Blue Light Perception in Plants

DETECTION AND CHARACTERIZATION OF A LIGHT-INDUCED NEUTRAL FLAVIN RADICAL IN A C450A MUTANT OF PHOTOTROPIN

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The LOV2 domain of Avena sativa phototropin and its C450A mutant were expressed as recombinant fusion proteins and were examined by optical spectroscopy, electron paramagnetic resonance, and electron-nuclear double resonance. Upon irradiation (420–480 nm), the LOV2 C450A mutant protein gave an optical absorption spectrum characteristic of a flavin radical even in the absence of exogenous electron donors, thus demonstrating that the flavin mononucleotide (FMN) cofactor in its photogenerated triplet state is a potent oxidant for redox-active amino acid residues within the LOV2 domain. The FMN radical in the LOV2 C450A mutant is N(5)-protonated, suggesting that the local pH close to the FMN is acidic enough so that the cysteine residue in the wild-type protein is likely to be also protonated. An electron paramagnetic resonance analysis of the photogenerated FMN radical gave information on the geometrical and electronic structure and the environment of the FMN cofactor. The experimentally determined hyperfine couplings of the FMN radical point to a highly restricted delocalization of the unpaired electron spin in the isalloxazine moiety. In the light of these results a possible radical-pair mechanism for the formation of the FMN-C(4a)-cysteinyl adduct in LOV domains is discussed.

Numerous phenomena in the life cycle of plants such as circadian timing, regulation of gene expression, and phototropism (the adaptive process whereby plants bend toward a light source to maximize light capture for photosynthesis) are responses to ambient light levels in the UV-A and blue spectral regions comprising wavelengths of about 320–500 nm (1–3). Currently, two classes of blue light photoreceptors have been identified in plants; they are the cryptochromes (4–6) and the phototropins (7, 8), both of which are flavoproteins. Phytotropins, the blue light photoreceptors for phototropic bending (9, 10), chloroplast relocation (11–13), and stomatal opening (14), have been identified in several plant species including Arabidopsis thaliana, Avena sativa (oat), Oryza sativa (rice), and Zea mays (corn) (8). Phototropin of A. sativa is a protein comprising 923 amino acids, which is specified by the phot1 gene (previously designated nph-1) (1, 9). The protein contains two 12-kDa flavin mononucleotide (FMN) binding domains. The FMN binding modules belong to the PAS (PER/ARNT/SIM) domain superfamily (15, 16) occurring in many regulatory proteins and have been designated as LOV1 domains; the acronym is based on the involvement in the signaling of light, oxygen, or voltage levels (10, 17). Very recently, the presence of a LOV domain with similarity to the photoactive LOV domains of the phototropin of higher plants has been identified in the non-photosynthetic soil bacterium Bacillus subtilis (18).

After illumination by blue light, recombinant LOV domains of phototropin undergo a transient and fully reversible bleaching of their optical absorption at 400–500 nm accompanied by an increase of absorption at 390 nm (19, 20). Based on the similarity of the spectral characteristics of the photoprotein 19 and that of a kinetically competent intermediate in mercuric ion reductase (21), Vincent Massey‡ suggested that the LOV photocycle comprises a light-induced addition of a thiol group (cysteine 450 of phototropin in the case of the LOV2 domain from A. sativa) to the C(4a) position of the flavin chromophore followed by the spontaneous fragmentation of the adduct in the dark (see Fig. 1). This hypothesis could later be confirmed by 13C NMR spectroscopy (22).

Recently, the crystal structure of the LOV2 domain of the phytochrome/phototropin chimeric photoreceptor phy3 from the fern Adiantum capillus-veneris was solved at a 2.7 Å resolution (23). The single LOV2 cysteine residue is located 4.2 Å from the flavin atom C(4a). Until now, however, the details of the mechanism of adduct formation have not been conclusively established. The ground state, an intermediate state resembling the FMN triplet (3FMN), and the adduct (19, 20) have been observed in phototropin by optical spectroscopy, whereas with 13C and 31P NMR (22) and x-ray crystallography (24) the ground state and the adduct could be characterized. Swartz et al. (20) propose an ionic reaction pathway for
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**Fig. 1. The formation of a cysteinyl-flavin-C(4a) covalent adduct in LOV2 after the absorption of blue light by the FMN cofactor.**

