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The Photoinduced Triplet of Flavins and Its Protonation States

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Abstract: The photogenerated triplet states of riboflavin and flavin mononucleotide (FMN) have been examined by time-resolved electron paramagnetic resonance (EPR) spectroscopy at low temperature ($T = 80$ K). Because of the high time resolution of the utilized EPR instrumentation, the triplets are for the first time observed in the nonequilibrated electron-spin polarized state and not in their equilibrated forms with the population of the triplet sublevels governed by Boltzmann distribution. The electron-spin polarization pattern directly reflects the anisotropy of the intersystem crossing from the excited singlet-state precursor. Spectral analysis of the resulting enhanced absorptive and emissive EPR signals yields the zero-field splitting parameters, $|D|$ and $|E|$, and the zero-field populations of the triplet at high accuracy. These parameters are sensitive probes for the protonation state of the flavin's isoalloxazine ring, as becomes evident by a comparison of the spectra recorded at different pH values of the solvent. The three protonation states of the flavins can furthermore be distinguished by the kinetics of the transient EPR signals, which are dominated by spin−lattice relaxation. The fastest decays are observed for the protonated FMN and riboflavin triplets, followed by the deprotonated flavin triplets. Slow decays are measured for the triplet states of neutral FMN and riboflavin. Because proton transfer is found to be slow on the time scale of spin-polarized triplet detection by transient EPR, the pH-dependent spin-relaxation and zero-field splitting parameters offer a novel approach to probe the protonation state of flavins in their singlet ground state through the characterization of their triplet-state properties.

Introduction

Flavins are the most frequently encountered organic cofactors in nature.[1−4] They can assume three different redox states: fully oxidized, one-electron reduced, and fully reduced.[5−7] Each of these redox states exists in a cationic, neutral, and anionic form depending on the pH of the solution. The fully oxidized redox state (Figure 1) is typically the energetically most stable one in flavoproteins. While proteins with flavin cofactors rarely utilize the electronic properties of photogenerated excited states, this chromophore is nevertheless widely used as a versatile photosensitizer in the oxidation of biologically important molecules, such as amino acids,[8,9] proteins,[10,11] DNA and nucleotides,[12−15] and EDTA.[16,17] In these photooxidations, three prominent features of the flavin's 7,8-dimethyl isoalloxazine ring are exploited:[18] the ground-state optical absorption in the visible region ranging from 400 to 500 nm, the high quantum yield for intersystem crossing (ISC),[19] and the high oxidation potential of the triplet state. These features are also prerequisite for the quantitative generation of the one-electron reduced semiquinone radical or the two-electron reduced hydroquinone forms of flavins in proteins mostly starting from the fully oxidized redox state of the flavin cofactor. Such photoreduction reactions are typically conducted in the presence of blue light and using an exogenous electron donor such as EDTA or dithionite.[20] However, intraprotein photoreduction processes have also been observed in the absence of external reductants, for example, in the DNA-repair enzyme photolyase.[21,22] In this redox reaction, the triplet state of the flavin adenine dinucleotide (FAD) chromophore is involved.[23,24]

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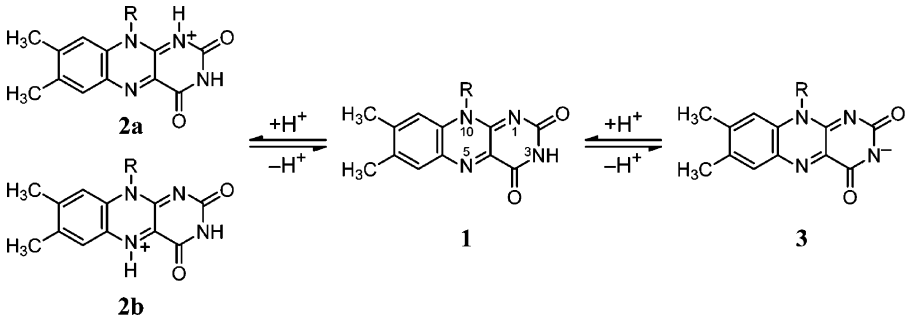

Figure 1. Structures and the IUPAC numbering scheme of cationic (**2a** and **2b**), neutral (**1**), and anionic (**3**) flavin species in the fully oxidized redox state. R = $-CH_2(CHOH)_3CH_2OH$ in riboflavin and R = $-CH_2(CHOH)_3CH_2OPO_3^{2-}$ in FMN.

