Low-temperature and time-resolved spectroscopic characterization of the LOV2 domain of *Avena sativa* phototropin 1

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ABSTRACT

The phototropins are plant 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 residue in light, oxygen or voltage (LOV) domains. The spectroscopic properties of the LOV2 domain of phototropin 1 of *Avena sativa* (oat) have been investigated by means of low-temperature absorption and fluorescence spectroscopy and by time-resolved fluorescence spectroscopy. The low-temperature absorption spectrum of the LOV2 domain showed a fine structure around 473 nm, indicating heterogeneity in the flavin binding pocket. The fluorescence quantum yield of the flavin cofactor increased from 0.13 to 0.41 upon cooling the sample from room temperature to 77 K. A pronounced phosphorescence emission around 600 nm was observed in the LOV2 domain between 77 and 120 K, allowing for an accurate positioning of the flavin triplet state in the LOV2 domain at 16900 cm^{-1}. Fluorescence from the cryotrapped covalent adduct state was extremely weak, with a fluorescence spectrum showing a maximum at 440 nm. Time-resolved fluorescence experiments utilizing a synchroscan streak camera revealed a singlet-excited state lifetime of the LOV2 domain of 2.4 ns. FMN dissolved in aqueous solution showed a pH-dependent lifetime ranging between 2.9 ns at pH 2.0 to 4.7 ns at pH 8.0. No spectral shifting of the flavin emission was observed in the LOV2 domain nor in FMN in aqueous solution.

1. 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.[1,2] The phototropins are serine/threonine kinases that undergo autophosphorylation in response to absorption of blue light,[3] and control several physiological responses such as phototropism, light-mediated chloroplast movement and stomatal opening.[3-6] 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 approximately 100 amino acids.[7-9] Besides in plant phototropins, LOV domains were recently identified in algal (*Chlamydomonas*) phototropin,[10,11] as photoreceptors involved in the regulation of plant and funghal circadian rhythms,[12,13] and in a number of prokaryotes, where their function remains unknown.[14,15]

Absorption of a blue photon in the LOV domain initiates a photocycle that leads to the formation of a long-lived flavin species that absorbs at 390 nm.[8,16,17] It was proposed that this species corresponds to a covalent cysteinyl-C(4a) adduct,[8] which was confirmed using NMR spectroscopy, X-ray crystallography and FTIR spectroscopy.[18-21] Thus, absorption of blue light leads to the transient formation of a covalent bond between the FMN cofactor and the protein, which slowly disrupts to regenerate the non-covalent dark ground state species.

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The light-driven reactions of the LOV domain have been studied by means of time-resolved absorption spectroscopy.[16,17] These experiments indicated that the formation of a spectroscopic photointermediate absorbing at 390 nm, corresponding to the covalent FMN-cysteine adduct, takes place on a μs timescale. This species has a lifetime on the order of minutes before it returns to the ground 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 nanosecond timescale at a high yield.[22] Based on earlier molecular orbital calculations,[23] this triplet state was proposed to be the reactive species that leads to adduct formation.[9,16,22] Recent work has challenged these ideas, and alternative models involving flavin-cysteine radical pairs have been put forward to explain the LOV photoreaction.[24-26]

In this contribution we further explore the spectroscopic properties of the LOV2 domain of *Avena sativa* (oat) phototropin 1 by low-temperature absorption and fluorescence spectroscopy. Moreover, we present the results of time-resolved fluorescence spectroscopy utilizing a multichannel synchroscan streak camera.

