Laser Flash Photolysis of DNA-Intercalated Ethidium Bromide in the Presence of Methylviologen

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Methylviologen quenches the fluorescence of DNA-intercalated ethidium bromide (EB) via electron transfer to yield reduced viologen. The majority of reduced viologen rapidly ($<10^{-9}$ s) recombines with oxidized EB on the DNA helix; however, a small (ca. 2%) fraction escapes from the helix into bulk solution. Recombination of this fraction then occurs via a second-order process ($k = (5.6 \pm 1.5) \times 10^9$ M^{-1} s^{-1}). The yield of reduced viologen which escapes the helix increases as the ionic strength of the solution rises. In the absence of methylviologen, excitation leads to small, long-lived absorptions in the visible region which are attributed to the first excited triplet state of EB. These are quenched by oxygen ($k = (3.8 \pm 0.6) \times 10^7$ M^{-1} s^{-1}) to yield oxidized EB.

Introduction

In our previous work on the photophysics and photochemistry of DNA intercalated ethidium bromide (EB) we concentrated on the fluorescence quenching of excited EB by transition-metal ions.[1,2] In particular we concluded that those ions which quenched EB fluorescence in aqueous solutions were capable of quenching the fluorescence of DNA-intercalated EB with greatly increased efficiency.[1] In the course of these studies we observed that DNA-intercalated EB fluorescence was also quenched by methylviologen (MV^{2+}) and that reduced viologen was formed on the time scale of the EB fluorescence decay.[2] Further, the reduced viologen was stable over hundreds of microseconds in nitrogen-saturated solution. This indicates that EB fluorescence is quenched by MV^{2+} via electron transfer from excited EB to MV^{2+} and also that some fraction of the reduced viologen is able to escape fast recombination with oxidized EB. A similar conclusion was reached recently by Fromherz and Rieger who used primarily steady-state methods to observe enhanced yields of electron transfer from EB excited singlet state to MV^{2+} in the presence of DNA.[3]

In this work we use transient absorption spectroscopy to follow the reactions of the products of this fluorescence quenching. Of particular interest is the environment and quantum yield of the reduced viologen which escapes rapid recombination with oxidized EB. Since to our knowledge no previous work on time-resolved absorption spectroscopy in the EB–DNA system has been reported, with the exception of our own nanosecond study,[4] we felt it necessary to investigate this system in addition to the system containing MV^{2+}. This is an extension of our earlier study to longer time scales. We have shown that the singlet excited state of EB absorbs in the visible and near-ultraviolet ($\lambda_{max} = 370$ nm);[4] however, this, having the same lifetime as the fluorescence (23 $\pm$ 1 ns in DNA solution,[5] less in the presence of MV^{2+}), therefore does not interfere with absorptions on the microsecond time scale.

Ethidium bromide is strongly intercalated within DNA (binding constant 2.1×10^6 M^{-1})[6] with little regard for base pair composition.[7] On binding, the fluorescence lifetime increases from 1.8 ns in pure water to 23 ns in DNA.[5] Methylviologen is also strongly bound to DNA (binding constant 1.8×10^5 M^{-1});[3] thus at the DNA, EB, and MV^{2+} concentrations used in this study the majority of EB and MV^{2+} are bound to DNA and we may be confident that the observable transient products are born of reactions occurring around the DNA matrix. Further consideration of this conclusion is given later in the text.

Experimental Section

Ethidium bromide and methylviologen were from Sigma and used as received. Tris(bipyridine)ruthenium dichloride was from G. F. Smith Chemical Co. and used as received. Sodium sulfate was MCB reagent grade. DNA (calf thymus type 1) was from Sigma and was purified by phenol extraction.[8] The salt concentration was always greater than 10^{-3} M to inhibit denaturation.[9] An extinction coefficient of 6.4×10^3 M^{-1} cm^{-1} for phosphate was used to standardize the DNA concentration. Water was purified by a Millipore filtration system. Samples were freshly made up on the day of use and all remaining DNA stock was stored in a refrigerator.

