An optomechanical transducer in the blue light receptor phototropin from *Avena sativa*

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The *PHOT1* (*NPH1*) gene from *Avena sativa* specifies the blue light receptor for phototropism, phototropin, which comprises two FMN-binding LOV domains and a serine/threonine protein kinase domain. Light exposure is conducive to autophosphorylation of the protein kinase domain. We have reconstituted a recombinant LOV2 domain of *A. sativa* phototropin with various ^{13}C/^{15}N-labeled isotopomers of the cofactor, FMN. The reconstituted protein samples were analyzed by NMR spectroscopy under dark and light conditions. Blue light irradiation is shown to result in the addition of a thiol group (cysteine 450) to the 4a position of the FMN chromophore. The adduct reverts spontaneously in the dark by elimination. The light-driven flavin adduct formation results in conformational modification, which was diagnosed by ^{1}H and ^{31}P NMR spectroscopy. This conformational change is proposed to initiate the transmission of the light signal via conformational modulation of the protein kinase domain conducive to autophosphorylation of *NPH1*.

Fig. 1. Proposed light-induced formation of a covalent cysteinyl-C(4a) adduct of FMN and LOV2 apoprotein.

Numerous phenomena in the life cycle of plants, including phototropism, stomatal opening, and circadian periodicity, are modulated by light. Photoreceptors responsive to UV, blue, red, and far red light have been reported. Together, they span the spectral range of about 280–800 nm.

Blue light-responsive processes have been known for a long time, but cognate receptors have been characterized only recently. Thus, cryptochromes characterized by sequence similarity to DNA photolyases are now believed to be involved in the synchronization of the circadian clock (1–3). Phototropins are involved in phototropism (4, 5) and have no sequence similarity with the cryptochrome group. FAD and FMN serve as chromophores for cryptochromes and phototropins, respectively (6).

Phototropin of *Avena sativa* is a protein with 923 amino acids specified by the *NPH1* gene (ref. 7; for review, see also ref. 8). The protein comprises two FMN-binding LOV domains and a serine/threonine protein kinase domain.

The LOV domains of phototropin are similar to PAS domains involved in light, oxygen, or voltage sensing (4) in a variety of regulatory as well as sensor proteins exhibiting diverse functions (4, 9, 10). Recombinant LOV1 and LOV2 domains of phototropins from different plants have been shown to bind FMN (11). The crystal structure of the LOV2 domain of PHY3 protein of *Adiantum capillus-veneris* has been published recently (12).

In this study, we reconstituted the LOV2 apoprotein from *A. sativa* with ^{13}C- and/or ^{15}N-labeled FMN. ^{1}H, ^{13}C, ^{15}N, and ^{31}P NMR spectra obtained in darkness and with blue light illumination identify the light-mediated reversible formation of a covalent flavin adduct as the trigger for conformational modulation of the protein.

Experimental Procedures

Materials. Stable isotope-labeled FMN samples were prepared by published procedures (13–15).
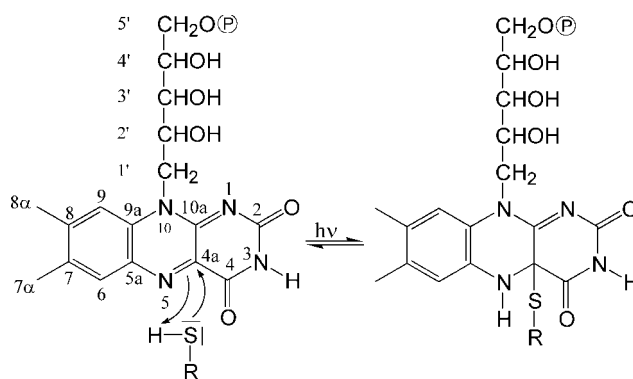
Recombinant LOV2 Domain of Phototropin. A recombinant fusion protein comprising the calmodulin-binding domain from myosin light chain kinase and the LOV2 domain from NPH1 protein of *A. sativa* was prepared as described earlier (16, 17). The fusion protein is subsequently designated recombinant LOV2 domain.

