a mw frequency of 9.66 GHz (1.58 μW mw power, 0.1 mT magnetic field modulation amplitude (100 kHz modulation frequency)). (c) and (d) show the cw-EPR spectra of $FMNH^{\bullet}$ bound in LOV1 C57S in H_2O and D_2O buffer, respectively. The spectra were recorded at 150 K and a mw frequency of 9.67 GHz (0.8 μW mw power, 0.1 mT magnetic field modulation amplitude (100 kHz modulation frequency)).

the chemical difference between the cysteine residue in the wild type and the methionine residue 57 in the mutant protein. Besides the additional CH_2-group between the backbone and the sulfur atom, methionine contains a terminal CH_3-group. In the wild type, protonation of FMN at N(5) by the cysteine's thiol group proton is postulated (*25*). In the C57M mutant, the corresponding reaction could be the transfer of the methionine's methyl group, leading to a nonexchangeable methylation at N(5). This could explain the invariance of the C57M-675 spectra upon H → D exchange in the buffer.

However, the structure of the EPR spectra of C57M-675 contradicts this hypothesis. A methyl group with its three magnetically equivalent protons results in a quartet splitting as opposed to the doublet splitting due to a single proton. Kurreck and co-workers (*26*) have shown that in liquid solution the methyl group in a N(5)-methylated neutral flavin radical has an isotropic hfc of (21.82 ± 0.02) MHz. Spectra simulations including a methyl group with such a hfc resulted in a total spectral width far wider than that observed for C57M-675. When reducing the hfc of a putative methyl group in the simulations to an extent where the total width of the calculated spectrum is in agreement with the experimental spectrum then the partially resolved structures are lost. We conclude, therefore, that the partially resolved doublet structures in the case of C57M-675 must arise from a single nonexchangeable proton.

To further characterize the nature of the radical species, C57M-675, we have also performed cw-EPR at W-band frequencies (93−95 GHz): the spectrum (solid line) is shown in Figure 3a. From a spectral simulation (dashed line), the principal values of the **g**-matrix, $g_X = 2.0053 \pm 0.0001$, $g_Y = 2.0038 \pm 0.0001$, and $g_Z = 2.0025 \pm 0.0001$ (*X*, *Y*, and *Z* are the principal axes of the **g**-tensor), can be extracted. These values and the shape of the W-band EPR spectrum of C57M-675 differ greatly from that of a typical protein-bound neutral flavin radical. For comparison, depicted in Figure 3b is the W-band EPR spectrum (solid line) of the flavin radical in a C57S mutant of LOV1. From a spectral simulation, the principal values of its **g**-matrix, $g_X = 2.0043 \pm 0.0001$, $g_Y = 2.0036 \pm 0.0001$, and $g_Z = 2.0022 \pm 0.0001$ are obtained. These values and the W-band EPR spectrum itself closely resemble those from the neutral flavin radical in a C450A mutant of LOV2 (*20*), and are typical of protein-bound neutral flavin radicals in general (*20*, *23*, *24*). In C57M-675 not only is the **g**-anisotropy much larger—resulting in the more rhombic than axial line shape—but the average *g*-value is much higher: 2.0039 ± 0.0001 compared with 2.0034 ± 0.0001 in LOV1 C57S. One possible reason for this increase could be spin−orbital coupling due a relatively heavy atom interacting with the unpaired electron: The sulfur atom from the methionine residue is an obvious candidate if it is not too remote from the unpaired electron spin.

ENDOR. To further corroborate the two conclusions based on the EPR spectra, (i) C57M-675 is a FMN-based radical, and (ii) C57M-675 contains a nonexchangeable proton that shows a hfc similar to the N(5)H proton of $FADH^{\bullet}$ we have performed ENDOR experiments on this species. Figure 4 shows the cw-ENDOR spectrum of C57M-675 in (a) protiated and (b) deuterated buffer. A group of signals with hfc's of less than 2.5 MHz in the so-called matrix region is the sum of contributions from weakly coupled protons of solvent water, protons of nearby amino acid residues and protons at the N(3), C(7), and C(9) positions of the isoalloxazine ring. Interactions resulting from exchangeable protons on the flavin moiety itself and neighboring molecules, and water may be identified by ENDOR on a C57M sample in which the protiated buffer is exchanged for a deuterated one (see Figure 4b). Thus, comparing Figure 4a,b, a significant collapse in signal intensity is observed in the matrix region, consistent with the presence of a large number of exchangeable protons close to the FMN cofactor. An unambiguous assignment of these couplings requires a more

