

The Primary Photophysics of the *Avena sativa* Phototropin 1 LOV2 Domain Observed with Time-resolved Emission Spectroscopy[†]

Ivo H. M. van Stokkum¹, Magdalena Gauden¹, Sean Crosson², Rienk van Grondelle¹, Keith Moffat^{2,3} and John T. M. Kennis^{*1}

¹Department of Physics and Astronomy, Faculty of Sciences, VU University, Amsterdam, The Netherlands

²Department of Biochemistry and Molecular Biology, University of Chicago, Chicago, IL

³Institute for Biophysical Dynamics, University of Chicago, Chicago, IL

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ABSTRACT

The phototropins are blue-light receptors that base their light-dependent action on the reversible formation of a covalent bond between a flavin mononucleotide (FMN) cofactor and a conserved cysteine in light, oxygen or voltage (LOV) domains. The primary reactions of the *Avena sativa* phototropin 1 LOV2 domain were investigated by means of time-resolved and low-temperature fluorescence spectroscopy. Synchroscan streak camera experiments revealed a fluorescence lifetime of 2.2 ns in LOV2. A weak long-lived component with emission intensity from 600 to 650 nm was assigned to phosphorescence from the reactive FMN triplet state. This observation allowed determination of the LOV2 triplet state energy level at physiological temperature at 16600 cm⁻¹. FMN dissolved in aqueous solution showed pH-dependent fluorescence lifetimes of 2.7 ns at pH 2 and 3.9–4.1 ns at pH 3–8. Here, too, a weak phosphorescence band was observed. The fluorescence quantum yield of LOV2 increased from 0.13 to 0.41 upon cooling the sample from 293 to 77 K. A pronounced phosphorescence emission around 600 nm was observed in the LOV2 domain between 77 and 120 K in the steady-state emission.

INTRODUCTION

Plant growth and development are to a great extent regulated by light. Plants have evolved several photoreceptors that are able to respond to both the blue and red regions of the solar spectrum (1–3). The plant phototropins are serine/threonine kinases that undergo autophosphorylation in response to absorption of blue light and control several physiological responses such as phototropism, light-mediated chloroplast movement and stomatal opening (1). The photochemistry in this class of photoreceptors takes place in two flavin mononucleotide (FMN)-binding light, oxygen or voltage (LOV) domains at the N-terminus of the protein, which comprise *ca* 100 amino acids (4–6). LOV domains form a subclass of the widely distributed Per-ARNT-Sim (PAS) family of signaling

and interaction proteins (7). LOV domains were also identified in algal (*Chlamydomonas*) phototropin (8,9), and in a number of prokaryotes (10–16). They act as signaling photoreceptors involved in the regulation of plant and fungal circadian rhythms (17,18). Because of the ubiquitous cellular bioavailability of flavins, it has been suggested that LOV domains may serve as genetically encoded switches and fluorescence sensors (19–23). LOV domains share such favorable bioengineering properties with BLUF domains (24), which also bind flavin, and bacteriophytochrome (25,26), which binds biliverdin. Thus, the photoactivation mechanisms of biological photoreceptors are of considerable interest to bio(medical) research and technology.

Absorption of a blue photon in the LOV domain initiates a photocycle that leads to the formation of a long-lived flavin species absorbing at 390 nm (5,27,28). It was proposed that this species corresponds to a covalent cysteinyl-C(4a) adduct (5) which was confirmed using NMR spectroscopy, X-ray crystallography and FTIR spectroscopy (29–32). Thus, absorption of blue light leads to the transient formation of a covalent bond between the FMN cofactor and the protein, which slowly ruptures in the dark to regenerate the noncovalent dark ground state species, presumably through a base-catalyzed mechanism (33,34). Absorption of near-UV light may also rupture the covalent adduct (35,36). Formation of a covalent bond to FMN bond triggers protein conformational changes on the surface of the PAS core which weaken interactions of this core with a C-terminal amphiphilic helix called J α , packed against its central β -sheet (37–39). Unfolding of the J α helix is the critical event which regulates C-terminal kinase activity of phototropin and downstream signal transduction (40).

The light-driven reactions of the LOV domain have been studied by means of time-resolved absorption spectroscopy (27,28,41,42). Formation of a spectroscopic photointermediate absorbing at 390 nm, corresponding to the covalent FMN-cysteine adduct, takes place on a microsecond timescale. The covalent adduct has a lifetime of seconds to hours before it returns to the dark state. An intermediate state preceding adduct formation absorbing in the red showed spectral features characteristic of a FMN triplet state. Ultrafast spectroscopy showed that indeed singlet-to-triplet intersystem crossing takes place on the FMN chromophore on the

*Corresponding author email: j.t.m.kennis@vu.nl (John T. M. Kennis)

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nanosecond timescale at high yield (43–45). Based on earlier molecular orbital calculations (46) this triplet state was proposed to be the reactive species that leads to adduct formation (6,27,43). Alternatively, a radical pair mechanism was invoked to explain the LOV photoreaction (47–49) with neutral Cys-flavin radicals forming a short-lived intermediate on the reaction coordinate toward the covalent adduct. Recent spectroscopic and computational work has favored the latter mechanism (45,50–54).

In this contribution we further explore the primary photo-physics of the LOV2 domain of *Avena sativa* (oat) phototropin 1 and FMN in aqueous solution by means of time-resolved fluorescence spectroscopy utilizing a multichannel synchroscan streak camera. The time-resolved emission data were published before in ref. (55). This work presents a reanalysis of these data and resolves phosphorescence from the reactive triplet state of FMN bound to LOV2 and in aqueous solution, which allows an accurate positioning of the FMN triplet state level at physiological temperature. We discuss the results in relation to low-temperature emission spectroscopy on LOV2 published earlier (55).

MATERIALS AND METHODS

Sample preparation. LOV2 from *A. sativa* phototropin 1 was expressed from a construct spanning residues 407–563 and contained an N-terminal fusion of protein G and a His-tag (37). Twelve liters of cells was grown at 37°C to an optical density of 0.4 at 600 nm, induced with 500 μ M Isopropyl β -D-thiogalactoside and grown for an additional 14 h after induction at 20°C. Cells were lysed *via* sonication. *A. sativa* phot1 LOV2 was purified on Ni-NTA resin (Qiagen). The protein was concentrated in a high-pressure stirred ultrafiltration cell with a 3000 molecular weight cutoff filter (Amicon). Prior to the experiments, the LOV2 domain was dissolved in 20 mM Tris/150 mM NaCl buffer at pH 8.0. For the time-resolved experiments, the absorbance of the sample was adjusted to 0.1 per mm at the absorption maximum of 447 nm. The sample was loaded in a flow system containing a cuvette of 1 mm path length, and flowed at a speed of *ca* 5 cm s⁻¹ by means of a peristaltic pump. The total volume of the flow system was 1.5 mL. For the low-temperature fluorescence measurements, the sample was diluted to an absorbance of 0.015 per mm, and contained in plastic cuvettes of 1 cm path length. FMN was purchased from Sigma Chemicals and used without further purification. FMN was dissolved in 20 mM Tris, acetate or formate buffers at pH 8.0, 5.0 or 3.0/2.0, respectively.