Reconstitution of LOV2 Domain with Isotope-Labeled FMN. Recombinant LOV2 domain (fusion protein) was depleted of FMN and subsequently reconstituted with isotope-labeled FMN by a procedure modified after van Berkel *et al.* (16). To a solution of recombinant LOV2 domain, ammonium sulfate was added to a final concentration of 60%. The precipitate was harvested by centrifugation and dissolved in 50 mM potassium phosphate, pH 7.5, containing 1 M ammonium sulfate (buffer A). The solution was passed through a column of a phenyl-Sepharose CL-4B, which had been equilibrated with buffer A. The column was washed with 50 mM potassium phosphate, pH 4.0, containing 1 M ammonium sulfate, and subsequently with buffer A. A solution containing 10 μM isotope-labeled FMN in buffer A was recycled through the column for 4 h at a flow rate of 1 ml/min. The column was then washed with buffer A and developed with 25 mM sodium/potassium phosphate, pH 7.0. Fractions were collected and concentrated by ultrafiltration. The yield was 15–25% based on recombinant protein.

NMR Spectroscopy. NMR spectra were measured at 27°C by using a four-channel Bruker (Billerica, MA) DRX 500 spectrometer equipped with pulsed-field gradient accessory. ^{1}H and ^{13}C NMR spectra were recorded at 500.1 and 125.8 MHz, respectively, with a 5-mm ^{1}H/^{13}C dual probehead. ^{15}N and ^{31}P NMR spectra were recorded at 50.7 and 202.5 MHz, respectively, with a 5-mm

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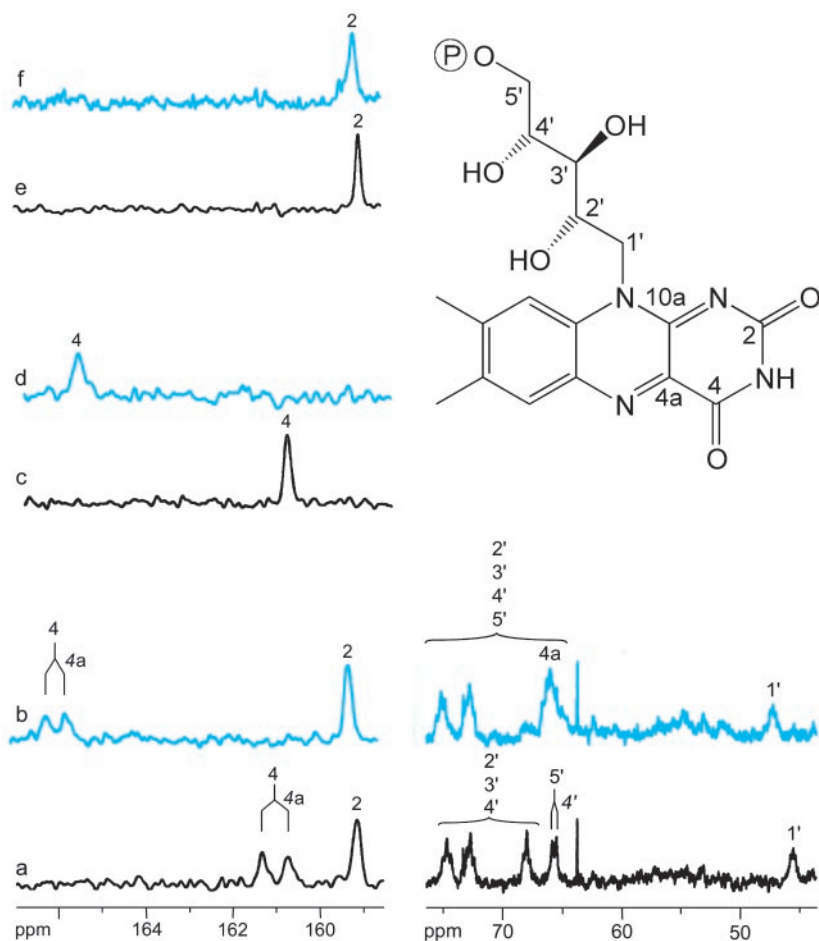
Fig. 2. ^{13}C NMR spectra of LOV2 domain with differently labeled FMN cofactors. (*a*, *c*, and *e*) Spectra measured in the dark. (*b*, *d*, and *f*) Spectra measured with blue light irradiation. The FMN isotopomers used were [U-[13]C$_{17}$,[15]N$_4$]FMN (*a* and *b*), [4,10a-[13]C$_2$]FMN (*c* and *d*) (14), and [2,4a-[13]C$_2$]FMN (*e* and *f*) (14).