2. MATERIALS AND METHODS

2.1 Sample preparation

LOV2 from *Avena sativa* phototropin 1 was expressed from a construct spanning residues 407 to 563 (construct provided by L. Neil and K. Gardner of UT-Southwestern and Medical Center, Dallas), and contained an N-terminal fusion of protein G and a His-tag.[27] Twelve liters of cells were grown at 37° C to an optical density at 600 nm of 0.4, induced with 500 μM Isopropyl ß-D-thiogalactoside and grown for an additional 14 h after induction at 20 °C. Cells were lysed via sonication. *Avena sativa* phot1 LOV2 was purified on Ni-NTA resin (Qiagen). The protein was concentrated in a high-pressure stirred ultrafiltration cell with a 3,000 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 (447 nm). The sample was loaded in a flow system containing a cuvette of 1 mm path length, and flowed at a speed of approximately 5 cm/s 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 0.15 per cm, and contained in plastic cuvettes of 1 cm path length. Flavin mononucleotide (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 2.0, respectively.

2.2 Absorption and fluorescence spectroscopy

Low temperature measurements were performed in 10 mM Tris-HCl buffer of pH 8.0, to which glycerol had been added (66% v/v). 77-K absorption spectra were recorded on a UV-VIS commercial spectrometer (Perkin-Elmer). To perform low temperature experiments the cuvette was placed in a liquid nitrogen cryostat, which fit into sample compartment of the spectrometer and fluorometer. Fluorescence spectra were measured with Aminco-Bowman series 2 fluorometer at an excitation power of 15 μW. Emission spectra were corrected for the detector sensitivity. The streak camera setup has been described earlier[28] and was applied to examine the fluorescence decay kinetics of the LOV2 domain and of FMN at pH 2.0, 5.0 and 8.0. The time resolved fluorescence kinetics were recorded upon excitation at 400 nm at an excitation power of 100 μW 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 (54.7^0) with a Hamamatsu C5680 synchroscan camera and a Chromex 250IS spectrograph. The streak images were recorded with a cooled (-55^0C) Hamamatsu C4880 CCD camera. The streak camera data were analyzed with a global analysis program using sums of exponentials,[29] Associated with each lifetime is a decay-associated spectrum (DAS). The instrument response function was fit to a gaussian (15 ps full width at half-maximum).

3. RESULTS AND DISCUSSION

3.1 Low-temperature absorption and fluorescence spectroscopy

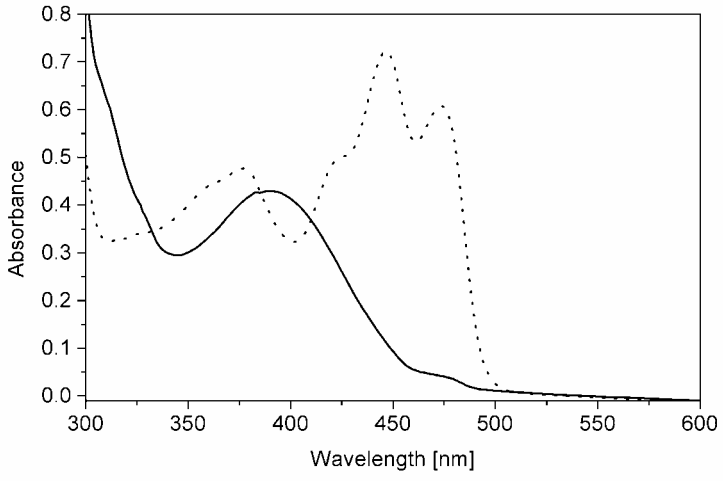

Figure 1. Dotted line: Absorption spectrum of the LOV2 domain of *Avena sativa* phototropin 1 at room temperature. Solid line: absorption spectrum of the LOV2 adduct state at 77K.

The room temperature absorption spectrum of dark-adapted *Avena sativa* LOV2 domain in aqueous buffer at pH 8.0 is shown in Figure 1 (dotted line). In accordance with the literature, this species is referred to as D_{447}.[16] The absorption spectrum exhibits a major peak at 447 nm, and two shoulders at 422 nm and 473 nm which are vibrational states of the lowest singlet excited state S_1 of the FMN chromophore. The vibronic structure within the S_0-S_1 absorption band is observed due to reduced solvent-induced inhomogeneous broadening in the FMN chromphore fixed in the protein binding pocket. The high energy band at 375 nm can be assigned to the higher-lying S_2 singlet excited state of FMN.

