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# LOV1 Protein From *Chlamydomonas Reinhardtii* is a Template For the Photoadduct Formation of FMN and Methylmercaptane.

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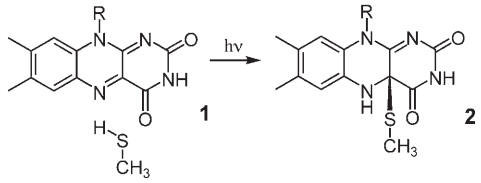
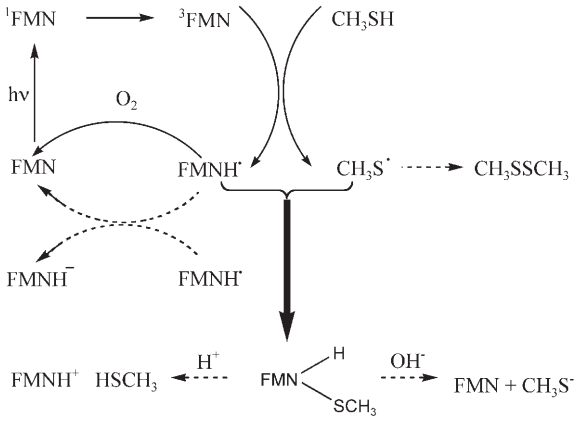
Nature has developed several photoactive proteins that function either as converters of solar energy into chemical energy (photosynthesis),[1] DNA photodamage repair enzymes (photolyases),[2] or light sensors.[3] Until recently, in all of these systems only two types of primary photochemical reactions have been identified: The photoexcited chromophore either undergoes electron transfer as chlorophyll in photosystems or flavin in photolyases. Or it performs a *cis/trans* isomerization around a double bond as in retinals, phytochromes, and xanthopsins.[4]

Three classes of blue-light sensing proteins have been described which contain a flavin cofactor as chromophore.[4] The cryptochromes,[5,6] which are frequently associated with circadian rhythms, contain flavin adenine dinucleotide (FAD), as do the BLUF (blue light sensing using flavin) proteins.[7] The third class are the phototropins,[8] which are composed of (usually two) LOV (light oxygen voltage sensitive) domains and a kinase domain. Each LOV domain noncovalently binds a flavin mononucleotide (FMN). The primary photoproduct is the covalent adduct of a cysteine residue to FMN.[9,10] The corresponding model reaction with methylmercaptane (MM) instead of cysteine is shown in Scheme 1.




**Scheme 1.** Photoreaction of FMN (**1**) with MM leading to the adduct (**2**) with a covalent bond between S and C4a. R is the ribityl-monophosphate group.

When this was discovered, it was a completely new reaction type for a biological photosensor system. The adduct has a sulfur atom with an aliphatic residue attached to the carbon atom C4a of flavin, and a hydrogen atom attached to N5. To the best of our knowledge, no stable compound with these structural characteristics has been reported so far. One can speculate that the protein is required to guide the reaction towards this product and to protect it from decomposition. Herein we show that compound **2**, that is, the adduct of FMN and MM, can indeed be synthesized inside the binding pocket of a LOV protein domain in which the cysteine residue of the wild type has been replaced by the photochemically inactive amino acid glycine. In the absence of the protein, the photoreaction between FMN and MM yields the fully reduced hydroquinone form $FMNH_2$ or the anion $FMNH^-$ (Scheme 2).




**Scheme 2.** Mechanism of the photoreaction of FMN with MM. Dotted arrows indicate reactions outside the protein, the bold array marks the specific reaction guided by the protein.

As a mold for the reaction the mutant LOV1-C57G of the first LOV domain of the photoprotein of the green alga *Chlamydomonas reinhardtii* was prepared. In this protein the cysteine at position 57 is replaced by glycine. Irradiation of this photoinactive mutant LOV1-C57G in the absence of reducing agents leads to the formation of the triplet state of FMN as the only intermediate, identified by the characteristic band at 715 nm.[10a] This decays back to the ground state with a time constant of about 5 μs in the presence of oxygen. When oxygen is removed, the decay time increases to about 27 μs. When β-mercaptoethanol (β-ME) is added and oxygen is removed, irradiation leads to the formation of a new species with absorption maxima at 575 and 615 nm which is assigned to the neutral FMNH radical.[10b] Isosbestic points at 490.5, 389.5, 380.5, and 340.5 nm indicate that the reaction leads to a single product (or a mixture of products with a fixed ratio): When oxygen is admitted to the sample, the original spectrum of FMN in LOV1-C57G is completely recovered.