Table 1. ^{13}C, ^{15}N, and ^{31}P NMR data of recombinant LOV2 domain of *A. sativa* phototropin reconstituted with [U-[13]C$_{17}$,U-[15]N$_4$]FMN in darkness (I) and under illumination with blue light (II)

| | Chemical shifts, ppm | | | | | | Coupling constants, Hz | | |
| | δ [13]C | | δ [15]N | | δ [31]P | | J$_{CC}$ | | J$_{CN}$ |
FMN-atom	I	II	I	II	I	II	I	II	II
2	159.2	159.3					(s)*	(s)	
4	161.0	165.9					75 (d)	52 (d)	
4a	134.5	65.0					65 (t)	(b)	
5a	136.3	130.3					60 (t)	(b)	
6	132.8	120.7					65 (t)	60 (t)	
7	139.0	130.3					55 (qua)	(b)	
7α	21.5	21.1					47 (d)	47 (d)	
8	150.7	136.2					55 (qua)	55 (qua)	
8α	22.5	21.8					44 (d)	44 (d)	
9	119.3	118.7					70 (t)	65 (t)	
9a	134.2	127.7					65 (t)	70 (t)	
10a	150.8	156.9					45 (d)	45 (d)	20 (d)
1′	45.8	47.3					40 (d)	(b)	
2′	68.1	66.8					40 (t)	(b)	
3′	72.8	72.9					40 (t)	(b)	
4′	74.8	75.3					40 (t)	40 (t)	
5′	65.8	66.3					40 (d)	(b)	
1			188.5	188.5					
3			157.5	164.5					
5			349.5	66.3					
10			155.5	164.5					
P					4.75	4.15			

*Signal multiplicity is given in parentheses. s, singlet; d, doublet; t, triplet; qua, quartet; b, broad signal.

broadband probehead. ^{13}C NMR chemical shifts were referenced to internal dioxane (67.84 ppm relative to tetramethylsilane), ^{15}N NMR chemical shifts were referenced to external [5-^{15}N]6,7-dimethyl-8-ribityllumazine (327 ppm relative to liquid ammonia), and ^{31}P NMR chemical shifts were referenced to external 85% (vol/vol) phosphoric acid (0 ppm).

The solvent was 25 mM sodium/potassium phosphate, pH 7.0, containing 10% (vol/vol) ^{2}H$_2$O, and the sample volume was 0.5 ml.

Composite pulse decoupling was used for ^{13}C and ^{31}P NMR experiments. All spectra were recorded by using a flip angle of 30°. The repetition time was 3.0 s for ^{13}C and ^{31}P NMR spectroscopy and 2.0 s for ^{15}N NMR experiments, respectively. Free induction decays were processed by zero filling and exponential multiplication. For ^{15}N NMR, 5–10 free induction decays (each comprising 20,000 scans) were added before Fourier transformation. The acquisition of ^{13}C and ^{31}P NMR spectra required 5–20 h; the acquisition of ^{15}N NMR spectra required more than 60 h.

When required, samples were illuminated inside the magnet via a flexible quartz optical fiber whose conical tip was immersed into the solution of the NMR tube 1.5 cm above the magnetic center (18). The light source was a 100-W mercury lamp (Oriel, Stamford, CT) operating at constant power of 100 W. The emitted light was filtered by using a pair of BG7 and GG420 filters from Schott (Mainz, Germany). Typical NMR samples were completely bleached within a period of 5 min. Spectra were recorded after that time with continuous illumination.