FMN-C(4a)-thiol adduct formation with the Cys-450 residue initially present as a thiolate, thus requiring a donor other than Cys-450 to protonate the light-generated FMN triplet (20). Recently, however, it has been shown by Fourier transform infrared spectroscopy that the cysteine residue in LOV2 is protonated in the ground state (25), implying that an ionic mechanism, which relies on the presence of a thiolate, is flawed. Thus other potential reaction pathways including a concerted mechanism and a radical-pair mechanism have to be considered. We have therefore investigated both the wild-type A. sativa LOV2 domain and a C450A mutant by electron paramagnetic resonance (EPR) spectroscopy. The formation of the FMN-thiol adduct is not possible in the C450A mutant due to the absence of a thiol group. In this case, as will be shown, FMN undergoes a photooxidation resembling that observed in photolases (26–28) and other flavoenzymes with the possible participation of a redox-active amino acid residue, resulting in the formation of a neutral flavin radical, FMNH₂. The implications of the observation of FMNH₂ are discussed in terms of a possible radical-pair mechanism for adduct formation in LOV domains. The characterization of FMNH₂ by EPR and electron-nuclear double resonance (ENDOR) spectroscopy (29) allowed us to probe the geometrical and electronic structure and the environment of the flavin cofactor in the LOV2 domain using the paramagnetic state as a natural spin label.

**EXPERIMENTAL PROCEDURES**

**Construction of an Expression Vector**—The open reading frame specifying hisactophin of Dicyostelium discoideum was amplified using the oligonucleotides described in Table 1 as primers and plasmid pH9252 as template. The amplified DNA fragment was digested with EcoRI and HindIII and ligated into a pNCO vector (30) that had been treated with the same restriction enzymes. The resulting plasmid pNCO-HISACT-BNH was transformed into Escherichia coli strain XL1-Blue.

**Construction of Expression Plasmids**—The gene segment specifying the A. sativa LOV2 domain was excised from the plasmid pCAL-LOV2 (19) by restriction with BamHI and HindIII. The fragment was ligated into the vector pNCO-HISACT-BNH, which had been treated with the same enzymes. The resulting plasmid designated pNCO-HISLOVVT specifies a fusion protein comprising the LOV2 domain of phot1 of A. sativa and hisactophin from D. discoideum (Fig. 2). An expression plasmid specifying the corresponding C450A mutant was obtained by the same approach starting from the plasmid pCAL-LOV2/C450A (19). The plasmids were electro-transformed into E. coli strain M15[pREP4].

**Protein Expression and Purification**—Bacterial cells were grown in LB medium supplemented with ampicillin (180 μg/ml) and kanamycin (15 μg/ml) to an optical density of 0.6 at 600 nm. Isopropyl-thio-β-D-galactopyranoside was added to a final concentration of 1 mM. The cultures were incubated for 5 h, harvested by centrifugation, and stored at −20 °C. Frozen cell mass (5 g) was thawed in 15 ml of buffer A (50 mM Tris hydrochloride, pH 8.0, containing 100 mM NaCl and 2 mM CaCl₂) supplemented with 20 mg of lysozyme. The suspension was sonicated and centrifuged. The supernatant was applied to a column of Chelating-Sepharose Fast Flow (column volume, 15 ml; Amersham Biosciences), which had been equilibrated with buffer B (50 mM sodium phosphate, pH 8.0, containing 300 mM NaCl) supplemented with 10 mM imidazole. The column was washed with 150 ml of buffer B and was then developed with a linear gradient of 10–500 mM imidazole in buffer B (total volume, 300 ml). Yellow fluorescent fractions were combined and concentrated by ultrafiltration (10-kDa membrane, Pall Gelman, Ann Arbor, MI). The solution was applied to a column of Superdex S75-prep grade (2.6 × 60 cm, Amersham Biosciences), which was developed with 50 mM potassium phosphate, pH 7.5. Yellow fluorescent fractions were combined and concentrated by ultrafiltration to a final concentration of 1 mM as determined photometrically (ε = 13,900 M⁻¹ cm⁻¹ (19)).

**Clavage of the LOV2 Cys-450 Hisactophin Fusion Protein by Thrombin**—10 mg of LOV2 C450A hisactophin fusion protein in buffer B supplemented with 2.5 mM CaCl₂ were mixed with 50 units of thrombin (Sigma) and incubated overnight at room temperature. The protein solution was applied to a column of Chelating-Sepharose Fast Flow (column volume, 15 ml) that had been equilibrated with buffer B supplemented with 10 mM imidazole. Cleaved LOV2 C450A protein was collected in the flow-through, whereas hisactophin protein and uncleaved fusion protein remained bound to the column. Yellow fluorescent fractions were combined and concentrated by ultrafiltration through microconcentrators (1-kDa membrane, Pall Gelman, Ann Arbor, MI). Protein homogeneity was monitored by SDS-PAGE electrophoresis.