Fluorescence spectroscopy. The streak camera setup has been described earlier (56,57) and was applied to examine the fluorescence decay kinetics of the LOV2 domain and of FMN at pH 2.0, 3.0, 5.0 and 8.0. The time-resolved fluorescence kinetics were recorded upon excitation at 400 nm at an excitation power of 100 μ W. The pulses were generated with a 50 kHz repetition rate using a regeneratively amplified titanium:sapphire laser (Coherent Mira-Rega). Fluorescence was collected with a right-angle detection geometry using achromatic lenses and detected through a sheet polarizer set at the magic angle (54.7°) with a Hamamatsu C5680 synchroscan camera and a Chromex 250IS spectrograph. The streak images were recorded with a cooled (-55°C) Hamamatsu C4880 CCD camera. The streak image represents the fluorescence intensity as a function of both time (vertical axis) and wavelength (horizontal axis). The image has a size of 1018 (vertical) \times 1000 (horizontal) pixels, corresponding to 2033.5 ps and 310 nm, respectively. The spectral resolution was 8 nm and the spectrometer was calibrated by means of a Hg lamp. The spectra were not corrected for the wavelength dependence of the spectrograph diffraction efficiency and streak camera sensitivity. The streak camera data were analyzed with a global analysis program using sums of exponentials (58,59). Associated with each lifetime is a decay-associated spectrum (DAS). The instrument response function was described by a gaussian (20 ps full width at half-maximum).

RESULTS AND DISCUSSION

Time-resolved fluorescence spectroscopy

FMN singlet excited-state dynamics in LOV2 and aqueous buffer. The room temperature absorption spectrum of dark-adapted *A. sativa* LOV2 domain (data not shown) agreed with those presented earlier. In accordance with the literature, this species is referred to as D₄₄₇ (27). The absorption spectrum exhibits a major peak at 447 nm and two shoulders at 422 and 473 nm, which correspond to vibronic states of the lowest singlet excited state S₁ of the FMN chromophore. The band at 375 nm can be assigned to the higher-lying S₂ singlet excited state of FMN.

The excited-state dynamics of the LOV2 domain and FMN in aqueous buffer at various pH were examined by means of time-resolved fluorescence spectroscopy. We performed synchroscan streak camera fluorescence measurements upon 400 nm excitation. Figure 1A,B shows the kinetics of the LOV2 domain at 524 and 620 nm, respectively, along with the result of a global analysis procedure. One lifetime is required to adequately describe the time-resolved fluorescence data. The DAS is shown in Fig. 1C (solid line) and shows vibronic maxima near 495 and 525 nm and a shoulder at 550 nm. We find a fluorescence lifetime of the LOV2 domain of 2.2 ns, which agrees well with our previous result from ultrafast spectroscopy of 2.0–2.2 ns (43,60) and that of others (44). It is significantly shorter than the fluorescence lifetime of free FMN in aqueous solution ([61,62] and see below), and shorter than the fluorescence lifetime for the LOV1 domain in *Chlamydomonas reinhardtii* of 2.9 ns (62). The shortened fluorescence lifetime of the LOV2 domain compared with free FMN in solution most likely results from enhanced intersystem crossing to the triplet state due to the proximity of the cysteine sulfur to the isoalloxazine ring (43,62,63). Enhancement of the intersystem crossing rate in LOV2 is induced through weak electron donation by the cysteine which mixes the FMN π -electrons with the sulfur orbitals (63). In agreement with this notion, the C57S mutant of *Chlamydomonas* LOV1 has a significantly longer fluorescence lifetime of 4.6 ns (62). Ultrafast IR and (time-resolved) FTIR experiments on plant LOV2 domains are consistent with an unprotonated FMN triplet as the primary photoproduct (45,53,54).

To date, all time-resolved work on LOV domains indicated strictly single-exponential fluorescence decay of the FMN chromophore in 2–4 ns (this work and refs. [43–45,62,64–66]). This contrasts with results on other flavin-binding photoreceptors such as BLUF domains and cryptochromes where rapid, multiphasic electron transfer processes from nearby aromatic side chains quench the oxidized flavin singlet excited state on the picosecond timescale (67–77). The single-exponential behavior is consistent with a highly ordered structure around the FMN chromophore in LOV domains, which is generally supported by their X-ray structures (6,32,78–80). However, it is noteworthy that multiple cysteine conformers were observed in the ground state in some X-ray structures (32,78) and by FTIR spectroscopy (81,82). If the reactive cysteine were to shorten the FMN singlet excited lifetime in LOV domains through a heavy atom effect, the single-exponential fluorescence decay of fluorescence would imply

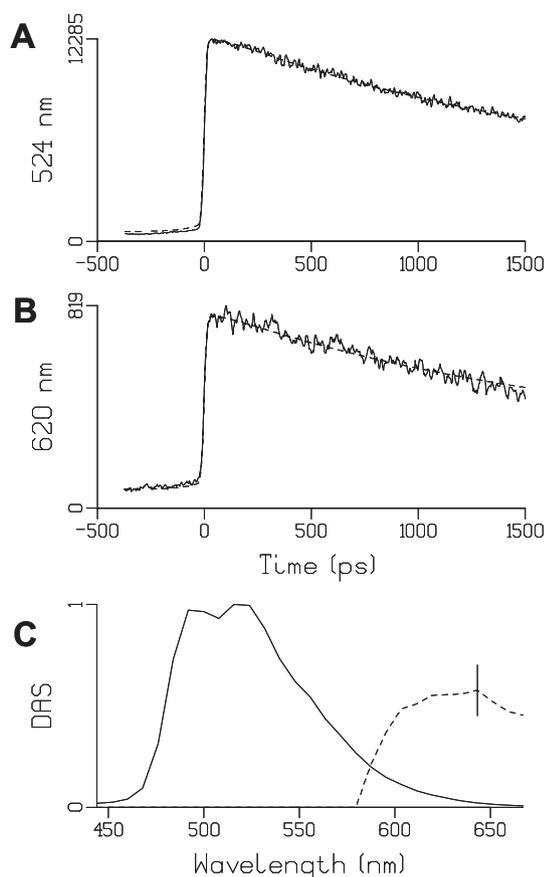


Figure 1. (A, B) Representative traces of light, oxygen or voltage 2 (LOV2) emission (solid) and their fits (dashed), at 524 nm (A) and 620 nm (B). Estimated lifetime was 2.2 ns. At 524 nm, the signal before time zero can be entirely attributed to the 2.2 ns decay in combination with the backsweep of the synchroscan streak camera system (59). At 620 nm, the relatively higher magnitude of the signal before time zero must be attributed to an additional long-lived component. For this we used the 2 μ s lifetime of the phosphorescence. During this very long lifetime the signal builds up from about a thousand forward and backward sweeps (the period of the synchroscan is 13 ns). See text for details. (C) Decay-associated spectra (DAS) of LOV2. The solid line denotes the 2.2 ns DAS, the dashed line the 2 μ s DAS. The dashed phosphorescence DAS has been multiplied by 5×10^4 . The vertical bar indicates the estimated standard error, which is negligible for the 2.2 ns DAS.

that cysteine conformer interconversion must be fast relative to the singlet excited-state lifetime of a few nanoseconds so that an averaged effect is observed. Indeed, molecular dynamics (MD) simulations have indicated that such side chain interconversions occur on the subnanosecond timescale (82).

