Electron Nuclear Double Resonance Differentiates Complementary Roles for Active Site Histidines in (6-4) Photolyase*

Received for publication, May 17, 2006, and in revised form, December 8, 2006 Published, JBC Papers in Press, December 12, 2006, DOI 10.1074/jbc.M604734200

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(6-4) photolyase catalyzes the light-dependent repair of UV-damaged DNA containing (6-4) photoproducts. Blue light excitation of the enzyme generates the neutral FAD radical, FADH_{\text{radical}}, which is believed to be transiently formed during the enzymatic DNA repair. Here (6-4) photolyase has been examined by optical spectroscopy, electron paramagnetic resonance, and pulsed electron nuclear double resonance spectroscopy. Characterization of selected proton hyperfine couplings of FADH\text{radical}, namely those of H^{3a} and H^{3b}, yields information on the micropolarity at the site where the DNA substrate is expected to bind. Shifts in the hyperfine couplings as a function of structural modifications induced by point mutations and pH changes distinguish the protonation states of two highly conserved histidines, His^{354} and His^{358}, in Xenopus laevis (6-4) photolyase. These are proposed to catalyze formation of the oxetane intermediate that precedes light-initiated DNA repair. The results show that at pH 9.5, where the enzymatic repair activity is highest, His^{358} is deprotonated, whereas His^{354} is protonated. Hence, the latter is likely the proton donor that initiates oxetane formation from the (6-4) photoproduct.

Ultraviolet light (λ ≈300 nm) damages cellular DNA by the formation of cyclobutane pyrimidine dimers (CPDs) and (6-4) photoproducts from adjacent pyrimidine bases on the same DNA strand (1). Such dimers are restored to their monomeric form by the action of two photolyase (300 < λ < 500 nm) damage-specific DNA repair enzymes, named CPD photolyase and (6-4) photolyase, collectively known as DNA photolyases (2–6). Both enzymes are found in various organisms, exhibit a 20–30% amino acid sequence identity (2, 7, 8), and share a common chromophore, FAD (9–12), although the two photolyases differ in DNA substrate specificity and repair mechanism.

For the CPD photolyase, the initial step in the proposed repair mechanism (13) is a photoinduced electron transfer from the fully reduced FAD cofactor (FADH\text{red}) to the CPD, resulting in the formation of a CPD anion radical and a neutral FADH\text{radical}. The cyclobutane ring of the unstable CPD radical opens, and subsequently the electron is transferred back to the FADH\text{radical}, thus restoring the initial redox states of both the FAD and the pair of pyrimidine bases in the DNA. Thus the entire process represents a true catalytic cycle with net-zero exchanged electrons.

Unlike CPD photolyase, (6-4) photolyases are not able to directly restore the original bases from the (6-4) photoproduct in UV-damaged DNA; rather, following binding of the lesion, the overall repair reaction consists of two distinct steps, one of which is light-independent and the other one light-dependent (Fig. 1) (14–16). In the initial light-independent step, a 6′-imino ion is thought to be generated via proton transfer induced by two histidines highly conserved among the (6-4) photolyases (His^{354} and His^{358} in Xenopus laevis (6-4) photolyase) (16) (Fig. 2). This intermediate spontaneously rearranges to form an oxetane intermediate by intramolecular nucleophilic attack. Such a mechanism requires that one histidine acts as a proton acceptor and the other as a proton donor (see Fig. 2), which implies that the two histidines should have markedly different pK_a values. However, until now it has not been established which histidine acts as an acid and which one functions as a base. The proposed reaction mechanism is based on a biochemical study using alanine-substituted mutants (16), and as yet spectroscopy has not been employed to address this stage of the reaction.

In the subsequent light-driven reaction, one electron is believed to be transferred from the fully reduced FAD cofactor (FADH\text{red}) to the oxetane intermediate thus forming a neutral FADH\text{radical} and an anionic oxetane radical, which spontaneously fractures. The excess electron is then back-transferred to the flavin radical restoring the fully reduced flavin cofactor and a pair of pyrimidine bases (see Fig. 1).

When the FAD cofactor is not in its catalytically active (fully reduced) redox state, another photoreaction takes place both in...
CPD photolyase and (6-4) photolyase (17–20). In this so-called photoreactivation (also called flavin-cofactor photoreduction), photolyases undergo reversible electron transfer reactions via amino acid residues to stepwise reduce their flavin cofactors to FADH /H 11002

in the range of 400–500 nm. The yellow species contains the flavin chromophore in its fully oxidized redox form (FAD ox) (19).

In this study, the stable blue radical form (FADH') of the flavin cofactor of (6-4) photolyase was generated by photoreduction of purified recombinant enzyme in the presence of an exogenously added electron donor, followed by aerial oxidation. This radical was examined by continuous-wave EPR spectroscopy and pulsed Davies-type electron nuclear double resonance (ENDOR). The hyperfine interactions arising from protons attached to the isoalloxazine moiety of the FAD cofactor were exploited to probe the immediate environment of the flavin, in particular hydrogen bonding and the micropolarity. The main focus of this study is to obtain information about the protonation states of His 354 and His 358, which are essential for DNA repair by (6-4) photolyase (16). This information is crucial for a thorough understanding of the light-independent catalytic steps preceding blue light-initiated enzymatic DNA repair, and the specific structural traits that distinguish (6-4) photolyase from the related CPD photolyase.

