Photoinduced Spin-Polarized Radical Pair Formation in a DNA Photolyase·Substrate Complex at Low Temperature†

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ABSTRACT: Electron spin polarization is a phenomenon characterized by anomalous line intensities (emission or enhanced absorption) in the EPR spectrum. It is highly diagnostic of radical pairs, such as those formed in photoinduced electron transfer reactions. Electron spin polarization behavior (E/A pattern) is observed in light-modulated EPR spectra obtained at 4 K with fully reduced DNA photolyase·substrate complexes. Similar results are obtained with complexes formed with native enzyme or reconstituted enzyme containing fully reduced flavin as its only chromophore. No signal is observed for fully reduced enzyme or substrate alone. The results suggest that the electron spin polarization signal is due to photoinduced formation of a flavin/substrate radical pair ($FADH^{\bullet}/T<>T^{\bullet-}$); splitting of $T<>T^{\bullet-}$ does not occur at 4 K, and the radical pair can only undergo back-electron-transfer reactions. The data are consistent with the proposal that electron transfer initiates DNA repair in the photolyase reaction.

Irradiation of DNA with ultraviolet light (200−300 nm) results in the formation of cyclobutane dimers between two adjacent pyrimidine residues in a (2 + 2) cycloaddition reaction. The damaged DNA can be repaired by irradiation with visible light in the presence of DNA photolyase (Jorns, 1990; Sancar, 1994). Extensive mechanistic studies have been conducted with DNA photolyase from *Escherichia coli.* The active form of the *E. coli* enzyme contains two chromophores: 1,5-dihydro-FAD,[1] probably in the anionic state ($FADH^-$), and 5,10-methenyltetrahydropteroylpolyglutamate (pterin) (Jorns et al., 1984, 1990; Wang et al., 1988; Wang & Jorns, 1989; Johnson et al., 1988; Kim et al., 1993). The pterin chromophore acts as an efficient antenna, harvesting light energy and transferring this energy to $FADH^-$. DNA repair is initiated by reaction of the pyrimidine dimer with the reduced flavin singlet, generated via singlet−singlet energy transfer from the pterin or by direct light absorption (Jordan & Jorns, 1988; Jorns et al., 1987a, 1990; Lipman & Jorns, 1992; Ramsey et al., 1992; Okamura et al., 1991; Kim et al., 1991).

The observed quenching of reduced flavin singlet by substrate has led to the proposal that dimer splitting may involve an electron transfer process (Okamura et al., 1991; Jordan & Jorns, 1988; Chanderkar & Jorns, 1991; Kim et al., 1991). Alternative modes of singlet quenching, such as energy transfer to pyrimidine dimer or enhanced intersystem crossing, can be ruled out (Chanderkar & Jorns, 1991; Ramsey & Jorns, 1992). On the other hand, model studies show that dimer radicals are unstable and that electron transfer to or from the dimer can initiate cleavage (Santus et al., 1972; Rokita & Walsh, 1984; Rustandi & Fischer, 1993; Hartman & Rose, 1992; Jorns, 1987; Yeh & Falvey, 1991; Roth & Lamola, 1972). Electron transfer between free reduced flavin singlet and pyrimidine dimer is thermodynamically feasible in only one direction, from excited flavin to the dimer (Heelis et al., 1992). Efficient photosensitized splitting of dimers requires the reduced flavin anion (Hartman & Rose, 1992) and is favored by nonpolar solvents (Hartzfeld & Rose, 1993). The flavin binding site in *E. coli* photolyase is hydrophobic (Jorns et al., 1990; Chanderkar & Jorns, 1991), and dimer repair is efficient (Ramsey et al., 1992). Very recent photo-CIDNP studies show that the photosensitized splitting of dimers with free reduced flavin involves a dimer radical anion intermediate (Pouwels & Kaptein, 1994).

