EPR Detection of the Transient Tyrosyl Radical in DNA Photolyase from *Anacystis nidulans*

Corinne Aubert,[†] Klaus Brettel,[†] Paul Mathis,[†]
André P. M. Eker,[‡] and Alain Boussac*,[†]

Section de Bioénergétique, DBCM
CEA Saclay, (URA CNRS 2096)
91191 Gif-sur-Yvette Cedex, France
Department of Cell Biology and Genetics
Medical Genetics Center, Erasmus University Rotterdam
P.O. Box 1738, 3000 DR Rotterdam, The Netherlands

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Amino acid radicals play important roles at catalytic centers in a number of proteins; tyrosyl radicals in particular are established to be involved in the enzyme mechanisms in class I ribonucleotide reductase, photosystem II (PSII), prostaglandin H synthase, and bovine liver catalase.[1] Recently, we provided evidence by kinetic absorption spectroscopy that a tyrosyl radical is transiently formed during photoactivation of DNA photolyase from *Anacystis nidulans*.[2] Here, EPR spectroscopy has been used to investigate this transient tyrosyl radical. Structural properties such as spin densities, protonation state, and β-CH_2 conformation were found to be similar to those of the tyrosyl radicals in PSII.[3]

DNA photolyases[4] use photonic energy of blue or near-UV light to repair UV-induced lesions in DNA. The repair function requires the flavin adenine dinucleotide (FAD) cofactor of the enzyme to be in the two-electron reduced form ($FADH^-$). In the purified enzyme, the FAD is typically present in the semiquinone radical form ($FADH^•$) but can be reduced to $FADH^-$ in a reaction induced by visible light (photoactivation).[4] In photolyase from *A. nidulans*, photoexcited $FADH^•$ abstracts an electron from a tryptophan residue in less than 500 ns; subsequent electron transfer from a tyrosine residue to the tryptophanyl radical ($t_{1/2} \approx 50\ \mu s$) creates a tyrosyl radical.[2] The tyrosyl radical is readily re-reduced by exogenous reductants, resulting in the $FADH^-$-containing, catalytically active form of the enzyme. In the absence of exogenous reductants, as in the present study, reverse electron transfer from $FADH^-$ to $Tyr^•$ ($t_{1/2} = 76$ ms) regenerates $FADH^•$ and Tyr.[2]

Kinetic absorption measurements (Figure 1) showed that the back-reaction between $FADH^-$ and $Tyr^•$ slowed from $t_{1/2} = 76$ to 198 ms (monoexponential fit)[5] when H_2O was replaced by D_2O in the buffer.[6] The difference spectrum of the 198-ms phase in D_2O (●, inset of Figure 1) is virtually identical to the spectrum of the 76-ms phase in H_2O previously reported[2] and corresponds to the sum of the $FADH^-$ minus $FADH^•$ difference spectrum (broken line, inset of Figure 1) and the $Tyr^•$ minus Tyr difference spectrum which peaks at 410 nm.[2,7]

