Articles

Origin of the Transient Electron Paramagnetic Resonance Signals in DNA Photolyase†

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ABSTRACT: DNA photolyase repairs pyrimidine dimer lesions in DNA through light-induced electron donation to the dimer. During isolation of the enzyme, the flavin cofactor necessary for catalytic activity becomes one-electron-oxidized to a semiquinone radical. In the absence of external reducing agents, the flavin can be cycled through the semiquinone radical to the fully reduced state with light-induced electron transfer from a nearby tryptophan residue. This cycle provides a convenient means of studying the process of electron transfer within the protein by using transient EPR. By studying the excitation wavelength dependence of the time-resolved EPR signals we observe, we show that the spin-polarized EPR signal reported earlier from this laboratory as being initiated by semiquinone photochemistry actually originates from the fully oxidized form of the flavin cofactor. Exciting the semiquinone form of the flavin produces two transient EPR signals: a fast signal that is limited by the time response of the instrument and a slower signal with a lifetime of approximately 6 ms. The fast component appears to correlate with a dismutation reaction occurring with the flavin. The longer lifetime process occurs on a time scale that agrees with transient absorption data published earlier; the magnetic field dependence of the amplitude of this kinetic component is consistent with redox chemistry that involves electron transfer between flavin and tryptophan. We also report a new procedure for the rapid isolation of DNA photolyase.

Cyclobutadipyrimidines (pyrimidine dimers, Pyr<>Pyr[1]) can be created from adjacent pyrimidine bases in DNA upon illumination with ultraviolet radiation (200−300 nm). This potentially lethal or mutagenic damage can be repaired either by the removal of the damaged bases by excision enzymes or by the enzymatic monomerization of the pyrimidine dimer in the presence of blue light. DNA photolyase has been shown to catalyze the monomerization process by using a light-induced electron transfer mechanism (*1*, *2*). The enzyme appears to be present in virtually all organisms.

Photolyase exists in only small amounts in nature, i.e., 10−20 molecules per cell (*3*), making the purification and in vitro study of the enzyme difficult. In 1978, the photolyase gene was cloned (*4*), and the protein was successfully overexpressed and purified from *Escherichia coli* (*5*, *6*). DNA photolyase is a monomeric protein of approximately 55 kDa. The enzyme has two noncovalently bound cofactors present: a 1,5-dihydroflavin adenine dinucleotide ($FADH_2$) cofactor (*7*, *8*), which is the catalytic site, and a second "antenna" molecule. Two types of antenna molecules have been found: methenyltetrahydrofolate with an absorption maximum of 360−390 nm and 8-hydroxy-5-deazaflavin with an absorption maximum of 430−460 nm (*2*). The antenna cofactor is not required for catalytic activity, but it is thought to harvest photon energy for transfer to the flavin (*9*).

The active form of the enzyme contains a fully reduced flavin cofactor (*10*), probably present as the flavin anion ($FADH^-$) as indicated by model studies (*11*, *12*). The flavin cofactor is required for both binding of the damaged DNA (*13*) and catalytic activity (*14*). The flavin cofactor is oxidized to the neutral radical semiquinone form of the flavin ($FADH^•$) upon isolation of the enzyme from the cell, rendering the enzyme catalytically inactive (*10*). This inactivation is reversible as discussed below. Further oxidation of the semiquinone can occur upon exposure to air or ferricyanide to produce the fully oxidized flavin (FAD). Scheme 1 summarizes the reactions needed to cycle between the active and inactive states.

The pathways of light-induced electron transfer within photolyase are crucially important to the mechanism of DNA repair. There are at least two different classes of photochemi-

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[1] Abbreviations: Pyr<>Pyr, cyclobutadipyrimidines; FAD, flavin adenine dinucleotide; $FADH^-$, fully reduced FAD anion; $FADH^•$, blue neutral FAD radical; CIDEP, chemically induced dynamic electron polarization; CRPP, correlated radical pair polarization; photo-CIDNP, photochemically induced dynamic nuclear polarization.

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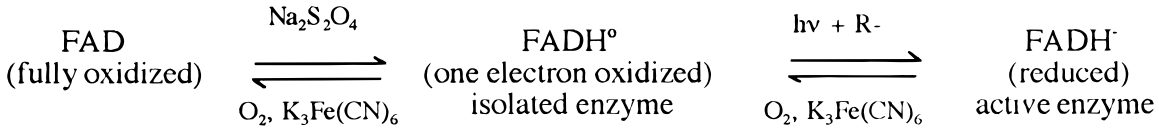
Scheme 1



cal reactions that occur within DNA photolyase: (a) photorepair of DNA by the fully reduced enzyme ($FADH^-$) and (b) photoreactivation of the semiquinone form ($FADH^•$) of the enzyme to form the fully reduced enzyme.

The crystal structure of DNA photolyase from *E. coli* has recently been determined to 2.3 Å resolution with the flavin cofactor in the semiquinone form (*15*). From the crystal structure, it appears that the pyrimidine dimer must flip out from the helix and into a binding site on the enzyme. The binding site and conformation of the flavin appear to be highly conserved as shown by the crystal structure of DNA photolyase from *Anacystis nidulans* (*16*). It is proposed that the enzyme binds to the DNA at the site of damage in the dark. Upon photoexcitation of the $FADH^-$ cofactor either directly or through the folate antenna molecule, the excited state chromophore donates an electron to the pyrimidine dimer to generate pyrimidine radical anion and $FADH^•$. The radical anion spontaneously splits into monomers with the back-donation of the electron to the $FADH^•$ cofactor as suggested by recent studies (*17*−*20*).

Insight into the electron transfer process can be gained by studying the photoreduction of the semiquinone flavin to the fully reduced active form of the enzyme. The photoreduction of the semiquinone must occur in vivo. The oxidation of the active enzyme to form the inactive state must occur readily within the cell since the concentration of O_2 in a cell in an aerobic environment can approach 250 μM. As mentioned above, the enzyme is isolated with the flavin cofactor in the neutral semiquinone form as shown by the steady state EPR signal (*21*).

The isolated semiquinone enzyme can be reactivated by two different methods: reduction by dithionite or photoreduction in the presence of thiol reagents (*14*, *22*). Optical spectroscopy has shown that the flavin is photoreduced by a nearby amino acid (*22*); Trp-306 has been implicated in the process through site-directed mutagenesis (*23*, *24*). In turn, the oxidized tryptophan is reduced by an external reducing agent, e.g., dithiothreitol or dithionite (*22*). In the absence of an external reducing agent, the electron is transferred back to the tryptophan from the flavin within a few milliseconds (*25*).