The results argue strongly in favor of a mechanism where photolyase-mediated DNA repair is initiated by formation of a neutral flavin/dimer anion radical pair ($FADH^{\bullet}/T<>T^{\bullet-}$). Subsequent rapid splitting of $T<>T^{\bullet-}$, followed by back-electron-transfer to $FADH^{\bullet}$, would repair the DNA and regenerate $FADH^-$. No evidence for $FADH^{\bullet}$ formation was obtained in time-resolved absorption spectroscopy studies at ambient temperature on a picosecond time scale (Okamura et al., 1991). A paramagnetic species was detected in ambient temperature flash EPR studies, but this unidentified species was long-lived and could not correspond to the initial reaction intermediate (Kim et al., 1992). The results suggested that a different approach might be needed to detect the putative initial radical pair.

Substrate quenches the fluorescence of $FADH^-$ in *E. coli* photolyase at 5 °C or 77 K, but dimer splitting does not occur at the lower temperature (Jordan & Jorns, 1988; Chanderkar & Jorns, 1991; Jorns, unpublished observations). This suggests that only the initial electron transfer step in the photolyase reaction can occur at 77 K or lower, consistent with model studies which show that splitting of $T<>T^{\bullet-}$ is subject to a thermal barrier that is not surmountable at or

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[1] Abbreviations: FAD, flavin adenine dinucleotide; $FADH^{\bullet}$, blue neutral FAD radical; $FADH^-$, fully reduced FAD anion; T<>T, thymine dimer; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; DPPH, 1,1-diphenyl-2-picrylhydrazyl radical; EPR, electron paramagnetic resonance; ESP, electron spin polarization or electron spin polarized; TM, triplet mechanism, RPM radical pair mechanism; CRPP, correlated radical pair polarization; CIDNP, chemically induced dynamic nuclear polarization.

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below 77 K (Kim & Rose, 1988, 1990). In this report we describe results obtained in light modulation EPR studies with a fully reduced DNA photolyase•substrate complex ($FADH^-$/T<>T). The experiments were conducted at low temperature (4 K) to eliminate possible complications due to the products of dimer splitting (e.g., $T^{\bullet -}$) and to slow spin lattice relaxation. A photoinduced electron spin polarization signal is observed with the enzyme•substrate complex but not with enzyme or substrate alone. The results suggest that the observed electron spin polarization signal is due to the formation of a flavin/substrate radical pair ($FADH^{\bullet}$/T<>$T^{\bullet -}$).

EXPERIMENTAL PROCEDURES

Materials. Oligothymidylate [$(dT)_9$] was purchased from Ransom Hill Bioscience, Inc. FAD was obtained from Sigma.

Holoenzyme and Reconstituted Enzyme Preparation. E. coli DNA photolyase was purified according to the procedure described previously (Jorns et al., 1987b). Apophotolyase was prepared and reconstituted with $FADH_2$ as described by Jorns et al. (1990). Stock solutions of holoenzyme and reconstituted enzyme (2.0×10^{-4} M) were stored at −80 °C in complete PRE buffer (50 mM Tris-HCl, pH 7.4, containing 50 mM NaCl, 1.0 mM EDTA, 10 mM dithiothreitol (DTT), and 50% glycerol). In each case, enzyme concentration was calculated on the basis of $FADH^{\bullet}$ absorption at 580 nm ($\epsilon_{580} = 4.8 \times 10^{-3}$ M^{-1} cm^{-1}) (Wang & Jorns, 1989). Absorption spectra were recorded at 5 °C using a Perkin-Elmer Lambda 3B spectrometer. Enzyme activity was measured as described previously (Lipman & Jorns, 1992).

EPR Studies. Oxidation of $FADH^-$ to $FADH^{\bullet}$ occurs during isolation of native enzyme or enzyme reconstituted with $FADH_2$ (Jorns et al., 1984, 1990; Payne et al., 1987). Enzyme containing fully reduced FAD was generated by irradiation under anaerobic conditions with yellow light for about 15−20 min at 4 °C in complete PRE buffer. The reaction was monitored by the appearance of $FADH^-$ fluorescence at 505 nm and/or by the disappearance of the EPR signal of $FADH^{\bullet}$. Fluorescence spectra were recorded using a Perkin Elmer LS5 luminescence spectrometer. Samples for photoreduction were prepared inside a glovebox which was flushed with nitrogen gas. The enzyme sample was initially placed in a small vial equipped with gas inlet and outlet ports; argon gas was allowed to bubble over the surface of the solution for about 45 min. The sample was then transferred to an EPR tube (3 mm i.d., 4 mm o.d.), previously flushed with argon gas and equipped with an air-tight ground glass stopcock.