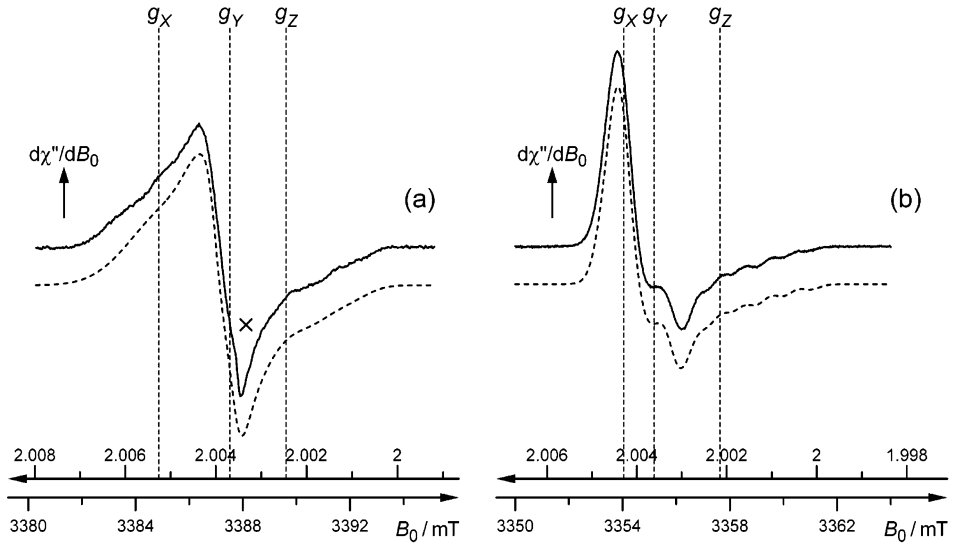

FIGURE 3: (a) W-band frozen-solution cw-EPR spectrum of C57M-675 in H_2O buffer (solid line) and simulation (dotted line). The spectrum was recorded at 210 K and a mw frequency of 95.00 GHz (5 μW mw power, 0.1 mT magnetic field modulation amplitude (8.22 kHz modulation frequency)). The × in the spectrum marks the position of a small signal contribution arising from the Mn(II)/MgO magnetic field standard. (b) W-band frozen-solution cw-EPR spectrum of the neutral flavin radical in C57S in H_2O buffer (solid line) and simulation (dotted line). The spectrum was recorded at 80 K and a mw frequency of 94.0896 GHz (50 μW mw power, 0.2 mT magnetic field modulation amplitude (100 kHz modulation frequency)). Simulations were performed using the computer program SIMPOW6 obtained from Mark Nilges and the Illinois EPR research center.

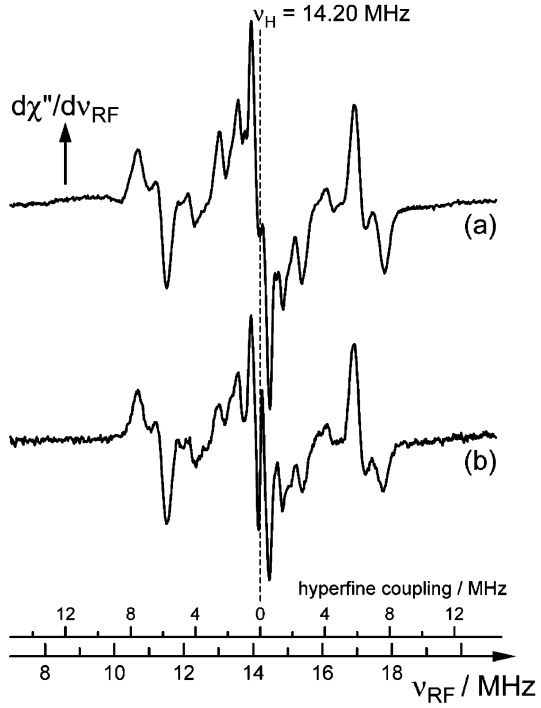

FIGURE 4: X-band frozen-solution cw-ENDOR spectrum of C57M-675 in (a) H_2O and (b) D_2O buffer. The spectra were recorded at 150 K with a mw frequency of 9.35 GHz at a magnetic field of 333.51 mT (2.4 mW mw power, 40 kHz RF modulation amplitude at 19 kHz RF modulation frequency).

systematic ENDOR examination of specifically isotope-labeled enzymes. This, however, is beyond the scope of the present contribution. Thus, here we will concentrate on the hfcs that can be readily assigned to specific groups within the flavin cofactor.