In previous work, the appearance of a spin-polarized EPR signal arising from the photoreduction of the flavin semiquinone in photolyase was reported (*23*, *26*). Since the enzyme appears to catalyze the repair of the DNA lesion by electron transfer to the thymine dimer, the pathway of electron transfer is crucially important to the understanding of the mechanism of the enzyme. Also, the photoreduction process is required to keep the enzyme in the active, fully reduced state within the organism. In this paper, we discuss the identity of the radicals involved in the spin-polarized transient signal and present new transient EPR signals observed with the enzyme during the photoreduction process. We also report a faster, higher-yield method for the purification of the enzyme.

EXPERIMENTAL PROCEDURES

Cell Growth and Harvest

Cultures of *E. coli* strain pMS969, the DNA photolyase-overproducing strain, were grown in Luria broth as described previously (*6*). Production of DNA photolyase was induced with the addition of 2 mM isopropyl thio-β-D-galactoside (Boehringer Mannheim GmbH) after the cells reached an optical density A_{600} of 0.65−0.8. The cells were then allowed to grow for an additional 16−18 h before being harvested. The cultures were spun for 15 min at 4500 rpm (4000*g*) in a DuPont GSA rotor. The cells were resuspended in lysis buffer [50 mM HEPES (pH 7.0) with 100 mM NaCl, 10% (w/w) sucrose, and 10 mM 2-mercaptoethanol] to a volume of approximately 5 mL of buffer per liter of culture. The suspension was homogenized and stored at −80 °C. Each liter of culture yielded approximately 3.5 g of cells (wet weight).

A typical preparation is described below. All solutions containing the enzyme were maintained at 4 °C.

Step 1: Lysis of Cells. Approximately 14 g of cells (from 4 L of medium) was harvested and frozen at −80 °C as described earlier. The cell suspension was thawed by placing the container in room-temperature water for 15 min. The cells were broken by two passes through a French pressure cell (SLM-Aminco, Urbana, IL) at 20 000 psi and 4 °C. The broken cells were centrifuged for 30 min at 19 000 rpm (43500*g*) in a DuPont SS-34 rotor at 4 °C to remove any unbroken cells and cell fragments. The clear brown supernatant was recovered with care taken not to disturb the pellet, since the cell fragments can interfere with the succeeding steps.

Step 2: Incubation and Precipitation. The supernatant was incubated with trace amounts of ribonuclease A (Sigma, from bovine pancreas) and deoxyribonuclease I (Sigma, from bovine pancreas) for 1 h at 4 °C while it was being stirred. Ammonium sulfate (0.43 g/mL of solution) was slowly added while the solution was being stirred to precipitate the enzyme. After the ammonium sulfate was completely dissolved, the milky gray suspension was centrifuged for 10 min at 19 000 rpm (43500*g*) in a SS-34 rotor at 4 °C. The ammonium sulfate pellet was resuspended in approximately 30 mL of buffer A [50 mM HEPES (pH 7.0) with 50 mM NaCl, 10% (v/v) glycerol, and 10 mM 2-mercaptoethanol]. The ammonium sulfate was removed from the olive brown protein solution with the use of desalting columns (Bio-Rad, P-6DG polyacrylamide gel columns with an exclusion size of 6000 Da). The desalting columns were first equilibrated by passing through approximately 30 mL of buffer A. The protein solution, 3.3 mL, was then loaded onto each column. The proteins were eluted in low-salt buffer by the addition of 4 mL of buffer A.

Step 3: Blue Sepharose Column. The desalted eluent was loaded onto a 3 cm × 10 cm Blue Sepharose CL6B (Pharmacia LKB Biotechnology) column equilibrated with

buffer A. After the loaded column was rinsed with 3 column volumes of buffer B [0.050 M HEPES (pH 7.0) with 10% (v/v) glycerol, 10 mM 2-mercaptoethanol, and 0.1 M KCl], the enzyme was eluted with buffer C [0.050 M HEPES (pH 7.0) with 10% (v/v) glycerol, 10 mM 2-mercaptoethanol, and 2.0 M KCl]. Fractions with blue color were selected and combined. The resulting blue solution was concentrated to <15 mL with an Amicon Ultrafiltration device equipped with a PM-10 membrane. Aliquots (3.3 mL) were loaded onto desalting columns equilibrated with buffer A as described previously, and the desalted protein was eluted from the column by the addition of 4 mL of buffer A.

Step 4: Heparin Column. The blue eluent from the desalting columns was loaded onto a 2 cm × 10 cm Heparin Sepharose CL6B column (Pharmacia LKB Biotechnology) equilibrated with buffer A. The column was rinsed with 3 column volumes of buffer B. DNA photolyase was eluted using a linear gradient composed of 100 mL of buffer B and 100 mL of buffer D [0.050 M HEPES (pH 7.0) with 10% (v/v) glycerol, 10 mM 2-mercaptoethanol, and 1.0 M KCl]. The purity of the individual fractions was assayed by SDS−PAGE. While there are small differences in the purities of the individual fractions, the fractions were usually combined and concentrated with an Amicon Ultrafiltration device with a PM-10 membrane. Additional glycerol was added to give a final concentration of 50% (v/v), and the protein was stored at −80 °C until further use.

Assay of Enzyme Activity

The activity of the purified enzyme was measured in two different buffers at different pHs by using the procedure described by Jorns at el. (*27*). The buffers included Tris at pH 7.2 and 7.4 and HEPES at pH 7.0, 7.2, and 7.4. Poly dT_{10} DNA was obtained from Macromolecular Structure Facility in the Biochemistry Department at Michigan State University. The UV damaging and repair photons were produced from a UVP model UVGL-58 (VWR Scientific) combination long-wave/short-wave UV lamp.

Sample Preparation

Just prior to use, the protein stored at −80 °C was thawed and exchanged into the appropriate buffer by using a desalting column (Bio-Rad, P-6DG polyacrylamide gel columns with an exclusion size of 6000 Da). The samples were concentrated further with Amicon microconcentrators (exclusion size of 30 kDa). If required, samples were deoxygenated with six alternating cycles of vacuum (to approximately 150 Torr) and argon gas. The samples were then transferred to a TE quartz flat cell under an argon atmosphere.

Sample integrity was monitored by using the absorption spectrum as obtained on a Perkin-Elmer Lambda 5 or an Aviv 14DS UV/vis double-beam spectrometer. An extinction coefficient of 4800 M^{-1} cm^{-1} was used to determine the concentration of the semiquinone form of the enzyme (*28*).