Except where noted, enzyme samples were mixed with substrate prior to photoreduction. Substrate was prepared by irradiating $(dT)_9$ in 1.0 mM Tris-HCl, pH 7.4, containing 0.05 mM EDTA with germicidal and blue/black light as described by Chanderkar and Jorns (1991). The dimer concentration in the stock substrate solution (1×10^{-3}) was determined by fluorescence titration (Jordan & Jorns, 1988) and enzyme assay (Jorns et al., 1985).

The EPR X-band measurements were done with a Varian E-9 spectrometer equipped with an Air Products low-temperature accessory. A 300 W xenon lamp (ILC) was modulated at 500 Hz, and the output passed through water and $\lambda > 300$ nm Pyrex filters. A lens was used to focus the xenon light beam on the EPR cavity. Electron spin-polarized (ESP) EPR signals were collected with a phase-sensitive detection system referenced to the modulated light source. The correct polarization phase at 500 Hz was calibrated by comparing it with a known ESP signal observed in a photosynthesis reaction center sample (Rustandi et al., 1992; Levanon, 1979). The photosynthesis reaction center sample was prepared as previously described (Biggins & Mathis, 1988). The *g*-factors were calibrated by comparing them with a powder DPPH sample ($g = 2.0037$).

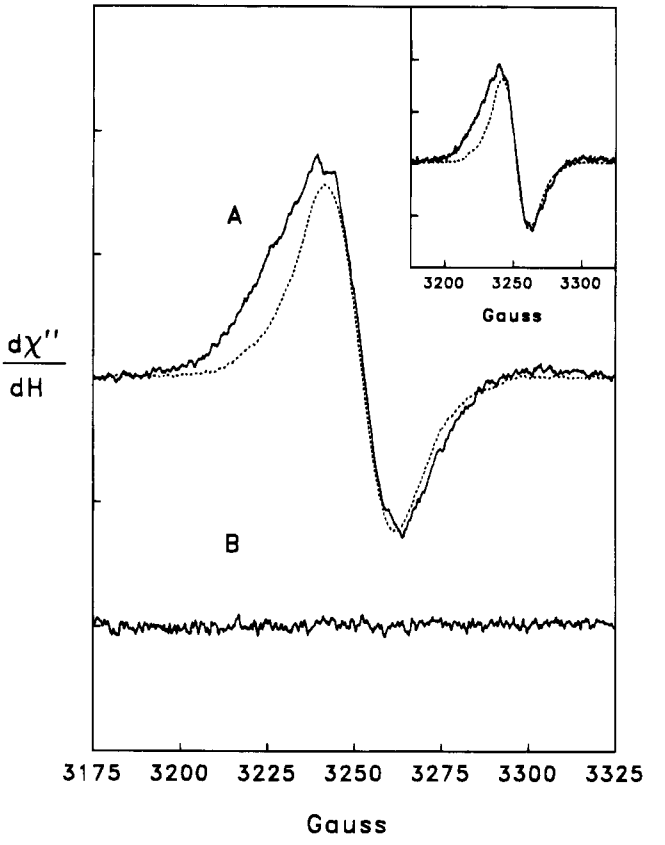


FIGURE 1: Effect of substrate or flavin radical reduction on the dark EPR signal observed with native DNA photolyase. The solid lines in panels A and B show X-band EPR signals observed with native enzyme (1.2×10^{-4} M with respect to $FADH^{\bullet}$) in complete PRE buffer containing UV-irradiated $(dT)_9$ (2.0×10^{-4} M with respect to dimer) before and after photoreduction with yellow light, respectively. The dashed spectrum in panel A was recorded for native enzyme (1×10^{-4} M) in the absence of substrate. Spectra were collected at 4 K, 4 scans, 0.05 mW microwave power, 2 G modulation amplitude (100 kHz field modulation), and 9.1161 and 9.1157 GHz microwave frequencies for solid and dashed lines in panel A, respectively. Inset: The solid line is the spectrum of the enzyme•substrate complex formed with reconstituted enzyme containing only $FADH^{\bullet}$ (1.2×10^{-4} M) in complete PRE buffer containing UV-irradiated $(dT)_9$ (2.0×10^{-4} M with respect to dimer). The dashed spectrum was recorded for reconstituted enzyme (1×10^{-4} M) in the absence of substrate. Spectra were collected at 9.1159 GHz (solid line) or 9.1161 GHz (dashed line) microwave frequencies under conditions otherwise identical to those described for native enzyme.

RESULTS

Oxidation of $FADH^-$ occurs during isolation of native DNA photolyase or enzyme reconstituted with fully reduced FAD to yield a stable radical form (Jorns et al., 1984, 1990; Payne et al., 1987). The solid line in Figure 1A shows the dark EPR signal due to $FADH^{\bullet}$ in the enzyme•substrate