The other characteristic features of the cw-ENDOR spectrum of the FMN radical appear in the frequency ranges of 10−11.5 MHz and 16.5−18 MHz and correspond to the hfc of the methyl group attached to position C(8) of the isoalloxazine moiety. The hfc of A_{iso} = 6.01 MHz with $A_{||}$= 7.14 MHz and $A_{\perp}$ = 5.45 MHz observed for C57M-675 is approximately 20% smaller than that of FADH• in CPD photolyase (*23*). The hfc due to the proton at C(6) could also be determined with A_{iso} = 4.01 MHz. Again, it is significantly smaller (18%) compared to that of FADH• in CPD photolyase (*23*).

In the case of FADH• in CPD photolyase, small but clearly discernible changes in the cw-ENDOR spectrum arise upon H → D buffer exchange due to the replacement of the exchangeable N(5)H proton (*23*). These changes are due to the overlap of resonances from the A_x component of the N(5) proton hfc tensor (*x*, *y*, and *z* are the principal axes of the H(5) hyperfine tensor) with those of the methyl group at position C(8). No such changes are observed in the case of C57M-675, again consistent with the findings by EPR. Neither for FADH• in CPD photolyase nor for C57M-675 can the complete tensorial pattern for the proton at N(5) be obtained by cw-ENDOR. However, this is possible by pulsed-ENDOR spectroscopy as has been recently shown for the FADH• cofactor of CPD photolyase (Weber et al., manuscript in preparation), where for the N(5)H proton a rhombic hfc tensor with principal values (A_x, A_z, A_y) = (−8.5, −25.0, −37.2 MHz) could be determined in good agreement with quantum-chemical computations of this hfc (*27*).

The pulsed-ENDOR spectrum of C57M-675 is shown in Figure 5. The most prominent features in the range of 10−11.5 and 16.5−18 MHz, again as in the cw-ENDOR spectrum, correspond to the C(8α) methyl group. Note that the pulsed-ENDOR spectrum shown in Figure 5 is recorded in absorption mode (echo amplitude as a function of ν_{RF}), while the cw-ENDOR spectrum (Figure 4) is recorded in derivative mode (dχ"/dν_{RF} as a function of ν_{RF}). The additional features in the range of 4−8 MHz and 22−26 MHz correspond to a hfc with an isotropic part of about (17.9 ± 1.1) MHz. This coupling can be assigned to the proton that gives rise to the partially resolved doublet splitting

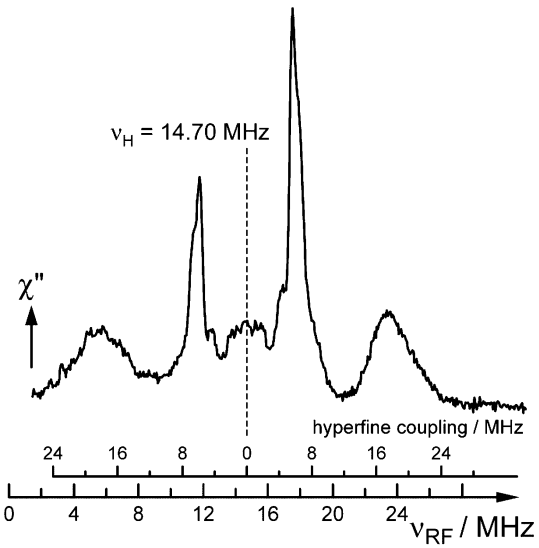

FIGURE 5: X-band frozen-solution pulsed ENDOR spectrum of C57M-675 in H_2O buffer. The spectrum was recorded at 80 K with a mw frequency of 9.68 GHz at a magnetic field of 345.27 mT.