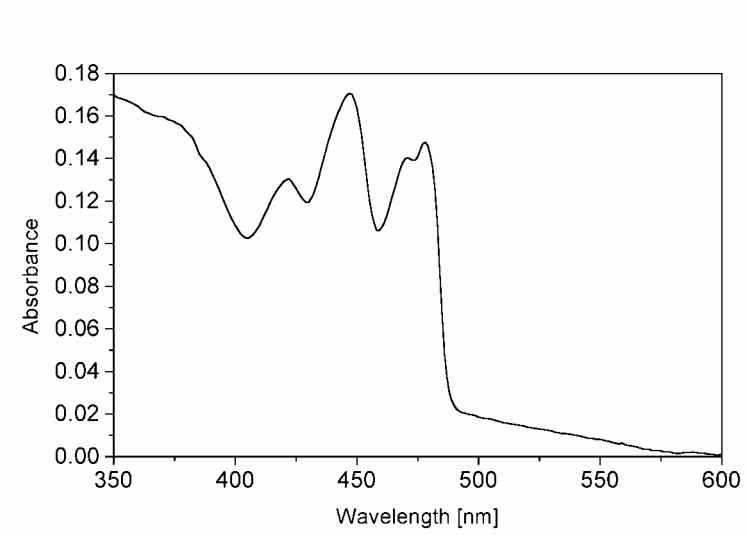

Figure 2. Absorption spectrum of the LOV2 domain of *Avena sativa* phototropin 1 at 77K.

As shown in Figure 2, lowering the temperature to 77 K gives rise to sharpening of the bands in the blue region of the spectrum. Interestingly, the 473 nm band splits in two distinct bands and a shoulder appears at 442 nm. This splitting indicates a heterogeneity in the LOV2 sample and the presence of two fractions of LOV2 domains which differ slightly by their absorption spectrum. Both forms can be photoconverted to the adduct state at low temperature. Possibly the double-band structure reflects multiple cysteine conformations as has been found in the LOV1 domain of *Chlamydomonas* phototropin.[21] We note that no double band structure at 475 nm was observed by Iwata et al. in their low-temperature experiments on *Adiantum* phy3 LOV2.[30]

Figure 3 shows steady-state temperature dependent fluorescence spectra of the *Avena sativa* LOV2 domain on excitation at 447 nm. The fluorescence emission spectra exhibit peaks at 485 nm and 520 nm at low temperature. Increasing the temperature to room temperature caused a shift of the peak positions to 495 nm and 525 nm, respectively. The vibrational progression visible in the absorption spectrum is also seen in the fluorescence spectra. However, the relative amplitudes of the emission bands differ from those of the corresponding absorption bands, and we conclude that there are deviations from mirror symmetry, indicating differences in the nuclear configurations of the relaxed ground and excited electronic states. The splitting of the 473 nm band in the absorption spectrum at 77 K is also reflected in the low-temperature fluorescence spectra, where a double band structure is observed at 485 and 495 nm.