complex formed with native enzyme and UV-irradiated $(dT)_9$. The EPR signal has a g-factor of around 2.0039, as expected for a blue neutral FAD radical, but the line width of 20.7 G is somewhat larger than the normal value of about 19 G (Palmer et al., 1971). The line width of the EPR signal for native enzyme in the absence of substrate (Figure 1A, dashed spectrum) is about 19 G, suggesting that substrate interaction with FADH• may broaden the signal by 1.7 G. Substrate–flavin interactions have previously been detected with reconstituted enzyme containing oxidized FAD and with native or reconstituted enzyme containing fully reduced FAD (Jordan & Jorns, 1988; Jorns et al., 1990). The dark EPR signal of the native enzyme•substrate complex disappeared after anaerobic photoreduction using yellow light, as expected for $FADH^-$ formation. Dimer repair does not occur during photoreduction because yellow light is absorbed only by FADH• and the radical form is catalytically inactive (Jorns et al., 1990). Similar results were obtained for the enzyme•substrate complex formed with reconstituted enzyme containing FADH• as its only chromophore (Figure 1, inset).

Evidence for light-induced formation of a flavin/substrate radical pair (FADH•/T<>$T^{\bullet-}$) was sought by recording light modulation EPR spectra at 4 K. In these studies, the fully reduced enzyme•substrate complexes described above were irradiated in the EPR cavity using a xenon arc lamp modulated at 500 Hz. Spectra were recorded with a phase-sensitive detection system referenced to the frequency of the modulated xenon lamp.

Figure 2 shows that similar light modulation EPR spectra are obtained with fully reduced enzyme•substrate complexes formed with native enzyme (curve a) or reconstituted enzyme containing only $FADH^-$ (curve b). In each case, the samples showed a transient EPR signal in the region of $g = 2.00$. The signal intensity was dependent on the detection phase at 500 Hz light modulation. Similar signals were obtained when the light was modulated at different frequencies. The most striking feature of these light modulation spectra is the emission/absorption (E/A) pattern. This pattern is clearly seen when the spectra are mathematically integrated, as illustrated in curve a′. As will be discussed, the E/A pattern is characteristic of a radical pair mechanism. These spectra have slightly asymmetric lobes and considerably narrower line widths than the dark EPR signal observed with FADH•-containing enzyme (see Figure 1). These slightly asymmetric line widths are not due to magnetic field overmodulation since a similar spectral shape at lower signal intensity was obtained when the experiment was repeated at lower field modulation (curve c). No light modulation EPR signal was observed with control samples containing substrate without enzyme (curve d) or reduced enzyme alone (curve e).