Figure 2 shows the kinetics of FMN fluorescence emission at 522 and 630 nm in aqueous solution at pH 2. Figure 3 shows the kinetics at these wavelengths for FMN in aqueous solution at pH 8. The DAS for FMN at pH 2, 3, 5 and 8 are shown in Fig. 4 and show a broad band with a single maximum near 520 nm. For FMN, monoexponential fluorescence lifetimes of 2.7 ns at pH 2, and 3.9, 4.1 and 3.9 ns at pH 3, 5, 8, respectively, were found. The fluorescence lifetime of FMN at pH 8 reasonably agrees with the literature value (61,62). At pH 2 there is a significant shortening of the FMN

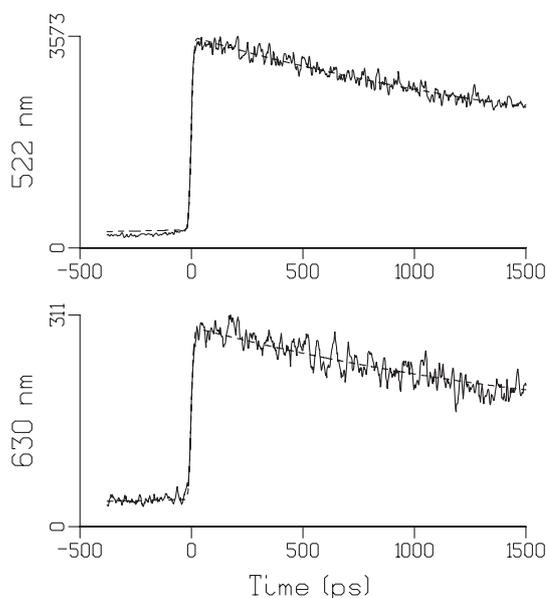


Figure 2. Representative traces of flavin mononucleotide emission at pH 2 (solid) and their fits (dashed) at 522 nm (top) and 630 nm (bottom). The estimated lifetime was 2.7 ns. At 522 nm, the signal before time zero can be entirely attributed to 2.7 ns decay in combination with the backsweep (after 6.5 ns) of the synchroscan streak camera system. At 630 nm, the relatively higher magnitude of the signal before time zero must be attributed to an additional long-lived component and is assigned to phosphorescence. See text for details.

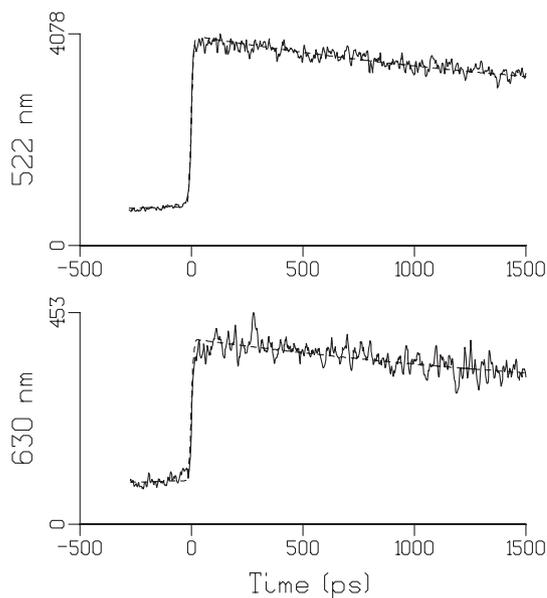


Figure 3. Representative traces of flavin mononucleotide emission at pH 8 (solid) and their fits (dashed) at 522 nm (top) and 630 nm (bottom). Estimated lifetime was 3.9 ns. At 522 nm, the signal before time zero can be entirely attributed to 3.9 ns decay in combination with the backsweep (after 6.5 ns) of the synchroscan streak camera system (59). At 630 nm, the relatively higher magnitude of the signal before time zero must be attributed to an additional long-lived component and is assigned to phosphorescence. See text for details.

singlet excited-state lifetime to 2.7 ns, as we have observed with ultrafast transient absorption spectroscopy (43). Similar results were reported for lumiflavin in aqueous solution, where

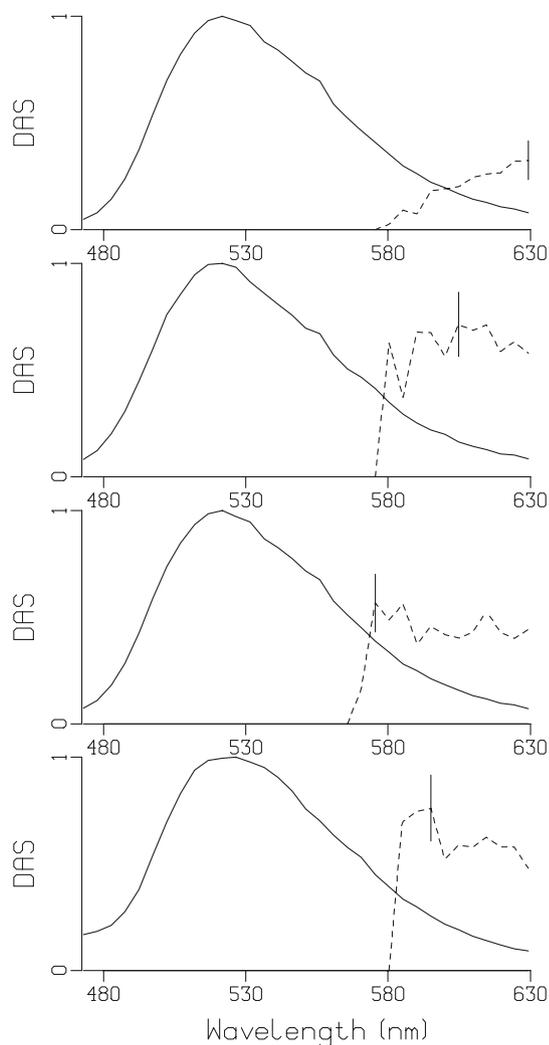


Figure 4. Decay-associated spectra (DAS) of flavin mononucleotide as a function of pH. From top to bottom pH 2, 3, 5, 8, with the solid lines denoting the 2.7, 3.9, 4.2 and 3.9 ns DAS (at pH 2,3,5,8, respectively), and the dashed lines denoting the $10 \mu\text{s}$ DAS. The dashed phosphorescence DAS has been multiplied by 10^5 . The vertical bar indicates the estimated standard error, which is negligible for the ≈ 4 ns DAS. The ordinate refers to the amplitude of the DAS.

the shortening of the fluorescence lifetime was assigned to hydronium ion (H_3O^+)-mediated conversion of neutral lumiflavin to cationic lumiflavin in the singlet excited state (83,84). Thus, although the LOV2 fluorescence lifetime and that of FMN at pH 2 are similar, there is no mechanistic relation between the two.