Confirming early indications,[25] the flavin triplet state has recently also been accepted as a reactive intermediate in the primary reaction of biological photoreception of blue light.[26] In the light-sensitive LOV domains of the phototropin blue-light receptor,[27] the triplet is generated via ISC from the excited singlet-state precursor.[28] It decays within a few microseconds by generating an adduct of the flavin mononucleotide (FMN) cofactor with a nearby cysteine residue.[29−31] Presently it is being discussed whether this primary photoreaction of blue-light sensing proceeds via an ionic mechanism,[26] or, in close analogy to the above-mentioned photooxidation of amino acids,[9] via a radical-pair mechanism with a triplet-configured radical pair converting to a singlet-configured radical pair as a precursor for covalent-bond formation.[32−34] In either case, it is of utmost importance to obtain more information on the electronic properties of flavin triplet states and, in particular, on the protonation state of the isoalloxazine core. Therefore, a detailed knowledge of the electronic structure of the flavin in the triplet state is required.

Despite some initial progress,[19,35−38] limited information on flavin triplets has been obtained from electron-paramagnetic resonance (EPR) thus far. To fill this gap, we have studied the lowest-lying photogenerated triplet states of FMN and riboflavin and their dependence on the pH of the surrounding medium. In the past, flavin triplets have been examined in their equilibrium state when the initial electron-spin polarization of the triplet sublevels arising from spin-selective ISC from the excited singlet-state precursor has decayed to the Boltzmann-equilibrium population.[35−38] Using high-time resolution EPR methods, we now show that it is possible to observe flavin triplets in their nonequilibrated electron-spin polarized state. To preserve as much as possible of the initial polarization, the triplets are detected in a frozen glassy matrix to slow relaxation to the equilibrium state. From these experiments, the spin−spin coupling parameters of the triplet, the so-called zero-field splitting (ZFS) parameters, as well as the zero-field populations are obtained. These parameters are important, for example, to judge the quality of the flavin-triplet wave functions calculated by modern quantum-chemical methods.[39−41] By our experiments, we furthermore can give a lower limit for the time scale of proton transfer in the frozen glassy solutions investigated; during the lifetime of electron-spin polarization, protonation or deprotonation of triplet-state flavin does not occur. Our studies thus reflect the protonation state of the electronic ground state of the flavin. The method described of using the photoexcited triplet as a time-resolved probe for the protonation state of the singlet ground state is novel and of potential value for the study of flavin-containing proteins.

Materials and Methods

Sample Preparation. Riboflavin and FMN were purchased from Sigma and were used without further purification. At pH 8.0, an aqueous buffer solution containing 10 mM sodium phosphate and 10 mM NaCl was used. The pH of the buffer was adjusted with HCl at low pH < 3 and with NaOH at high pH > 8. Glycerol was added to all buffer solutions (60% concentration (v/v)) to yield a transparent glass in the frozen state. FMN and riboflavin were then dissolved at a concentration in the range 0.08−0.7 mM, and the final pH values were measured with a digital pH meter. Optical absorption spectra of the different FMN and riboflavin solutions were recorded on a Shimadzu UV-1601PC spectrophotometer (data not shown) and were found to agree well with those published.[42] All samples were then deoxygenated, transferred into EPR suprasil quartz tubes (3-mm inner diameter) under an argon atmosphere, and rapidly frozen in liquid nitrogen.

EPR Instrumentation. Time-resolved (tr) EPR experiments were performed using a laboratory-built spectrometer consisting of an AEG electromagnet, a Bruker ER041 MR (Rheinstetten, Germany) microwave bridge in conjunction with a Bruker ER4118X-MD-5W1 dielectric resonator, which was immersed in a laboratory-built helium-gas flow cryostat. The temperature of the sample was controlled to ±1 K by a

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LakeShore Cryotronics 321 (Westerville, OH) auto-tuning temperature controller. The microwave (mw) frequency was measured by an EIP 548 (Milpitas, CA) frequency counter, and the magnetic field was measured by a Bruker ER035MR NMR gaussmeter. The time-dependent EPR signal elicited by pulsed laser excitation was amplified in a broadband low-noise preamplifier and detected directly without magnetic-field modulation using a Tektronix TDS-520A digitizing oscilloscope. The time resolution of the spectrometer is in the 40−100 ns range.

Optical excitation of the samples was provided by a Nd:YAG (neodymium yttrium/aluminum garnet) laser-pumped dye laser BMI AL.152C (Thomson-CSF, Buyancourt, France) with a wavelength of 440 nm (laser dye, coumarin-120), a pulse duration of about 6 ns, a pulse energy of 1 mJ, and a laser repetition rate of 1 Hz.