Results and Discussion

The recombinant LOV2 domain used in this study has absorbance maxima at 380, 425, 450, and 475 nm (11, 19). The photobleached form obtained by blue light irradiation has an absorbance maximum at 390 nm, suggesting the formation of a 4a thiol adduct of the FMN chromophore. Replacement of the conserved cysteine 450 by alanine affords an FMN-binding mutant protein that is no longer susceptible to photobleaching (19). Comparison with the spectral properties of model systems suggested that photobleaching involves the formation of a covalent adduct of the sulfhydryl group of cysteine 450 to C-4a of the flavin chromophore (Fig. 1) (20).

A recombinant fusion protein comprising the calmodulin-binding domain from myosin light chain kinase followed by the LOV2 domain of the *NPH1* gene of *A. sativa* was selected for NMR experiments for reasons of solubility and stability (11, 19). The recombinant fusion protein was reconstituted with various isotope-labeled FMN samples by a procedure modified after van Berkel *et al.* (16). The absorption spectrum of the reconstituted protein was virtually identical to that of the native recombinant protein before reconstitution. Moreover, the reconstituted protein samples showed the same photobleaching behavior as the native protein.

^{1}H, ^{13}C, ^{15}N, and ^{31}P NMR spectra were recorded without illumination and subsequently with continuous blue light illumination of the sample. Chemical shift modulations caused by blue light irradiation in the ^{1}H, ^{13}C, ^{15}N, and ^{31}P spectra were fully reversible in the dark. No significant irreversible photodamage was noted during NMR acquisition.

^{13}C signals of the protein-bound FMN chromophore were broadened to around 25 Hz. Despite the line broadening, many signals of the protein reconstituted with [U-^{13}C$_{17}$,U-^{15}N$_4$]FMN appear as doublets or multiplets because of ^{13}C^{13}C coupling (Fig. 2, Table 1).

Signal assignments were based on (*i*) comparison of proteins reconstituted with different isotope-labeled FMN samples, (*ii*) ^{13}C^{13}C coupling patterns, (*iii*) comparison with other flavoproteins (17, 21–23), and (*iv*) comparison with flavin model compounds (24–26).
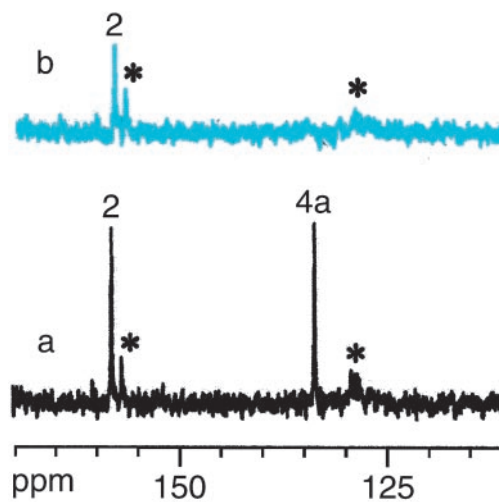
Fig. 3. ^{13}C NMR spectra of LOV2 domain with [2,4a-^{13}C$_2$]FMN (*a*) in the dark and (*b*) under illumination with blue light. Impurities are marked by asterisks.

More specifically, the signals of carbon atoms 2, 4, 4a, and 10a under dark conditions could be easily assigned on the basis of experiments with proteins reconstituted with [2,4a-^{13}C$_2$]FMN and [4,10a-^{13}C$_2$]FMN, respectively (Figs. 2 and 3, Table 1). The ^{13}C NMR signal of C-4 appears as a singlet in the sample with [4,10a-^{13}C$_2$]FMN (Fig. 2 *c* and *d*) and as a doublet in the sample with universally ^{13}C,^{15}N-labeled FMN because of coupling (75 Hz) with C-4a (Fig. 2 *a* and *b*). The signals of carbon atoms 7α and 9 were unequivocally assigned on the basis of the protein samples reconstituted with [7α,9-^{13}C$_2$]FMN (13). The signal of the 7α methyl group (21.5 ppm) appears as a doublet in the sample with universally ^{13}C,^{15}N-labeled FMN because of ^{13}C^{13}C coupling (47 Hz) with C-7. C-9 appears as a pseudotriplet because of coupling to C-8 and C-9a via single bonds. A doublet