LOV2 phosphorescence at physiological temperature. To describe observation of LOV2 phosphorescence by the streak camera system, some explanation of the data collection method is required. The synchroscan streak camera is synchronized to the pulse train emitted by the Ti:sapphire oscillator (operating at 76 MHz) that seeds the Rega amplifier (operating at 50 kHz). Thus, the streak camera sweeps back and forth every 13 ns while excitation of the sample takes place every $20 \mu\text{s}$. For this reason, emission that is longer-lived than the (maximum) 2 ns time window may be recorded on the streak camera image as a constant contribution that is not time

resolved. Its presence becomes apparent as a nonzero signal before time zero, essentially a “baseline” signal that in such a case has a distinct physical origin.

With the 2.2 ns fluorescence lifetime of LOV2, it is expected that such a nonzero signal appears on the streak image as a result from the first “back sweep” at 6.5 ns, because the fluorescence signal has not entirely decayed to zero at that time. In fact, the amplitude of this so-called back sweep signal can be used to accurately estimate fluorescence lifetimes that are significantly longer than the maximum 2 ns time basis of the streak camera (59), as we do here for LOV2 and FMN in aqueous solution. This is illustrated in Fig. 1A, where a kinetic trace is shown with detection at 524 nm. It shows a signal before time zero that has a finite value, and the subsequent rise and decay of the fluorescence. The dashed line indicates a fit with an estimated single-exponential lifetime of 2.2 ns which also takes into account the back sweep effect on the streak camera. The fit accurately describes fluorescence decay and the prezero signal.

Figure 1B shows the streak camera signal at 620 nm. Interestingly, the prezero signal is higher relative to the fluorescence signal after time zero (amplitude ratio 1:10) as compared to that observed at 524 nm of Fig. 1A (amplitude ratio 1:25). We conclude that a long-lived emission component, additional to the 2.2 ns fluorescence decay, also contributes to the streak camera signal at this longer wavelength. With the laser system operating at 50 kHz ($20 \mu\text{s}$ pulse-to-pulse interval), the streak camera sweeps more than 4000 times between consecutive excitation pulses and accumulates the long-lived emission shown as the finite prezero signal in Fig. 1B. Hence, the absolute emission amplitude of the additional component may be very weak. Since we were aware that phosphorescence from the FMN triplet state might be present, we analyzed the data with an additional emission component with a lifetime of $2 \mu\text{s}$, the FMN triplet state lifetime in the *A. sativa* LOV2 domain (27). Figure 1C shows the DAS of the LOV2 fluorescence (solid line) and the additional long-lived component (dashed line), which was expanded 5×10^4 times. It shows a band spanning 600–650 nm, which indeed is typical of flavin phosphorescence (85). Thus, this experiment provides the first experimental observation of phosphorescence from the reactive FMN triplet state in LOV domains at physiological temperature. With a modeled decay time of $2 \mu\text{s}$, the integrated area under the phosphorescence band as compared to that of the fluorescence band provides an estimate of the dipole strength of the spin-forbidden $^3\text{FMN} \rightarrow ^1\text{FMN}$ transition. It amounts to roughly 10^{-5} of that of the optical singlet-state emission, corresponding to the spin-forbidden nature of the triplet-singlet transition. The observation of phosphorescence allows determination of the LOV2 triplet state energy level at $16\,600 \text{ cm}^{-1}$. Losi *et al.* (86) determined the energy content of a transient species absorbing at 660 nm, which is presumed to correspond to the triplet state, in the *Bacillus subtilis* YtvA protein by calorimetric methods. They estimated the energy content of the triplet state species to be 198 kJ mol^{-1} or about 16800 cm^{-1} , in excellent agreement with our present value.

For FMN in aqueous solution, phosphorescence phenomena similar to those in LOV2 were observed. The kinetic traces of FMN at pH 2 and 8 show a relatively higher prezero signal at 630 nm than at 522 nm (Figs. 2 and 3). Global analysis of

the time-resolved data yielded the DAS of fluorescence (solid line) and phosphorescence (dashed line) shown in Fig. 4 for FMN at pH 2, 3, 5 and 8. Because the FMN triplet lifetime in aqueous solution is *a priori* unknown (we did not attempt to control the oxygen concentration), we arbitrarily adjusted the lifetime to 10 μ s to give a DAS amplitude similar to that of LOV2. For FMN at pH 2, the phosphorescence is not well resolved, which may be related to the low triplet yield under these conditions (43).

We also performed streak camera experiments on LOV2 on a short time basis of 200 ps with 3 ps time resolution (data not shown). As for the long time basis of 2 ns, a single fluorescence decay time constant of about 2 ns was observed, without appreciable spectral shifting and a DAS identical to that shown in Fig. 1C. The lack of spectral redshifting of the fluorescence on a picosecond timescale is consistent with the notion that the solvation response of a protein matrix to creation of an excited state on the chromophore is very rapid and occurs on the (sub) 100 fs timescale (87–89). In similar experiments on BLUF domains, no spectral redshifting of the fluorescence spectrum was observed (70,77,90).

Low-temperature absorption and fluorescence spectroscopy

To compare the observation of phosphorescence in the streak camera data, it is useful to consider low-temperature steady-state fluorescence data of the *A. sativa* LOV2 domain, reproduced from ref. (55) shown in Fig. 5. The excitation wavelength was 447 nm. The fluorescence emission spectra exhibit peaks at 485 and 520 nm at low temperature. Increasing the temperature to room temperature shifts the peak positions to 495 and 525 nm, respectively. The slightly different intensity ratios of the 495 and 525 nm bands between the room temperature steady-state fluorescence spectrum (1.2:1) and that of the streak camera (0.95:1, Fig. 1C) arise because the latter was not corrected for wavelength sensitivity of the detection system. The splitting of the 473 nm band in the absorption spectrum at 77 K (33,39) is also reflected in the low-temperature fluorescence spectra, where a double band

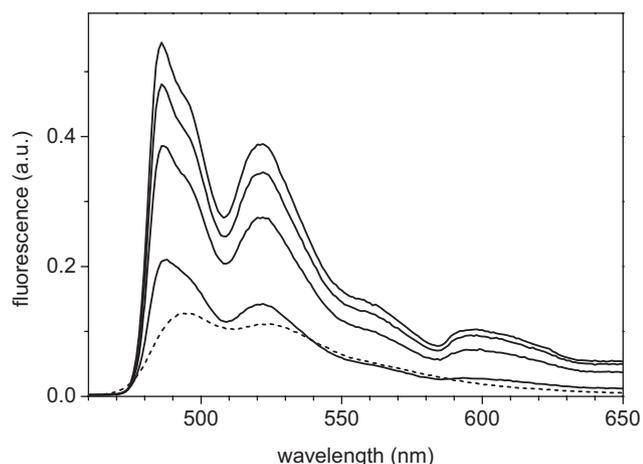


Figure 5. Fluorescence emission spectra of the LOV2 domain of *Avena sativa* phototropin 1 at, from top to bottom, 77 K, 90 K, 120 K, 150 K and 293 K (dashed line). The excitation wavelength was 447 nm.

structure is observed at 485 and 495 nm. The double peak structure was assigned to two different FMN C(4) = O conformer populations, coexisting in the dark state and characterized by C(4) = O carbonyl frequencies at 1712 and 1694 cm^{-1} . These arise from a single H-bond or double H-bonds to this site, from Gln-513 or Asn-492, respectively (39).