EPR Experiments

Steady state and transient EPR studies were carried out on a Varian E-4 EPR instrument interfaced with a personal computer. A TM_{001} cavity equipped with a 60% transmission slotted faceplate was used with either a TM quartz flat cell for aerobic samples or a TE quartz flat cell for anaerobic samples (Wilmad, Buena, NY). Steady state EPR spectra were recorded with a Metrabyte WAAG data acquisition board to collect the analogue signal from the instrument. Kinetic traces were collected with in-house software with data acquisition and timing controlled by a Metabyte Waag board and a MetraByte CTM-05 timing board as described in earlier work (*29*). The EPR, with a 100 kHz modulated detection system, had an instrument rise time of approximately 30 μs. Transient EPR spectra were obtained by using an internally triggered Stanford Research Systems SR250 gated integrator and boxcar averager as described earlier (*29*). The frequency of the cavity was monitored with a Hewlett-Packard 5245L counter equipped with a Hewlett-Packard 5255A frequency converter. Liquid samples in the TM flat cell were cooled to −60 °C with a quartz dewar by using N_2 gas cooled with a dry ice/propanol bath. The temperature within the EPR cavity was monitored by using a thermocouple.

Two different light sources were used: a broadband source and a Nd:YAG laser. The broadband source was a xenon flash lamp with a 17 μs pulse (fwhm) manufactured in-house and described previously (*26*). The wavelength of light reaching the EPR cavity could be regulated by the use of long- and short-pass filters (Andover Corp., Salem, NH). The Quanta-Ray DCR-11 pulsed Nd:YAG laser (Spectra-Physics, Mountain View, CA) was equipped with both doubling and tripling crystals to provide 532 or 355 nm light pulses approximately 10 ns (fwhm) in duration.

RESULTS

Purification Procedure. In our initial work, we encountered difficulty reproducing earlier EPR results (*23*, *26*) with enzyme obtained from the purification procedure described in ref *8*. At times, the transient polarized EPR signal, which had been attributed to a radical pair process involving the flavin semiquinone, could not be obtained from protein that had large semiquinone steady state signals. Moreover, two-electron oxidation of the reduced flavin and denaturation occurred readily. After EPR experiments were completed, a mixture of semiquinone and fully oxidized flavin was often observed. We were also concerned that components of the buffer system were possibly interfering with the photoreduction experiment; flavin can be photoreduced by EDTA (*30*). By modifying the published isolation procedure, we obtained a preparation that was more resistant to air oxidation as described in Experimental Procedures. The procedure also requires significantly less time to obtain pure enzyme, which further limits the exposure to air.

Yields of 3.0−8.5 mg of enzyme per gram of cells were obtained with the preparation described above, whereas a yield of approximately 0.5 mg of enzyme per gram of cells is typical with the earlier procedure (*6*). We eliminated all dialysis steps since the enzyme appears to be very susceptible to air oxidation under dialysis conditions. The buffer system was also changed to a HEPES buffer at a lower pH that minimized oxidation of the enzyme to the fully oxidized form.

The activity of the protein (*27*) was assayed in two different buffer systems to ensure that we were not operating under conditions in which the protein was inactive. The

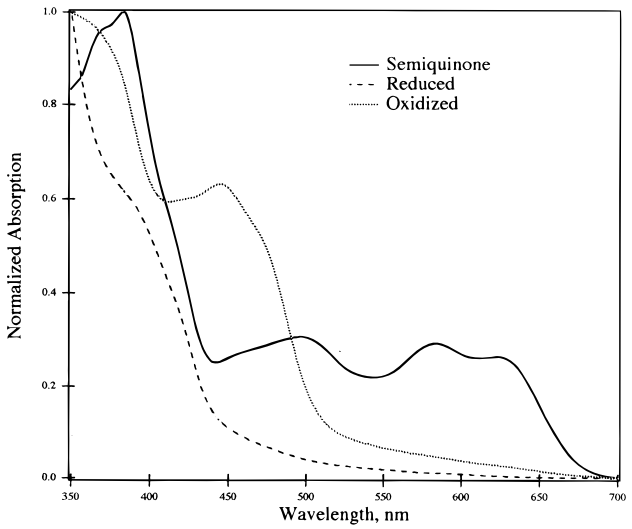


FIGURE 1: Normalized absorption spectra of the enzyme in different oxidation states. The spectrum of the semiquinone form was acquired as soon as possible after the protein eluted from the final column in the purification procedure. The semiquinone is in a buffer of 50 mM HEPES (pH 7.2) with approximately 1 M KCl and 10% glycerol. The fully reduced enzyme was obtained by photoreducing the semiquinone form in the presence of dithiothreitol. The spectrum for the fully oxidized enzyme was obtained by exchanging the protein into 50 mM Tris (pH 7.6) with 10% glycerol. The protein was then allowed to incubate overnight at 4 °C to air oxidize.

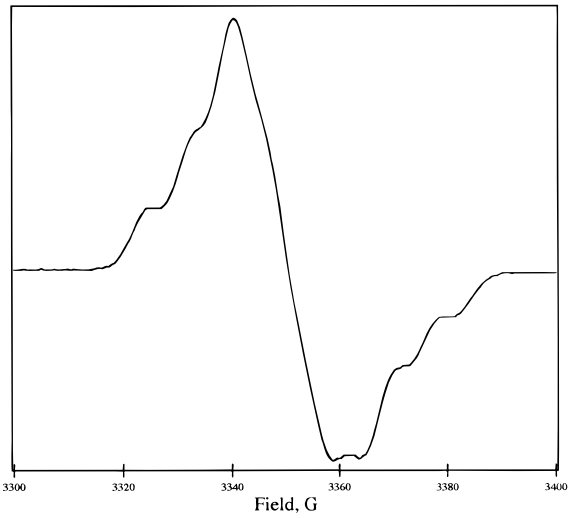


FIGURE 2: Steady state EPR spectrum of the semiquinone form of the DNA photolyase. The enzyme is in 50 mM HEPES (pH 7.0) with 0.25 M $(NH_4)_2SO_4$ and 50% (v/v) glycerol. No oxidized protein was present, as judged from the UV/vis absorption spectrum. The EPR settings were as follows: TE cavity, 9.4120 GHz microwave frequency, 2.5 G modulation, and 6.3 mW microwave power. The temperature was 20 °C. The spectrum was slightly smoothed to remove steps due to digitization of the data.

buffers used included Tris at pH 7.2 and 7.4 and HEPES at pH 7.0, 7.2, and 7.4. The measured activity of the enzyme was essentially identical in the five buffer systems; the lower pH did not appear to inhibit the enzyme.