EXPERIMENTAL PROCEDURES

Protein Preparation—X. laevis wild-type and mutant (6-4) photolyases were overproduced in E. coli, purified as described previously (10, 19), and stored in liquid nitrogen. The concentration of the enzyme was determined on the basis of the oxidized FAD cofactor's absorbance at 450 nm (ε 450 = 1.12 × 10 4 M -1 cm -1 ) (10). For the present experiments, typically 0.5 mM (6-4) photolyase in a buffer containing 0.1 M NaCl, 0.05 M Tris HCl (pH 6 to 9.5) and 30% (v/v) glycerol was used. The redox state of the flavin cofactor was monitored by recording the ground state optical absorption spectrum from 300 to 800 nm using a Shimadzu UV-1601PC spectrophotometer.

Preparation of a Stable Flavin Radical—Recombinantly produced oxidized (6-4) photolyase samples were supplemented with 10 mM EDTA, illuminated at 4 °C for 1 min with blue light (Halolux 30HL, Streppel, Wermelskirchen-Tente, Germany) selected with a 420–470-nm band filter (Schott, Mainz), and then incubated for about 20 min at 4 °C with air to reoxidize the light-generated dihydroflavin form of the flavin cofactor. The concentration of the resultant blue flavin radical (FADH') was estimated based on its absorbance at 580 nm (ε 580 = 0.48 × 10 4 M -1 cm -1 ) (27).
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Buffer Exchange—Samples were transferred into the desired buffer (usually 0.1 M NaCl, 0.05 M Tris-HCl, or 0.05 M HEPES (pH 6–9.5) in 50% (v/v) glycerol) by dilution and ultrafiltration through Amicon C30 microconcentrators at 4 °C. Experiments in D2O were carried out at pH 8.0 (uncorrected glass electrode reading) by performing a gel filtration chromatography step (HiPrep Desalting, GE Healthcare) at 4 °C.

EPR Sample Preparation—The enzyme preparations were transferred into EPR quartz tubes (3 mm inner diameter) under an argon inert-gas atmosphere in the dark. The enzyme was frozen rapidly with liquid nitrogen and stored therein. No changes in the signal line shape and intensity have been observed over a period of several months.

EPR/ENDOR Instrumentation—Continuous-wave X-band (microwave frequency, 9.67 GHz/magnetic field, ~0.345 T) EPR spectra were obtained using a laboratory-built spectrometer described previously (25). X-band pulsed ENDOR spectra were recorded using a commercial pulse EPR spectrometer Bruker E580 (Bruker BioSpin GmbH, Rheinstetten, Germany) in conjunction with a dielectric-ring ENDOR resonator Bruker ER 4118X-MD5-EN. For Davies-type ENDOR (28), a microwave pulse sequence \( \pi-t-\pi/2-\pi-\pi \) using 64- and 128-nm \( \pi/2 \) and \( \pi \)-pulses, respectively, and a radio frequency pulse of a 10-\( \mu \)s duration starting 1 \( \mu \)s after the first microwave pulse was used. The separation times \( t \) and \( \tau \) between the microwave pulses were set to 13 \( \mu \)s and 500 ns, respectively. To avoid saturation effects because of long relaxation times, the entire pulse pattern was repeated with a frequency of only 200 Hz.

ENDOR Spectra Analysis—For doublet state radicals, two ENDOR lines were expected per group of magnetically equivalent nuclei according to the first-order resonance condition, \( n_{\text{ENDOR}} = n_{\text{nu}} \pm (A/2) \), where \( n_{\text{nu}} = g_{\text{n}}B_{\text{n}}B_{\text{o}}/\hbar \) is the Larmor precessional frequency of the nucleus at the respective magnetic field \( B_{\text{o}} \), and \( g_{\text{n}} \) and \( B_{\text{n}} \) are the nuclear \( g \) values and the Bohr magnetons, respectively. In the weak coupling case, \( |A| < 2n_{\text{nu}} \), the separation of the line pair is determined by the orientation-dependent hyperfine coupling constant, \( A \), that quantifies the interaction of the nuclear magnetic moment with the electron magnetic moment.

The hyperfine couplings from so-called \( \alpha \)-protons bonded directly to \( \pi \)-conjugated carbon (or nitrogen) atoms are typically anisotropic, and the interaction symmetry is rhombic, i.e. \( A_x \neq A_y \neq A_z \), where \( x, y, \) and \( z \) are the principal axes of the hyperfine tensor. With respect to the C–H (or N–H) bond and the aromatic \( \pi \)-plane, \( x, y, \) and \( z \) are aligned along the C–H (or N–H) bond, in-plane perpendicular to the C–H (or N–H) bond, and parallel to the plane normal, respectively. If one extracts the isotropic part \( A_{\text{iso}} = (A_x + A_y + A_z)/3 \) from the tensor, the traceless anisotropic hyperfine contributions remain \( T_i = A_i - A_{\text{iso}}, \ i \in \{x, y, z\} \). These are largely determined by the electron-nuclear dipolar interaction. Following arguments outlined previously for the N6-\( \text{H}^2 \)-system fragment in FADH* (26), short N–H bond lengths lead to a symmetric rhombic hyperfine tensor with \( T_x \rightarrow 0 \) and \( T_z = -T_y \). When the N-to-H bond length is increased, e.g. as a result of hydrogen bonding of the flavin to the protein backbone, the hyperfine tensor is expected to become more axially symmetric with \( T_x = T_y = T_{\parallel}, \) and \( T_z = T_{\perp} = -2T_{\parallel} \).