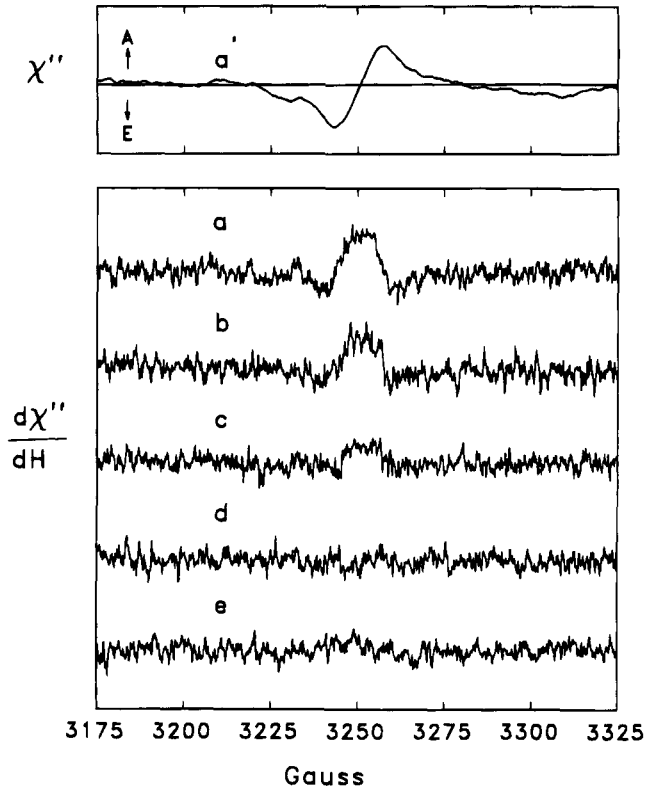


FIGURE 2: Light-modulated EPR spectra obtained for fully reduced photolyase•substrate complexes at 4 K. Unless otherwise noted, X-band ESP EPR signals were collected at 500 Hz light modulation, 25 scans, 0.5 mW microwave power, 5 G modulation amplitude (100 kHz field modulation), and 9.1116, 9.1131, and 9.1143 GHz microwave frequencies for curves a, b, and c, respectively. Enzyme•substrate complexes with fully reduced native photolyase or reconstituted enzyme containing only $FADH^-$ (no pterin) and control samples were prepared as described in the legend to Figure 1. Spectra obtained for native and reconstituted enzyme•substrate complexes (1.2×10^{-4} M with respect to $FADH^-$) in complete PRE buffer containing UV-irradiated $(dT)_9$ (2.0×10^{-4} M with respect to dimer) are shown in curves a and b, respectively. The spectrum obtained for the native enzyme•substrate complex (curve a) is also shown in integrated form in curve a′. Curve c is the spectrum of the native enzyme•substrate complex recorded at 2.5 G modulation amplitude and 0.1 mW microwave power. Control reactions containing only substrate (2.0×10^{-4} M with respect to dimer) to fully reduced native enzyme (1.2×10^{-4} M with respect to $FADH^-$) alone are shown in curves d and e, respectively.

DISCUSSION

Light-modulated EPR spectra exhibiting an E/A pattern are obtained at 4 K with fully reduced enzyme•substrate complexes formed with native or reconstituted DNA photolyase and UV-irradiated $(dT)_9$ but not with reduced enzyme or substrate alone. Similar spectra are obtained with native and reconstituted enzymes. This shows that the pterin chromophore does not contribute to the observed signal since the reconstituted enzyme contained $FADH^-$ as its only chromophore. It also confirms previous studies that $FADH^-$ in reconstituted enzyme occupies the same binding site as in native enzyme (Jorns et al., 1990) since a different orientation would be reflected in the EPR spectrum (Rustandi et al., 1992).

The E/A pattern in the light-modulated EPR spectra provides clear evidence for electron spin polarization (ESP), a phenomenon characterized by anomalous line intensities (emission or enhanced absorption) in the EPR spectrum. This polarization is due to a non-Boltzmann spin distribution in a radical pair. Spectra exhibiting ESP are highly diagnostic of radical pairs, such as those formed in photoinduced electron transfer reactions. The detailed theories describing ESP have been reviewed (Adrian, 1979; Norris et al., 1990).