Irradiation of FMN-loaded LOV1-C57G protein in the presence of MM ($CH_3SH$) with light of 460 nm wavelength resulted in bleaching of the flavin absorption bands as shown in Figure 1. Three isosbestic points at 323.5, 375, and 403 nm indicate that this reaction is also unique. Hence the data were analyzed in terms of a two component model

$$A(\lambda,t) = c_0\,\varepsilon_1(\lambda)\,x_1(t) + c_0\,\varepsilon_2(\lambda)\,x_2(t) \quad (1)$$

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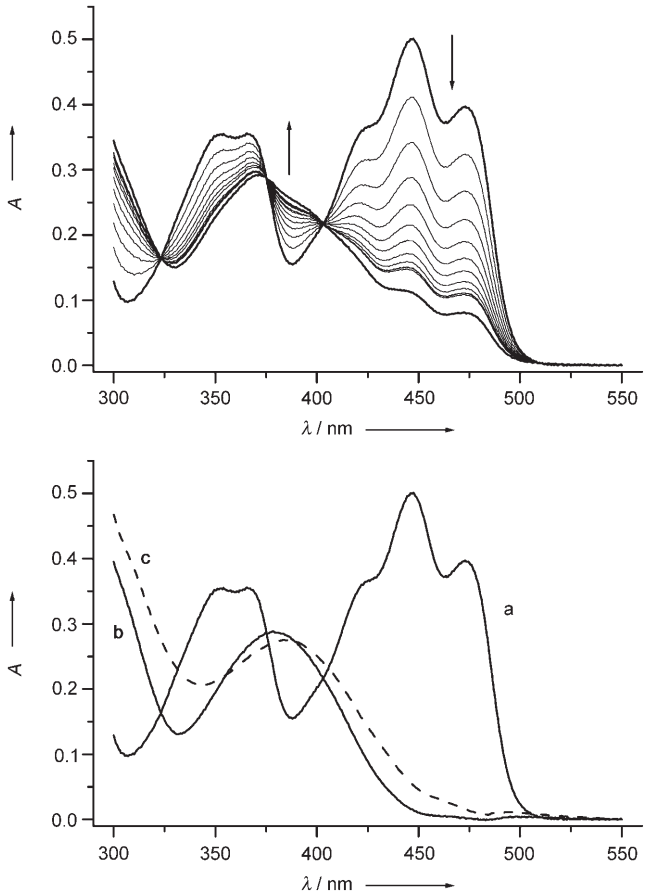


**Figure 1.** Top: Sequence of absorption spectra of LOV1-C57G with MM. Irradiation occurred in steps of 1.05 s duration, the last spectrum was recorded after a total irradiation time of 16.6 s. Below: Spectra of reactant a and product b extracted from the data by principal component analysis. Curve c is the spectrum of the photoadduct in LOV1-wt.

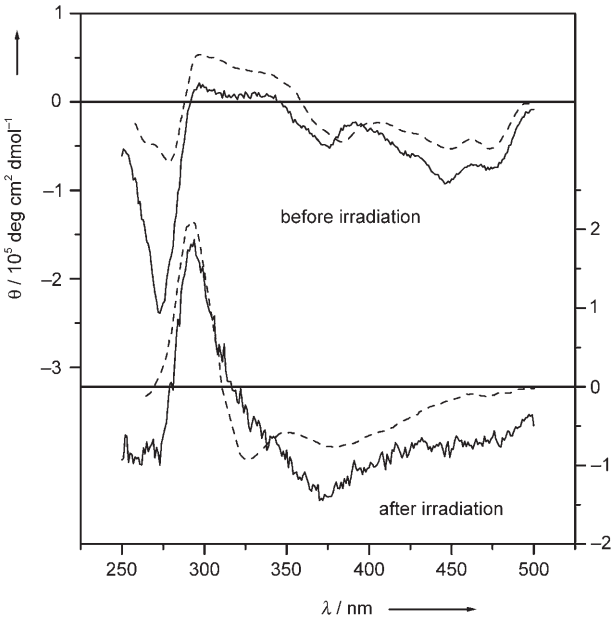


**Figure 2.** CD-Spectra of the system LOV1-C57G + MM (full lines) and wild-type LOV2 (dashed lines, adapted from ref. [11]) before (left) and after (right) irradiation.