The isotropic hyperfine coupling of a \( \beta \)-proton is related to the spin density \( \rho_i \) of the neighboring carbon (\( X = \text{C} \)) or nitrogen (\( X = \text{N} \)) atom in the \( \pi \)-plane, and according to the McConnell relation (29–32), \( A_{\text{iso}}(\text{H}^\beta) = \rho_i^2 \left( B' + B'\cos^2\theta \right) \), depends on the spatial orientation of the side chain. From the two empirical constants, \( B' \) and \( B'' \), \( B' \) is typically very small, i.e. \( B' \approx 0 \), \( \theta \) is the dihedral angle between the plane normal of the \( \pi \)-system and the projected C\( ^6 \)H\( ^\beta \) bond. The hyperfine anisotropy of \( \beta \)-protons is typically small and approaches axial symmetry: \( T_x \approx T_y = T_{\parallel}, \) and \( T_z = T_{\perp} \).

Spectral Simulations—For ENDOR spectral simulations, the signal amplitudes were normalized to the integral of the signal arising from the hyperfine coupling of H6 in FADH*. This proton is nonexchangeable, and hence, the intensity of its ENDOR signal is expected to remain constant throughout different buffer conditions. Furthermore, H6 is more than 1 nm distant from the proposed substrate-binding pocket. Hence, changes of the protonation states of the histidines close to the substrate-binding site are not expected to alter the intensity or shape of the H6 ENDOR signal pair. To precisely extract the principal values of the hyperfine couplings of the H6\( ^\text{iso} \) and H6\( ^1 \) probes from the ENDOR data, spectral simulations were performed using the EPR spectral simulation software package EasySpin (33) in conjunction with the MATLAB routine Isqcurvefit (The MathWorks, Natick, MA). This program calculates ENDOR powder spectra arising from two hyperfine tensors and fits their principal values to achieve the best possible agreement with the experimental data.

RESULTS

UV-visible Spectroscopic Analyses of the Flavin Cofactor Redox State—FAD cofactors often show characteristic optical absorption properties in all three biologically relevant redox states as follows: fully reduced (FADH– or FADH\( _2 \)), semiquinone radical (FAD\( ^* \) or FADH\( ^* \)), and fully oxidized (FAD\( ^{\text{ox}} \)). The (6-4) photolyase enzyme from \( X. \text{laevis} \) is isolated in a yellow form characteristic of a flavoprotein with a fully oxidized FAD\( ^{\text{ox}} \). The protein has absorption maxima at 448 and 366 nm that represent the \( S_0 \rightarrow S_1 \) and \( S_0 \rightarrow S_2 \) transitions of its flavin cofactor, respectively (Fig. 3). Well resolved vibration side bands at 425 and 475 nm and 356 and 372 nm, respectively, are indicative of tight and well defined noncovalent binding of the chromophore to the highly ordered protein structure. After illumination with blue light (420 < \( \lambda < 470 \) nm) in the presence of EDTA as an exogenous electron donor, the formation of the fully (two-electron) reduced FADH\( ^* \) form of the flavin chromophore is observed (see Fig. 3). A flavin radical, which exhibits absorption maxima at 590 and 635 nm that represent the \( S_0 \rightarrow S_1 \) and \( S_0 \rightarrow S_2 \) transitions of its flavin cofactor, respectively (Fig. 3). Well resolved vibration side bands at 425 and 475 nm and 356 and 372 nm, respectively, are indicative of tight and well defined noncovalent binding of the chromophore to the highly ordered protein structure. After illumination with blue light (420 < \( \lambda < 470 \) nm) in the presence of EDTA as an exogenous electron donor, the formation of the fully (two-electron) reduced FADH\( ^* \) form of the flavin chromophore is observed (see Fig. 3). A flavin radical, which exhibits absorption maxima at 590 and 635 nm that represent the \( S_0 \rightarrow S_1 \) and \( S_0 \rightarrow S_2 \) transitions of its flavin cofactor, respectively (Fig. 3). Well resolved vibration side bands at 425 and 475 nm and 356 and 372 nm, respectively, are indicative of tight and well defined noncovalent binding of the chromophore to the highly ordered protein structure. After illumination with blue light (420 < \( \lambda < 470 \) nm) in the presence of EDTA as an exogenous electron donor, the formation of the fully (two-electron) reduced FADH\( ^* \) form of the flavin chromophore is observed (see Fig. 3). A flavin radical, which exhibits absorption maxima at 590 and 635 nm that represent the \( S_0 \rightarrow S_1 \) and \( S_0 \rightarrow S_2 \) transitions of its flavin cofactor, respectively (Fig. 3).
us to conclude that in (6-4) photolyase the flavin radical is also present in the H5-protonated neutral form, FADH\(^{+}\). Typical for neutral flavin radicals are the absorption bands at 635 nm, which are assigned the D\(0 \rightarrow D_2\) transition, and the weak shoulder at around 510 nm that arises from the D\(0 \rightarrow D_1\) transition \(\text{(35)}\). A closer examination of the absorption spectrum of (6-4) photolyase reveals significant red shifts of 10, 10, and 13 nm for the red-most absorption bands at 590 and 635 nm relative to those of \textit{E. coli}, yeast, and \textit{Thermus thermophilus} CPD photolyase, respectively \(\text{(34, 36, 37)}\). Such bathochromic shifts in chromophores are normally caused by more extended delocalization of the π-electron system of aromatic molecules thus implying that FADH\(^{+}\) of (6-4) photolyase has altered electronic and hence redox properties as compared with the CPD photolyases, which could be important for its function.