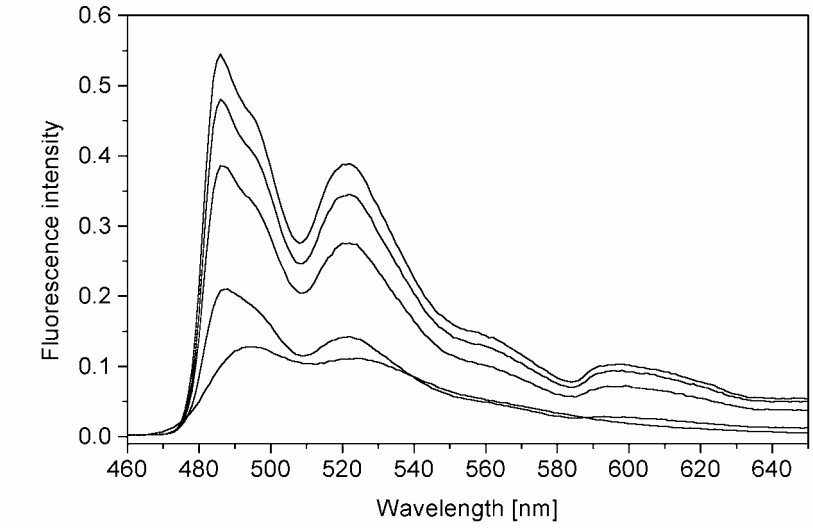

Figure 3. Fluorescence emission spectra of the LOV2 domain of *Avena sativa* phototropin 1 at, from top to bottom, 77K, 90 K, 120 K, 150 K and 293 K. The excitation wavelength was 447 nm.

As can be clearly observed in Figure 3, lowering the temperature from 298 to 77K leads to a marked increase of the fluorescence intensity. Integration of the fluorescence spectra shows that the fluorescence yield at 77K is 3.7 times higher than at room temperature. Given a room temperature fluorescence quantum yield of 0.13 in the LOV2 domain,[22] this implies that the fluorescence quantum yield at 77 K amounts to 0.41. Such a sizable increase of fluorescence yield at low temperature may well occur at the expense of the flavin triplet yield, leading to an overall decreased efficiency of adduct formation at low temperature. The increase of fluorescence yield probably follows from internal properties of the protein-bound FMN chromophore, as Sun et al. have reported that the fluorescence emission of flavins in solution increases in a similar way upon lowering the temperature to 77 K.[31]

A conspicuous feature of the low temperature emission spectrum is the small but distinct band near 600 nm. This band can be assigned to phosphorescence of the protein-bound flavin.[31] The phosphorescence is observed in the 77, 90 and 120 K spectra, and to a less extent in the spectrum at 150 K. At room temperature no phosphorescence is observed. 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, indicating that the phosphorescence in the LOV2 domain is relatively long-lived. The observation of phosphorescence allows for an accurate determination of the energy level of the protein-bound FMN triplet state at 16900 cm^{-1}. Losi et al. have determined the energy content of the transient species absorbing at 660 nm, which is presumed to correspond to the triplet state, in the *Bacillus subtilis* YtvA protein by calorimetric methods. The energy content of the 660 nm species was estimated to be 198 kJ/mole, or about 16800 cm^{-1},[32] in excellent agreement with our present findings.

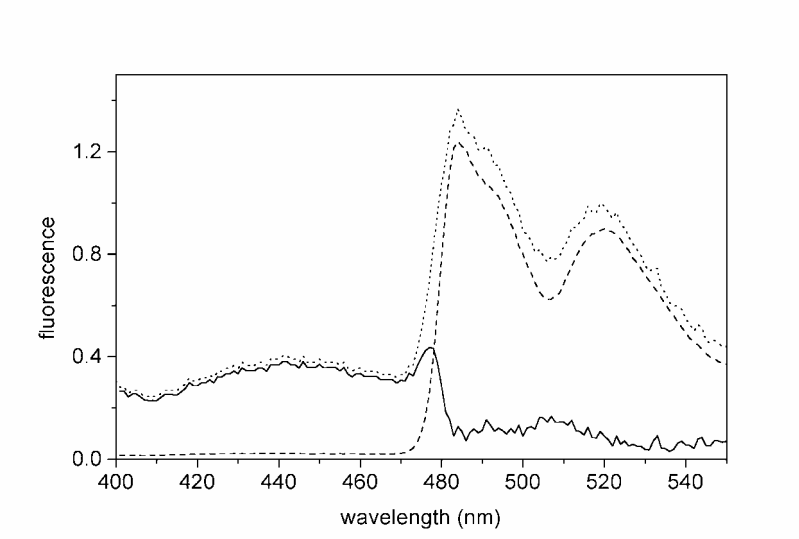

Figure 4. Dotted line: fluorescence spectrum of the adduct state of the LOV2 domain of *Avena sativa* phototropin 1 at 77 K which was cryotrapped as described in the text. Dashed line: fluorescence spectrum of the D447 state at 77 K . Solid line: The 'pure' fluorescence spectrum of the adduct state that results from the subtraction procedure as described in the text.