in the EPR spectrum. In contrast to the case of $FADH^{\bullet}$ in CPD photolyase where a rhombic hfc tensor is found, we obtain here a rather isotropic hfc. The magnitude of this coupling is smaller than the isotropic average of −23.6 MHz for the N(5)H proton coupling of $FADH^{\bullet}$ in CPD photolyase, and for the methyl protons in a N(5)-methylated neutral flavin radical, (21.82 ± 0.02) MHz, obtained by Kurreck and coworkers (*26*). The shape of the line in the pulsed ENDOR spectrum of C57M-675 indicates a hfc of rather small anisotropy which is, however, subject to inhomogeneous broadening. Such a line cannot be assigned to an α-proton but instead to a β-proton. The magnitude of the isotropic part a as a function of the dihedral angle θ between the direction of the p_z-orbital carrying the unpaired spin density and the N−H bond of the hfc of β-protons is given by the Heller-McConnell relation (*28*), $a(\theta) = C_0 + C_1 \cos^2\theta$. The coefficient C_0 is usually small, and C_1 reflects the spin density in the p_z-orbital. The maximum value $a(0)$ for a β-proton is typically rather similar to the isotropic value of an α-proton. If we conclude that the hfc of 17.9 MHz is due to a β-proton in a CH_2-group, the question arises why we observe only a single hfc from one proton. This can be simply explained by the angular dependence expressed in the Heller-McConnell relation: Assuming that the second proton of a putative CH_2-group attached to N(5) makes a dihedral angle of about 90° with respect to the p_z-axis, then the hfc of this second proton tends to zero. The remaining two possible orientations for the other proton of a CH_2-group then correspond to a dihedral angle θ of about 30° or 60°. Since $\cos^2(30°) = 0.75$, a β-proton making a dihedral angle of 30° with respect to the p_z-orbital shows a hfc with only 25% reduction compared to the maximum value at $\theta = 0$. To substantiate this qualitative line of arguments, we have performed DFT calculations for a N(5)-ethyl substituted flavin neutral radical. These calculations corroborate the discussion given above and yield a hfc for one β-proton consistent with the experimentally obtained value of 17.9 MHz for a dihedral angle of 45.4°, while the dihedral angle of the second proton was 72.4° with a hfc of 4.9 MHz. The transitions due to this second β-proton would thus be overlapping with lines originating from other protons in the ENDOR spectra, and it is therefore not possible to unambiguously assign them. In summary, our EPR and ENDOR results show that the radical species in C57M-675 is a N(5)-alkylated neutral flavin radical.

DISCUSSION

In the related study (*21*), it has been found that in the C57M-675 species the FMN cofactor is covalently bound to the protein and cannot be extracted by the procedures leading to a cofactor release in the wild-type LOV1 and the C57M mutant ground-state form. Combining these findings with our results from EPR and ENDOR, we conclude that the alkylation of the FMN radical in C57M-675 is due to a covalent bond between the protein and the N(5) position of the FMN cofactor, and that the bridge is formed from the methionine residue. Such an N(5)-alkyl substitution is also consistent with the substantial shift of the absorption band in the red spectral region to longer wavelengths (*21*, *29*), the shift in the g-factor, and the relatively small hfc's. Taken together, these measurements point to a somewhat distorted flavin radical. This is to be expected if the FMN radical in C57M-675 is bound to the protein not only via hydrogen bonds to the hydroxyl groups of the ribityl side chain and O(2), N(3)H, and O(4), but also directly to the backbone of residue 57 via an ethylene bridge to N(5). The latter is likely to distort the hybridization at N(5) from sp^2, as is usual in N(5)-protonated neutral flavin radicals, toward sp^3 in the N(5)-alkylated neutral flavin radical. Such a distortion occurring at a position in the molecule with a high unpaired electron-spin density certainly affects the electronic structure. It is therefore no surprise that the spectroscopic observables, such as optical absorption, g-factor and hfcs, are also modulated in comparison with those of a typical neutral flavin radical.

Reaction Mechanism. A likely reaction scheme leading to the suggested N(5)-alkylation and covalent attachment of the FMN cofactor to the protein is shown in Figure 6. Following blue-light photoexcitation, the FMN cofactor in the LOV1 C57M mutant undergoes ISC into the triplet state, ^{3}FMN (*12*, *13*). In the wild type, ^{3}FMN has been postulated to be subsequently protonated at the N(5) position to give the $^3FMNH^+$ cation, which has a formally positive charge at the C(4a) position. This appears plausible as FMN in its triplet state is far more basic than FMN in the singlet ground state, which is only protonated at pH ≈ 0 (*30*). The thiolate would be then the nucleophile that attacks the flavin C(4a) carbocation thus forming the photoadduct. In the C57M mutant, due to the absence of a hydrogen bound to the sulfur, adduct formation cannot proceed via this pathway. Nevertheless, results presented in ref *21* unequivocally show that adduct formation still occurs.