**Characterization of the Flavin Radical State by EPR Spectroscopy**—To obtain a more detailed characterization of the electronic structure of the flavin radical in \textit{X. laevis} (6-4) photolyase, EPR experiments were performed with a sample that was frozen in liquid nitrogen after generation of the flavin radical. The continuous-wave detected X-band EPR signal (see Fig. 4) reveals a radical signature centered at \(g = 2.0034 \pm 0.0005\). The experimentally observed peak-to-peak line width of (2.00 \pm 0.01) mT is typical of a neutral flavin radical, FADH\(^{+}\). The overall line width and the line shape of the signal are attributed to the mostly unresolved contributions of hyperfine couplings of the unpaired electron spin with \(^1\text{H}\) and \(^{14}\text{N}\) nuclei of the isoalloxazine moiety of FADH\(^{+}\) as well as of the protein environment. Upon buffer deuteration, the exchangeable protons at N\(^5\) and N\(^3\) are replaced with deuterons. This results in a significant reduction of the inhomogeneous line width, which is clearly visible in Fig. 4.

**Characterization of the Flavin Radical State by ENDOR Spectroscopy**—We have performed ENDOR experiments to further characterize the electronic structure of the FADH\(^{+}\) cofactor in (6-4) photolyase. In Fig. 5, the X-band pulsed ENDOR spectrum of the FADH\(^{+}\) radical cofactor in protiated buffer is shown. The spectrum was recorded at a magnetic field value corresponding to the center magnetic field position of the X-band continuous-wave EPR signal shown in Fig. 4. In brief, ENDOR signals in five spectral regions between 1 and 37 MHz can be identified. Nearly all signals in this range are symmetrically distributed in pairs around the free proton Larmor frequency, \(v_H = 14.75\) MHz. Hence, most proton hyperfine couplings are detected in the weak-coupling limit.

(i) The central so-called matrix-ENDOR signal extends from about 13.2 to 16.2 MHz and includes hyperfine couplings from protons whose nuclear spins are only weakly interacting with the unpaired electron spin, which is delocalized over the aromatic π-plane of the FADH\(^{+}\) isoalloxazine moiety. Small hyperfine couplings originate from protons in the protein backbone within the cofactor binding pocket, from protons of water molecules surrounding the flavin, and also from weakly coupled protons directly attached to the 7,8-dimethyl isoalloxazine ring, namely H\(^5\), H\(^{10}\), and H\(^9\).
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(ii) Two prominent features are observed in the flanking 10.5–12- and 17.5–19-MHz radio frequency ranges. The tensorial line shapes are of almost axial symmetry and arise from the hyperfine couplings of the β-protons of the methyl group attached to C8. Typically, methyl groups rotate about their C–C bond at elevated temperatures. Hence, if this rotation is fast on the ENDOR time scale, one common hyperfine tensor for all three protons of the methyl group is observed. Signals of the H8α hyperfine tensor are in general easily detected in proton-ENDOR spectroscopy on flavins (25, 38–41) and are considered to be sensitive probes of the electron spin density on the outer xylene ring of the flavin isoalloxazine moiety (see also below). Furthermore, theory shows that the size of this coupling responds sensitively to polarity changes of the protein surroundings (42).

(iii) Flanking the H8α signals at around 9–10.5 and 19–20.5 MHz are found the transitions of one of the two β-protons, H5', attached to C1' in the ribityl side chain of the isoalloxazine ring. In flavins, an analysis of this hyperfine coupling together with the one from the weaker-coupled second β-proton at C1' yields information on the geometry of the ribityl chain attached to N10 as has been shown for the case of E. coli CPD photolyase (25).

(iv) Signals arising from the hyperfine coupling of the H6 proton are found at around 12 and 17.5 MHz. These show a smaller hyperfine anisotropy, in line with recent quantum-chemical calculations (22, 43).