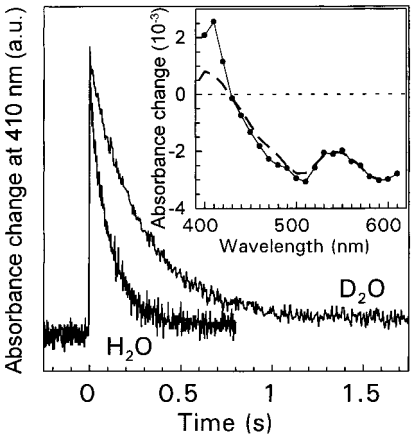

Figure 1. Hydrogen isotope effect on the decay of the flash-induced absorbance changes at 410 nm, at 10 °C, attributed to the back-reaction $FADH^-$-$Tyr^•$ → $FADH^•$-Tyr in photolyase from *A. nidulans*. $FADH^•$ was excited at time zero by a nonsaturating laser flash (635 nm, 7 ns, ∼30 mJ/cm^2 for the "H_2O" sample and ∼10 mJ/cm^2 for the "D_2O" sample). The time resolution was 2 ms for the "H_2O" sample and 5 ms for the "D_2O" sample. The "H_2O" sample contained 90 μM enzyme in buffer A (0.2 M NaCl, 20 mM Tris·HCl, pH 7.4, in H_2O) and 15% (v/v) glycerol. The "D_2O" sample contained 92 μM of enzyme and was prepared from a fresh "H_2O" sample by buffer exchange with polyacrylamide chromatography microcolumns (15% glycerol was added afterward). The exchange buffer was prepared by drying buffer A and adding D_2O to reach the same volume as before drying. Amplitudes of the two traces were normalized. Inset: Spectrum of the amplitude of the 198 ms phase in the D_2O sample (●). The broken line is the $FADH^-$ minus $FADH^•$ difference spectrum in *A. nidulans* photolyase reported previously;[2] its amplitude has been normalized to the spectrum of the 198-ms phase at 610 nm.

Taking advantage of the isotope effect on the lifetime of the tyrosyl radical, EPR measurements on photolyase from *A. nidulans* were performed in D_2O. Traces a and b in Figure 2 show flash-induced EPR signals at two different magnetic field positions. The kinetically nonresolved rise of the signals is attributed to the $FADH^•$-Tyr → $FADH^-$-$Tyr^•$ reaction.[10] The signals then decayed with $t_{1/2} = 190$ ms (monoexponential fit shown as lines trough the experimental traces a and b in Figure 1), which is close to the value found by absorption change measurements. This decay is attributed to the $FADH^-$-$Tyr^•$ → $FADH^•$-Tyr back-reaction.

The EPR spectrum of the flash-induced transients (spectrum c, Figure 2) was obtained by plotting the amplitude of the 190-

* Corresponding author.
[†] Section de Bioénergétique, DBCM, CEA Saclay.
[‡] Erasmus University Rotterdam.

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(5) The fit function included a constant to account for the small, very long-lived absorption changes. The latter resembles the $FADH^-$ minus $FADH^•$ difference spectrum. Presumably, a small fraction of $Tyr^•$ was reduced by an electron donor other than $FADH^-$.

(6) Implications of this kinetic isotope effect for the reaction mechanism will be discussed in a forthcoming paper. For a recent treatise of isotope effects on enzymatic reactions, see: Cook, P. F., Ed. *Enzyme Mechanism from Isotope Effects*; CRC Press: Boca Raton, FL, 1991.

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(8) Previous EPR experiments showed that 532-nm light excites selectively the semiquinone form of FAD.[9]

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(10) The tryptophanyl radical is too short-lived to be detected at our instrumental time resolution of 10 ms.

(11) This procedure should diminish possible distortions of the spectrum due photodegradation of the samples (∼1.3% per 100 flashes, as estimated from the amplitude of the $FADH^•$ spectrum when the excitation flashes were stopped).

(12) This constant may have the same origin as the constant in the transient absorption measurements.[5]

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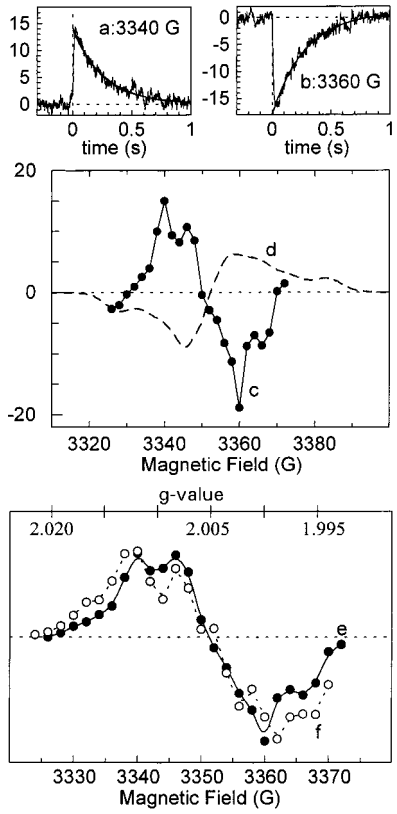