The normalized UV/vis absorption spectra of the three oxidation states of DNA photolyase are shown in Figure 1. The absorption spectra are virtually identical to the spectra published earlier (*14*). The protein with fully reduced flavin has very little absorption above 400 nm. The fully oxidized flavin has an absorption band at 445 nm, and the semiquinone form has three bands at approximately 500, 580, and 625 nm. The folate (the antenna cofactor present in *E. coli*) absorbs at 384 nm in all three oxidation states; the ratio of flavin to folate is not constant in the samples shown in Figure 1, as commonly observed with the purified enzyme (*31*). As folate is not essential for enzyme activity (*9*), no special procedures were developed to stabilize the antenna pigment/flavin cofactor ratio.

Results of EPR Spectroscopy. The steady state EPR spectrum of DNA photolyase in the semiquinone form isolated by the procedure described above is shown in Figure 2 and is identical to the EPR spectrum published earlier (*23*). The spectrum has a peak to peak width consistent with the neutral radical form of the flavin radical (*21*). The transient spectrum was captured by using a 48 μs boxcar window starting with a delay of 4 μs after the lamp was triggered. The spin-polarized transient EPR signal, attributed in earlier papers to photochemistry involving the flavin semiquinone state and Trp-306 of photolyase (*23*, *26*), is shown in panels A and B of Figure 3 in the derivative form obtained from the instrument; the integrated signal is shown in Figure 3C. The signal was identified as a spin-polarized signal on the basis of the alternating pattern of emission and absorption. More specifically, the pattern of E/A/E/A/E/A is indicative of a correlated radical pair as discussed in detail elsewhere (*32*, *33*). As examined in earlier work (*23*), the transient spectrum clearly shows at least two different splittings: a strong 15 G splitting and a 5 G splitting, with both splittings resulting from spin density on the tryptophan species. Also, as shown clearly in Figure 3B, we detect no other transient signal from 2850 to 3850 G.

The kinetic trace of the transient, spin-polarized signal is given in Figure 4. The time course of the kinetic trace is unchanged in the temperature range of 20 to −60 °C (not shown). The rise and initial fall of the signal are limited by the instrument response time. The appearance of the trace is independent of the light source used; the 10 ns light pulse from the Nd:YAG laser (355 nm line) and the 17 μs flash from the xenon flash lamp produce kinetic traces that are superimposable.

Origin of the Spin-Polarized Signal. As described above, we were not able to obtain consistent results in generating the spin-polarized signal with enzyme prepared according to the earlier purification procedure. In particular, we were often not able to detect a sizable transient signal, despite having a large steady state EPR signal that indicated that the flavin was in the semiquinone state. In addition, we were not successful in obtaining a spin-polarized transient signal from the 532 nm Nd:YAG laser line, although it could be generated easily with the 355 nm line. This was puzzling since, if the transient signal originated from the flavin semiquinone, which absorbs strongly at 532 nm as shown in Figure 1, green light should be optimally effective in driving the photochemistry.

To examine the origin of the spin-polarized signal more closely, a flashing light study was carried out with the transient signal in which the excitation wavelength range was modified selectively by appropriate filters. The results are summarized in Table 1. The amount of light reaching the cavity with each filter in place was determined in a separate experiment by integrating the response of a photodiode placed inside the EPR cavity. When wavelengths of 500 nm or greater were used to excite the photolyase sample in the

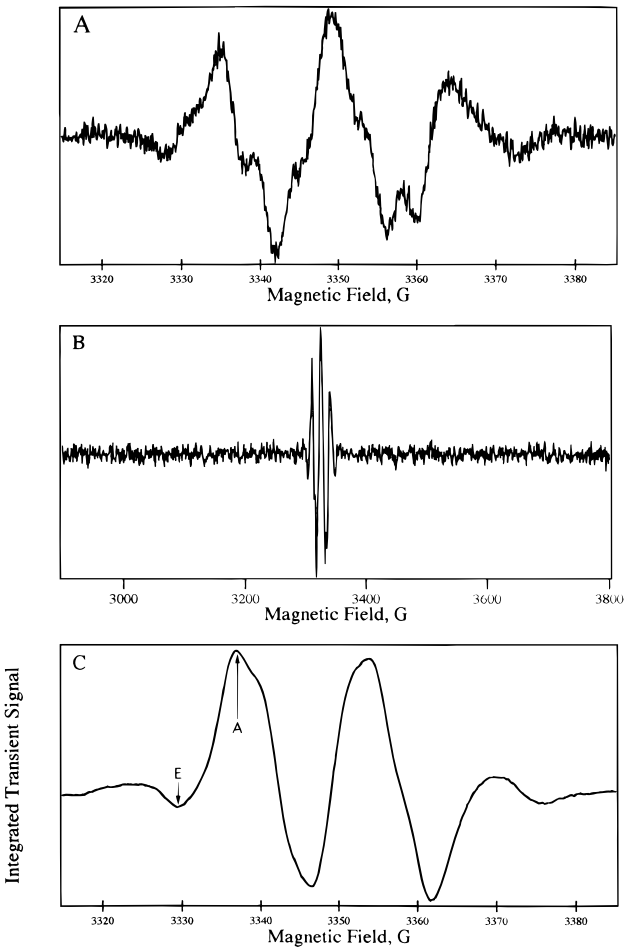


FIGURE 3: Transient spin-polarized EPR spectrum of DNA photolyase. (A) The protein is in 50 mM Tris (pH 7.4) with 5 mM 2-mercaptoethanol, approximately 1 M KCl, 50% (v/v) glycerol, and 20 mM potassium ferricyanide. The EPR settings were as follows: TM cavity, 9.39908 GHz microwave frequency, 6.3 mW microwave power, 2.5 G modulation, and the magnetic field swept from 3300 to 3400 G. The boxcar integrator was set to capture data with a 40 μs delay after the lamp flash and a 435 μs window. The signals were passed through a 10 Hz high-pass filter. A single spectrum is divided into 1024 field points with 10 decays averaged at each point. The data shown are the average of 10 spectra. The temperature was 20 °C. (B) The protein is in 50 mM HEPES (pH 7.0) with 0.25 M $(NH_4)_2SO_4$, 2 mM potassium ferricyanide, and 10% (v/v) glycerol. The EPR settings were as follows: TM cavity, 9.39650 GHz microwave frequency, 6.3 mW microwave power, 2.5 G modulation, and the magnetic field swept from 2850 to 3850 G. The boxcar integrator was set to capture data with a 50 μs delay after the lamp flash and a 220 μs window. The signals were passed through a 10 Hz high-pass filter. A single spectrum is divided into 1024 field points with 10 decays averaged at each point. The data shown are the average of 15 spectra. The temperature was 20 °C. (C) Transient spectrum in panel A integrated.