Simulation of EPR Spectra. The EPR powder spectra have been analyzed using a computer program for simulation and fitting of spectra with isotropic *g*-factor and dipolar coupling tensor **D**. The spectra were calculated by computing the resonant magnetic-field positions at the given mw frequency by numerical diagonalization of the Hamiltonian *H*, which was approximated by the sum of the Zeeman and electron dipolar interactions:

$$H(\Omega) = g\beta_e \mathbf{B}\cdot\mathbf{S} + \mathbf{S}\cdot\mathbf{D}(\Omega)\cdot\mathbf{S} \qquad (1)$$

The latter contribution depends on the Euler angles $\Omega = (\alpha, \beta, \gamma)$, which specify the orientation of the triplet-state principal axes with respect to the external magnetic-field vector **B**. The electronic spin-vector operator of the triplet state ($S = 1$) is denoted **S**, and β_e is the Bohr magneton. The orientation dependence of the dipolar-coupling term is that of a rank-two tensor,

$$\mathbf{D}(\Omega) = \mathbf{R}(\Omega)\cdot\mathbf{D}_d\cdot\mathbf{R}(\Omega)^{-1} \qquad (2)$$

where $\mathbf{R}(\Omega)$ is a Cartesian rotation operator. The diagonal tensor $\mathbf{D}_d$ is traceless and parametrized in terms of two ZFS parameters, *D* and *E*: $\mathbf{D}_d = \mathrm{Diag}(D/3 - E, D/3 + E, -2D/3)$. The magnitude of the ZFS parameter $|D|$ is a measure of the delocalization of the molecular orbitals involved in the triplet state and is proportional to $\langle r^3\rangle^{-1}$, where *r* is the distance between the unpaired electrons and $\langle ... \rangle$ denotes the average over the electron distribution. The magnitude of the ZFS parameter $|E|$ is related to the degree of distortion from axial symmetry. The quotient $3|E|/|D|$ lies in the range $0 \le 3|E|/|D| \le 1$, where the two extremes represent axial symmetry ($E = 0$) and orthorhombic symmetry ($3|E|/|D| = 1$).[43,44]

In the spectral analysis, effects due to spin relaxation and molecular motion were neglected. This is adequate as long as spectra are analyzed that were extracted from the two-dimensional data set $S(B_0, t)$ at times soon after the laser pulse (i.e., $t \ll T_1, T_2$).

The detected EPR line shape is the cumulative signal from all photogenerated flavin triplets in the sample. As the flavin molecules have random orientation and the excitation laser pulse is effectively unpolarized, the spectrum appears as the sum over all orientations of the triplets with respect to the external magnetic field. The two resonance magnetic-field positions for a specific orientation Ω are found from the energy splittings between the eigenstates of the Hamiltonian (eq 1). The relative weights of these transitions are calculated from the population differences between the corresponding eigenstates. The level populations in the presence of the magnetic field are computed from the populations p_X, p_Y, and p_Z of the Cartesian triplet sublevels T_X, T_Y, and T_Z in zero-magnetic field in first-order perturbation treatment.[45]

Generally, the shape of the powder spectrum does not strongly depend on the numerical value of the intrinsic line width of the two-line spectrum for one single orientation Ω. This leaves essentially a total of four independent parameters in the model, namely the ZFS parameters, *D* and *E*, and the three zero-field level populations constrained by the normalization condition, $p_X + p_Y + p_Z = 1$, to two independent parameters. It should be noted that a fundamental ambiguity exists as to the sign of *D* and *E* since the EPR line positions depend only on their relative signs. Therefore, only absolute values of the ZFS parameters, $|D|$ and $|E|$, are given.

A nonlinear least-squares fitting routine based on an interior-reflective Newton method (Matlab, The Mathworks, Natick, MA) was used to find the optimum parameter set for simulating a given experimental spectrum. The starting parameter set was systematically varied over a wide range of physically plausible values to avoid convergence of the optimization procedure into a local minimum. The error margins of the fitted parameters were estimated from the confidence intervals obtained from the fitting routine.

Results and Discussion

EPR Spectra of the Lowest Triplet State of Flavin Derivatives. Samples of FMN and riboflavin in frozen aqueous solutions at 80 K were irradiated with 440-nm laser pulses of about 6-ns pulse duration. For repetitive signal accumulation, a low pulse-repetition frequency of 1 Hz was chosen to ensure that the flavins are relaxed to the ground state prior to each laser pulse. This is important as the triplet lifetime of FMN in the temperature range around 80 K in a frozen glycerol matrix is in the 100-ms range.[19,35] The time evolution of the transverse magnetization after pulsed laser excitation was monitored for various static magnetic fields. The complete experimental data set consists of transient EPR signals taken with a maximum time resolution of 10 ns per data point (as determined by the transient digitizer) at equidistant magnetic-field points covering the whole spectrum. This implies a two-dimensional variation of the signal intensity with respect to both the magnetic field (B_0) and the time axis (t). In contrast to previous EPR observations of flavin triplets,[35−38] the tr-EPR experiments presented here were performed without magnetic-field modulation but in the direct-detection mode instead. Hence, the signals have a nonderivative line shape where the sign of $S(B_0, t)$ directly reflects the electron-spin polarized nature of the EPR transition. Positive signals indicate enhanced absorptive (A) and negative emissive (E) spin polarization.