Figure 2. (a, b) Transient EPR signals at the indicated magnetic field positions induced by flash excitation of photolyase from *A. nidulans* at 8 ± 2 °C. Excitation was provided by a Nd:YAG laser (8 ns, 200 mJ; Spectra Physics GCR-230-10) at 532 nm.[8] The sample (360 μM) was prepared as the "D_2O" sample in Figure 1 and was introduced into a small flat cell inserted in a nitrogen gas flow system (Bruker, B-VT-1000). EPR data were recorded with a Bruker ESP300 X-band spectrometer. Instrument settings: microwave frequency, 9.4 GHz; modulation amplitude, 2.8 G; time constant, 10 ms; microwave power, 20 mW; each trace is the average of 128 accumulations on six different samples. Each sample was used to record 64 transients at each of the 24 field positions going from low field to high field, and another 64 transients at the same field positions going from high field to low field.[11] The lines through the experimental kinetics correspond to a monoexponential fit ($t_{1/2}$ = 190 ms, plus a small constant[12]). (c) Amplitude of the 190-ms phase versus the magnetic field. (d) Spectrum of FADH$^\bullet$ in *A. nidulans* photolyase recorded in the dark; the spectrum was inverted and scaled as described in the text. (e) Spectrum c minus spectrum d. (f) $Tyr_Z^\bullet$ spectrum measured in Mn-depleted PSII prepared as previously described.[14] Instrumental conditions and fitting procedure as for DNA photolyase.

ms phase recorded at different magnetic field positions. Spectrum c corresponds to the sum of (i) a contribution due to the formation of the tyrosyl radical and (ii) a contribution due to the disappearance of FADH$^\bullet$. To obtain the pure Tyr$^\bullet$ EPR spectrum, contribution ii must be removed from spectrum c. For that, the pure FADH$^\bullet$ spectrum was recorded in darkness; this spectrum was inverted and scaled (spectrum d)[13] to yield the same amplitude at 3326 G as that in spectrum c. This scaling is based on the assumption that Tyr$^\bullet$ did not contribute to spectrum c at 3326 G.[3] Subtraction of spectrum d from spectrum c yields spectrum e, which we attribute to the pure Tyr$^\bullet$ EPR spectrum in DNA photolyase from *A. nidulans*. It was checked to which extent the form of spectrum e is affected by the scaling of spectrum d. Varying this scaling by a factor between 0.5 and 2 did not significantly change the hyperfine line spacing of spectrum e (not shown), so that we can safely attribute this hyperfine line spacing to the tyrosyl radical.

Since the Tyr$^\bullet$ spectrum in photolyase from *A. nidulans* could so far only be obtained as point per point spectrum with a 2.8-G resolution, reasonable structural information may only be deduced by comparison with other known systems. For an accurate comparison, the $Tyr_Z^\bullet$ spectrum has been measured in Mn-depleted PSII[14] with instrumental conditions and a fitting procedure identical to those used with DNA photolyase (spectrum f, Figure 2). The hyperfine structure observed in spectrum e is very similar to that of the $Tyr_Z^\bullet$ signal (see also ref 3b), indicating similar structural properties (spin density distribution on the ring carbons and dihedral angle θ_1 of the β-methylene proton). Furthermore, the *g*-value of approximately 2.005 strongly indicates that the tyrosyl radical in photolyase from *A. nidulans* is deprotonated.[15] This is in line with the isotope effect on the kinetics of the reverse electron transfer from FADH$^-$ to Tyr$^\bullet$ (Figure 1).

The results presented above provide a first EPR characterization of the tyrosyl radical involved in photoactivation of the photolyase from *A. nidulans*. The time-resolved EPR spectrum indicates that the structural properties of Tyr$^\bullet$ in photolyase from *A. nidulans* are similar to those of the tyrosyl radicals in PSII and in ribonucleotide reductase from *S. typhimurium*.[16] Obtaining refined structural information (e.g., existence of hydrogen bonds and chemical nature of surrounding amino acids) will require the trapping at helium temperature of the tyrosyl radical for studies by pulsed EPR. Such information is essential for a detailed analysis of electron transfer involving the tyrosyl radical in the photoactivation process of photolyase from *A. nidulans*.

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(13) For unknown reasons, our FADH spectrum shows less hyperfine structure in the 3300−3370 G region than the FADH spectrum in photolyase from *E. coli*.[9]

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