Lowering the temperature from 298 K to 77 K leads to a marked increase of the fluorescence intensity. Integration of the fluorescence spectra shows that the fluorescence yield at 77 K is 3.7 times higher than at room temperature. With a room temperature fluorescence quantum yield of 0.13 in the LOV2 domain (43), this observation suggests that the fluorescence quantum yield at 77 K is 0.41. The increase probably follows from internal properties of the FMN chromophore rather than from specific protein–chromophore interactions, as Sun *et al.* have reported that the fluorescence emission of free flavins in solution similarly increases upon lowering the temperature to 77 K (85).

A conspicuous feature of the low-temperature emission spectrum is the small but distinct band near 600 nm. Following our streak camera observations of Fig. 1, this band can be assigned to phosphorescence of the protein-bound flavin (85). The phosphorescence is observed in the 77 K, 90 K and 120 K spectra and to a lesser extent at 150 K. At room temperature no phosphorescence is observed in steady state. Thus, while at room temperature the phosphorescence band is only observed in a time-resolved measurement, lowering the temperature leads to ready observation of phosphorescence in the steady-state luminescence. This observation allows accurate positioning of the flavin triplet state in the LOV2 domain at 16 900 cm^{-1} at cryogenic temperatures.

The question arises why phosphorescence from the FMN triplet state is not observed in the steady-state emission spectrum at physiological temperature, yet is so prominent at cryogenic temperatures. The observation of a phosphorescence band at 77–120 K indicates that (with the forbidden nature of phosphorescence), the FMN triplet state must have a long lifetime. At 5 K, where no photoconversion to the adduct state occurs upon blue-light illumination (data not shown), an orange-red glow lasting for a fraction of a second emerged from the sample upon illumination and subsequent quick blocking of the light (55), indicating that phosphorescence indeed has a relatively long lifetime. Sun *et al.* observed phosphorescence for flavin in frozen solution at 77 K (85), indicating that such long FMN triplet lifetimes are an intrinsic property of flavin. At room temperature, the FMN triplet state is the precursor to the adduct state and has a short lifetime of only 2 μ s, and hence phosphorescence is not observed in steady state. At low temperature, LOV2 only partly photoconverts to the adduct state, at a 28% fraction at 77 K and 78% at 150 K (50,82). Sato *et al.* demonstrated that the partial photoconversion is related to the aforementioned multiplicity of cysteine conformers in LOV2. At such low temperatures, only limited thermal interconversion between the conformers takes place and only those conformers that have a favorable molecular configuration with respect to the conserved cysteine form the covalent adduct upon illumination (82). Hence, the FMN triplet states of those LOV2 domains that do not undergo adduct formation obtain a long lifetime and phosphorescence becomes observable in steady-state emission.