The complete transient EPR signal of photoexcited FMN is shown in Figure 2. A similar data set has been obtained for riboflavin (data not shown). Each spectrum consists of a narrow emissively electron-spin polarized feature centered at $g \approx 4$ around 168.5 mT and a broad signal contribution centered at $g \approx 2$ in the magnetic-field range 280 mT $< B_0 <$ 410 mT with emissive spin polarization below $g \approx 2$ and enhanced absorptive spin polarization above $g \approx 2$. Such a pattern is typical of a triplet state from a photoexcited aromatic hydrocarbon where the lowest triplet is populated via ISC from an excited singlet-state precursor.[46,47] The narrow lines at 168.5 mT are the so-called half-field transitions arising from formal "$\Delta M_S = \pm 2$" resonances between the lowest and highest energy levels in an intermediate magnetic-field regime in which the eigenfunctions of the spin Hamiltonian become linear combinations of the high-field states and $M_S \in \{-1, 0, +1\}$ are no longer adequate quantum numbers. In the past, using continuous-wave EPR with

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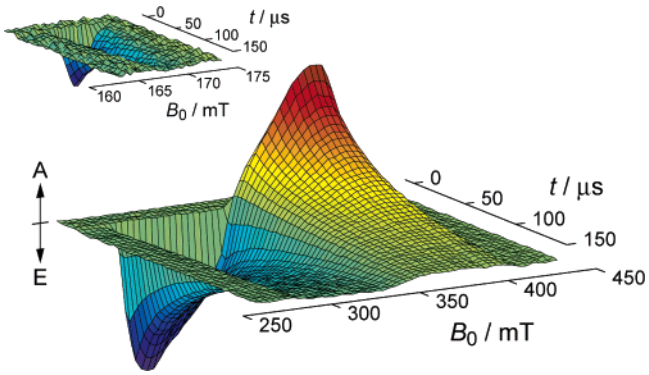

Figure 2. Complete transient EPR data set $S(B_0, t)$ of FMN in frozen aqueous solution (pH 8.0) measured at $T = 80$ K. The data set has been obtained by measuring EPR time profiles at equidistant values of the magnetic field B_0. Each time profile is the average of 25 acquisitions recorded with a laser-pulse repetition rate of 1 Hz. Other parameters: microwave frequency, 9.68 GHz; microwave power, 25 dB (0.63 mW); detection bandwidth, 100 MHz.

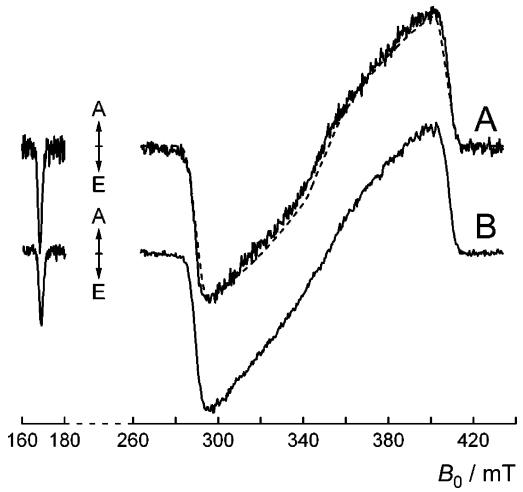

Figure 3. Transient EPR spectra of the photoexcited triplet state of FMN recorded at a temperature of 80 K at two different pH values: (A) pH 8.0 and (B) pH 2.8. The signal intensity of each time trace of the complete data set $S(B_0, t)$ was integrated over a time window of 500 ns centered at $t = 1$ μs after pulsed laser excitation. Parameters are as those in Figure 2. The dashed line in (A) represents a spectral simulation using the parameters of Table 1.

first-derivative detection of the signal with respect to B_0 (as a consequence of magnetic-field modulation), observation of flavin triplets was mostly restricted to the "$\Delta M_S = \pm 2$" transition centered at $g \approx 4$ because of the high intensity of this line in first-derivative mode.[35−38] The comparatively low intensity of the first derivative of the anisotropic and, hence, broad $\Delta M_S = \pm 1$ transitions in the range 280 mT $< B_0 <$ 410 mT and centered about $g \approx 2$ typically preclude the observation of the complete flavin triplet signal by continuous-wave EPR. We have found only one report in the literature in which the entire continuous-wave EPR spectrum of a protonated FMN triplet state is described; however, it is detected not in its spin-polarized state but rather in its quasi-stationary (Boltzmann) equilibrated state. In an EPR experiment with high time resolution, however, which is for the first time performed here on the lowest FMN and riboflavin triplets, the full electron-spin polarization of all transitions between the non-Boltzmann populated energy levels is exploited. Hence, a characteristic powder pattern is observed in which the $\Delta M_S = \pm 1$ transitions at the canonical orientations of the flavin-triplet principal axes (X, Y, and Z) are separated by $|D'| + 3|E'|$, $|D'| - 3|E'|$, and $2|D'|$ (with $|D'| = |D|\ hc/(g\beta_e)$ and $|E'| = |E|\ hc/(g\beta_e)$; h and c are the Planck constant and the vacuum speed of light, respectively) and centered about the g-value of the triplet. Unlike previous EPR observations of the lowest triplet state of FMN,[38] no narrow signal was detected in the center of the $\Delta M_S = \pm 1$ region. Such a signal could, in principle, arise from stable FMN radicals generated as a consequence of irreversible FMN photochemistry under high-intensity illumination conditions or from a double-quantum transition in the triplet manifold connecting the lowest and the highest energy levels. A double-quantum transition can be induced by the simultaneous absorption of two microwave quanta. However, its transition probability is low, and hence, it is typically not observed in direct-detection tr-EPR of triplet states of organic molecules.