Thus, we prefer to consider a radical-pair mechanism (*19*, *20*) (for a review on radical pair mechanisms, see ref *31*) comprising an initial electron transfer from the sulfur to the ^{3}FMN, which is consistent with adduct formation in both wild-type LOV and the C57M mutant.

Following excitation to the lowest excited singlet state, a flavin typically undergoes rapid ISC to the lowest excited triplet state (*32*, *33*). ^{3}FMN is an extremely efficient oxidizing agent in the presence of electron donors, such as EDTA or

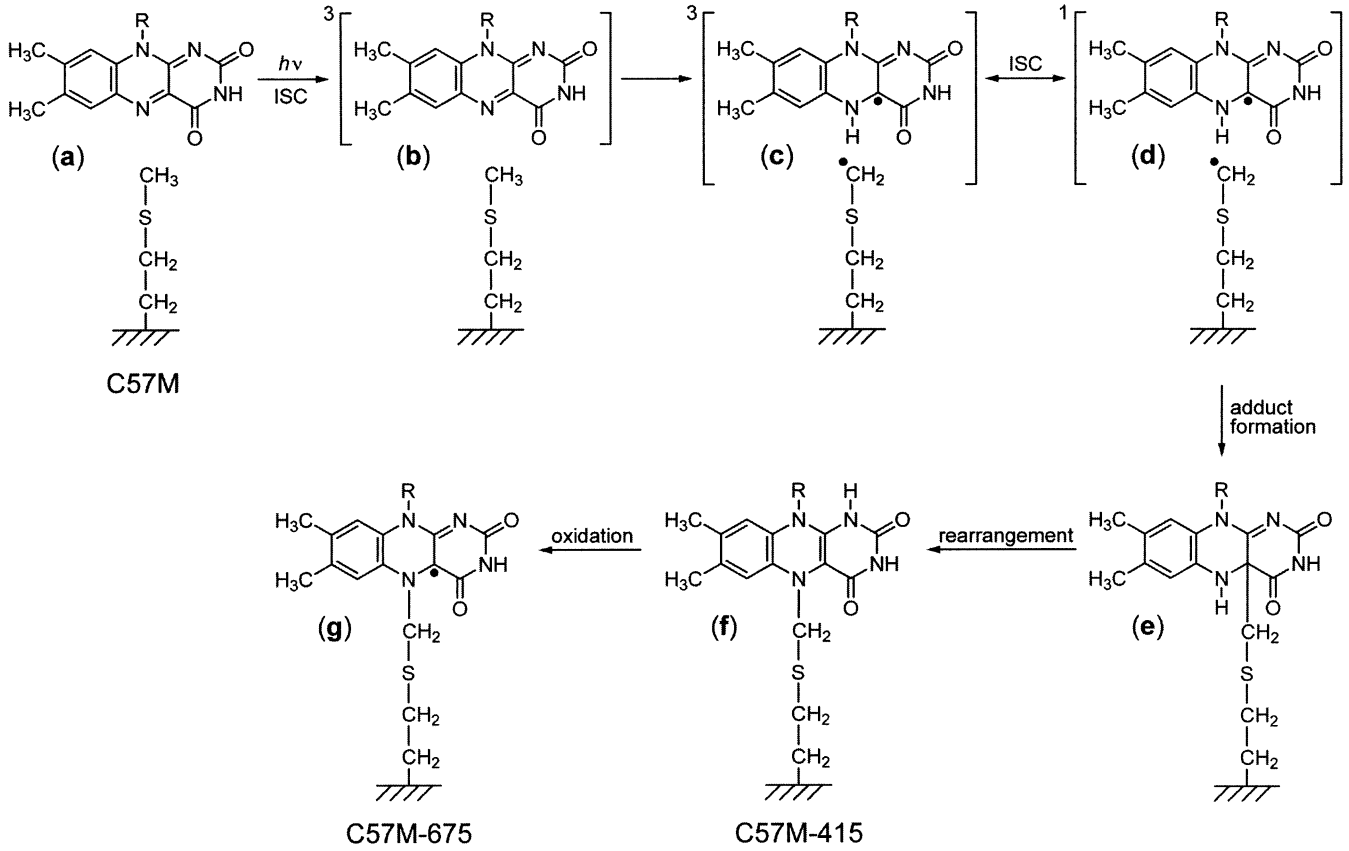

FIGURE 6: Suggested reaction mechanism for the formation of the covalently N(5)-bound FMN• radical (LOV C57M-675) in the LOV1 C57M mutant following the absorption of blue light and thermal decomposition of the intermediate. The putative radical mechanism is initiated by photoexcitation of FMN followed by ISC to form the FMN triplet state (a → b). Electron transfer followed by proton transfer generates a neutral radical pair in the triplet state (b → c) that is converted to a singlet-state radical pair by ISC (c → d). Subsequently, C(4a)-S bond formation occurs (d → e). Rearrangement results in the C57M-415 intermediate with alkylation at N(5) (e → f). Oxidation yields the observed radical species C57M-675 (f → g).