Table 2. ^{13}C NMR chemical shifts of free FMN (27), FMN bound to LOV2 domain in darkness (I) and under illumination with blue light (II) (this study), 3-methyl-4a-benzyl-4a,5-dihydrolumiflavin (III) (25, 26), and 3-methyl-4a-allyl-4a,5-dihydrolumiflavin (IV) (26)

	FMN*	I†	II†	III‡	IV‡
2	159.8	159.2	159.3	161.7	162.0
4	163.7	161.0	165.9	169.1	167.7
4a	136.2	134.5	65.0	59.0	63.0
5a	136.4	136.3	130.3	130.2	130.3
6	131.8	132.8	120.7	117.3	116.9
7	140.4	139.0	130.3	125.6	133.4
7α	19.9	21.5	21.1	19.4	19.4
8	151.7	150.7	136.2	128.5	133.9
8α	22.2	22.5	21.8	19.4	19.7
9	118.3	119.3	118.7	117.5	124.9
9a	133.5	134.2	127.7	132.2	134.3
10a	152.1	150.8	156.9	155.6	160.0
1′	48.8	45.8	47.3		
2′	70.7	68.1	66.8		
3′	73.1	72.8	72.9		
4′	74.0	74.8	75.3		
5′	66.4	65.8	66.3		

*Measured in D$_2$O.
†Measured in 25 mM sodium/potassium phosphate, pH 7.0, containing 10% (v/v) ^{2}H$_2$O.
‡Measured in CDCl$_3$ (28).
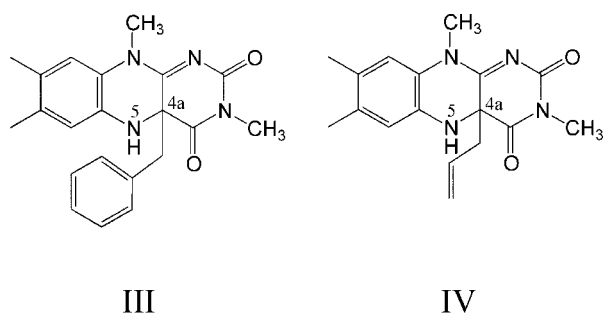
Fig. 4. Structures of 3-methyl-4a-benzyl-4a,5-dihydrolumiflavin (III) and 3-methyl-4a-allyl-4a,5-dihydrolumiflavin (IV).

at 22.5 ppm can be assigned to the 8α methyl carbon on the basis of the chemical shift and the coupling pattern (44 Hz).

The multiplets at 74.8, 72.8, and 68.1 ppm observed in the sample with universally $^{13}C,^{15}N$-labeled FMN represent the ribityl side chain carbons 2′ to 4′ on the basis of their chemical shifts (by comparison with free FMN) and coupling patterns (Fig. 2 a and b). The doublet at 65.8 ppm (coupling constant, 40 Hz) and the broad line at 45.8 ppm in Fig. 2a most probably represent C-5′ and C-1′ of the ribityl side chain that resonate at 66.4 and 48.8 ppm in free FMN, respectively (Table 2).

Constant light illumination caused substantial spectral modulation. Generally, the signal-to-noise ratio in these spectra obtained under blue light irradiation of the samples was lower as compared with the dark condition spectra. This may be the result of several cooperating factors such as incomplete photobleaching and modified dynamic features of the flavin in the photobleached protein. The spectra clearly show line broadening in the illuminated state, which contributes to the lower apparent signal-to-noise ratio (Fig. 2). This could be caused by aggregation in the illuminated state, but it could also possibly be caused by a short lifetime of the photo-excited state.