**UVVisible Measurements**—Protein samples (concentration = 0.05 mg/ml) were transferred into an optical cuvette (path length, 1 mm; Hellma, Mullheim, Germany) and supplemented with 0.5 mM EDTA depending on the sample and/or deoxygenated and then illuminated for up to 4 hr with 420–440 nm light from a filtered Xe lamp (JLC, PS8005W-1) at room temperature. Decay of the radical was measured in a Shimadzu UV-1601PC (Shimadzu Scientific Instruments, Columbia, MD) spectrophotometer.

**Buffer Exchange**—Samples were transferred into the desired buffer (usually 70 mM sodium/potassium phosphate, pH 7.0) in H₂O or D₂O by dilution and ultrafiltration through C10 microconcentrators at 4 °C. The cycle was repeated 5 times to give a final D₂O enrichment of 93–97%.

**EPR Sample Preparation**—The enzyme preparations were transferred into EPR quartz tubes (3-mm inner diameter for X-band (9–10 GHz) EPR, 0.6-mm inner diameter for W-band (95 GHz) EPR) under an anaerobic nitrogen gas atmosphere. They were then illuminated with light of 420–480 nm from a filtered Xe lamp and frozen rapidly in liquid nitrogen.

**EPR Instrumentation**—Continuous-wave (cw) EPR spectra were X-band frequencies (9–10 GHz) were obtained using a laboratory-built spectrometer. It consists of a Bruker ER414MR microwave (mw) bridge (Bruker, Rheinstetten, Germany) and an AEG-20 electromagnet. Samples were placed in a Bruker ER4118X-MS-SW1 dielectric resonator, which was immersed in a laboratory-built helium gas flow cryostat controlled by a LakeShore 321 temperature controller. W-band cw-EPR spectra were recorded with a laboratory-built high field EPR spectrometer operating at 94–98 GHz and equipped with a cylindrical TE011 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.

**ENDOR Instrumentation**—X-band cw-ENDOR spectra were recorded using a laboratory-built spectrometer consisting of an AEG-20 electromagnet and a Bruker ER414MR mw bridge. A radio frequency synthesizer (Hewlett Packard 8647A) in conjunction with a high power radio frequency amplifier (ENI A-300) was used to generate the cw radio frequency field in the laboratory-built TM110 ENDOR resonator (Q = 1800, 1 turn/mm of NMR coil). The temperature was adjusted using a nitrogen-gas flow controlled by a Bruker ER4111VT temperature controller.

**Calculations**—To assist in the assignment of experimentally determined hyperfine couplings (hfs) to individual nuclei in the 7,8-dimethyl isoxazoline moiety of FMNH₂, density-functional theory calculations were performed with lumiflavin (7,8,10-trimethyl isoxazoline) using the program package Gaussian 98 (31). Lumiflavin is considered as a valid model because the ribityl side chain has no significant influence on the electronic structure of the FMN moiety bound in an extended conformation in the LOV domain. The geometry of the FMNH₂ state was optimized at the unrestricted B3LYP/EPR-II level of theory, and single-point calculations of hfs and electron densities were performed at the same level.

**RESULTS**

**Optical Spectroscopy**—Expression vectors specifying fusion proteins comprising the A. sativa LOV2 domain (amino acid residues 412–516) or its C450A mutant and the actin binding
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Table I: Microorganisms, plasmids, and primers used

| E. coli strain/plasmid | Relevant characteristics | Reference |
|------------------------|--------------------------|-----------|
| M15(pREP4)            | lac,ara,gal,mlt,recA<sup>+</sup>,uvr[pREP4,lac,kan<sup>+</sup>] | 60        |
| XL1-Blue              | recA1endA1gyrA96, thi-1, hsdR17, supE44, rfiAlac[F<sup>+</sup>,proAB,lacI<sup>+</sup>,lacZΔM15,Tn10(tet<sup>+</sup>)] | 61        |
| pNCO113               | Expression vector for E. coli | 30, 62    |
| pNCO-HISACT-BNH<sup>a</sup> | pNCO113 with the gene coding for hisactophilin from D. discoideum | This study |
| pNCO-HISLOVWT         | pNCO-HISACT-BNH with the gene coding for LOV2 domain of phototropin from A. sativa | This study |
| pNCO-HISLOVC450A      | pNCO-HISACT-BNH with the gene coding for LOV2C450A mutant domain of phototropin from A. sativa | This study |
| p1MS5/c516            | pUC19 with the gene coding for hisactophilin from D. discoideum | 63        |