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REFERENCES

- Briggs, W. R., T. S. Tseng, H. Y. Cho, T. E. Swartz, S. Sullivan, R. A. Bogomolni, E. Kaiserli and J. M. Christie (2007) Phototropins and their LOV domains: Versatile plant blue-light receptors. *J. Integr. Plant Biol.* **49**, 4–10.
- Kami, C., S. Lorrain, P. Hornitschek and C. Fankhauser (2010) Light-regulated plant growth and development. *Curr. Top. Dev. Biol.* **91**, 29–66.
- Möglich, A., X. J. Yang, R. A. Ayers and K. Moffat (2010) Structure and function of plant photoreceptors. *Annu. Rev. Plant Biol.* **61**, 21–47.
- Christie, J. M., M. Salomon, K. Nozue, M. Wada and W. R. Briggs (1999) LOV (light, oxygen, or voltage) domains of the blue-light photoreceptor phototropin (nph1): Binding sites for the chromophore flavin mononucleotide. *Proc. Natl Acad. Sci. USA* **96**, 8779–8783.
- Salomon, M., J. M. Christie, E. Knieb, U. Lempert and W. R. Briggs (2000) Photochemical and mutational analysis of the FMN-binding domains of the plant blue light receptor, phototropin. *Biochemistry* **39**, 9401–9410.
- Crosson, S. and K. Moffat (2001) Structure of a flavin-binding plant photoreceptor domain: Insights into light mediated signal transduction. *Proc. Natl Acad. Sci. USA* **98**, 2995–3000.
- Möglich, A., R. A. Ayers and K. Moffat (2009) Structure and signaling mechanism of Per-ARNT-Sim domains. *Structure* **17**, 1282–1294.
- Huang, K. Y., T. Merkle and C. F. Beck (2002) Isolation and characterization of a *Chlamydomonas* gene that encodes a putative blue-light photoreceptor of the phototropin family. *Physiol. Plant.* **115**, 613–622.
- Huang, K. Y. and C. F. Beck (2003) Phototropin is the blue-light receptor that controls multiple steps in the sexual life cycle of the green alga *Chlamydomonas reinhardtii*. *Proc. Natl Acad. Sci. USA* **100**, 6269–6274.
- Losi, A., E. Polverini, B. Quest and W. Gartner (2002) First evidence for phototropin-related blue-light receptors in prokaryotes. *Biophys. J.* **82**, 2627–2634.
- Crosson, S., S. Rajagopal and K. Moffat (2003) The LOV domain family: Photoresponsive signaling modules coupled to diverse output domains. *Biochemistry* **42**, 2–10.
- Purcell, E. B., D. Siegal-Gaskins, D. C. Rawling, A. Fiebig and S. Crosson (2007) A photosensory two-component system regulates bacterial cell attachment. *Proc. Natl Acad. Sci. USA* **104**, 18241–18246.
- Purcell, E. B. and S. Crosson (2008) Photoregulation in prokaryotes. *Curr. Opin. Microbiol.* **11**, 168–178.
- Swartz, T. E., T. S. Tseng, M. A. Frederickson, G. Paris, D. J. Comerci, G. Rajashekar, J. G. Kim, M. B. Mudgett, G. A. Splitter, R. A. Ugalde, F. A. Goldbaum, W. R. Briggs and R. A. Bogomolni (2007) Blue-light-activated histidine kinases: Two-component sensors in bacteria. *Science* **317**, 1090–1093.
- Losi, A. and W. Gartner (2008) Bacterial bilin- and flavin-binding photoreceptors. *Photochem. Photobiol. Sci.* **7**, 1168–1178.
- Krauss, U., B. Q. Minh, A. Losi, W. Gartner, T. Eggert, A. von Haeseler and K. E. Jaeger (2009) Distribution and phylogeny of light-oxygen-voltage-blue-light-signaling proteins in the three kingdoms of life. *J. Bacteriol.* **191**, 7234–7242.
- He, Q. Y., P. Cheng, Y. H. Yang, L. X. Wang, K. H. Gardner and Y. Liu (2002) White collar-1, a DNA binding transcription factor and a light sensor. *Science* **297**, 840–843.
- Imaizumi, T., H. G. Tran, T. E. Swartz, W. R. Briggs and S. A. Kay (2003) FKF1 is essential for photoperiodic-specific light signalling in Arabidopsis. *Nature* **426**, 302–306.
- Drepper, T., T. Eggert, F. Circolone, A. Heck, U. Krauss, J. K. Guterl, M. Wendorff, A. Losi, W. Gartner and K. E. Jaeger (2007) Reporter proteins for in vivo fluorescence without oxygen. *Nat. Biotechnol.* **25**, 443–445.
- Chapman, S., C. Faulkner, E. Kaiserli, C. Garcia-Mata, E. I. Savenkov, A. G. Roberts, K. J. Oparka and J. M. Christie (2008) The photoreversible fluorescent protein iLOV outperforms GFP as a reporter of plant virus infection. *Proc. Natl Acad. Sci. USA* **105**, 20038–20043.
- Strickland, D., K. Moffat and T. R. Sosnick (2008) Light-activated DNA binding in a designed allosteric protein. *Proc. Natl Acad. Sci. USA* **105**, 10709–10714.
- Möglich, A., R. A. Ayers and K. Moffat (2009) Design and signaling mechanism of light-regulated histidine kinases. *J. Mol. Biol.* **385**, 1433–1444.
- Wu, Y. L., D. Frey, O. I. Lungu, A. Jaehrig, I. Schlichting, B. Kuhlman and K. M. Hahn (2009) A genetically encoded photo-activatable Rac controls the motility of living cells. *Nature* **461**, U104–U111.
- Schroder-Lang, S., M. Schwarzel, R. Seifert, T. Strunker, S. Kateriya, J. Looser, M. Watanabe, U. B. Kaupp, P. Hegemann and G. Nagel (2007) Fast manipulation of cellular cAMP level by light in vivo. *Nat. Methods* **4**, 39–42.
- Shu, X. K., A. Royant, M. Z. Lin, T. A. Aguilera, V. Lev-Ram, P. A. Steinbach and R. Y. Tsien (2009) Mammalian expression of infrared fluorescent proteins engineered from a bacterial phytochrome. *Science* **324**, 804–807.
- Toh, K. C., E. A. Stojkovic, I. H. M. van Stokkum, K. Moffat and J. T. M. Kennis (2010) Proton-transfer and hydrogen-bond interactions determine fluorescence quantum yield and photochemical efficiency of bacteriophytochrome. *Proc. Natl Acad. Sci. USA* **107**, 9170–9175.
- Swartz, T. E., S. B. Corchnoy, J. M. Christie, J. W. Lewis, I. Szundi, W. R. Briggs and R. A. Bogomolni (2001) The photocycle of a flavin-binding domain of the blue light photoreceptor phototropin. *J. Biol. Chem.* **276**, 36493–36500.
- Kotke, T., J. Heberle, D. Hehn, B. Dick and P. Hegemann (2003) Phot-LOV1: Photocycle of a blue-light receptor domain from the green alga *Chlamydomonas reinhardtii*. *Biophys. J.* **84**, 1192–1201.
- Salomon, M., W. Eisenreich, H. Durr, E. Schleicher, E. Knieb, V. Massey, W. Rudiger, F. Muller, A. Bacher and G. Richter (2001) An optomechanical transducer in the blue light receptor phototropin from *Avena sativa*. *Proc. Natl Acad. Sci. USA* **98**, 12357–12361.
- Crosson, S. and K. Moffat (2002) Photoexcited structure of a plant photoreceptor domain reveals a light-driven molecular switch. *Plant Cell* **14**, 1067–1075.
- Swartz, T. E., P. J. Wenzel, S. B. Corchnoy, W. R. Briggs and R. A. Bogomolni (2002) Vibration spectroscopy reveals light-induced chromophore and protein structural changes in the LOV2 domain of the plant blue-light receptor phototropin I. *Biochemistry* **41**, 7183–7189.
- Fedorov, R., I. Schlichting, E. Hartmann, T. Domratcheva, M. Fuhrmann and P. Hegemann (2003) Crystal structures and molecular mechanism of a light-induced signaling switch: The Phot-LOV1 domain from *Chlamydomonas reinhardtii*. *Biophys. J.* **84**, 2474–2482.
- Alexandre, M. T. A., J. C. Arents, R. van Grondelle, K. J. Hellingwerf and J. T. M. Kennis (2007) A base-catalyzed mechanism for dark state recovery in the *Avena sativa* phototropin-1 LOV2 domain. *Biochemistry* **46**, 3129–3137.
- Zoltowski, B. D., B. Vaccaro and B. R. Crane (2009) Mechanism-based tuning of a LOV domain photoreceptor. *Nat. Chem. Biol.* **5**, 827–834.
- Kawaguchi, Y., Y. Nakasone, K. Zikihara, S. Tokutomi and M. Terazima (2010) When is the helix conformation restored after the reverse reaction of phototropin? *J. Am. Chem. Soc.* **132**, 8838.
- Kennis, J. T. M., N. H. M. van Stokkum, S. Crosson, M. Gauden, K. Moffat and R. van Grondelle (2004) The LOV2 domain of phototropin: A reversible photochromic switch. *J. Am. Chem. Soc.* **126**, 4512–4513.