EPR cavity, no discernible transient signal was observed. From the data in Table 1, we conclude that the bulk of the signal arises from light with a wavelength in the range of 400−450 nm. In contradiction to earlier interpretations (*23*, *26*), this study indicates that the spin-polarized EPR signal does not originate from the semiquinone form of the enzyme.

Further evidence for the lack of involvement of the semiquinone form was obtained by measuring the amplitude of the transient signal as a function of the amplitude of the steady state spectrum (Figure 5). As the steady state amplitude decreases as the result of oxidation of the semiquinone upon exposure to air, the transient signal increases in amplitude. This observation provides evidence that the transient signal does not originate from the steady state, EPR detectable semiquinone form of the enzyme.

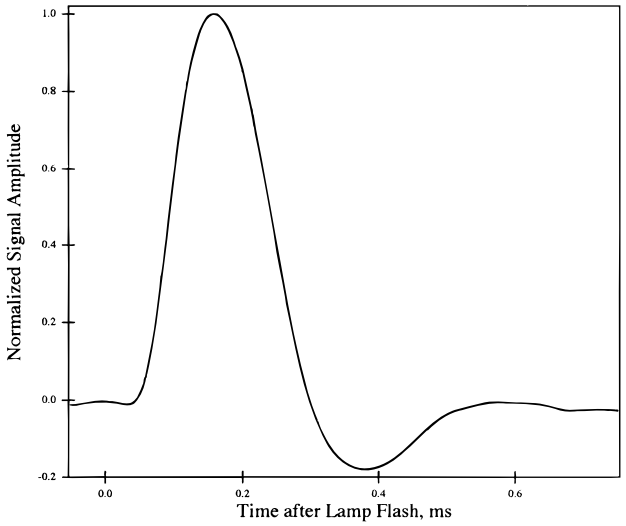


FIGURE 4: Kinetic trace of the transient signal. The protein is in 100 mM HEPES (pH 7.0) with 0.50 M $(NH_4)_2SO_4$ and 1 mM potassium ferricyanide. The EPR settings were as follows: TM cavity, 9.39523 GHz microwave frequency, 6.3 mW microwave power, 2.5 G modulation, and 3349 G magnetic field. The xenon flash lamp was triggered 250 μs after data acquisition was started. Two thousand five hundred decays were averaged at a frequency of 0.7 Hz. The temperature was 20 °C.

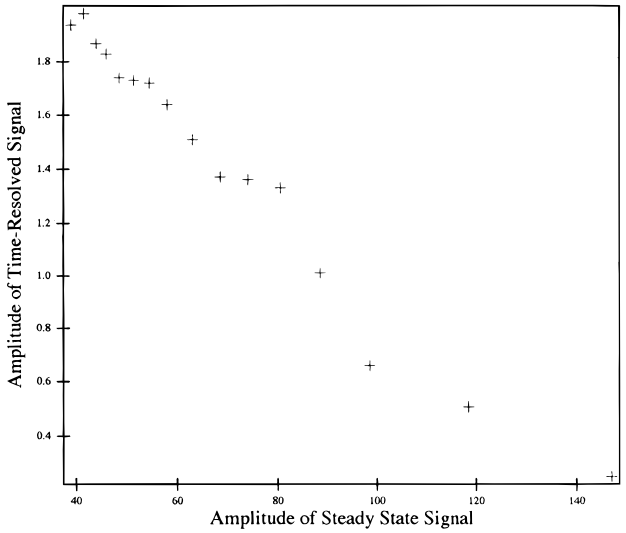


FIGURE 5: Amplitude of the transient signal vs amplitude of the steady state signal. The protein was anaerobic in 100 mM HEPES (pH 7.0) with 0.50 M $(NH_4)_2SO_4$ and 1 mM potassium ferricyanide. The EPR settings are as follows: TM cavity, 9.39679 GHz microwave frequency, 6.3 mW microwave power, and 2.5 G modulation. The amplitude of the steady state is a measure of the peak to trough signal. The transient signal was generated from 500 flashes (xenon lamp) with the magnetic field at 3349 G.

Table 1: Correlation of the Exciting Wavelength and EPR Signal of the Transient Signal

wavelength of exciting light	relative intensity of light reaching the EPR cavity	relative amplitude of the transient signal
white light (no filter)	1.00	1.00
$\lambda > 450$ nm	0.90	0.13
$\lambda > 400$ nm	0.97	0.91
$\lambda > 500$ nm	0.81	0.00

Instead, we conclude that the spin-polarized EPR signal originates from the fully oxidized flavin, as shown by the increase in the transient signal as the flavin quinone is produced from the semiquinone form by air oxidation.

We also investigated the possibility of the signal originating from the fully reduced flavin. Fully reduced flavin was generated from the semiquinone form by using either dithionite or dithiothreitol and light. With the fully reduced flavin cofactor, the steady state semiquinone EPR signal disappeared, and no transient signal could be found.

The spin-polarized signal is independent of the presence of the folate cofactor. We found the folate cofactor to degrade during the transient EPR experiments as shown by monitoring the absorption of the folate. The amplitude of the spin-polarized signal was independent of the folate absorption.

The buffer conditions were carefully examined to ensure that the transient signal originates from the enzyme alone and is not related to anything in the buffer solution. Under the buffer conditions used in the earlier work [50 mM Tris buffer (pH 7.4) with 0.1 M NaCl and 10% (v/v) glycerol], the flavin cofacter in photolyase is rapidly oxidized at room temperature. The transient EPR signal appears immediately from this sample, and no induction time is required. If the buffer conditions are changed to 50 mM HEPES buffer (pH 7.0) with 0.5 M $(NH_4)_2SO_4$ and 50% (v/v) glycerol, the semiquinone state is relatively resistant to air oxidation. In this case, there is no transient signal unless potassium ferricyanide (5 mM) is added to oxidize the flavin cofactor.

The transient signal is also generated in the absence of glycerol and salt, although the protein denatures readily under those conditions. The presence of low concentrations (5−10 mM) of EDTA or 2-mercaptoethanol has no effect on the transient signal. The signal is also independent of oxygen as long as the flavin is already in the fully oxidized form. The transient signal decreases with the addition of 0.1 M KI, a triplet quencher.