(v) The broad feature extending from 21 to 34 MHz in the pulsed ENDOR spectrum is typically not observed in continuous-wave ENDOR experiments (25, 26). Its counterpart spanning the corresponding low radio frequency range is not resolved because of the overlap with ENDOR signals of the nitrogen nuclei. The broad 21–34-MHz pulsed ENDOR signal disappears when the enzyme is transferred from protiated into deuterated buffer (data not shown) (26). The symmetry of the underlying hyperfine coupling is clearly rhombic, i.e., $A_x \neq A_y \neq A_z$. As inferred from EPR spectroscopy and quantum-chemical calculations (22), this hyperfine coupling in the pulsed ENDOR spectrum is assigned to the proton bound to N5 (44). Its contribution to the overall spectrum is easily discriminated from that of other protons in the isoalloxazine moiety because of the exchangeability of H5 with a deuteron upon buffer deuteration. The only other exchangeable proton in the isoalloxazine moiety of flavins is that at N3. Its hyperfine coupling, however, is considerably smaller than that of H5, as has been shown by previous ENDOR experiments and quantum-chemical calculations (22).

The amplitude and the anisotropy of the H5 hyperfine coupling has been shown recently to yield information on the strength of hydrogen bonding at H5 of the flavin to the protein backbone (26). The $A_x$ and $A_z$ components of this coupling are easily extracted from the peak position of the proton-ENDOR signal and the outer inflection point of the tensor pattern, respectively. The value of the $A_x$ component has been obtained from deuteron ENDOR (44).

In Table 1, the proton hyperfine couplings from X. laevis (6-4) photolyase that have been obtained from pulsed ENDOR spectroscopy are summarized and compared with the proton hyperfine couplings of E. coli CPD photolyase.

### Table 1

<table>
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<tr>
<th>Atom</th>
<th>X. laevis (6-4) photolyase</th>
<th>E. coli CPD photolyase</th>
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<tbody>
<tr>
<td>H5</td>
<td>$A_x = -13.7^a$</td>
<td>$-15.0^a$</td>
</tr>
<tr>
<td>H5</td>
<td>$A_y = -38.6$</td>
<td>$-37.0^a$</td>
</tr>
<tr>
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<td>$A_z = -26.1$</td>
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<td>$A_z = +16.50$</td>
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<tr>
<td>H5'</td>
<td>$A_{mix} = +13.85$</td>
<td>$+19.0^c$</td>
</tr>
</tbody>
</table>

* Data were scaled from deuteron ENDOR (44).

** Data were from Ref. 26.

Data were from Ref. 25.

The pH Dependence of the H8α Hyperfine Coupling—Two histidines, His354 and His356, in X. laevis (6-4) photolyase that are unique to (6-4) photolyases and consequently are not found in CPD photolyases are critical for DNA repair activity. These histidines were proposed to play a pivotal role in the formation of the oxetane intermediate from the (6-4) photoproduct (Fig. 2), a step that is believed to occur prior to light-induced reductive cleavage (16). A theoretical structure model of (6-4) photolyase, which was built based on the crystallographic structure of *E. coli* CPD photolyase (45), suggests that these histidines are located close to (−0.4 nm) the 8α-methyl group of the isoalloxazine moiety. Furthermore, if these histidines indeed play a functional role, then one would expect that changing the pH might readily cause a change of their protonation state. To test this model, we have recorded pulsed ENDOR spectra for the wild-type enzyme over the pH range between 6 and 9.5.

In Fig. 6, a series of difference ENDOR spectra for the wild-type protein as a function of pH are depicted. In the spectral region where the H8α protons show resonances, significant spectral shifts and intensity changes are observed. Other differences are mostly restricted to the matrix-ENDOR, region but they are very small. Notably, the signals of the other protons such as H6 or H5 remain virtually unchanged. Hence, in the following Figs. 7 and 8, we only show the high frequency parts of the ENDOR spectra in the 17.8–21.2-MHz range. These spectra were normalized with respect to the ENDOR signal of H6. It is immediately apparent that the intensity of the H8α ENDOR signal changes significantly as a function of pH (see Fig. 7, panel A). Similar experiments carried out with HEPES-buffered protein in order to exclude potential buffer-dependent artifacts (46) yielded virtually identical results.

For data analysis, it has to be taken into account that the signal of H8α overlaps with the one arising from H1. We have therefore simulated the data from Fig. 7, panel A, using a least squares fitting program that calculates ENDOR spectra arising from these two nonequivalent protons with anisotropic hyperfine tensors. In the simulations, the hyperfine principal values...
of both tensors were then adjusted such that the best possible agreement with the experimental data was obtained. As an illustration, the experimental and simulated ENDOR spectra of the wild-type enzyme recorded at pH 8 are shown in Fig. 7, panel B. In general, the agreement between experiment and simulation is excellent. Thus precise principal values for the two hyperfine tensors of H^{8α} and H^1 could be obtained (see Table 2). Both the principal values of the H^{8α} hyperfine coupling tensor and the overall signal intensity (the integral over the entire tensorial pattern; see Fig. 7, panel C) are affected by changes of the pH. This is not unexpected because it is well documented that changes in the micropolarity or the pH of the surroundings of a paramagnetic molecule may alter both the hyperfine couplings and the relaxation behavior of the nuclei (47–50). Furthermore, changes in pH often cause small but distinct geometrical reorientations of protein side chains. These structural changes may influence the free rotation of methyl groups. When the motion is fast on the time scale of the observer, the hyperfine couplings of the three protons coalesce and average to give a single purely axial hyperfine coupling tensor. When the motion slows, the averaging is incomplete, thus rendering the tensor slightly rhombic.