redox-active amino acids, in solution (*34*, *35*) and in various proteins, such as CPD photolyases (*36*−*38*). In all cases, a flavin semiquinone, neutral, or anionic depending on the pH, is thus formed from ^{3}FMN in a one-electron photoreduction (*39*).

Hence, it is conceivable that in both the wild type and the C57M mutant protein ^{3}FMN could abstract an electron from the cysteine/methionine sulfur and a spin-correlated radical pair consisting of a flavin anion radical, $FMN^{\bullet-}$, and a sulfur-centered radical, $RS^{\bullet+}H/CH_3$, would be formed (see Figure 6). In the wild-type protein, there could also be simultaneous or sequential proton transfer of the thiol proton to the N(5) of FMN, resulting in the formation of an neutral radical pair consisting of a neutral flavin radical, $FMNH^{\bullet}$, and a sulfur-centered radical, $RS^{\bullet}$. In the methionine mutant, there is no formally labile proton; however, the terminal methyl group could deprotonate upon electron abstraction, thus stabilizing the sulfur radical, $RS^{\bullet} = CH_2 \leftrightarrow RSCH_2^{\bullet}$. Again, the result is the formation of a neutral radical pair. The alternative is that the neighboring CH_2 group deprotonates. Although this might be expected, the structural data in the accompanying manuscript (*21*) rule out this possibility.

A radical pair created from a triplet-state precursor has initially the same spin state (i.e., $^3[FMNH^{\bullet} \cdots RS^{\bullet}/RSCH_2^{\bullet}]$), due to the conservation of angular momentum, and thus cannot form a covalent bond. This may only form if the spin state of the radical pair evolves to have singlet character by singlet−triplet mixing: $^3[FMNH^{\bullet} \cdots RS^{\bullet}/RSCH_2^{\bullet}] \leftrightarrow {}^1[FMN^{\bullet-} \cdots RS^{\bullet}/RSCH_2^{\bullet}]$. Due to strong spin−orbit coupling in sulfur-containing radicals (spin−orbit coupling constant, $\xi = 382$ cm^{-1}) a mechanism for efficient singlet−triplet interconversion exists. As soon as the radical ion pair is in its singlet state, covalent bond formation can occur, and, as depicted in Figure 6, the FMN-cysteinyl adduct is formed.

In the case of the wild type, the C(4a) adduct LOV-390 represents the product of the photoreaction. In the C57M mutant, however, additional reaction steps are required to yield to the final product C57M-675 (see Figure 6 (e → f, f → g) and (*21*)). Rearrangement of the initial C(4a) adduct to an N(5) adduct (C57M-415) is conceivable (*40*, *41*). The N(5) photoadduct can then slowly be oxidized to form the stable flavin radical C57M-675 (*40*).

CONCLUSIONS

In ref *21*, a covalent attachment of the FMN cofactor to the protein in the C57M-415 state has been established. Here, we have shown by EPR and ENDOR spectroscopy that the blue radical species, C57M-675, which occurs in the LOV1 C57M after thermal decomposition of the initial photoproduct, C57M-415, is an N(5)-alkylated neutral flavin radical. Taking these results together, we conclude that the neutral FMN radical is covalently bound to the protein via the alkyl chain of the methionine residue to N(5). The combination of multi-frequency EPR and ENDOR proved to be an important tool for identification and discrimination of

structurally distinct flavin radicals via their **g**-tensors and their hfcs, respectively.

The demonstration that the methionine residue can also form an adduct with the photoexcited FMN cofactor in a LOV domain strongly suggests that adduct formation in FMN-based photoreceptors does not necessarily require initial proton transfer from a cysteine to N(5). Instead, a radical mechanism comprising an initial electron transfer from the sulfur to the ^{3}FMN should be considered as a general primary step for adduct formation (*19*, *20*).

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