No part of the kinetic trace could be fit by using an exponential decay or rise time, indicating that the data shown are a convolution of EPR signals of the radical pair and the instrument response function. The negative going signal is reproducible and real. It is probably not due to the response of the instrument since we were not able to reproduce this feature in the fast decay of the spin-polarized signal from duroquinone in a 4:1 mixture of 2-propanol and triethylamine (*35*). Also, the negative going feature is not a Torrey oscillation (*36*); the kinetic trace is independent of the microwave power up to 14 mW.

We attempted to capture a spin-polarized EPR signal from a flavin system that is known to generate a spin-polarized NMR signal (*34*). We were not able to detect a transient signal either aerobically or anaerobically by using a mixture of 4 mM *N*-acetyltryptophan with 0.2 mM FAD in HEPES (pH 7.0) with 0.50 M $(NH_4)_2SO_4$ and 20% glycerol.

Transient Signals Originating from the Flavin Semiquinone. As described above, the transient spin-polarized EPR signal from DNA photolyase does not originate from the semiquinone form of the enzyme. Further attempts were made to reproduce the results of the transient absorption work on the photoreduction of the semiquinone (*22, 26, 37*−*39*). We limited our light source to the 532 nm line from the Nd:YAG laser to minimize the generation of transient signal from any fully oxidized flavin present. No signal could be generated from the fully oxidized protein with the 532 nm line; therefore, the strong spin-polarized signal will not interfere with any signals generated from the semiquinone state.

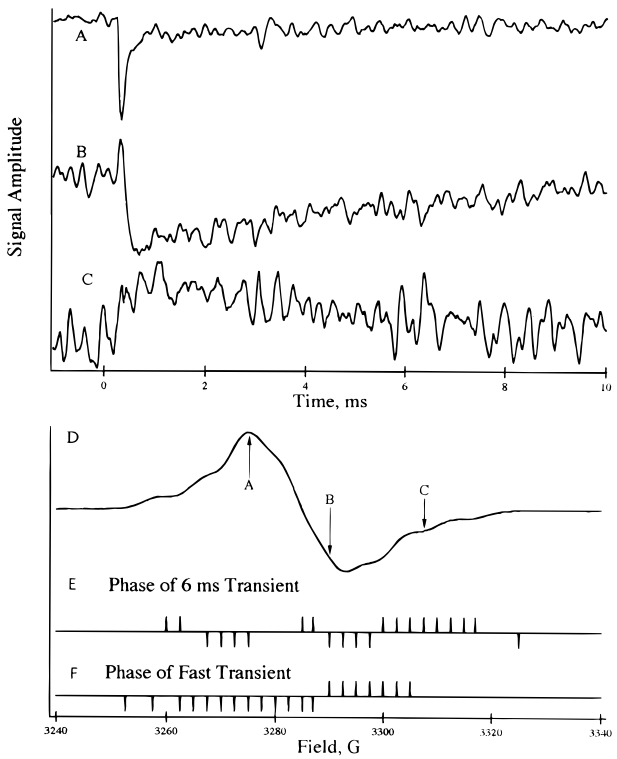
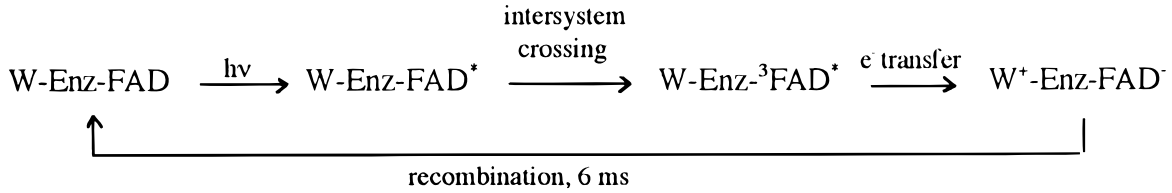


FIGURE 6: Transients generated from the semiquinone form of the enzyme. The protein was in 50 mM HEPES (pH 7.0) with 0.25 M $(NH_4)_2SO_4$ and 50% (v/v) glycerol. The EPR settings were as follows: TM cavity, 9.22618 GHz microwave frequency, 6.3 mW microwave power, and 2.5 G modulation. The sample and cavity were cooled to −19 °C. The sample was excited by using the 532 nm line from the Nd:YAG laser with an average pulse energy of 20 mJ and a pulse frequency of 4 Hz. Approximately 300 pulses were averaged for each trace. The laser was triggered 2 ms after the start of the data acquisition, and 2000 data points were acquired at a sampling time of 10 μs per point. (A) The magnetic field was set to 3275 G. (B) The magnetic field was set to 3290 G. (C) The magnetic field was set to 3307.5 G. (D) The steady state spectrum under identical conditions. (E) The sign of the 6 ms transient as a function of magnetic field. The data were acquired as described for traces a−c. (F) The sign of the fast transient as a function of magnetic field. The data were acquired as described for traces a−c.

Typical kinetic traces at selected magnetic fields are shown in Figure 6A−C. Two kinetic components were observed: a fast component whose kinetics are limited by the instrument response time and a second slower component with an exponential decay of approximately 6 ms. The field settings for the kinetic traces are indicated in the steady state spectrum in Figure 6D. These transient signals could be reproduced under specific conditions: concentrated enzyme (>500 μM) in the semiquinone form at a temperature lower than 5 °C in an aerobic atmosphere. These conditions provide insight into the origin of the transient species observed.

Although we initiated the time-resolved experiments with the enzyme in essentially 100% semiquinone form, we noticed that, under signal averaging conditions, a mixture of all three oxidation states developed. The amount of fully oxidized and fully reduced enzyme that was produced during the experiment depended strictly on the number of laser pulses the sample received, increasing in proportion to the

Scheme 2



number of flashes incident on the sample. The presence of fully oxidized protein in the flat cell was confirmed by the presence of the 445 nm band in the UV/vis absorption spectrum of the protein in the EPR flat cell recorded immediately after the EPR experiment. The protein was then washed out of the flat cell for more rapid exposure to oxygen, and the absorption spectrum was monitored with time. The presence of the fully reduced enzyme was indicated by the increase in the absorbance of the semiquinone bands with time as the fully reduced enzyme was reoxidized to the semiquinone state. From this behavior, we conclude that one of the transient signals is generated by a reaction in which the semiquinone state reacts to form fully oxidized and fully reduced species.