<sup>a</sup> The nucleotide sequence has been deposited in the GenBank<sup>™</sup> database under GenBank<sup>™</sup> accession number AF544403.

hisactophilin protein from D. discoideum were constructed as described under “Experimental Procedures.” E. coli strains harboring one of these plasmids formed copious amounts of the recombinant fusion protein (about 15% of soluble cell protein), which could be purified easily by affinity chromatography using a nickel-chelate column. The fusion proteins bind tightly to the chelating-Sepharose matrix as a consequence of the abundant histidine residues at the surface of hisactophilin. LOV2 C450A domain, from which the hisactophilin module was cleaved, was prepared with a yield of ~80% as described under “Experimental Procedures.” LOV2 C450A domain fused with calmodulin-binding protein was expressed and purified according to published procedures (19).

The optical absorption spectra of the LOV2 C450A domains from the samples under investigation before and after irradiation with blue light (420 < λ < 480 nm) are shown in Fig. 3. Both fusion proteins have virtually the same ground-state absorbance properties (Fig. 3, <i>B</i> and <i>C</i>, solid lines) as LOV2 protein cleaved from hisactophilin (Fig. 3A, solid line) but show enhanced stability and solubility. All three proteins have absorption maxima at 363 and 447 nm, characteristic of an FMN chromophore in the oxidized redox state. Shoulders at 425 and 474 nm are vibrational contributions that are well resolved. This is indicative of tight binding between the noncovalently bound FMN and the highly ordered protein structure as well as of the nonpolar nature of the flavin binding pocket.

After continuous blue light irradiation all three LOV2 C450A proteins show the formation of a flavin radical characterized by absorption maxima at 570 and 605 nm (Fig. 3, <i>A</i>, <i>C</i>–<i>D</i>) and in the presence of an exogenous electron donor such as EDTA (Fig. 3D). Radical formation occurs in the absence (Fig. 3, <i>A</i>–<i>C</i>) and in the presence of an exogenous electron donor such as EDTA (Fig. 3D). Furthermore, the radical formation could be observed either in the presence (Fig. 3, dotted lines) or in the absence (Fig. 3, dashed lines) of oxygen. However, inspection of Fig. 3 clearly shows that the yield of flavin radical differs depending on the protein construct and on the photooxidation conditions. By comparison with the absorption spectra of other flavin radicals in a protein matrix (see, e.g. Ref. 27), we conclude that in the fusion protein comprising the LOV2 C450A domain and hisactophilin, supplemented with a 10-fold excess of EDTA, the FMN cofactor is fully converted from the oxidized to the one-electron reduced semiquinone form (Fig. 3D, dotted line). To compare the amount of flavin semiquinone radical formed in the various other samples, we compare their absorbances at 605 nm normalized to the LOV2 C450A hisactophilin fusion protein sample supplemented with EDTA (Fig. 3D, dotted line). The salient points of these data are that the presence of oxygen reduces the radical yield, whereas the presence of either EDTA or the protein fusion partner hisactophilin enhances the radical yield and reduces the oxygen dependence (see Table II). In all samples investigated, the flavin radical is completely reoxidized in the dark to regenerate oxidized FMN on a time scale of minutes depending on the protein construct and buffer conditions (see Table II). We have monitored this process by observing the absorbance changes at 605 nm as a function of time after blue light illumination (see Fig. 4). Under anaerobic conditions (squares) the flavin radical in the cleaved LOV2 C450A domain (Fig. 4A) slowly decays with a 1/e time constant of (78 ± 7) min, whereas under aerobic conditions (circles) the
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TABLE II

Decay of the light-induced flavin radical in various A. sativa LOV2 C450A constructs

| Protein sample                  | Condition | Radical yield/% | Radical decay/\(\text{min}\) |
|---------------------------------|-----------|-----------------|-----------------------------|
| LOV2 C450A-hisactophilin        | Aerobic   | 90              | 8 ± 2                       |
|                                 | Anaerobic | 92              | 43 ± 5                      |
|                                 | Aerobic + EDTA | 97             | 28 ± 4                      |
|                                 | Anaerobic + EDTA | 100           | 31 ± 4                      |
| LOV2 C450A-calmodulin-binding protein | Aerobic | 29              | ND                          |
|                                 | Anaerobic | 57              | ND                          |
|                                 | Aerobic   | 20              | 7 ± 1                       |
|                                 | Anaerobic | 56              | 78 ± 7                      |
|                                 | Aerobic + EDTA | ND            | 71 ± 3                      |
|                                 | Anaerobic + EDTA | ND        | 72 ± 3                      |

* Typical error; ±5%.