Transient EPR spectra can be extracted from the two-dimensional data sets $S(B_0, t)$ at any fixed time after the laser pulse as slices parallel to the magnetic-field axis. Likewise, the time evolution of the transverse magnetization may be obtained for any given field as a slice along the time axis (see below). The rise time of the triplet EPR signal is limited by the instrumental time response of the spectrometer, which is in the range of 40−100 ns. The exponential decay of the electron-spin polarized signal does not reflect the lifetime of the flavin triplet states, which, for flavins in a glassy matrix at 77 K, has been reported to be in the 100-ms range.[19,35] Rather, for very low microwave power, the effective decay time can be identified with the spin−lattice relaxation time T_1 characterizing the decay of the electron-spin polarization to Boltzmann-equilibrium (see also below).[48] Because of the sensitivity loss as a consequence of the direct signal detection, a relaxed triplet state in Boltzmann-equilibrium population is usually not accessible by transient EPR, and therefore, information on the triplet lifetime cannot be given from our experiments.

From a spectral simulation of the electron-spin polarized powder pattern of the $\Delta M_S = \pm 1$ transitions, the ZFS parameters, $|D|$ and $|E|$, of the triplet state are obtained at high accuracy. For this purpose, EPR spectra have been extracted at the maximum of the signal amplitude (Figures 3 and 4) to avoid spectral line-shape distortions due to anisotropic (i.e., magnetic-field dependent) spin relaxation. The ZFS parameters obtained from line-shape fittings are summarized in Table 1. The $|D|$ value obtained for the FMN triplet at pH 8.0, $|D| = (0.0568 \pm 0.0006)$ cm^{-1}, is comparable to the one reported earlier from optically detected magnetic resonance (ODMR), $|D| = 0.058$ cm^{-1}.[49] The corresponding $|E|$ value ($|E| = (0.0173 \pm 0.001)$ cm^{-1}), however, is smaller than that obtained previously, $|E| = 0.019$ cm^{-1}. The same trend is observed for the ZFS parameters of the riboflavin triplet state (Table 1). Because only part of the primary data used to extract the ZFS parameters is presented in the earlier ODMR study and no quantitative error margins have been given,[49] it is difficult to judge the accuracy of the values reported previously. We have tried to reproduce our experimental spectra with simulations using the ZFS parameters from ODMR; however, we did not receive a good agreement between experiment and simulation. We are nevertheless confident that our ZFS values are reliable and accurate,