Steady-state absorption and emission measurements were made on a Hewlett Packard 8540A UV–visible spectrophotometer and a Perkin-Elmer LS5 fluorescence spectrophotometer, respectively. For time-resolved absorption measurements excitation was provided by the second harmonic (532-nm, 11-ns pulse) from either a Quantel YG481 or Quantel YG581 Q-switched Nd:YAG laser. The beam energy was attenuated as necessary either by using calibrated metal screens or by passing the beam through crossed calcite Glan-Laser polarizers whose crossing angle could be continuously varied. Beam energies up to a maximum of 20 mJ per pulse were employed. Transient absorptions were measured perpendicular to the excitation beam by using a conventional lamp, monochromator, photomultiplier tube arrangement. For time scales shorter than ca. 400 μs a xenon arc lamp was used as the analyzing source and the output from the photomultiplier tube (Hamamatsu R928) was terminated across a 50-ohm load before being sent to a Biomation 8100 transient recorder. If required the xenon lamp could be pulsed to high intensity for a short (a few milliseconds) period which allows measurement with a smaller bandwidth. For longer time scales a tungsten lamp provided the analyzing source and the photomultiplier output was terminated across 10 kohms before the Biomation. A DEC PDP 11/70

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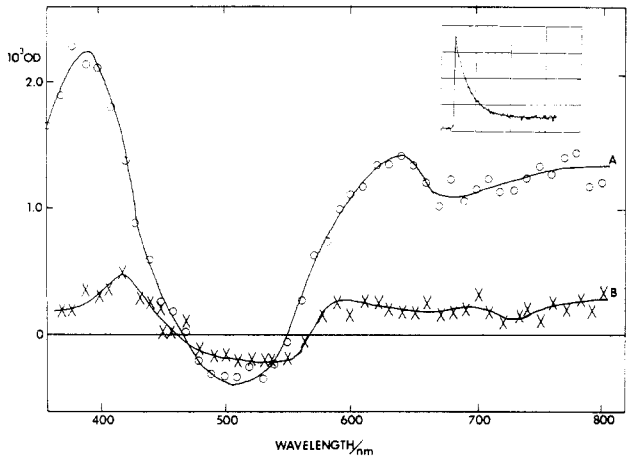

Figure 1. Absorption spectra observed after 532-nm irradiation of N_2-saturated 3.6×10^{-4} M DNA, 4×10^{-5} M EB, and 2.5×10^{-3} M Na_2SO_4: (A) 200 μs after the pulse; (B) 7.3 ms after the pulse. Inset: decay of absorption at 390 nm. One horizontal division is 3 ms. Solid line is the best single-exponential fit to a nonzero base level.

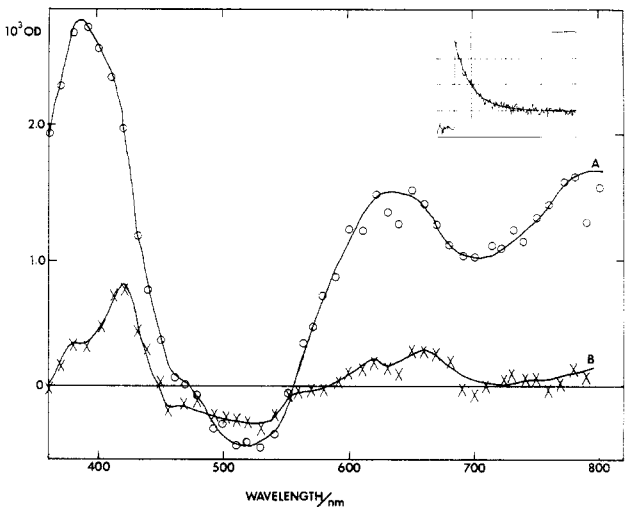

Figure 2. Absorption spectra observed after 532-nm irradiation of 3.6×10^{-4} M DNA, 4×10^{-5} M EB, and 2.5×10^{-3} M Na_2SO_4 saturated with a mixture of 10% oxygen in nitrogen: (A) 15 μs after the pulse; (B) 1.4 ms after the pulse. Inset: decay of absorption at 390 nm. Solid line is the best double-exponential fit to the data. One horizontal division is 0.5 ms.