where $\varepsilon_1(\lambda)$ and $\varepsilon_2(\lambda)$ are the absorption spectra of the reactant and the product, $c_0$ is the initial concentration of the reactant, and $x_1(t)$, $x_2(t)$ are the corresponding mole fractions. The result of this deconvolution is shown in the bottom part of Figure 1. The spectrum of the product has a single absorption band in the near UV with a broad maximum at 379 nm. The spectrum of the photoadduct of FMN and cysteine occuring in the wild-type (that is, functioning) LOV domain is shown as curve c.[10] It has a maximum at 390 nm. Based on the similarity of the spectra we assign curve b to the photoadduct of MM and FMN inside the LOV protein. This assignment is supported by the CD spectra measured before and after irradiation. As seen in Figure 2, these spectra are very similar to the corresponding CD spectra measured for the dark state and adduct state of wild-type LOV2.[11] The negative peak of the CD signal at 370 nm indicates that the corresponding species is chiral. As flavin and MM are both nonchiral compounds, a reaction in free solution would result in a nonchiral product.

The absorption band of the flavin chromophore recovers in the dark on a time scale of about 10 h. The photoproduct decomposes, one product being the original flavin. Attempts to separate the photoadduct from the protein mold through denaturation with 1% of SDS or addition of urea lead to immediate appearance of the characteristic spectrum of free water solvated FMN, showing broad bands and a loss of vibrational fine structure. Apparently the photoadduct decomposes within seconds when the protecting protein cage is removed. All attempts to identify the species by mass spectrometry failed. The mass spectrum of the reaction mixture, measured after replacement of the phosphate buffer by an ammonium acetate buffer, did not show the pattern typical for the various ionization states of a protein, and could hence not be deconvoluted. We believe that this is due to the known strong tendency of LOV domains to form dimers and higher aggregates. When the reaction mixture was passed through a column immediately before mass spectrometry analysis, the mass spectrum of the protein was obtained, but the chromophore was lost.

Irradiation of a sample containing FMN and MM in the absence of LOV protein leads to the spectrum of the doubly reduced hydroquinone form of FMN as the only product (Figure 3). When this reaction mixture is stirred in the presence of air, the hydroquinone is immediately oxidized back to the quinone form of FMN. On the other hand, oxygen does apparently not disturb the adduct formation inside the protein. If oxygen is removed from a sample of LOV1-C57G with MM before irradiation, the photoadduct is still formed, but the FMN semiquinone radical is observed as a byproduct. In the presence of oxygen, this semiquinone radical is oxidized back to the fully oxidized FMN, thus increasing the yield of the adduct.

The thermal backreaction in LOV1-wt is catalyzed by a base.[10] The instability of the photoadduct **2** might be due to acid- or base-catalyzed decomposition. We performed ab initio calculations at the RHF and DFT–B3LYP level with the 6–31G**

  

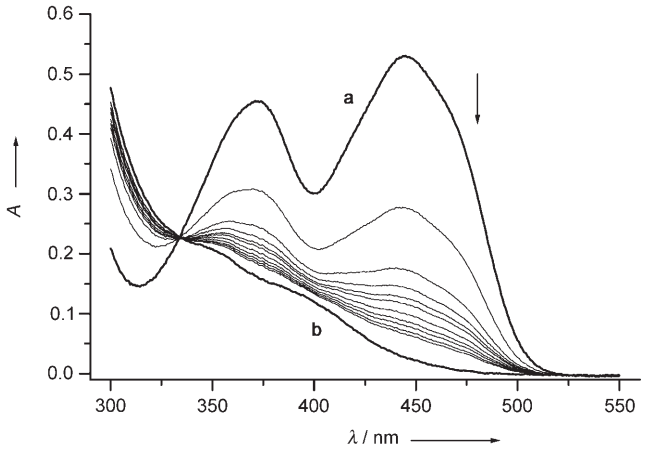


**Figure 3.** Sequence of absorption spectra of FMN in aqueous solution in the presence of MM during photolysis with the light of a blue LED at 460 nm. Irradiation occurred in steps of 5 s duration. The thick lines are the spectrum of free FMN (a), spectrum of the hydroquinone (b, extrapolated from the data).

basis set for the photoadduct as well as the N5-deprotonated compound and the S-protonated compound using the GAMESS[12] program. In these calculations the ribityl chain and phosphate group of FMN were replaced by a methyl group, that is, FMN was represented by lumiflavin. Whereas the structure of the neutral adduct could be optimized with either RHF or DFT methods, no structure with a bond between C4a and S could be obtained for the N5-deprotonated or S-protonated species.

