three analyses, the enthalpic differences among the Pro-Ala, Pro-Gly, and Pro-Val systems are significant. Statistical analyses of protein crystal structures indicate a decreasing tendency for β-turn formation across Pro-Gly, Pro-Ala, and Pro-Val sequences.[2,16,17]

The conformational behavior of these model peptides in a solvent of low dielectric constant is relevant to the protein folding problem because the interior of a folded protein is thought to be characterized by a relatively low dielectric constant[18] and because many proteins adopt their native conformations is nonpolar locales (e.g., the interior of a biomembrane). The thermodynamic data we have provided should also be useful for evaluating the accuracy with which computational methods reproduce the balance of noncovalent forces that controls peptide conformation.[5]

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Supplementary Material Available: Figures pertaining to the van't Hoff analyses of β-turn formation in the Pro-Ala, Pro-Gly, and Pro-Val dipeptides (7 pages). Ordering information is given on any current masthead page.

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Evidence from Photoinduced EPR for a Radical Intermediate during Photolysis of Cyclobutane Thymine Dimer by DNA Photolyase

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Ultraviolet light (200–300 nm) induces adjacent pyrimidines in DNA to undergo a symmetry-allowed [2 + 2] cycloaddition reaction, which produces pyrimidine dimers (Pyr⟨⟩Pyr).[1] DNA photolyases repair the Pyr⟨⟩Pyr by utilizing photonic energy of near-UV and visible light (300–500 nm).[2] All photolyases characterized to date contain $FADH_2$ as the catalytic cofactor in addition to a second chromophore of high extinction coefficient which functions as a photoantenna, absorbing 300–500 nm photons and transferring the excitation energy to $FADH_2$.[3-7] On the basis of model reactions with a variety of photosensitizers,[8] it has been proposed that enzymatic Pyr⟨⟩Pyr splitting is initiated by electron transfer between $FADH_2$ and the photodimer.[2a,7a] A recent picosecond flash photolysis study revealed the build-up of a new absorption band (λ ≈ 400 nm) following the quenching of the excited singlet state of $FADH_2$ by Pyr⟨⟩Pyr.[9] The 400-nm species was not identified, but it was attributed to a reaction intermediate produced during catalysis. In this communication, we use time-resolved EPR to show that a radical intermediate is generated during flash-induced dimer repair by DNA photolyase.

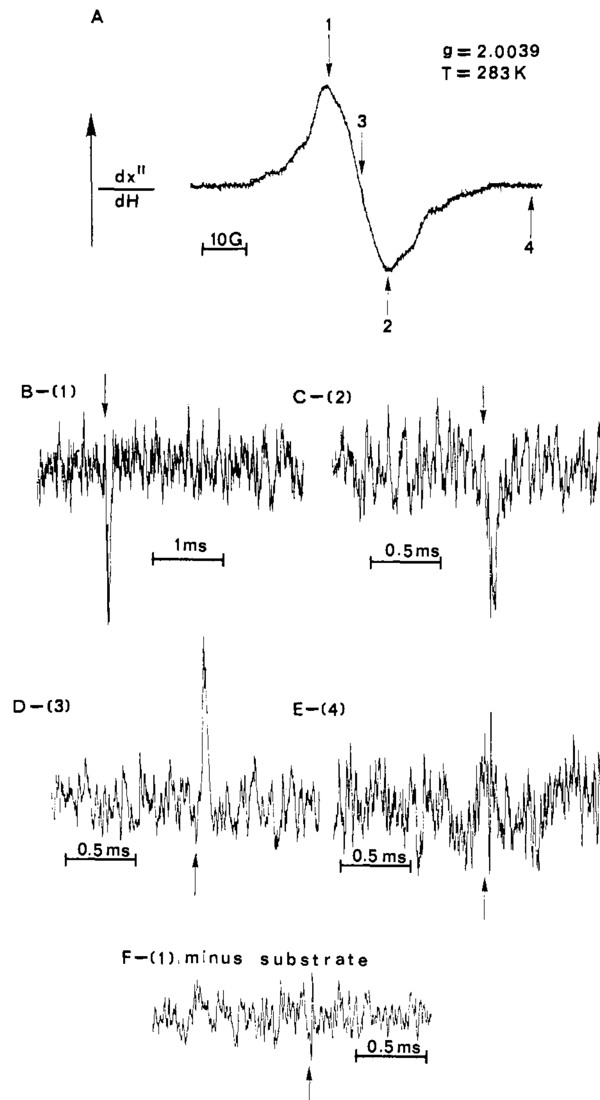

Figure 1. EPR detection of radical transients in *E. coli* DNA photolyase catalyzed pyrimidine dimer splitting reaction. A is the dark-stable EPR spectrum of E–FADH•, and B–E are the kinetic transients of E–$FADH_2$ + T⟨⟩T at field positions 1–4, respectively. F is the kinetic transient of E–$FADH_2$ at field position 1 in the absence of T⟨⟩T. At each field position, 1200 flashes (positions indicated by arrows in B–F) were averaged. The spectrometer gain was 1.6×10^6 for all kinetic traces. For each measurement, fresh sample was used.