minicomputer controlled the data acquisition and analysis as has been previously described.[10]

Results and Discussion

Figure 1 shows the difference absorption spectrum 200 μs after 532-nm irradiation of a N_2-saturated solution containing 3.6×10^{-4} M DNA, 4×10^{-5} M EB, and 2.5×10^{-3} M Na_2SO_4. The main features are a maximum at ca. 380–390 nm, ground-state bleaching between 470 and 550 nm, and a broad absorption reaching into the red beyond 800 nm. On the nanosecond time scale this absorption is observed as a small (<10% at 400 nm) residual absorption subsequent to the decay of EB excited singlet state,[4] showing that either the yield or extinction coefficient of this species is small with respect to that of the singlet excited state. The inset in Figure 1 shows the decay profile at 390 nm fit as a single exponential to a small nonzero base level. The rate constant for decay is given as $(8.2 \pm 0.8) \times 10^2$ s^{-1}. The decay of the residual absorption, whose spectrum is also shown in Figure 1 (spectrum B, 7.3 ms after the pulse), is difficult to measure due to its low absorbance and long time scale but has a half-life in excess of 10 ms. The possible identity of this species will be discussed later.

Figure 2 shows the difference absorption spectrum obtained when the solution is bubbled with a mixture of 10% oxygen in nitrogen. The initial absorption (spectrum A, 15 μs after the pulse) is similar to the initial absorption in Figure 1 but now there is a more rapid decay to yield an absorption which is red-shifted, having a maximum at ca. 420 nm. This latter absorption (spectrum B, 1.4 ms after the pulse) decays slowly via what appears to be a second-order process with $k/\epsilon = (9 \pm 3) \times 10^4$ cm^{-1} s^{-1} at 420 nm.

Aeration or saturation of the solution with oxygen further increases the decay rate of the 390-nm absorbing species and increases the residual absorption at 420 nm. Plotting the rate of decay of the 390-nm absorbing species vs. oxygen concentration, for N_2-saturated, 10% oxygen, aerated (20%), and oxygen-saturated solutions, indicated that the decay was first order in oxygen. The slope of the plot yielded a bimolecular rate constant for oxygen quenching of $(3.8 \pm 0.6) \times 10^7$ M^{-1} s^{-1}. We assign the 390-nm absorption to the triplet excited state of EB on the basis of our earlier considerations[4] and the reaction of this species with oxygen.

We rule out the anion of EB, which could be formed by photoionization followed by reaction of the hydrated electron with ground-state EB, for the following reasons. We fail to observe the characteristic absorption of the hydrated electron in the red[11] decaying over the appropriate time scale. Whillans[12] reports a rate of reaction of 2×10^9 M^{-1} s^{-1} for DNA-intercalated EB with hydrated electrons, which under our conditions translates to a first-order decay rate of 8×10^4 s^{-1}. A decay on this time scale would be easily observable if present in our systems. In addition the spectra are essentially identical when 5% v/v *tert*-butyl alcohol is added and the solution is saturated with N_2O. If hydrated electrons were formed they would be rapidly scavenged by N_2O to give OH radicals, which would in turn react with *tert*-butyl alcohol. We should then see the disappearance of any absorptions which result from the reaction of hydrated electrons with EB. Also it is difficult to understand how reaction of oxygen with the anion of EB would increase the absorbance at 420 nm since O_2^- does not absorb in the visible.

To our knowledge observation of the EB triplet state has not been reported, though its existence has been inferred from studies of the photooxidation of 1,3-diphenylisobenzofuran using EB as sensitizer.[13] A quantum yield of 2.5×10^{-3} was measured in methanol solution and this may be expected to rise a factor of about four when EB is intercalated into DNA. The decay rate of EB triplet was estimated as $<10^4$ s^{-1} in methanol on the basis that the rate of photooxidation was unchanged between aerated and oxygen-saturated solutions.[13] Both these observations are in accord with the present work.