To further characterize the the LOV2 adduct state spectroscopically, we have recorded the fluorescence spectrum of the cryotrapped adduct. First the adduct was formed by blue-light illumination at 190 K, resulting in a near-complete conversion of D_{447} to the adduct, which is also referred to as S_{390} (Figure 1, solid line). Subsequently the sample was cooled to 77 K and the fluorescence spectrum was recorded upon excitation at 390 nm. Figure 4 shows the result (dotted line). The fluorescence spectrum recorded in this way strongly resembles that of the D_{447} state (Figure 3), indicating that even though hardly any LOV2 domains in the D_{447} state are left in the cryotrapped sample (< 5%), these still dominate the fluorescence. This shows that the S_{390} state has a very low fluorescence quantum yield, in accordance with earlier reports.[14,33] The low fluorescence quantum yield of S_{390} is consistent with our results obtained from ultrafast spectroscopy,[34] where we found a sub-ps to ps excited-state lifetime of the photoexcited adduct. We do see features, however, that can be assigned to fluorescence from S_{390} because the fluorescence spectrum also shows a broad, weak band near 440 nm. The dashed line shows the fluorescence spectrum of the D_{447} state under identical conditions. The latter spectrum lacks the 440 nm band, indicating that in the cryotrapped adduct state's spectrum, it originates from the S_{390} state and not from D_{447}. In order to get the pure fluorescence spectrum of S_{390}, the D_{447} spectrum was appropriately scaled and subtracted. The subtracted spectrum (solid line) is broad and featureless, like the S_{390} absorption, and shows a maximum around 440 nm. From 470 to 500 nm there is an anomalous behavior due to an incomplete subtraction procedure.

3.2 Time-resolved fluorescence spectroscopy

The excited-state dynamics of the LOV2 domain and FMN and various pH has been examined by means of time-resolved fluorescence spectroscopy. We have performed synchroscan streak camera fluorescence measurements upon 400 nm excitation. Streak camera experiments result in two-dimensional data sets, and Figure 5 shows a typical streak image obtained on LOV2 projected on a 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) ×1000 (horizontal) pixels, corresponding to 2033.5 ps and 310 nm, respectively. As can be seen in the image, the spectral

evolution of the LOV2 domain is particularly simple, and it becomes obvious that the FMN chromophore in LOV2 has a singlet-state lifetime of several nanoseconds during which it shows no spectral shifts.

Figure 5. Streak camera image taken of the LOV2 domain *of Avena sativa* upon excitation at 400 nm, displaying the intensity of the fluorescence as a function of time (vertical axis) and wavelength (horizontal axis).