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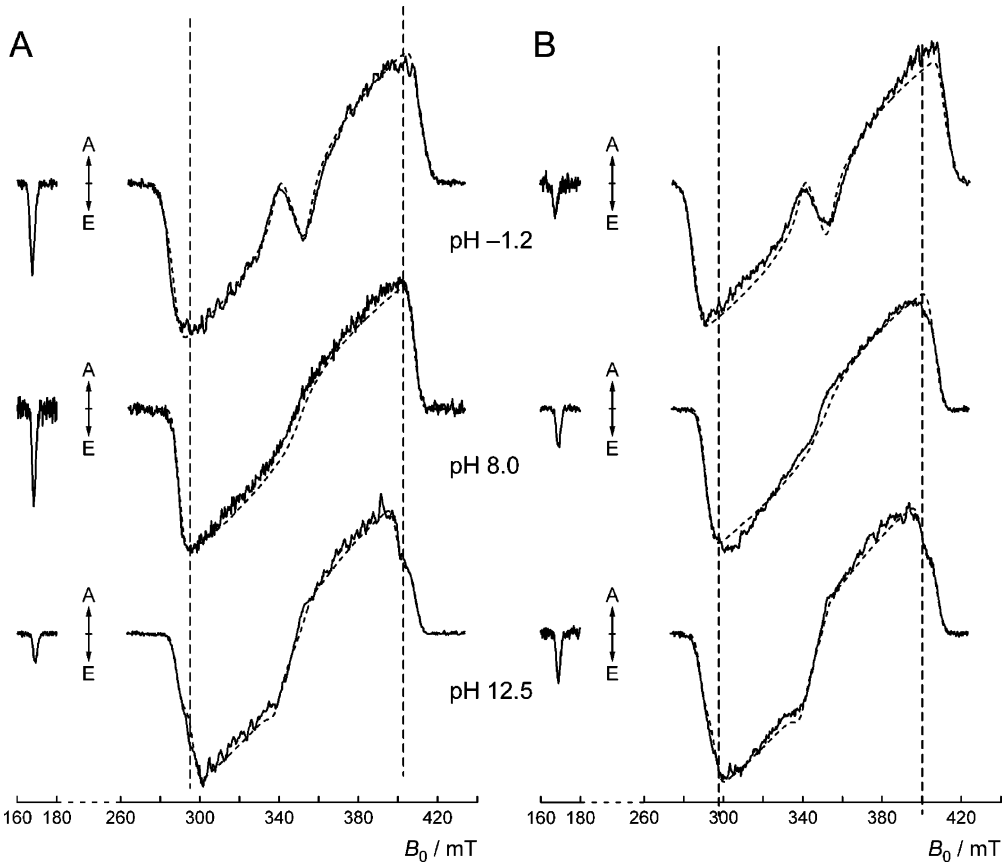

Figure 4. Transient EPR spectra of FMN (A) and riboflavin (B) measured at 80 K (drawn lines) at three different pH values: pH −1.2 (top), pH 8.0 (middle), and pH 12.5 (bottom). The spectra have been obtained as described in Figure 2. The dashed lines represent spectral simulations using the parameters given in Table 1. The vertical lines are drawn to assist the reader in recognizing the changes in spectral line width as a function of buffer pH.

Table 1. Triplet Zero-Field Splitting and Population Parameters of Free FMN and Riboflavin in Frozen Aqueous Solution ($T = 80$ K) Obtained by Simulation of the Time-Resolved EPR Spectra of Figures 3A and 4[a]

	pH	$\|D\|/(10^{-4}$ cm$^{-1})$	$\|E\|/(10^{-4}$ cm$^{-1})$	p_X	p_Y	p_Z
FMN	−1.2	629	172	0.50	0	0.50
	8.0	568	173	0.33	0.67	0
	12.5	565	132	0.40	0.60	0
riboflavin	−1.2	634	177	0.50	0	0.50
	8.0	557	165	0.33	0.67	0
	12.5	560	138	0.42	0.58	0

[a] Estimated error ranges for the $|D|$ and $|E|$ values are $\pm 6 \times 10^{-4}$ cm^{-1} and $\pm 1 \times 10^{-3}$ cm^{-1}, respectively, and for the zero-field populations the value is $(p_i) \pm 0.04$.

as in cases where $|D| \approx 3|E|$ already very subtle variations of $|D|$ and $|E|$ give rise to significant changes of the spectral line shape.

Within experimental error, the $|D|$ and $|E|$ values of FMN and riboflavin at pH −1.2 and 12.5 are very similar. At pH 8.0, however, the riboflavin $|D|$ value is significantly smaller than that of FMN (Table 1). Hence, because the $|D|$ parameter is a measure for the delocalization of the two unpaired electron spins in the triplet and is inversely proportional to the cubed distance between the spin centers ($|D| \propto \langle r^3 \rangle^{-1}$), this indicates more delocalized unpaired electron spins in the riboflavin triplet compared to that in the FMN triplet. One reason for this finding might be that because of the inferior water solubility of riboflavin compared to FMN, some aggregation might take place with the 7,8-dimethyl isoalloxazine moieties of two or more riboflavin molecules getting in close distance to each other. By π-stacking interactions, the triplet-spin configuration then could be partially delocalized over two or more isoalloxazine rings, thus increasing the average distance r between the two unpaired spins, which in turn results in a smaller $|D|$ value. Such overlapping is frequently observed in crystal structures of riboflavin and its related compounds.[50,51] It could be an energetically favorable packing mode for the isoalloxazine ring.