ESP can arise from either the triplet mechanism (TM) or the radical pair mechanism (RPM). ESP due to the TM is possible only when the radical pair is created from a spin-polarized, triplet state precursor. Both radicals in the pair are identically polarized and exhibit spectra in *either* emission or enhanced absorption but not an E/A (or A/E) pattern. The TM cannot be operative in the photolyase reaction which is

known to proceed via the singlet state of $FADH^-$ (Jordan & Jorns, 1988; Chanderkar & Jorns, 1991; Kim et al., 1991).

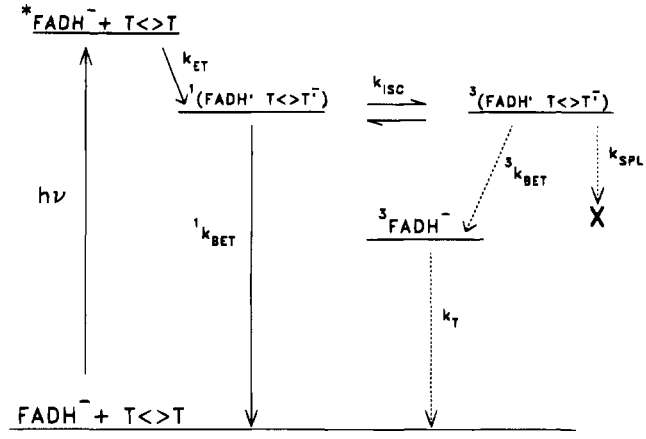
Polarization in the RPM arises from various interactions in spin-correlated radical pairs and can be observed for either singlet- or triplet-born radical pairs. In the conventional (or noncorrelated) RPM, the EPR spectrum is recorded after the spin-polarized radicals have separated to a point where they no longer experience exchange interaction ($J = 0$), a reasonable assumption when experiments involve small radicals in mobile solution (Adrian, 1979). In the correlated RPM (usually referred to as CRPP or correlated radical pair polarization), polarization develops under conditions where radical diffusion is restricted (e.g., charge separation in photosynthesis) and the EPR spectrum is recorded for the correlated radical pair itself ($J \neq 0$) (Thurnauer & Norris, 1980; Closs et al., 1987; Buckley et al., 1987).

In either the conventional RPM or CRPP, development of polarization in a spin-correlated radical pair depends on differences in g-factors (net effect) and/or nuclear hyperfine interactions (multiplet effect) of the radicals and requires an exchange interaction. The g-factor of $FADH^•$ is known to be 2.0039. The g-factor for $T<>T^{•-}$ is not known but may be estimated on the basis of the g-factor of glycine anhydride anion ($g = 2.0030$) which has a similar radical anion structure. Rustandi and Fischer (1993) have used this value for $T<>T^{•-}$ to correctly explain the CIDNP polarization pattern in their model system. Assuming this is the case, $FADH^•$ polarization contributes the low-field component of the photolyase ESP spectrum (emission) and the high field of the spectrum (absorption) is due to $T<>T^{•-}$ polarization. The asymmetric line width with the low-field emission lobe slightly broader than the high-field absorption lobe is consistent with this proposal since $FADH^•$ line width is expected to be larger than that of $T<>T^{•-}$. Since the estimated Δg is small, the net effect will be small and the multiplet effect should dominate, as observed for most organic radicals.

In either conventional RPM due the multiplet effect or in CRPP, an E/A pattern is predicted for *each* radical in a singlet-born radical pair when J is positive. This can generate an E/A/E/A pattern (Norris et al., 1990). However, when Δg is small, the central peaks may overlap and cancel each other out, resulting in an apparent E/A pattern. The rather weak E/A polarization signals observed in these studies may be partly due to cancellation between overlapping $FADH^•$ and $T<>T^{•-}$ polarizations and may also reflect the small singlet−triplet ($S-T_0$) mixing characteristic of singlet-born radical pairs. Additional studies are needed to further evaluate the polarization mechanism responsible for the E/A pattern observed with photolyase which includes g-anisotropy and dipolar interaction.