The transients could be generated with the enzyme under an anaerobic atmosphere, but the transients survived fewer laser pulses since the fully reduced enzyme was not cycled back to semiquinone by exposure to oxygen. Under these anaerobic conditions, exposure to laser light eventually produced samples that contained only the oxidized and reduced enzyme. As before, the fully reduced enzyme returned to the semiquinone state upon exposure to air.

We were not successful in obtaining time-resolved spectra of the species responsible for the transient signals. Due to the dismutation process that occurred with each laser pulse, we could not reliably estimate the amplitudes of the components at different fields. However, we are able to distinguish the field dependence of the phase of the signal, i.e., whether the signal is positive or negative going, as shown in traces E and F of Figure 6. The two components display different behavior as a function of the magnetic field. The fast kinetic component correlates inversely with the steady state EPR spectrum; where the steady state spectrum has positive amplitude, the fast component has negative amplitude. Thus, we conclude that the fast component corresponds to the loss and recovery of the semiquinone absorption. We were able to determine the relationship between the laser pulse power and the amplitude of this component. With a photolyase concentration of approximately 1 mM, the amplitude of the fast component is at a maximum at a laser pulse power of 20 mJ or greater. At that incident energy with our experimental geometry, we estimate that approximately one photon is absorbed per protein molecule per laser pulse.

The slower kinetic component has a more complicated relationship with the magnetic field as shown in Figure 6E; it alternates between negative and positive amplitude across the spectrum. The signal is a convolution of the loss and recovery of the semiquinone absorption along with the generation of a paramagnetic intermediate.

DISCUSSION

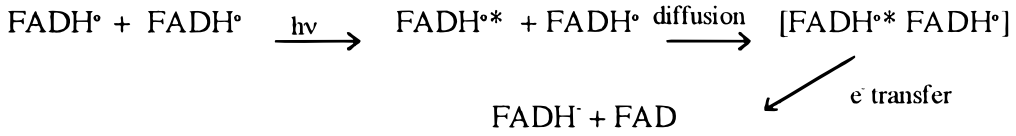
Origin of the Spin-Polarized Transient Signal. We have found the redox chemistry and EPR spectroscopy of DNA photolyase to be complex. The spin-polarized signal, which our data show originates from the fully oxidized flavin, not the semiquinone state of the enzyme, arises from a non-Boltzmann distribution of spin states, thus causing EPR signals to be orders of magnitude larger than the EPR signals from a thermally populated system. Thus, the spin-polarized signal is much more prominent than the thermalized signals arising from the semiquinone state, and a small amount of contamination by the fully oxidized enzyme can produce signals that dominate in the EPR experiments.

Our results show that the spin-polarized signal originates from the fully oxidized flavin, rather than from the semiquinone form. The signal is not generated with wavelengths of 500 nm or greater, light absorbed by the semiquinone, but rather with light with a wavelength of 400−450 nm, which is absorbed by the fully oxidized flavin. The folate antenna molecule appears to play a minimal role in the production of the spin-polarized signal.

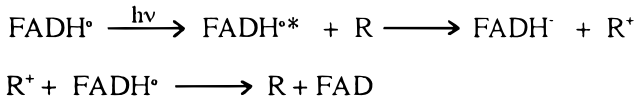
The spin-polarized signal cannot be explained by conventional chemically induced dynamic electron polarization (CIDEP) theory, which accounts for the population inequalities of a radical pair that has diffused apart. In CIDEP, EPR lines appear only during emission or absorption. In contrast, the spin-polarized signal observed with photolyase has EPR lines with half of each line in emission and half in absorption, indicating that the radical pairs are still interacting during the time that the EPR is probing the spin states. This type of situation has been discussed by Norris et al. (*33*), and they have designated the mechanism responsible as correlated radical pair polarization (CRPP). CRPP has been observed in radicals trapped in micelles and in biological systems in which the radicals are held by protein, e.g., photosynthetic reactions centers. Typically, the members of the radical pair must be constrained from rapidly diffusing apart by an immobilizing medium, such as a protein matrix, to observe CRPP signals (*32*).

We propose the mechanism in Scheme 2 for the generation of the CRPP signal from oxidized photolyase. The fully oxidized flavin is excited and subsequently intersystem crosses to the triplet state. The flavin triplet then abstracts an electron from a nearby tryptophan to produce a flavin anion radical and tryptophan cation radical; Trp-306 has been implicated in this process (*23*, *24*). The radical pair generated by the electron transfer cannot diffuse apart since both members are held by the protein matrix; thus, they are still interacting when the system is probed by EPR to give a CRPP signal. After some time, the electron is transferred back to the tryptophan, and the original species are regenerated. There is precedent in the literature for our proposed mechanism. Similar mechanisms have been put forward to explain the photochemically induced dynamic nuclear polarization (photo-CIDNP) signals observed when tryptophan reacts with photoexcited flavin (*34*, *40*−*42*).

Scheme 3



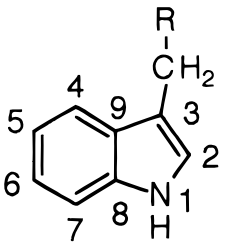
Scheme 4



Moreover, evidence for the presence of the flavin triplet is shown by the disappearance of the CRPP signal with the addition of KI, a triplet quencher. KI has been shown to be an effective triplet quencher for free flavins (*43*). Oxygen does not appear to significantly quench the transient signal at the concentrations studied.

In the proposed mechanism, the correlated radical pair is the tryptophan cation radical and flavin anion radical. We have not observed any transient signals from the flavin doublet anion; it is possible that the tryptophan signal obscures the flavin signal.

Our mechanism for the production of the flavin doublet/tryptophan doublet radical pair invokes an electron abstraction, not a hydrogen abstraction, from the tryptophan on the basis of the results obtained from the isotopic labeling studies carried out earlier (*23*). The 5 G splitting in the transient signal was assigned to the hydrogen on C(2) of the tryptophan, and the large 15 G splitting was assigned to the C(3) β-methylene protons of the tryptophan from isotopic labeling studies. The numbering used in the tryptophan molecule is shown in structure **1**.



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Examination of the isotopic labeling work indicates that the signal is produced by a tryptophan radical cation based on the study with deuterium labels at positions C(2) and C(5). With deuterium at those positions, the CRPP signal is altered by the disappearance of the 5 G splitting. Both calculations by this laboratory (*23*) and by Walden and Wheeler (*44*) indicate that very little spin density should be present at C(5) for either the neutral or the cation radical radicals, and that a significant amount of density will be present at the C(2) position for the cation radical. The disappearance of the 5 G splitting indicates that there is spin density at the C(2) position, indicating a tryptophan radical cation is formed as a result of electron abstraction.