Two mutant proteins, H354A and H358A, were also examined by pulsed ENDOR spectroscopy at two extreme pH values, pH 6 and 9.5, to identify the origin of the pH dependence of the principal values of the H^{8α} hyperfine tensor. Both mutant enzymes are inactive in photorepair (16), but the photoreduction reaction is still possible. In Fig. 8, the pulsed ENDOR spectra and the spectral simulations for the mutant proteins are shown and compared with the data from the wild type. A comparison of the wild type and the mutants at different pH conditions reveals characteristic differences both in the hyperfine principal values and the signal intensities.

For the wild-type enzyme, the ENDOR signal arising from the H^{8α} hyperfine coupling has axial symmetry, i.e., \( A_{ij}(H^{8α}) = A_{ij}(H^{8α}) \equiv A_1(H^{8α}) + A_3(H^{8α}) \equiv A_2(H^{8α}) \) (see Table 2) as is expected for a rapid (on the ENDOR time scale) methyl group rotation about the C8=C8α bond in FADH^+. The H^1 hyperfine coupling tensor on the other hand is slightly rhombic, \( A_1(H^1) \neq A_2(H^1) \neq A_3(H^1) \) as is predicted from quantum-chemical calculations (22). Within experimental error, the principal values of the hyperfine tensors of H^{8α} and H^1 remain constant from pH 9.5 to pH 6. In contrast, the signal intensity of H^{8α} is pH-dependent with an observed maximum at pH 7.

The overall shapes of the ENDOR spectra of the H358A mutant protein largely resemble those of the wild type at the respective pH conditions. The slight rhombicity of the H^{8α} hyperfine splitting measured at pH 9.5 reveals a perturbation of the flavin-binding site at this position induced by the replacement of His^{358} by alanine. Furthermore, the isotropic hyperfine coupling of H^{8α} at pH 9.5 (Table 2) increases from 7.05 MHz (wild type) to 7.16 MHz (H358A) indicating that the modification of the environment renders the surroundings of H^{8α} slightly more polar. The small shift observed in the hyperfine coupling of H^1 likely reflects a small reorientation of the ribityl side chain, although no appreciable changes in the principal values of the hyperfine tensor are observed upon reduction of the buffer pH.

The H354A protein, in contrast to the wild type or the H358A mutant, exhibits significant pH-dependent changes in its ENDOR spectra (Fig. 8). At pH 9.5, a substantial reduction of the H^1 hyperfine coupling is observed accompanied by a clear symmetry change of the H^{8α} signal. Furthermore, the principal hyperfine values of both protons change significantly upon altering the pH. Thus replacement of His^{354} with alanine leads to significant modifications of the cofactor-binding site at the 8α-methyl group and at the linkage of the ribityl side chain. These modifications render the principal components of
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the H₈α hyperfine tensor more sensitive to pH changes as compared with the wild-type enzyme and the H358A mutant. Virtually identical results were obtained from samples where the pH has been adjusted by HEPES instead of Tris buffer.

DISCUSSION

Comparison of the Electronic Structure of the Neutral Flavin Radicals in (6-4) Photolyase and CPD Photolyase—Differences in the proton hyperfine couplings of FADH⁺ bound to (6-4) photolyase versus CPD photolyase (Table 1) can be interpreted in light of sequence and proposed structural differences in the flavin-binding sites of these DNA repair enzymes. Isotropic hyperfine couplings of α- and β-protons of aromatic radicals, such as FADH⁺, reflect the unpaired electron spin density on the carbons and nitrogens to which they are bonded. This electron spin density distribution in turn reflects the protein environment. In Fig. 9, the amino acid alignment of the C-terminal parts of E. coli CPD photolyase and X. laevis (6-4) photolyase highlights the 14 amino acids within 0.5 nm of the isoalloxazine moiety of FADH⁺ in E. coli CPD photolyase (45). Nine of these

14 are conserved in X. laevis (6-4) photolyase; two have similar electronic properties, and three are dissimilar. Among the (6-4) photolyases, two of these are highly conserved histidines (His354 and His358 in X. laevis), and the third is the polar Asn394 (X. laevis) replacing of Gly381 in CPD photolyase (E. coli).

H₈α-proton hyperfine coupling is known to be an excellent probe for sensing subtle differences in the surroundings of the xylene region of the isoalloxazine up to distances of about 0.6 nm (39, 42). The size of the H₈α coupling depends on the micropolarity as well as on the aromaticity of the amino acids binding the flavin. Although the H₈α-proton hyperfine coupling of FADH⁺ in E. coli CPD photolyase is one of the smallest reported for an enzyme with a neutral flavin radical (25, 40), that of FADH⁺ in (6-4) photolyase is even smaller (see Table 1). This decrease in H₈α-proton hyperfine coupling implies a less polar and/or more aromatic environment for FADH⁺ in (6-4) photolyase, due to one or both of the following changes: (i) a smaller substrate-binding pocket for (6-4) photolyase that would limit the number of water molecules that could approach the redox-active flavin cofactor in the absence of a DNA lesion; and (ii) the two aromatic histidine residues, His354 and His358, predicted to replace nonaromatic Asn and Met in the E. coli CPD photolyase active site (16).