The overall line width and line shape of the signal is attributed to the mostly unresolved contributions of hfc of the unpaired electron spin with \(^1\)H and \(^{14}\)N nuclei of the isoalloxazine moiety of FMN as well as of the protein environment.

To try to detect the EPR signal of a possible second radical species that ought to be created upon electron transfer to FMN in the LOV2 C450A mutant protein, EPR experiments at higher microwave frequencies (95 GHz) and correspondingly larger magnetic fields were also performed (Fig. 6), because overlapping signal contributions from organic radicals are better separated in high magnetic fields due to their differences in g-factor.

Even at 95 GHz, only the spectrum arising from one radical species was observed. Scanning over a wider magnetic field range did not reveal any additional signals except for the hyperfine lines of the Mn(II)/MgO standard used for g-factor calibration. The signal shown in Fig. 6A resembles the frozen-solution spectrum of a neutral flavin radical and is similar to the flavin adenine dinucleotide semiquinone radical observed in DNA photolyase (32). From spectral simulation of the rhombic symmetry of the signal, the principal values of the g-matrix, \(g_\text{x} = 2.0042 ± 0.0001, g_\text{y} = 2.0035 ± 0.0001\), and \(g_\text{z} = 2.0020 ± 0.0001\) \((X, Y, Z\) are the principal axes of the g-tensor) may be extracted.

Some hyperfine structure emerges in both W-band and X-band EPR spectra (Figs. 5A and 6A). Although not fully re-
solved, a spacing of 1.05 mT (29 ± 3 MHz) between adjacent shoulders in the signals could be determined. After the procedure outlined previously (32), this hyperfine splitting has been assigned to the hfc tensor component \( A_{5} \) of H (5) \( x \), \( y \), and \( z \) are the principal axes of the H (5) hyperfine tensor based on the following findings. (a) The splitting disappears when H (5) is replaced with a deuteron in an exchange of the protonated for deuterated buffer (Figs. 5B and 6B); (b) in the high field EPR experiment, the center of the hyperfine structure shifts toward resonance field values where molecules with their \( g \) axis aligned parallel to the external magnetic field are in resonance (Fig. 6A), which is characteristic for an \( \alpha \)-proton; and (c) the exchangeable \( \alpha \)-proton at N (3) is expected to contribute only marginally to the overall EPR line width due to its small hfc in the 1–2-MHz range (35).

**ENDOR Spectroscopy**—To characterize in greater detail the electronic structure of the FMN radical, we have also performed ENDOR experiments. For doublet-state radicals, two ENDOR lines are expected per proton hfc tensor component, \( A \). When \( A < 2 \nu_{H} \), the resonance frequencies are

\[
\nu_{\text{ENDOR}} = \nu_{H} \pm \frac{A}{2} ,
\]  
(Eq. 1)

or, when \( A > 2 \nu_{H} \), the resonance frequencies are

\[
\nu_{\text{ENDOR}} = \frac{A}{2} \pm \nu_{H} ,
\]  
(Eq. 2)

where \( \nu_{H} = g_{H} \nu_{L} B_{0} / h \) is the proton Larmor frequency at the magnetic field \( B_{0} \), and \( \nu_{L} \) and \( g_{H} \) are the Bohr magneton of a proton and its \( g \)-value, respectively.

In Fig. 7, A and B, the X-band cw-ENDOR spectra of the FMN radical cofactor in protonated and deuterated buffer are shown, recorded at a magnetic field of 333.86 mT, corresponding to \( g_{\text{iso}} = 2.0032 \). The frozen samples give rise to powder-type spectra that are symmetrically centered at the proton Larmor frequency, \( \nu_{H} \). A group of overlapping signals with hfc \( A \leq 2.0 \) MHz forms the so-called “matrix” ENDOR line and is the sum of contributions from the weakly coupled protons at C(7\( \alpha \)) and C(9) of the isoalloxazine moiety as well as protons of solvent water and amino acid residues near the cofactor-binding site. Their individual signal contributions are not readily assigned. One conclusion, however, may be drawn from a comparison of the matrix lines of the protein in protonated and deuterated buffer (Fig. 7, A and B); the remarkably small differences in intensity and shape indicate that the cofactor-binding site comprises mostly non-exchangeable protons.