The assignment of carbon atoms 2, 4, 4a, 7α, 9, and 10a in the light state is again straightforward on the basis of comparison between the spectra of proteins that had been reconstituted with differently labeled FMN (Figs. 2 and 3). Notably, in the spectrum of the protein reconstituted with $[U-^{13}C_{17},U-^{15}N_4]FMN$, the coupling constant of the doublet representing C-4 (due to $^{13}C^{13}C$ coupling with C-4a) is 52 Hz in the light state as compared with 75 Hz in the dark state (Fig. 2 a and b, Table 1).
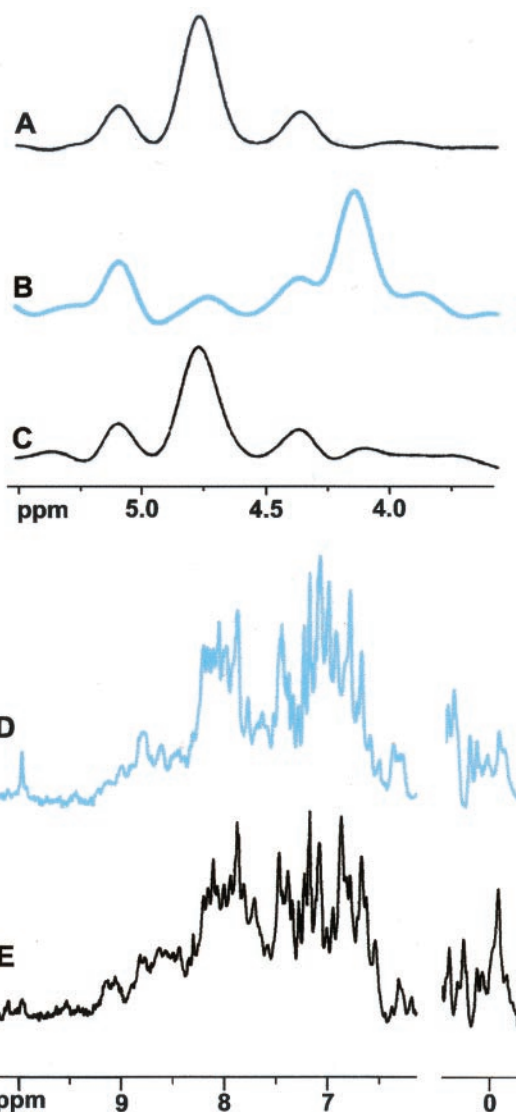
Fig. 6. ^{31}P NMR spectra (*A–C*) and ^1H-NMR spectra (*D* and *E*) of LOV2 domain. (*A–C*) Spectra were measured consecutively in the dark, under illumination, and in the dark, respectively. Spectrum *E* was measured in the dark, and spectrum *D* was measured under blue light illumination (see Fig. 2).
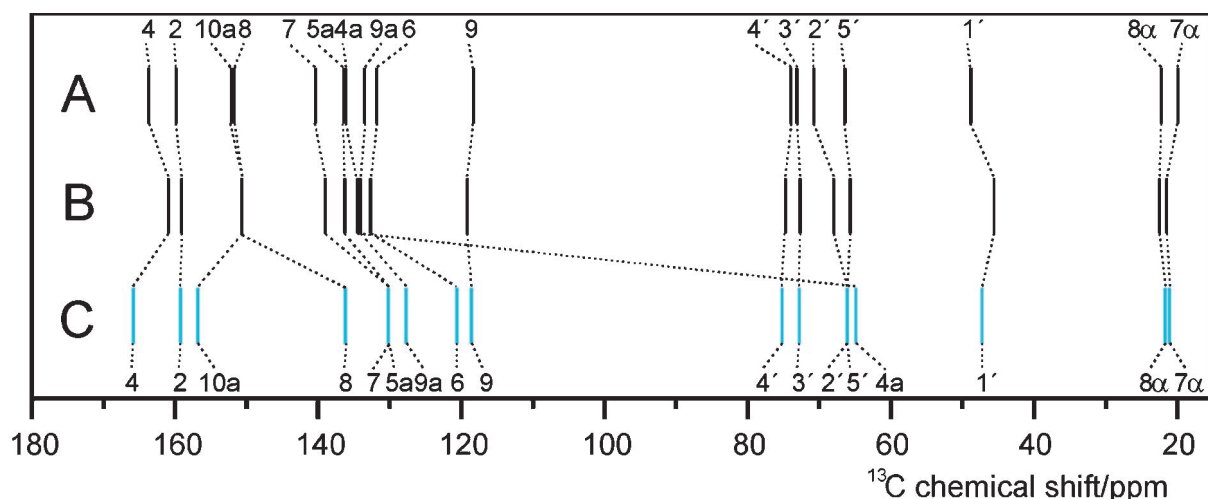
Fig. 5. ^{13}C chemical shifts of FMN (*A*) in solution, (*B*) bound to LOV2 domain in the dark, and (*C*) bound to LOV2 domain under illumination with blue light.