Analysis of the crystal structure of a CPD photolyase bound to a repaired CPD-damaged DNA double strand showed that the 8α-methyl group of FAD is oriented toward the substrate binding cavity (51). The structure of (6-4) photolyase is predicted to be largely similar (16); however, because of the different geometry of the (6-4) photoproduct (52, 53), one would expect a smaller substrate cavity as compared with the CPD photolyase. We have previously shown that the displacement of water molecules upon substrate binding to E. coli CPD photolyase alters the polarity of the flavin cofactor surroundings (42). In these experiments, however, the proton hyperfine shifts detected upon binding of a DNA substrate were minute; the isotropic hyperfine coupling of H₈α in substrate-bound photolyase was only about 1% smaller than that of the free enzyme. Thus, it is very likely that the small H₈α hyperfine coupling in (6-4) photolyase is predominantly because of differences in the immediate environment of H₈α introduced by His354 and His358, rather than to the smaller substrate cavity.

Hydrogen Bonding of the Flavin Chromophore to the Protein Environment—By further inspection of the hyperfine couplings of FADH⁺ listed in Table 1, and by comparison of these values

![FIGURE 8. X-band (9.7318 GHz) frozen solution (80 K), pulsed ENDOR spectra of FADH⁺ bound to wild-type and mutant X. laevis (6-4) photolyases at different pH values. All experimental spectra (dots) were recorded at a magnetic field of 347.07 mT. The simulations have been performed using the parameters given in Table 2. The red and the blue curves show the contributions of the H₆α and the H₈α hyperfine couplings to the overall calculated ENDOR (dashed black curves) spectra, respectively. The vertical lines show the positions of the principal hyperfine components of the hyperfine tensors.](image-url)

### TABLE 2

H₆α and H₈α proton hyperfine couplings of the FADH⁺ cofactors obtained from simulations of the pulsed ENDOR spectra of wild-type and mutant X. laevis (6-4) photolyase at different pH values

<table>
<thead>
<tr>
<th>Protein</th>
<th>pH 6</th>
<th>pH 9.5</th>
<th>H₆α</th>
<th>pH 6</th>
<th>pH 9.5</th>
<th>H₈α</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>A₁</td>
<td>6.46</td>
<td>A₆₆</td>
<td>6.50</td>
<td>A₆₆</td>
<td>7.05</td>
</tr>
<tr>
<td></td>
<td>A₂</td>
<td>6.50</td>
<td>A₆</td>
<td>6.50</td>
<td>A₆</td>
<td>7.05</td>
</tr>
<tr>
<td></td>
<td>A₃</td>
<td>6.83</td>
<td>A₈</td>
<td>8.14</td>
<td>A₈</td>
<td>8.14</td>
</tr>
<tr>
<td>H354A</td>
<td>A₁</td>
<td>6.43</td>
<td>A₆₆</td>
<td>6.50</td>
<td>A₆</td>
<td>7.11</td>
</tr>
<tr>
<td></td>
<td>A₂</td>
<td>6.76</td>
<td>A₆</td>
<td>6.83</td>
<td>A₆</td>
<td>7.11</td>
</tr>
<tr>
<td></td>
<td>A₃</td>
<td>8.32</td>
<td>A₈</td>
<td>8.37</td>
<td>A₈</td>
<td>8.37</td>
</tr>
<tr>
<td>H358A</td>
<td>A₁</td>
<td>6.58</td>
<td>A₆₆</td>
<td>6.52</td>
<td>A₆</td>
<td>7.16</td>
</tr>
<tr>
<td></td>
<td>A₂</td>
<td>6.64</td>
<td>A₆</td>
<td>6.71</td>
<td>A₆</td>
<td>7.16</td>
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<td>8.24</td>
<td>A₈</td>
<td>8.26</td>
<td>A₈</td>
<td>8.26</td>
</tr>
</tbody>
</table>

All values are in MHz; signs of hyperfine couplings have not been determined experimentally but were taken from theoretical calculations. The experimental errors are ±0.02 MHz for the A₁ components, and ±0.04 MHz for the A₂ and A₃ components.
The Positions and Protonation States of the Functional Histidines His\(^{354}\) and His\(^{358}\) — In 1994, Kim et al. (14) proposed that formation of a four-membered ring intermediate precedes thymine-dimer splitting in (6-4) photolyase. Later it was suggested that two highly conserved histidines His\(^{354}\) and His\(^{358}\) catalyze the formation of this oxetane intermediate (16). (6-4) photolyases, in contrast to CPD photolyases, exhibit a pronounced pH dependence for DNA repair. The repair activity is highest at around pH 9; the reaction rate decreases by nearly 50% at pH 7 (16). The interpretation of this phenomenon led to discussions about the protonation states of His\(^{354}\) and/or His\(^{358}\). Because the function of histidine is markedly influenced by its protonation state, it is likely that the histidines at the solvent-exposed active site cause the unusual pH dependence in the (6-4) photolyase repair activity \textit{in vitro} (16). The protonation of a histidine alters its polarity, which may be indirectly probed by proton-ENDOR spectroscopy using FADH\(^+\) as an observer. The principal values of the hyperfine coupling tensors of His\(^{1}\) and H\(^{86}\), the symmetry of their hyperfine interaction, and the relaxation behavior of the nuclear spins as reflected in the ENDOR signal intensities depend on structural modifications introduced by point mutations and on the pH. Hence, proton-ENDOR experiments performed on the wild-type enzyme and the two mutant proteins, in which the two histidines are separately replaced by alanine, allow a detailed picture of the microenvironment at the putative substrate-binding site to be painted (Fig. 10).