A pair of lines with an hfc of 2.3 ± 0.1 MHz is located next to the matrix region (Fig. 7A). Based on the disappearance of these signals upon buffer deuteration (Fig. 7B) they are assigned to the proton at N(3), see Table III.

When comparing the protonated and deuterated samples in the region where the resonances of H(6) and the methyl protons H(8\( \alpha \)) are expected, two changes become obvious. First, there is a feature at 10.2 MHz that does not appear at 18.2 MHz in the protonated sample (Fig. 7A). At first glance this might be due to a large nitrogen hfc tensor. Upon deuteration, however, the spectrum is restored to symmetry, demonstrating that this feature is due to a proton hfc tensor component (Fig. 7B). It must be a very large hfc tensor component that has been reflected through zero frequency, because it only appears on one side of \( \nu_{H} \). From Equation 2, a hfc of 48.8 ± 1 MHz may be estimated. This can only be the \( A_{1} \) component of H (5). There should be a partner transition at 38.6 MHz, but it has not been detected. Second, the features at 10.7 and 17.7 MHz decrease in intensity on going from protonated to deuterated buffer. This indicates that another hfc component, again from an exchangeable proton, has resonances at these frequencies. This hfc
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Signs of hyperfine couplings have not been determined experimentally but given in comparison with density-functional theory calculations (35).

| Atom position | A /MHz | A /MHz | A /MHz |
|---------------|-------|-------|-------|
| H(3) \(a\)   | 0.9   | 0.5   | 0.9   |
| H(5) \(a\)   | 0.6   | 0.5   | 0.6   |
| H(6) \(a\)   | 0.4   | 0.8   | 0.4   |
| H(7a) \(a\)  | 0.5   | 0.5   | 0.5   |
| H(8a) \(a\)  | 0.4   | 0.5   | 0.4   |

* All hyperfine couplings are accurate within ±0.1 MHz except for the \(A_{iso}\) components of H(8a) for which the errors are ±0.1 and ±0.2 MHz, respectively.

* From EPR spectra (error ± 3 MHz).

The observation of FMNH \(2\) is independent of protein construct and buffer conditions such as the presence of an exogenous reductant. This is in contrast to the well known photoreduction of flavoproteins (described by Massey and Palmer (39)) in which supplemented electron donors are required for flavin semiquinone generation. Therefore, we conclude that in the LOV2 C450A domains the flavin radical is formed by intraprotein electron transfer from a redox-active amino acid. Tryptophan, histidine, or tyrosine are likely candidates for electron transfer to flavin radicals (43). Thus, either additional donating capabilities of the hisactophilin moiety, which contains 31 histidines and three tyrosines as potentially redox-active amino acid residues (43), or the electron transfer pathway being initial electron transfer to flavin radicals (40, 41).

The aligned electron reactivation of the radical is observed upon laser flash photolysis (40, 41).

The amount of FMNH \(2\) produced is different for the various LOV2 samples, and these variations deserve some comment. In principle, the radical yield reflects the competition between radical formation and its decay, the latter occurring via either back electron transfer or reoxidation of FMNH \(2\) by molecular oxygen to form FMN and O\(2\) (42). Radical formation is enhanced in the LOV2 C450A hisactophilin construct compared with the cleaved LOV2 C450A domain and the calmodulinfused protein. This may be understood by the superior electron-donating capabilities of the hisactophilin moiety, which contains 31 histidines and three tyrosines as potentially redox-active amino acid residues (43). Thus, either additional electron transfer pathways are operating or the initial electron hole generated by electron transfer to flavin radicals (40, 41).
azine ring of FMN (23). Although only the FMN radical but not its redox partner has been detected, its observation is nevertheless consistent with an intraprotein electron transfer. We conclude that the redox partner is either degraded or that more than one amino acid may act as an electron donor to FMN.

Reoxidation of FMNH\textsuperscript{−}/H\textsubscript{18528} by molecular oxygen is expected to be similar for all three LOV2 samples, as is indeed observed experimentally (Fig. 4). Under aerobic conditions, this is clearly the preferred process for regeneration of oxidized FMN and, therefore, dominates flavin radical decay kinetics. Under anaerobic conditions, radical decay occurs via back electron transfer to regenerate the thermodynamically more stable oxidized FMN. Again, the hisactophilin-fused LOV2 domain provides more efficient electron transfer pathways compared with the cleaved LOV2 domain, and therefore, radical decay is enhanced in the recombinant fusion protein.