Our observations can be rationalized by the following reaction mechanism (see Scheme 2): Irradiation at 460 nm excites FMN to the first excited singlet state, which decays by intersystem crossing to the lowest triplet state within a few ns.[10] The reactive triplet species $^3$FMN abstracts a hydrogen atom from $CH_3SH$, leading to the product radicals $CH_3S^\bullet$ and $FMNH^\bullet$. This reaction might occur in two separate steps, as the sequence of an electron transfer and a proton transfer. Inside the protein, the proton might be provided not by the species $CH_3SH^{\bullet+}$ but by an amino acid residue, and the two radicals $CH_3S^\bullet$ and $FMNH^\bullet$ are not necessarily produced in close proximity. Hence, in addition to the adduct formation by recombination of these radicals, other reactions must be taken into account. Two $CH_3S^\bullet$ radicals can recombine and form the stable disulfide $CH_3$–SS–$CH_3$. In aerated solution, $FMNH^\bullet$ is readily oxidized back to FMN by molecular oxygen. Outside of the protein, two $FMNH^\bullet$ radicals can disproportionate into FMN and the fully reduced hydroquinone form. At pH 8 the latter will exist in the deprotonated form $FMNH^-$. Disproportionation is not possible inside the protein because of the constraints imposed upon the minimum distance between two flavin units. Hence, if no $CH_3S^\bullet$ radical is available in the immediate neighborhood of the $FMNH^\bullet$ radical, the latter will accumulate as a byproduct. This is indeed observed either after prolonged irradiation when all oxygen in the solution has been consumed, or immediately when the experiment is performed in oxygen free solution. If two radicals $RS^\bullet$ and $FMNH^\bullet$ encounter, the adduct can be formed. This is observed with MM, but not with βME. Apparently, βME is too large to enter the binding pocket of the FMN in the LOV protein. Protonation of the adduct at the sulfur atom leads to spontaneous dissociation into $CH_3SH$ and $FMNH^+$ without barrier. Deprotonation of the adduct at N5 results in decomposition into FMN and the thiolate anion $CH_3S^-$, again in a barrierless process.

The adduct postulated by us is a flavin derivative with a thioalkyl residue at the C4a-position and a hydrogen atom at the N5-position. We conclude that such compounds are only stable in a protein cage[13] but not in aqueous solution. There are three reports on compounds with a bond between a sulfur atom and C4a of a flavin.[14–16] In no case could the compound be isolated, and one report has been questioned by later work.[17] In summary, no evidence exists that the structural unit **2** is stable in aqueous solution. We propose that it is readily hydrolyzed either by protonation at the sulfur atom or by deprotonation at N5. However, it can be formed and stabilized for many hours in the flavin binding pocket of a LOV protein domain. We conclude that the protein not only plays a decisive role in guiding the reaction towards this product, but also in keeping it stable. We assume that the reaction proceeds along the same mechanism as the cysteine adduct formation in the wild-type protein, albeit with lower quantum yields and rate constants. Although the semiquinone form of FMN was observed under oxygen free reaction conditions, this is not yet conclusive proof that it is an intermediate in adduct formation. Time-resolved measurements are under way which will hopefully resolve this question.

## Experimental Section

The mutated gene fragment of LOV1 was a kind gift of Prof. Hegemann (Humboldt University Berlin). It was digested with EcoRI and HindIII and cloned into the His-p2x-vector, which was derived from the pMalp2x-vector (New England Biolab) by excising the MBP-encoding segment and inserting a DNA sequence encoding ten histidines into the EcoRI site. Then the fusion protein was expressed in *E. coli* strain BL21 and purified via a nickel-nitrilotriacetate (Ni-NTA) column according to the instructions of the supplier (Quiagen, Hilden, Germany). The protein was diluted in 10 mM phosphate buffer, pH 8, containing 10 mM NaCl, and 100 μM phenylmethanesulfonyl fluoride. Saturated solutions of methylmercaptane (Aldrich) were prepared by bubbling through the buffer.

For irradiation two high-intensity LEDs (Conrad, Luxeon III Emitter LXHL-PBO9) with a power of 1 W at 460 nm were mounted opposite to each other and with a spacing of 1 cm to allow the insertion of a standard quartz cuvette (rectangular cuvette: 10×2 mm). After every irradiation step of 1–15 s duration an absorption spectrum was recorded with a Lambda 9 spectrophotometer (Perkin–Elmer). The temperature was maintained at 20 °C by a thermostat.

Mass spectra were obtained with a SSQ 7000 (Finnigan MAT) equipped with an ESI source. A C4 column (50×2, 1 mm Vydac-214TP, 5 Micrometer) was used in the GC-MS coupling.

  

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