Oxygen quenching of the EB triplet state leads to the formation of a species whose absorption peaks at ca. 420 nm. We assign this absorption to one-electron-oxidized EB (EB^+) on the basis of results to be presented in the next section. If this is the case then the reaction of EB triplet with oxygen must lead, at least in part, to the formation of EB^+ and presumably O_2^-. The assumption in the photooxidation experiments was that triplet quenching of EB by oxygen proceeds via energy transfer to yield singlet oxygen, which then oxidizes 1,3-diphenylisobenzofuran.[13] Our observations indicate that either oxygen quenching of the EB triplet proceeds via both mechanisms or that O_2^- is responsible for bleaching 1,3-diphenylisobenzofuran. If triplet quenching proceeds via both mechanisms and O_2^- does not bleach 1,3-diphenylisobenzofuran then the triplet quantum yield is in fact higher than reported.

The rate constant for oxygen quenching of the EB triplet is measured as 3.8×10^7 M^{-1} s^{-1}, 2 orders of magnitude less than the typical rate constants for oxygen quenching of triplet states in homogeneous solution.[14] We attribute this to the protection

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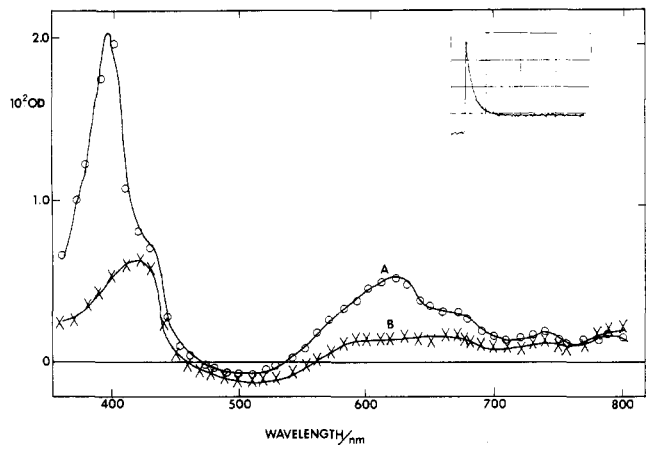

Figure 3. Absorption spectra observed after 532-nm irradiation of aerated 3.6×10^{-4} M DNA, 4×10^{-5} M EB, 2.5×10^{-3} M Na_2SO_4, and 2×10^{-4} M MV^{2+}: (A) 0.9 μs after the pulse; (B) 27 μs after the pulse. Inset: decay at 395 nm. One horizontal division is 26 μs and the solid line is the best single-exponential fit to a nonzero base level.

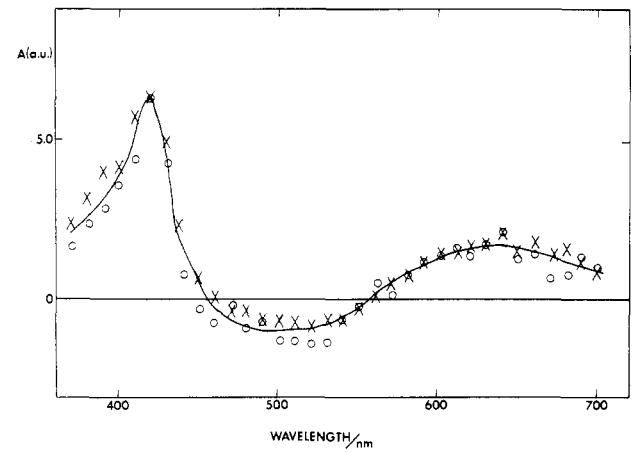

Figure 4. Absorption spectra observed after 532-nm irradiation of oxygen-saturated (×) 3.6×10^{-4} M DNA, 4×10^{-5} M EB, and 2.5×10^{-3} M Na_2SO_4, 200 μs after the pulse. (○) Same solution as × with 2×10^{-4} M MV^{2+}, 13 μs after the pulse. Spectra have been normalized at 420 nm and the vertical scale is absorbance in arbitrary units.

effect of DNA intercalation, oxygen having reduced access to the interior of the DNA where the EB triplet is located. This is not without precedent; Whillans[12] reports a factor of 15 decrease in the rate of reaction of the hydrated electron with EB when the latter is intercalated in DNA. Also a factor of 30 decrease in the rate constant for oxygen quenching of the EB excited singlet state due to DNA intercalation has been reported.[15]

The small residual absorption subsequent to triplet decay in N_2-saturated solution (Figure 1, spectrum B) is similar to that observed after quenching of the triplet by oxygen. It may be that trace amounts of oxygen remain in our solutions even after N_2 saturation and are responsible for the formation of a small amount of EB^+.