The situation is different in the presence of EDTA. EDTA is a well known reducing agent for photoexcited flavin triplet states (39). In solution studies, triplet flavin abstracts a hydrogen atom from EDTA, forming a neutral flavin radical, whereas the ETDA then decarboxylates and fragments to produce stable products (44, 45). However, in the photoreduction of LOV2 C450A, EDTA has only minor influence on the measured radical yields (Fig. 3, C and D), which indicates that FMNH\textsuperscript{−} is almost exclusively formed by intraprotein electron transfer rather than by reaction of 3FMN with EDTA. On the other hand, in the presence of EDTA, the reoxidation of FMNH\textsuperscript{−} becomes independent of oxygen content (Fig. 4, diamonds and triangles). Qualitatively, this finding may be understood in terms of the EDTA sequestering trace amounts of transition metal ions (that are unavoidably present as impurities from the protein preparation) that, if not chelated, may act as catalysts in the activation of molecular oxygen (46, 47). Thus, EDTA plays its well known antioxidant role by preventing reoxidation of the flavin.

Once FMNH\textsuperscript{−} is generated, its decay is extremely slow (Fig. 4). This is even more evident when taking into account the small size of the LOV2 domain (23). Electron transfer time constants in the sub-microsecond range are observed in other flavoproteins; for example, in photolyases in which electrons are transferred over distances exceeding the diameter of LOV domains (28). This demonstrates that, in contrast to many other flavoproteins, low activation energy electron transfer pathways do not exist in the LOV2 domain or that endergonic steps in the electron transfer pathway have to be considered. Nevertheless, both redox forms of the FMN chromophore, oxidized and one-electron reduced, are well stabilized by the protein matrix. Hence, overall the excited-state flavin is optimized
to undergo electron transfer but the electron transfer pathways leading to the surface of the LOV2 domain are inefficient. This may be necessary so that adduct formation with Cys-450 in the wild-type, which takes place on a microsecond time scale (20, 48), could dominate alternative electron transfer processes. From this study we cannot directly draw conclusions about the time scale or quantum yield of the forward electron transfer observed in the LOV2 C450A domain. A slowdown of electron transfer by roughly an order of magnitude is expected for every 2-Å increase of distance between the redox partners, if other parameters such as protein packing density, free energy, and reorganization energy remain comparable (49). Thus, in comparison to a possible electron transfer from Cys-450 (which is 4.2 Å away from C(4a) of FMN) to 3FMN in the wild-type a roughly 104-fold decreased rate of electron transfer from one of the other (more distant) redox-active amino acids in the LOV2 domain to 3FMN would be expected. This decrease, however, is predicted to make the electron transfer rate so slow that 3FMN would be mostly converted back to the ground state before electron transfer could happen. Thus, the quantum yield of electron transfer should be rather low. This is most likely the reason why in previous laser-flash photolysis studies no flavin radical formation was observed, whereas by using continuous irradiation in this study, a flavin radical was generated at high yield. Taken together, these observations have several major implications for adduct formation in LOV domains, which will now be considered.

Swartz et al. (20) propose an ionic reaction pathway with the Cys-450 residue initially present as a thiolate. In this mechanism, 3FMN is protonated at N(5) by a nearby and as yet unidentified proton-donating group in the protein to give the FMNH\(^+\) cation. Upon protonation of N(5) (which occurs in the ground state FMN only at pH \(\approx 0\)), the electron density distribution of the isoalloxazine ring is altered due to the non-bonding pair of electrons at N(5) becoming a bonding pair with the additional proton. The FMNH\(^+\) carbocation, which formally has a positive charge at C(4a), is the electrophile that can then form a bond with the nucleophilic thiolate, thus generating the adduct. The hypothetical ionic mechanism also requires that Cys-450 is present as a thiolate and simultaneously that 3FMN is protonated before adduct formation occurs. Yet in solution it has been demonstrated that flavin triplet protonation occurs only at pH < 4.4 (50–52). At higher pH, 3FMN undergoes electron transfer followed by protonation when the pH is <8.3 to form a neutral flavin radical (50) rather than the anion radical, FMN\(^-\). These facts seem to be somewhat contradictory, and although it is not unreasonable that the local pH at the FMN-binding site could be rather low given the proximity of FMN and C450, it seems unlikely that the latter could act at the same time be deprotonated (\(pK_a\) of cysteine in solution, 8.37). Furthermore, it has recently been shown by Fourier transform infrared spectroscopy that the cysteine residue is protonated in the ground state (25).