37. Harper, S. M., L. C. Neil and K. H. Gardner (2003) Structural basis of a phototropin light switch. *Science* **301**, 1541–1544.
38. Yamamoto, A., T. Iwata, Y. Sato, D. Matsuoka, S. Tokutomi and H. Kandori (2009) Light signal transduction pathway from flavin chromophore to the J alpha helix of Arabidopsis phototropin1. *Biophys. J.* **96**, 2771–2778.
39. Alexandre, M. T. A., R. van Grondelle, K. J. Hellingwerf and J. T. M. Kennis (2009) Conformational heterogeneity and propagation of structural changes in the LOV2/J alpha domain from *Avena sativa* phototropin 1 as recorded by temperature-dependent FTIR spectroscopy. *Biophys. J.* **97**, 238–247.
40. Harper, S. M., J. M. Christie and K. H. Gardner (2004) Disruption of the LOV-J alpha helix interaction activates phototropin kinase activity. *Biochemistry* **43**, 16184–16192.
41. Guo, H. M., T. Kottke, P. Hegemann and B. Dick (2005) The Phot LOV2 domain and its interaction with LOV1. *Biophys. J.* **89**, 402–412.
42. Corchnoy, S. B., T. E. Swartz, J. W. Lewis, I. Szundi, W. R. Briggs and R. A. Bogomolni (2003) Intramolecular proton transfers and structural changes during the photocycle of the LOV2 domain of phototropin 1. *J. Biol. Chem.* **278**, 724–731.
43. Kennis, J. T. M., S. Crosson, M. Gauden, I. H. M. van Stokkum, K. Moffat and R. van Grondelle (2003) Primary reactions of the LOV2 domain of phototropin, a plant blue-light photoreceptor. *Biochemistry* **42**, 3385–3392.
44. Schuttrigkeit, T. A., C. K. Kompa, M. Salomon, W. Rudiger and M. E. Michel-Beyerle (2003) Primary photophysics of the FMN binding LOV2 domain of the plant blue light receptor phototropin of *Avena sativa*. *Chem. Phys.* **294**, 501–508.
45. Alexandre, M. T. A., T. Domratheva, C. Bonetti, L. van Wilderen, R. van Grondelle, M. L. Groot, K. J. Hellingwerf and J. T. M. Kennis (2009) Primary reactions of the LOV2 domain of phototropin studied with ultrafast mid-infrared spectroscopy and quantum chemistry. *Biophys. J.* **97**, 227–237.
46. Song, P. S. (1968) On basicity of excited state of flavins. *Photochem. Photobiol.* **7**, 311–313.
47. Kay, C. W. M., E. Schleicher, A. Kuppig, H. Hofner, W. Rudiger, M. Schleicher, M. Fischer, A. Bacher, S. Weber and G. Richter (2003) Blue light perception in plants—Detection and characterization of a light-induced neutral flavin radical in a C450A mutant of phototropin. *J. Biol. Chem.* **278**, 10973–10982.
48. Bittl, R., C. W. M. Kay, S. Weber and P. Hegemann (2003) Characterization of a flavin radical product in a C57M mutant of a LOV1 domain by electron paramagnetic resonance. *Biochemistry* **42**, 8506–8512.
49. Kottke, T., B. Dick, R. Fedorov, I. Schlichting, R. Deutzmann and P. Hegemann (2003) Irreversible photoreduction of flavin in a mutated Phot-LOV1 domain. *Biochemistry* **42**, 9854–9862.
50. Schleicher, E., R. M. Kowalczyk, C. W. M. Kay, P. Hegemann, A. Bacher, M. Fischer, R. Bittl, G. Richter and S. Weber (2004) On the reaction mechanism of adduct formation in LOV domains of the plant blue-light receptor phototropin. *J. Am. Chem. Soc.* **126**, 11067–11076.
51. Dittrich, M., P. L. Freddolino and K. Schulten (2005) When light falls in LOV: A quantum mechanical/molecular mechanical study of photoexcitation in Phot-LOV1 of *Chlamydomonas reinhardtii*. *J. Phys. Chem. B* **109**, 13006–13013.
52. Domratheva, T., R. Fedorov and I. Schlichting (2006) Analysis of the primary photocycle reactions occurring in the light, oxygen, and voltage blue-light receptor by multiconfigurational quantum-chemical methods. *J. Chem. Theory Comput.* **2**, 1565–1574.
53. Pfeifer, A., T. Majerus, K. Zikihara, D. Matsuoka, S. Tokutomi, J. Heberle and T. Kottke (2009) Time-resolved Fourier transform infrared study on photoadduct formation and secondary structural Changes within the phototropin LOV domain. *Biophys. J.* **96**, 1462–1470.
54. Sato, Y., T. Iwata, S. Tokutomi and H. Kandori (2005) Reactive cysteine is protonated in the triplet excited state of the LOV2 domain in *Adiantum* phytochrome3. *J. Am. Chem. Soc.* **127**, 1088–1089.
55. Gauden, M., S. Crosson, I. H. M. van Stokkum, R. van Grondelle, K. Moffat and J. T. M. Kennis (2004) Low-temperature and time-resolved spectroscopic characterization of the LOV2 domain of *Avena sativa* phototropin. In *Femtosecond Laser Applications in Biology* (Edited by S. Avrillier and J. M. Tualle), pp. 97–104. SPIE, Bellingham.
56. Gobets, B., I. H. M. van Stokkum, M. Rogner, J. Kruip, E. Schlodder, N. V. Karapetyan, J. P. Dekker and R. van Grondelle (2001) Time-resolved fluorescence emission measurements of photosystem I particles of various cyanobacteria: A unified compartmental model. *Biophys. J.* **81**, 407–424.
57. Gobets, B., J. T. M. Kennis, J. A. Ihalainen, M. Brazzoli, R. Croce, L. H. M. van Stokkum, R. Bassi, J. P. Dekker, H. van Amerongen, G. R. Fleming and R. van Grondelle (2001) Excitation energy transfer in dimeric light harvesting complex I: A combined streak-camera/fluorescence upconversion study. *J. Phys. Chem. B* **105**, 10132–10139.
58. van Stokkum, I. H. M., D. S. Larsen and R. van Grondelle (2004) Global and target analysis of time-resolved spectra. *Biochim. Biophys. Acta-Bioenerg.* **1657**, 82–104.
59. van Stokkum, I. H. M., B. van Oort, F. van Mourik, B. Gobets and H. van Amerongen (2008) (Sub)-picosecond spectral evolution of fluorescence studied with a synchroscan streak-camera system and target analysis. In *Biophysical Techniques in Photosynthesis Vol. II* (Edited by T. J. Aartsma and J. Matysik), pp. 223–240. Springer, Dordrecht, The Netherlands.
60. Kennis, J. T. M. and M. T. A. Alexandre (2006) Mechanisms of light activation in flavin-binding photoreceptors. In *Flavins: Photochemistry and Photobiology* (Edited by E. Silva and A. M. Edwards), pp. 287–319. The Royal Society for Chemistry Publishing, Cambridge.
61. Visser, A., A. vanHoek, N. V. Visser, Y. Lee and S. Ghisla (1997) Time-resolved fluorescence study of the dissociation of FMN from the yellow fluorescence protein from *Vibrio fischeri*. *Photochem. Photobiol.* **65**, 570–575.
62. Holzer, W., A. Penzkofer, M. Fuhrmann and P. Hegemann (2002) Spectroscopic characterization of flavin mononucleotide bound to the LOV1 domain of Phot1 from *Chlamydomonas reinhardtii*. *Photochem. Photobiol.* **75**, 479–487.
63. Alexandre, M. T. A., R. van Grondelle, K. J. Hellingwerf, B. Robert and J. T. M. Kennis (2008) Perturbation of the ground-state electronic structure of FMN by the conserved cysteine in phototropin LOV2 domains. *Phys. Chem. Chem. Phys.* **10**, 6693–6702.
64. Holzer, W., A. Penzkofer and P. Hegemann (2005) Absorption and emission spectroscopic characterisation of the LOV2-His domain of phot from *Chlamydomonas reinhardtii*. *Chem. Phys.* **308**, 79–91.
65. Holzer, W., A. Penzkofer, T. Susdorf, M. Alvarez, S. D. M. Islam and P. Hegemann (2004) Absorption and emission spectroscopic characterisation of the LOV2-domain of phot from *Chlamydomonas reinhardtii* fused to a maltose binding protein. *Chem. Phys.* **302**, 105–118.
66. Alexandre, M. T. A., E. B. Purcell, R. van Grondelle, B. Robert, J. T. M. Kennis and S. Crosson (2010) Electronic and protein structural dynamics of a photosensory histidine kinase. *Biochemistry* **49**, 4752–4759.
67. Brazard, J., A. Usman, F. Lacombe, C. Ley, M. M. Martin, P. Plaza, L. Mony, M. Heijde, G. Zabolon and C. Bowler (2010) Spectro-temporal characterization of the photoactivation mechanism of two new oxidized cryptochrome/photolyase photoreceptors. *J. Am. Chem. Soc.* **132**, 4935–4945.
68. Gauden, M., I. H. M. van Stokkum, J. M. Key, D. C. Luhrs, R. Van Grondelle, P. Hegemann and J. T. M. Kennis (2006) Hydrogen-bond switching through a radical pair mechanism in a flavin-binding photoreceptor. *Proc. Natl Acad. Sci. USA* **103**, 10895–10900.
69. Gauden, M., J. S. Grinstead, W. Laan, H. M. van Stokkum, M. Avila-Perez, K. C. Toh, R. Boelens, R. Kaptein, R. van Grondelle, K. J. Hellingwerf and J. T. M. Kennis (2007) On the role of aromatic side chains in the photoactivation of BLUF domains. *Biochemistry* **46**, 7405–7415.
70. Bonetti, C., T. Mathes, I. H. M. van Stokkum, K. M. Mullen, M. L. Groot, R. van Grondelle, P. Hegemann and J. T. M. Kennis (2008) Hydrogen bond switching among flavin and amino acid side chains in the BLUF photoreceptor observed by ultrafast infrared spectroscopy. *Biophys. J.* **95**, 4790–4802.
71. Bonetti, C., M. Stierl, T. Mathes, I. H. M. van Stokkum, K. M. Mullen, T. A. Cohen-Stuart, R. van Grondelle, P. Hegemann and