No three-dimensional structure of a (6-4) photolyase enzyme is available to date, but the strong shift of the isotropic hyperfine coupling of His\(^{1}\) in H354A, as compared with the wild type...
or the H358A protein, both at pH 9.5 and pH 6 (see Fig. 8), suggests that His$^{354}$ is close to H$^{1}$ (see Fig. 10). The changes in $A_{iso}(H^{1})$ may be rationalized in terms of a ribityl side-chain reorientation, which happens when His$^{354}$ is replaced by the smaller alanine. This reorientation results in an altered direction of the C$^{-}$–H$^{1}$ bond with respect to the $\pi$-plane of the isoalloxazine ring, thus changing the dihedral angle $\theta$ (see above) and hence the H$^{1}$ hyperfine coupling. Only small changes on the H$^{1}$ coupling are observed in the H358A mutant, which shows that His$^{358}$ must be further removed from H$^{1}$.

At pH 9.5 the shift of the isotropic hyperfine coupling of H$^{8}_{aa}$, with respect to the wild type, is larger in the H358A mutant than in H354A, which shows that His$^{358}$ is located close to H$^{8}_{aa}$. The line shape of the H$^{8}_{aa}$ ENDOR signal in the H354A mutant is quite different from that of the other two proteins. In H354A, a more rhombic symmetry is observed: $A_{1} \neq A_{2} \neq A_{3}$, whereas in the others the hyperfine tensor is nearly axial symmetric, i.e. $A_{1} \approx A_{2} \approx A_{3}$. Furthermore, the H$^{8}_{aa}$ ENDOR signal in the H354A mutant is also strongly pH-dependent, an effect that must originate from a protonation change of His$^{358}$ when going from pH 9.5 to 6. For steric reasons, it is likely that at pH 9.5, the (deprotonated) His$^{358}$ residue turns toward the smaller Ala$^{354}$. The axial symmetry of the hyperfine tensor, which is due to free methyl group rotation in the wild type, might be affected by this His$^{358}$ reorientation such that the rotation about the C$^{8}_{a}$–C$^{8}_{a}$ bond is hindered and thus the rhombicity of the H$^{8}_{aa}$ hyperfine coupling is increased. At pH 6, the H$^{8}_{aa}$ hyperfine coupling remains slightly rhombic, although the anisotropy between $A_{1}$ and $A_{2}$ is significantly reduced presumably due to a further reorientation of His$^{358}$, which is protonated at pH 6 (Fig. 10). In the wild type, these effects are not observed presumably because reorientation of the histidine residue is not possible because of mutual direct or indirect hydrogen bonding.

The pH changes are also reflected in the signal intensities of the H$^{8}_{aa}$ hyperfine coupling. The integrals of this signal at two different pH values, pH 9.5 and 6, for all three proteins are compared graphically in Fig. 11.

In the wild type and the mutants, the H$^{8}_{aa}$ ENDOR signal intensity generally increases when going from pH 9.5 to pH 6 (see Figs. 7 and 8). However, although the wild type and the H354A mutant protein exhibit virtually the same intensity changes as the proton concentration is raised, the H$^{8}_{aa}$ ENDOR signal of the H358A mutant shows a larger increase (Fig. 11, panel A). This is more clearly observed if one corrects the gradients of H354A and H358A for the wild-type effect (Fig. 11, panel B). The same quantitative changes in the relaxation behavior of the H354A mutant and the wild type imply that His$^{354}$ undergoes the same change of protonation state in both the wild-type and H354A mutant proteins. In the H358A mutant, His$^{358}$ could be protonated at pH 6 and deprotonated at pH 9.5, but the effect of a possible protonation change on the H$^{8}_{aa}$ ENDOR signal is expected to be small because His$^{354}$ is closer to H$^{1}$. However, the principal values of the H$^{1}$ and H$^{8}_{aa}$ hyperfine couplings in the H358A mutant show (within experimental error) virtually no pH dependence. This implies that His$^{354}$ does not change its protonation state when going from pH 9.5 to 6. Hence, the protonated histidine that catalyzes oxet-
ENDOR on (6-4) Photolyase

Chenphyschem. 6, 292–299
Electron Nuclear Double Resonance Differentiates Complementary Roles for Active Site Histidines in (6-4) Photolyase
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J. Biol. Chem. 2007, 282:4738-4747. doi: 10.1074/jbc.M604734200 originally published online December 12, 2006

Access the most updated version of this article at doi: 10.1074/jbc.M604734200

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