In summary our results show that 532-nm irradiation of DNA-intercalated EB results in a low yield of triplet state absorbing in the visible with a peak at 390 nm. Oxygen quenches the triplet state with a rate constant of 3.8×10^7 M^{-1} s^{-1}, at least in part, via an oxidative mechanism to yield EB^+ and O_2^-. EB^+ decays via second-order kinetics presumably due to reaction with O_2^-.

Addition of Methylviologen

Addition of 2×10^{-4} M MV^{2+} to the N_2-saturated solution of 3.6×10^{-4} M DNA, 4×10^{-5} M EB, and 2.5×10^{-3} M Na_2SO_4 results in considerably larger absorptions on irradiation at 532 nm (ca. 5 times larger at 400 nm), as shown in Figure 3 for an aerated sample. The initial spectrum has the familiar features of reduced viologen (a peak around 400 nm and a broad absorption around 600 nm) in addition to a slight shoulder at 420–430 nm, bleaching in the region 470–500 nm, and an absorption into the red past 800 nm. In nitrogen-saturated solution all absorptions decay practically to zero via second-order kinetics (k/ϵ = (7.2 ± 1.5) × 10^5 cm^{-1} s^{-1} at 610 nm).

Reduced viologen (MV^+) must be formed via oxidative quenching of EB excited states by MV^{2+} yielding MV^+ and EB^+. We see no indication of the EB triplet state in the presence of MV^{2+} and must assume MV^{2+} quenches triplet EB. Although it would be difficult to separate the absorption of triplet EB from that of MV^{2+} under nitrogen-saturated conditions, saturation of the solution with oxygen reveals no species decaying with a rate characteristic of the decay of triplet EB. Given the low (10^{-2}) quantum yield of triplet state,[13] and the observation that at these salt and MV^{2+} concentrations 78% of the EB fluorescence is quenched (this will be discussed further in the next section), we conclude that the vast majority of the MV^+ we observe is due to MV^{2+} quenching of the EB excited singlet state. Also the quenching of EB excited singlet states by MV^{2+} will lead to a corresponding decrease in the yield of triplet states.

In aerated solution the decay of MV^+ is accelerated (see Figure 3) due to reaction of oxygen with MV^+ to yield MV^{2+} and superoxide (O_2^-). Figure 3 also shows the residual absorption, which we attribute to EB^+, after completion of this reaction. This absorption has a peak at 420 nm, bleaching in the region of EB ground-state absorption (460–560 nm), and a broad absorption into the red past 700 nm. It is very similar to the absorption present after oxygen quenching of the EB triplet state in the absence of MV^{2+}. This is shown more clearly in Figure 4 where we have plotted the spectrum observed after oxygen quenching of the EB triplet state and that observed after oxygen quenching of MV^+ in oxygen-saturated solutions. The spectra were normalized at 420 nm. Although the spectrum observed after MV^+ quenching appears slightly narrower around the 420-nm peak, a faster time scale and smaller bandwidth were employed here than for the spectrum after quenching of the EB triplet. Further evidence for the equivalence of these absorbing species comes from their similar decay kinetics (second order with k/ϵ = (5 ± 3) × 10^4 cm^{-1} s^{-1} at 420 nm for the absorption in Figure 3, spectrum B). These observations led us to believe that oxygen quenching of EB triplet yields EB^+.