The signal of C-4a experiences an upfield shift of 69.5 ppm on illumination but appears with a relative intensity of 0.4 as compared with the signal of C-2, whereas the signals of C-2 and C-4 have similar amplitudes in the dark. The observed upfield shift, as well as the decreased coupling constant between C-4 and C-4a, provides strong evidence for sp^3 hybridization of C-4a. The 4a-substituted 4a,5-dihydroflavins display ^{13}C NMR chemical shifts for C-4a in the range of 60–85 ppm (25, 26) (Fig. 4, Table 2). The detected chemical shift of 65.0 ppm for C-4a in LOV2 is therefore well in line with a C-4a-cysteinyl adduct.

Substantial chemical shift changes occur with the carbon atoms 5a, 6, 7, 8, 9, and 9a, which are clustered around the 125-ppm region. Because the signals can as yet not be assigned on the basis of chemical editing data due to the lack of appropriately labeled FMN samples, they can be assigned only tentatively by comparison with other flavins and flavoproteins (Tables 1 and 2).

Chemical shifts of all carbon atoms of FMN are shown schematically in Fig. 5. The positions of most signals are shifted by the protein environment, but by far the largest shift on illumination is that of the signal of C-4a, supporting the formation of a (C-4a) adduct. The formation of a 4a thiol adduct is well in line with the x-ray structure of the LOV2 domain in the dark state, where the sulfhydryl group of cysteine 450 is located close to the 4a position of the isoalloxazine chromophore (12).

^{15}N NMR observation of signals of a sample reconstituted with universally ^{13}C,^{15}N-labeled FMN suggests that the signal of N-5 undergoes an upfield shift of 283 ppm under illumination (Table 1), confirming the 4a,5-dihydroflavin motif.

The light-induced chemical shift changes of the isoalloxazine chromophore of protein-bound enzyme result from the structural changes described. However, the ribityl side chain carbon atoms also undergo substantial chemical shift changes (Table 1), which are proposed to result from conformational modification. Similarly, the reversible upfield shift of 0.6 ppm experienced by the phosphate residue of FMN on illumination indicates conformational modification of the phosphate environment (Fig. 6). Last but not least, significant differences between the dark and light state are observed in ^{1}H NMR spectra (Fig. 6). Major changes can be seen in the NH region, where backbone NH protons resonate (6–10 ppm).

The light-induced chemical shift changes of the ribityl carbon atoms, the phosphate moiety, and the ^{1}H resonances of the protein signify changes in the environment of a large number of atoms, which are best explained by conformational changes that are secondary effects caused by the structural changes involved in covalent chromophore–protein linkage. PAS domains are known to act as dimerization domains (29, 30). We propose therefore that the conformational changes of LOV domains initiate dimerization or multimerization of NPH1 protein, which results in autophosphorylation of the protein by the kinase domain. Thus, the adduct formation could act as an optomechanical switch that triggers the blue light signal cascade.

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