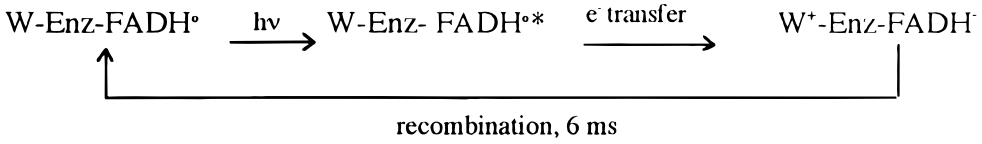
We were unable to find any examples in the literature of EPR-detected flavin/tryptophan systems exhibiting spin polarization, although we found several examples of such systems displaying photo-CIDNP as detected by NMR (*34*, *40*−*42*). The photo-CIDNP was generated from cyclic electron transfer reactions between photoexcited free flavin and free tryptophan or tryptophans on the surface of a protein. We attempted to reproduce some of the photo-CIDNP experiments by using EPR detection, but without success, probably due to the difference in relaxation times for electron and nuclear spins. Photolyase is a unique system in that both the flavin and the tryptophan are held securely by the protein, which will not allow for diffusion of the radical pair. Thus, as with micellular and viscous solutions, the radical pair lifetime is longer than in less viscous solutions (*32*), allowing us to detect the spin-polarized EPR signal with our instrument.

Origin of the Thermally Populated Transient Signals. Transient EPR signals could be obtained from the semiquinone form of photolyase by using concentrated (1 mM) solutions. The signals were produced most efficiently under conditions in which any fully reduced flavin could be oxidized back to semiquinone, and production of fully oxidized flavin due to air oxidation was minimized. Even under these conditions, the signals were present only fleetingly; a significant amount of the protein undergoes an irreversible process with each flash. Eventually all of the semiquinone exposed to laser light can be converted to the fully oxidized form or fully reduced form.

There appear to be two independent kinetic components present. The fast component is more sensitive to the laser pulse power; it dominates when the laser power is adjusted to give approximately one photon per protein molecule per laser flash. The amplitude of the short-lived component appears to correlate well with the disappearance and recovery of the steady state semiquinone signal. We tentatively assign the fast component to two possible reactions: a dismutation reaction or an electron transfer with an unidentified intermediate. Although both the fully oxidized and fully reduced flavins are EPR silent, we can follow this reaction by monitoring the recovery of the semiquinone EPR signal. We do not detect the presence of any paramagnetic intermediates in this reaction.

In the postulated dismutation reaction mechanism (Scheme 3), a semiquinone is photoexcited. During the semiquinone excited state lifetime, collisions between this species and

Scheme 5



ground state semiquinone species occur that facilitate electron transfer to produce fully reduced and fully oxidized enzyme. Clearly, this diffusional process will have a low yield, and most of the flavin pairs undergo a back transfer reaction to regenerate the semiquinones. Nonetheless, a fraction will produce fully oxidized and reduced states according to Scheme 3. This dismutation reaction appears to be quite common with flavin semiquinones (*45*).

A second possibility exists for the production of fully reduced and fully oxidized enzyme as outlined in Scheme 4. A semiquinone is photoexcited. During the semiquinone excited state lifetime, the semiquinone abstracts an electron from a molecule, R, present in the buffer to form fully reduced flavin. $R^{+\bullet}$, the molecule which lost an electron to the excited state semiquinone, then goes on to abstract an electron from a ground state semiquinone to produce the fully oxidized flavin. Once again, the reaction produces both fully oxidized and fully reduced flavin.

The longer-lived component, with a lifetime of approximately 6 ms, correlates with some of the transient absorption data published earlier on photolyase on the photoreactivation of the semiquinone to form the fully active reduced enzyme. Heelis, Payne, and Sancar studied the photoreactivation of the flavin semiquinone in the absence of extrinsic electron donors by using transient absorption optical spectroscopy. They reported that 92% of the semiquinone absorbance was recovered after 12 ms (*25*). Using their data, we calculate a lifetime for the recombination process of approximately 5 ms which is in agreement with our 6 ms component. Heelis et al. ascribe the recovery of the semiquinone absorption to the reversal of the initial electron transfer from a nearby amino acid to the flavin semiquinone, which is postulated to occur in the photoreactivation of the enzyme in the absence of external reducing agents (Scheme 5).

The 6 ms component appears to have a complicated transient spectrum as shown by the phase variations of the signal in Figure 6E. The transient spectrum can be ascribed to a convolution of the production of a signal from a paramagnetic intermediate along with the loss and recovery of the semiquinone steady state signal. Keeping in mind the relatively poor spectral quality of the data, the paramagnetic intermediate appears to have a *g* value slightly smaller than that of the flavin as the 6 ms component has a positive amplitude at the zero field crossing of the semiquinone signal. We measure the *g* value to be 2.004 for the flavin semiquinone in the enzyme. Tryptophan cation and neutral radicals typically have *g* values in the 2.003−2.004 range (*46*). Thus, we tentatively conclude that our data for the paramagnetic intermediate produced during the photoreduction of the flavin semiquinone do not contradict its assignment as a typtophan cation radical (see Scheme 5 and ref *25*), consistent with the mechanism proposed by Heelis et al.

In summary, the spin-polarized EPR signal reported earlier (*23*, *26*) originates from the fully oxidized protein, rather than the semiquinone form. Although there are several examples of CIDNP signals generated from tryptophan/flavin systems, this is the first example of a spin-polarized flavin/tryptophan signal detected with EPR. After changing the purification procedure and the buffer system to minimize the flavin oxidation, we obtained two transient EPR signals from the semiquinone form of the enzyme. One paramagnetic transient with a lifetime of approximately 6 ms resembles the transient obtained earlier with transient absorption, while the other transient, which decays with a half-time of less than 30 μs, appears to originate from a dismutation reaction of the semiquinone.

Upon absorption of 400−450 nm light, fully oxidized photolyase abstracts an electron from a nearby tryptophan to form a tryptophan cation radical and flavin semiquinone anion reversibly. Upon absorption of 532 nm light, the neutral semiquinone form of photolyase generates a paramagnetic intermediate with a lifetime that is consistent with a tryptophan radical cation first proposed from transient absorption studies (*25*). We tentatively conclude that tryptophan is likely to have an important role in electron transfer in the enzyme during the photoreactivation process.

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