The pH Dependence of EPR Spectra of Flavin Derivatives. To study protonated and deprotonated flavins in the triplet state we first examined the protonation behavior in the glassy glycerol matrix at 80 K. For this purpose, the FMN triplets recorded at pH 8.0 (Figures 3A and 4) and at pH 2.5 (Figure 3B) have been compared. At the higher proton concentration, the isoalloxazine ring in the triplet state of FMN is expected to get easily protonated, as the threshold for flavin-triplet protonation is at p$K = 4.5$.[52,53] Within experimental error, however, the two spectra were found to be identical (Figure 3). The same findings have been obtained for the riboflavin triplet states recorded at pH 8.0 and 2.5 (results not shown). Therefore, we conclude that flavin-triplet protonation under low-temperature conditions is either inhibited or drastically slowed to a time scale far longer than that of the decay of electron-spin polarization. A second explanation could be that the electron distribution of (unpro-

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tonated) FMN in the triplet-spin configuration (compound ^{3}**1**, see Figure 1; the superscript ^{3} or ^{1} denotes the spin multiplicity of the triplet and the singlet state, respectively), and hence, the ZFS parameters are identical to those of protonated FMN in the triplet state (either compound ^{3}**2a** or ^{3}**2b**). This is, however, very unlikely for the following reason: The one-electron reduced forms of **1** and **2**, that is, the FMN anion and neutral radicals, respectively, show very different hyperfine couplings in EPR/ENDOR experiments.[54−57] The hyperfine couplings probe the (singly occupied) frontier orbital of the radical at the positions of the various magnetic nuclei (i.e., protons and/or ^{14}N or ^{15}N nuclei). To a first approximation, the one-electron reduced radical forms of **1** and **2** and the triplet states ^{3}**1** and ^{3}**2**, respectively, share the same LUMO. Therefore, having different hyperfine couplings in the anion and neutral radical states, it is very unlikely that the triplet states could be identical.

As the triplet state of FMN and riboflavin in frozen aqueous solution do not become protonated on the time scale of our experiments at pH values lower than the p*K* of triplet protonation, we followed a different strategy to examine the triplet states of flavins in the various protonation states. Prior to freezing, the pH of the samples is adjusted such that the proton concentration is well above the one necessary for protonation of the ground state (i.e., pH < 0) or below the threshold for deprotonation (i.e., pH > 10). We therefore expect to observe protonated and deprotonated flavin triplets that reflect the protonation state of the ground-state molecules. The results of these experiments are summarized in Figure 4. Clearly, the spectra taken at the different pH values are very distinct. Not only the width of the triplet signals varies, but also the electron-spin polarization pattern in the spectral region around $g \approx 2$. All three spectra of FMN and riboflavin, respectively, can be simulated with the formalism outlined in the Materials and Methods section. The ZFS parameters and the zero-field populations that determine the shape of the electron-spin polarization pattern are summarized in Table 1. While the FMN and riboflavin triplets that are deprotonated at N(3) show the smallest ZFS parameters corresponding to considerable delocalization of the unpaired electron spin, the triplet states of the compounds **1** and **2** show larger $|D|$ values. This is indicative of a stronger confinement of the unpaired electron spins in the triplet states ^{3}**1** and ^{3}**2**. Both FMN and riboflavin follow the same trend. Hence, by tr-EPR the protonation state of the triplet and, for the reason outlined above, the ground states can be probed by measurement of the ZFS parameters and zero-field populations.

In the ground state of the oxidized flavin, the N(1)-protonated configuration (i.e., compound ^{1}**2a**) is energetically more stable than the N(5)-protonated configuration (i.e., ^{1}**2b**). Although there is no experimental evidence, it appears generally accepted that in the triplet state, on the other hand, the N(5)-protonated form, ^{3}**2b**, appears to be energetically favored over the N(1)-protonated form, ^{3}**2a**.[18] Using density-functional theory calculations at the UB3LYP/6-31G** level of theory performed with Gaussian03[58]

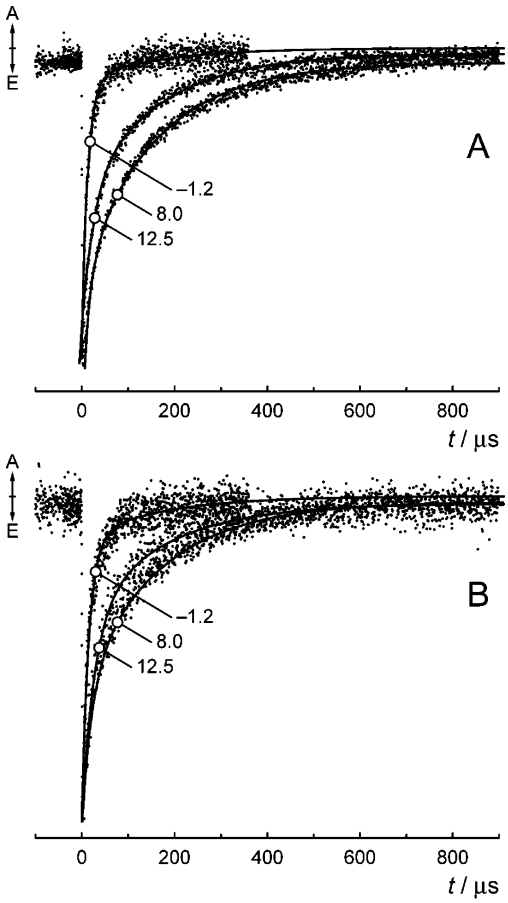

Figure 5. EPR time traces of FMN (A) and riboflavin (B) measured at 80 K (drawn lines) at three different pH values: pH −1.2, pH 8.0, and pH 12.5. The time traces have been recorded at the magnetic field positions 298.0, 291.0, and 285.0 mT, respectively. Parameters are as those in Figure 2 except for the microwave power: 55 dB (0.63 μW). The drawn lines represent fittings with biexponential decay functions using the parameters given in Table 2.