An ESP signal due to the cation radical of tryptophan 306 ($Trp^{•+}$) has been observed upon excitation of $FADH^•$ in the *radical form* of *E. coli* photolyase (Essenmacher et al., 1993; Kim et al., 1993). Electron transfer to excited $FADH^•$ (quartet state) generates $Trp^{•+}$ and the triplet state of $FADH^-$ ($^3FADH^-$); the ESP signal arises through interaction between $Trp^{•+}$ and $^3FADH^-$. Could the ESP signal observed in our studies with the *fully reduced* enzyme•substrate complex be due to $Trp^{•+}$? This would require a biphotonic process: (1) formation of $FADH^•$ by initial electron transfer from fully reduced flavin singlet to substrate and (2) a second electron transfer from Trp 306 to flavin radical quartet. This possibility appears unlikely since our ESP spectra differ from that reported for $Trp^{•+}$: our ESP spectra (E/A pattern) are slightly asymmetric and exhibit 3−4 G larger total line width as compared with the signal observed for $Trp^{•+}$ (E/A/E/A/E/A pattern). Finally, could the ESP signal observed in our studies be due to electron transfer from reduced flavin singlet to Trp 306, creating a $FADH^•/Trp^{•-}$ radical pair in a monophotonic process? This possibility also appears unlikely since it is difficult to explain why the reaction would occur only in the presence of substrate, a requirement which is not observed for electron transfer from Trp 306 to excited $FADH^•$ (Essenmacher et al., 1993; Kim et al., 1993).

Scheme 1: Low-Temperature Photocycle with Fully Reduced DNA Photolyase•Substrate Complex[a]



[a] Electron transfer from excited flavin to substrate generates a flavin/substrate radical pair in the singlet state. This species may undergo back-electron-transfer, returning the system to ground state. Dimer splitting, shown by a dotted arrow, does not occur at low temperature. Intersystem crossing from the singlet to the triplet state of the flavin/substrate radical pair is arbitrarily shown as an isoenergetic process. Back-electron-transfer may occur after intersystem crossing but is shown by a dashed arrow since a signal attributable to $^3FADH^-$ was not observed in light-modulated EPR studies with photolyase in the presence or absence of substrate.

In summary, we demonstrate that excitation of the fully reduced photolyase•substrate complex ($FADH^-•T<>T$) in light-modulated EPR studies at 4 K generates an E/A polarization signal. We propose that this signal is due to the formation of a flavin/substrate radical pair, $^1(FADH^•/T<>T^{•-})$, in a photoinduced electron transfer reaction (Scheme 1). Since $T<>T^{•-}$ cannot split at low temperature, the flavin/substrate radical pair can only undergo back-electron-transfer reactions under these conditions. Back-electron-transfer with the radical pair in the singlet state regenerates $FADH^-•T<>T$ in a cyclic reaction. Back-electron-transfer with the radical pair in the triplet state might populate a lower lying triplet state of $FADH^-$ ($^3FADH^-$), but an EPR signal due to $^3FADH^-$ was not detected. [Lhoste et al. (1966) also failed to detect the triplet state with free reduced flavins in EPR studies and suggested poor triplet yield and/or high zero-field splitting as possible explanations.] The ESP behavior observed with *E. coli* photolyase is consistent with the proposal that DNA repair is initiated by electron transfer from the excited reaction center chromophore ($^1FADH^-$) to the dimer. A similar electron transfer mechanism appears likely with other photolyases since, although the antenna chromophore may vary, all known enzymes contain the same reaction center chromophore (Jorns, 1990). The photocycle proposed to account for the

results obtained with photolyase at low temperature is somewhat analogous to the photocycle exhibited by the photosynthetic reaction center when steps beyond initial radical pair formation are blocked by prior reduction or removal of the secondary (quinone) electron acceptor (Snyder & Thurnauer, 1993).

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