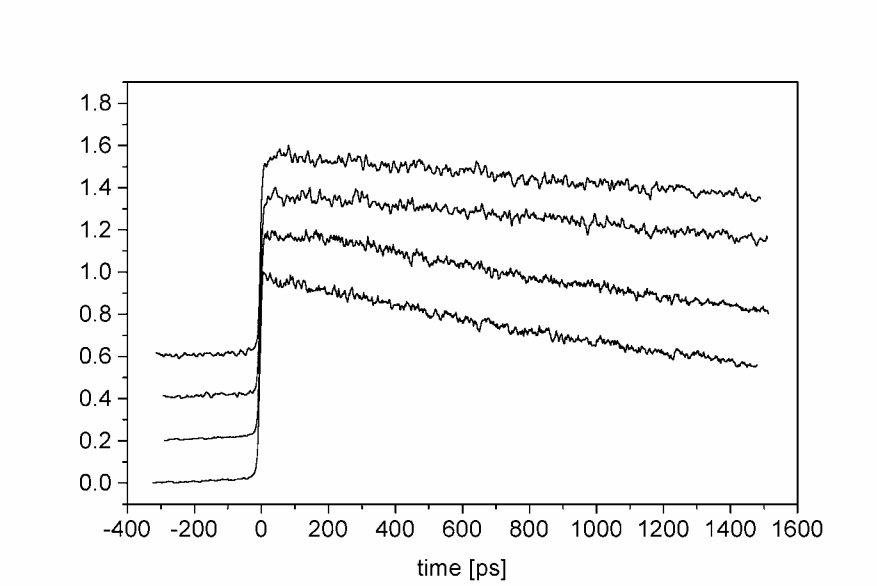

Figure 6. Fluorescence kinetic traces of, from top to bottom, FMN at pH 8.0, 5.0, 2.0 and of the LOV2 domain of *Avena sativa* phototropin 1, respectively. The excitation wavelength was 400 nm.

Figure 6 shows fluorescence kinetic traces of the LOV2 domain and of FMN at pH 2.0, 5.0, 8.0 at the maximum of their fluorescence spectra. Global analysis of the streak images showed that all datasets could be described by a single component. Figure 7 shows the result of the global analysis in the form of decay-associated spectra. For FMN, single fluorescence lifetimes of 2.9 ns at pH 2.0 and 4.7 ns at pH 5.0 and pH 8.0 were found. The fluorescence lifetime of

FMN at pH 8 agrees with the values reported in the literature.[35,36] At pH 2 there is a significant shortening of the FMN singlet excited-state lifetime, as we have observed with ultrafast transient absorption spectroscopy.[22] The reason for that could be an enhanced internal conversion resulting from singlet state protonation, or a proton collisional quenching. The decay-associated spectrum of the LOV2 domain is blue-shifted and narrower with respect to those of FMN in solution. Moreover, vibrational fine structure is observed with a maximum near 495 nm and a shoulder at 525 nm. We find a fluorescence lifetime of the LOV2 domain of 2.4 ns, which fairly agrees with our previous result from ultrafast spectroscopy (2.0 ns)[22] and that of others.[37] It is significantly shorter than the fluorescence lifetime of its chromophore in aqueous solution, and shorter than the fluorescence lifetime for the LOV1 domain in *Chlamydomonas reinhardtii* (2.9 ns).[36] The shortened fluorescence lifetime of the LOV2 domain compared with free FMN in solution most likely results from an enhanced intersystem crossing to the triplet state due to the vicinity of the sulfur atom of the cysteine residue to the isoalloxazine ring.[22,36]

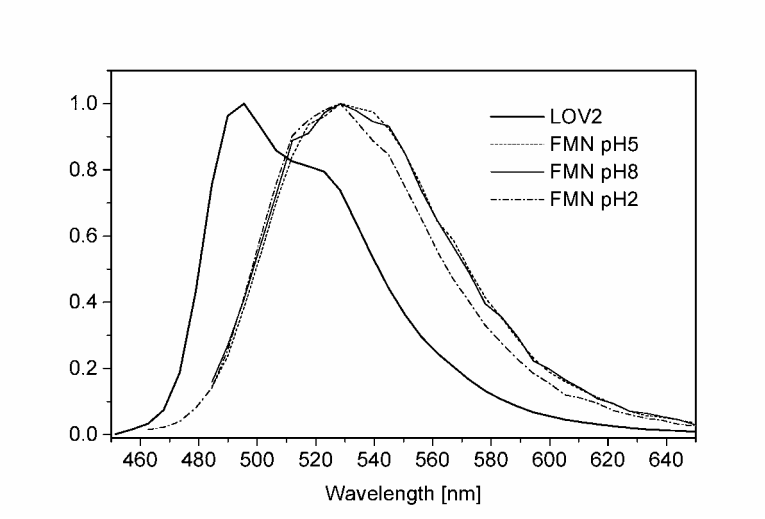

Figure 7. Decay-associated spectra (DAS) of fluorescence decay that follow from a global analysis of streak camera experiments on the LOV2 domain of *Avena sativa* (thick solid line) and FMN in aqueous solution at the pH indicated (thin solid, dashed and dotted lines).

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