- J. T. M. Kennis (2009) The role of key amino acids in the photoactivation pathway of the synechocystis Slr1694 BLUF domain. *Biochemistry* **48**, 11458–11469.
72. Kennis, J. T. M. and M. L. Groot (2007) Ultrafast spectroscopy of biological photoreceptors. *Curr. Opin. Struct. Biol.* **17**, 623–630.
 73. Dragnea, V., M. Waegle, S. Balascuta, C. Bauer and B. Dragnea (2005) Time-resolved spectroscopic studies of the AppA blue-light receptor BLUF domain from *Rhodobacter sphaeroides*. *Biochemistry* **44**, 15978–15985.
 74. Zirak, P., A. Penzkofer, T. Schiereis, P. Hegemann, A. Jung and I. Schlichting (2005) Absorption and fluorescence spectroscopic characterization of BLUF domain of AppA from *Rhodobacter sphaeroides*. *Chem. Phys.* **315**, 142–154.
 75. Shibata, Y., Y. Murai, Y. Satoh, Y. Fukushima, K. Kajima, M. Ikeuchi and S. Itoh (2009) Acceleration of electron-transfer-induced fluorescence quenching upon conversion to the signaling state in the blue-light receptor, TePixD, from *Thermosynechococcus elongatus*. *J. Phys. Chem. B* **113**, 8192–8198.
 76. Toh, K. C., I. H. M. van Stokkum, J. Hendriks, M. T. A. Alexandre, J. C. Arents, M. A. Perez, R. van Grondelle, K. J. Hellingwerf and J. T. M. Kennis (2008) On the signaling mechanism and the absence of photoreversibility in the AppA BLUF domain. *Biophys. J.* **95**, 312–321.
 77. Gauden, M., S. Yeremenko, W. Laan, I. H. M. van Stokkum, J. A. Ihalainen, R. van Grondelle, K. J. Hellingwerf and J. T. M. Kennis (2005) Photocycle of the flavin-binding photoreceptor AppA, a bacterial transcriptional antirepressor of photosynthesis genes. *Biochemistry* **44**, 3653–3662.
 78. Halavaty, A. S. and K. Moffat (2007) N- and C-terminal flanking regions modulate light-induced signal transduction in the LOV2 domain of the blue light sensor phototropin 1 from *Avena sativa*. *Biochemistry* **46**, 14001–14009.
 79. Moglich, A. and K. Moffat (2007) Structural basis for light-dependent signaling in the dimeric LOV domain of the photosensor YtvA. *J. Mol. Biol.* **373**, 112–126.
 80. Zoltowski, B. D., C. Schwerdtfeger, J. Widom, J. J. Loros, A. M. Bilwes, J. C. Dunlap and B. R. Crane (2007) Conformational switching in the fungal light sensor vivid. *Science* **316**, 1054–1057.
 81. Bednarz, T., A. Losi, W. Gartner, P. Hegemann and J. Heberle (2004) Functional variations among LOV domains as revealed by FT-IR difference spectroscopy. *Photochem. Photobiol. Sci.* **3**, 575–579.
 82. Sato, Y., M. Nabeno, T. Iwata, S. Tokutomi, M. Sakurai and H. Kandori (2007) Heterogeneous environment of the S-H group of cys966 near the flavin chromophore in the LOV2 domain of *Adiantum neochrome* 1. *Biochemistry* **46**, 10258–10265.
 83. Drossler, P., W. Holzer, A. Penzkofer and P. Hegemann (2002) pH dependence of the absorption and emission behaviour of riboflavin in aqueous solution. *Chem. Phys.* **282**, 429–439.
 84. Tyagi, A. and A. Penzkofer (2010) pH dependence of the absorption and emission behaviour of lumiflavin in aqueous solution. *J. Photochem. Photobiol. A, Chem.* **215**, 108–117.
 85. Sun, M., T. A. Moore and P. S. Song (1972) Molecular luminescence studies of flavins. I. The excited states of flavins. *J. Am. Chem. Soc.* **94**, 1730–1740.
 86. Losi, A., B. Quest and W. Gartner (2003) Listening to the blue: The time-resolved thermodynamics of the bacterial blue-light receptor YtvA and its isolated LOV domain. *Photochem. Photobiol. Sci.* **2**, 759–766.
 87. Homoelle, B. J., M. D. Edington, W. M. Diffey and W. F. Beck (1998) Stimulated photon-echo and transient-grating studies of protein-matrix solvation dynamics and interexciton-state radiationless decay in alpha phycocyanin and allophycocyanin. *J. Phys. Chem. B* **102**, 3044–3052.
 88. Kennis, J. T. M., D. S. Larsen, K. Ohta, M. T. Facciotti, R. M. Glaeser and G. R. Fleming (2002) Ultrafast protein dynamics of bacteriorhodopsin probed by photon echo and transient absorption spectroscopy. *J. Phys. Chem. B* **106**, 6067–6080.
 89. van Stokkum, I. H. M., B. Gobets, T. Gensch, F. van Mourik, K. J. Hellingwerf, R. van Grondelle and J. T. M. Kennis (2006) (Sub)-picosecond spectral evolution of fluorescence in photoactive proteins studied with a synchroscan streak camera system. *Photochem. Photobiol.* **82**, 380–388.
 90. Laan, W., M. Gauden, S. Yeremenko, R. van Grondelle, J. T. M. Kennis and K. J. Hellingwerf (2006) On the mechanism of activation of the BLUF domain of AppA. *Biochemistry* **45**, 51–60.