(geometries of ^{1}**2a**, ^{1}**2b**, ^{3}**2a**, and ^{3}**2b** have been optimized at the UB3LYP/6-31G* level), we have confirmed the results of previous calculations performed at a less computationally expensive level. In the triplet state, ^{3}**2b** is 79.6 kJ/mol lower in energy than ^{3}**2a**, whereas in the ground state, ^{1}**2b** is 34.5 kJ/mol higher in energy than ^{1}**2a**. On the basis of these predictions and on our findings that protonation and deprotonation are drastically slowed at 80 K, we conclude that the protonated triplet states observed by tr-EPR at very high proton concentrations (i.e., pH −1.2) arise from the N(1)-protonated flavin rather than the N(5)-protonated form.

The pH Dependence of EPR Decay Curves of Flavin Derivatives. Differences between the various flavin triplets can

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Table 2. EPR Signal Decay Time Constants of Free FMN and Riboflavin in Frozen Aqueous Solution ($T = 80$ K) Obtained by Fitting of the Respective EPR Time Profiles with Biexponential Decay Functions $S(t) = A_{fast} \exp(-t/T_{fast}) + A_{slow} \exp(-t/T_{slow})$[a]

	pH	$T_{fast}/\mu s$	$T_{slow}/\mu s$	A_{fast}/A_{slow}
FMN	−1.2	12	160	64
	8.0	19	160	0.05
	12.5	22	160	1.07
riboflavin	−1.2	16	168	6.3
	8.0	23	168	0.07
	12.5	27	168	1.63

[a] Estimated error range is ±5%.

also be monitored by comparing the decay times of the electron-spin polarized EPR signals (Figure 5). The respective spin−lattice relaxation times extracted from the decay curves recorded at low microwave power (Table 2) do not exhibit any significant magnetic-field (B_0) anisotropy. Nevertheless, to ensure comparability between the different protonation states, all time constants have been obtained from the analysis of time traces at B_0 positions corresponding to the minimum amplitude of the electron-spin polarized signal.

The individual EPR time traces have been analyzed by fitting to biexponential decay functions. The results of our analysis are summarized in Table 2. In each case there is a major component reflecting the spin−lattice relaxation time of the respective protonation state of the triplet. A minor contribution is due to the presence of a remainder of the acid−base couple from the protonation equilibrium. Hence, three characteristic relaxation times are sufficient to describe all three decay curves of the differently protonated FMN and riboflavin triplets. The longest decays are observed for the triplets of the neutral state, ^{3}**1** (160 and 168 μs for FMN and riboflavin, respectively). Faster decay times are found for the protonated (cationic) ^{3}**2** (12 μs and 16 μs) and the deprotonated (anionic) ^{3}**3** (22 μs and 27 μs) triplet states. Although there is no direct connection between the lifetime and the spin−lattice relaxation time of the triplet (see also above), the different decay times of the spin polarization for the three protonation states of the flavin triplets, ^{3}**1**, ^{3}**2**, and ^{3}**3**, follow a trend that has been previously observed for the pH dependence of the triplet lifetimes of FMN measured under similar conditions.[35] While the triplet lifetime is governed by the intersystem-crossing rate from the excited triplet state to the singlet ground state, the spin−lattice relaxation rate depends on fluctuations of magnetic parameters with spectral components near the microwave frequency. Apparently, both processes have a similar dependence on the protonation states of the nitrogens N(1), N(3), and N(5).

Conclusions

By time-resolved EPR we have demonstrated that the protonation state of the 7,8-dimethyl isoalloxazine moiety of flavins has a significant influence on the delocalization of the unpaired electron spins in the triplet state. This becomes evident when the spectral line shapes and the decays of the electron-spin polarization of the triplets are compared at different proton concentrations. Protonation or deprotonation of the triplet seems impossible in the frozen solution at 80 K on the microsecond time scale of the decay of electron-spin polarization. Therefore, by transient EPR, the protonation state of the singlet ground state is observed. This opens a novel approach to probe the protonation state of the ground state of flavin cofactors in proteins by examination of their photogenerated triplet states in an EPR experiment of appropriate time resolution. First applications of this method to the FMN cofactor in LOV domains of phototropin and several other flavoproteins are currently being examined in our laboratory using this method.

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