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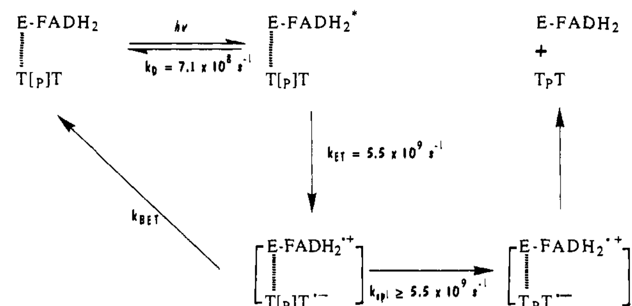
Scheme I. A General Kinetic Scheme for Pyrimidine Dimer Splitting by *E. coli* DNA Photolyase



Escherichia coli photolyase containing only flavin and no second chromophore (methenyl tetrahydrofolate) was prepared by selective photodecomposition of folate.[10] The substrate, T⟨⟩T, was prepared by acetone-photosensitized irradiation of TpT at 313 nm.[11] The reaction solution contained 5×10^{-5} M photolyase and 1×10^{-2} M T⟨⟩T in 5×10^{-2} M Tris-HCl, pH 7.5, 5×10^{-2} M NaCl, 1×10^{-3} M EDTA, 1×10^{-2} M dithiothreitol, and 10% (v/v) glycerol. The EPR measurements were performed at room temperature with a Bruker ER-200 spectrometer and xenon flashlamp excitation.[12] The flavin cofactor of photolyase is oxidized to the paramagnetic blue neutral radical (FADH$^\bullet$) during purification.[3b] The EPR spectrum of this stable radical is shown in Figure 1A. To detect light-induced radicals, the enzyme was photochemically reduced[3b] to the $FADH_2$ form at 10 °C under argon atmosphere and mixed with substrate anaerobically. A control scan on this reaction mixture with the photoreactivating lamp off showed a flat base line (data not shown), as expected.

Direct EPR detection of transient radicals produced by repetitive flashlamp signal averaging methods[12b] on the enzyme–substrate complex is shown in Figure 1B–D. The kinetic traces,[12c] which rise rapidly and decay with the 35-μs instrument time constant, were obtained at various field values, as indicated in Figure 1A; together, they show that the transient species does not result from oxidation of the $FADH_2$ in the course of the experiment. Thus, while the negative signal at field position 2 (Figure 1C) is consistent with the FADH$^\bullet$ spectrum, the negative and positive signals at field positions 1 (Figure 1B) and 3 (Figure 1D) are not. Moreover, at field position 4, we observed no flash-induced EPR signal (Figure 1E). We conclude that the transient signals in Figure 1B–D are not due to flash artifact and/or dark-stable flavin radical. The transients shown in Figure 1B–D were gradually reduced and eventually not detectable after prolonged flash photolysis. The decrease in signal results from repair and is not due to enzyme degradation, as evidenced by the fact that the dark radical flavin signal (generated by exposure of the enzyme–substrate or product mixtures to air) before and after the experiments differs by no more than 10%. Furthermore, no transient was observed when either T⟨⟩T (Figure 1F) or enzyme (data not shown) was omitted from the reaction mixture. The lack of transient signals in these latter controls provides compelling evidence that the transients in Figure 1B–D arise from the reaction intermediates during catalysis.

While the direction of electron transfer between photolyase and Pyr⟨⟩Pyr dimer has not been determined experimentally, studies with model systems have shown that both electron abstraction from the dimer[13] and electron donation to the dimer[14] led to efficient dimer splitting. However, both thermodynamic[15] and chemical[11] consideration of Pyr⟨⟩Pyr splitting by DNA photolyase strongly suggest electron transfer from photoexcited reduced flavin to Pyr⟨⟩Pyr. Assuming this to be the case, we would like to know the identity of the flash-induced paramagnetic species in our system. The rapid decay of the radical, however, limited signal intensity[12c] and, in combination with the requirement for high substrate (T⟨⟩T) concentration, precluded attempts to obtain a higher resolution spectrum of the transient species. Nonetheless, the kinetic scheme shown in Scheme I based on this study and the photoexcited state decay kinetics[7d] of E-$FADH_2$ narrows down the candidates.

In this scheme,[16] excitation of E–$FADH_2$ is followed by an efficient electron transfer to T⟨⟩T, which produces a charge-separated radical pair (E–$FADH_2^{\bullet+}$...T⟨⟩T$^{\bullet-}$). The dimer radical anion within the radical pair then undergoes splitting in competition with back electron transfer. The high quantum efficiency of splitting,[17] together with data from the secondary deuterium isotope effect on splitting,[18] implies that the T⟨⟩T$^{\bullet-}$ species is short-lived, and therefore, the transients observed in our time-resolved EPR in Figure 1B–D must arise from the final state (E–FADH$^{\bullet+}$...T–T$^{\bullet-}$) rather than the initial state (E–$FADH_2^{\bullet-}$...T⟨⟩T$^{\bullet-}$) of catalysis.

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(16) It is conceivable that the reduced flavin in photolyase is an anion (E–$FADH^-$). In this case, one-electron oxidation of the flavin produces the neutral radical FADH$^\bullet$.

(17) The observed repair quantum yield of T⟨⟩T ($\Phi_r = 0.90$) is related to the quantum efficiency (ϕ_{css}) of formation of the initial radical pair (E–$FADH_2^{\bullet+}$...T⟨⟩T$^{\bullet-}$) and the quantum efficiency (ϕ_{spl}) of splitting by $\Phi_r = \phi_{css} \times H_{spl}$, and $\phi_{css} = 1 - (\tau_1/\tau_0) = 0.9$ where $\tau_1 = 0.16$ ns and $\tau_0 = 1.6$ ns are the fluorescence lifetimes[7d] of E–$FADH_2$ in the presence and absence of T⟨⟩T, respectively.

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