An alternative that is consistent with our experimental observations is a radical pair mechanism (see Fig. 8B; for review, see Ref. 53). 3FMN is an extremely efficient oxidizing agent in the presence of electron donors such as EDTA or redox-active amino acids in solution (40, 41) and in various proteins, including the LOV2 domain, as demonstrated in this study. A flavin semiquinone is formed from 3FMN in a one-electron photoreduction that may be followed by proton transfer depending on the pH to give the anionic or neutral flavin radical (39).

In the wild-type LOV2 domain 3FMN could abstract an electron from Cys-450 and a spin-correlated radical pair consisting of an anionic flavin radical, FMN\(^-\), and a sulfur-centered radical, RS\(^-\)H, would be formed. This could undergo subsequent proton transfer to give the neutral flavin radical, FMNH, and a sulfur-centered radical, RS. Alternatively, 3FMN could directly abstract a hydrogen atom from cysteine, giving the same product, a neutral radical pair. A radical pair created from a triplet state precursor has initially the same spin state (i.e. 3FMNH \(\sim\) RS\(^-\)), due to the conservation of angular momentum, and thus cannot form a covalent bond. At first glance this might be thought to rule out adduct formation via a radical-pair mechanism or that radical pairs should have been detected in the wild type, which has not been the case to date. A covalent bond and, hence, the FMN-cysteinyl adduct may only form if the spin state of the radical pair evolves to obtain a singlet character by singlet-triplet mixing: \(3\text{FMN} \sim\) RS\(^-\) \(\rightarrow\) [3FMN \(\sim\) RS]. Because of strong spin-orbit coupling in the flavin-centered radicals (spin-orbit coupling constant, 382 cm\(^{-1}\)), however, a mechanism for extremely rapid singlet-triplet interconversion exists. At room temperature, in solution, these radicals have been shown to have relaxation times in the nanosecond regime (54). Hence, if spin mixing occurs on this time scale or even faster, the covalent adduct will be formed on the same time scale, which implies that the lifetime of the intermediate radical pair will be too short for detection by EPR. That they were not detected by optical methods simply implies that their short-lived absorption may be swamped by the background of relatively long-lived 3FMN and adduct.

The spin chemistry is also important for the hypothetical ionic mechanism discussed by Swartz et al. (20) and for a possible concerted mechanism. Given that adduct formation proceeds via 3FMN, then FMNH\(^+\) and the adduct must also be created in the triplet state. That the adduct would be created in a triplet state is rather unlikely, but if it were, its spin state would have been evident from its effect on the NMR line widths, yet no line broadening was observed (22). The only possibility is that FMNH\(^+\) converts to the singlet ground state before the adduct is formed. This is certainly possible under the influence of the sulfur atom, but the singlet ground state would almost certainly deprotonate before adduct could form (recall that the ground-state flavin is a much weaker base than the excited state (55)).

In a hypothetical radical-pair mechanism these requirements are relaxed. Electron transfer may occur with either a protonated or deprotonated cysteinyl residue (40, 41). Flavin protonation at N(5) from either the cysteine or another donor group can occur either during the radical-pair lifetime or after the adduct has been formed. An important point is that protonation of 3FMN is not necessarily the rate-determining step in a radical-pair mechanism, whereas it should be in an ionic mechanism.

The minimal differences in the matrix region of the ENDOR spectra in protonated and deuterated buffer are also of interest. Although the x-ray structure does not show the presence of any water molecules close to the FMN isoalloxazine ring, this does not necessarily prove that the cofactor is in a water-free environment. Often in flavoenzymes the matrix region collapses upon buffer deuteration. This is especially true where a substrate approaches the flavin cofactor very closely to facilitate, for example, hydrogen ion transfer (56). In DNA photolyases, the matrix region is slightly altered, and it is known from its x-ray structure (57) that the cofactor is mostly buried in the protein and isolated from solvent water. In the C450A mutant of phototropin, the changes in the ENDOR spectrum are minimal. No exogenous substrate needs to approach the FMN cofactor, because the cysteine is an integral part of the protein. The flavin triplet state is a very reactive species that must be isolated so that it may only perform its biological function; that is, adduct formation. In LOV2 domains, 3FMN is protected
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