Reduced viologen and oxidized EB must be formed in equal concentrations. Thus after the decay of MV^+ in the presence of oxygen we are left with an equivalent concentration of EB^+ to the concentration of MV^+ which has decayed away. We have carefully measured the relative contributions of EB^+ and MV^+ to the total absorptions at 395 and 610 nm in both aerated and oxygen-saturated solutions by extrapolation of both the faster (MV^+) and slower (EB^+) components of the decay to time zero. Measurements in both aerated and oxygen-saturated solutions gave the same results within experimental error. Taking extinction coefficients of 3.8×10^4 M^{-1} cm^{-1} and 1.15×10^4 M^{-1} cm^{-1} for MV^+ at 395[16] and 610 nm,[17] respectively, we calculate extinction coefficients for EB^+ of 8.4×10^3 M^{-1} cm^{-1} at 395 nm, 4.3×10^3 M^{-1} cm^{-1} at 610 nm, and 1.7×10^4 M^{-1} cm^{-1} at 420 nm. Using these values we calculate a bimolecular rate constant of (5.6 ± 1.5) × 10^9 M^{-1} s^{-1} for the reaction of MV^+ with EB^+ measured at 610 nm, and a bimolecular rate constant for reaction of EB^+ with O_2^- of (1.2 ± 0.3) × 10^9 M^{-1} s^{-1}, an average of our two determinations. The former rate constant may be faster than the latter because EB^+ is bound to the DNA helix. The negative charge on the helix will have a repulsive effect on O_2^- but an attractive effect on MV^+.

The rate constant for the decay of MV^+ was measured as a function of oxygen concentration at both 395 and 610 nm and a plot of these data is shown in Figure 5. The best straight line

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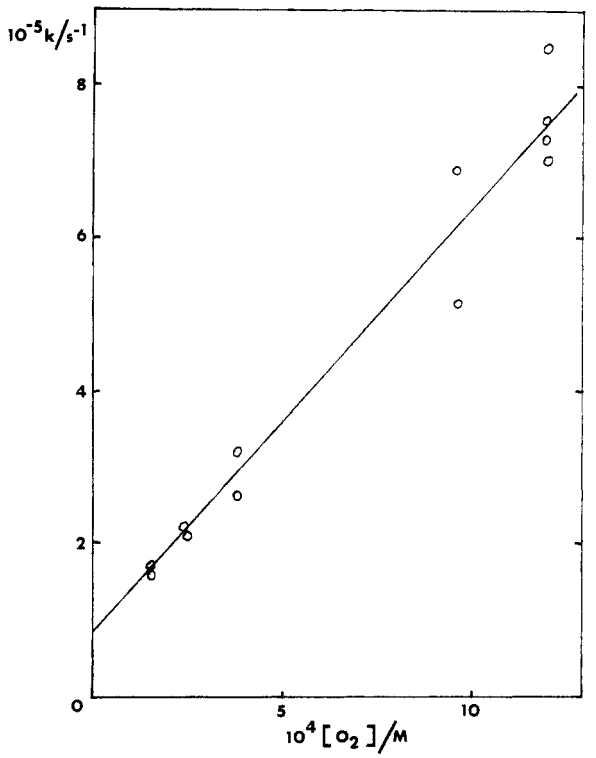

Figure 5. Plot of the observed rate constant, k, for decay of MV^+ as a function of oxygen concentration.

through these points yields a bimolecular rate constant of (5.6 ± 0.4) × 10^8 M^{-1} s^{-1} for the reaction of MV^+ with oxygen. This is to be compared with a value of 6.0 × 10^8 M^{-1} s^{-1} measured for the same reaction in DNA-free aqueous solution.[18] The reaction of oxygen with MV^+ results in a net change in the product of the charges of the reactants and products from 0 to −2, and hence the rate constant will be dependent on the dielectric constant of the medium. This has been shown previously in methanol/water mixtures where the rate constant was found to decrease from 6.0 × 10^8 M^{-1} s^{-1} in pure water (D = 79) to ca. 2 × 10^7 M^{-1} s^{-1} in a mixture of D = 44.[18] The dielectric constant in the environment of the DNA helix will be different from that in the bulk solution, and the equivalence of the rate observed in the present system to that in the absence of DNA tells us that we are observing MV^+ which has escaped from the DNA helix. The same conclusion can be reached from the decay kinetics of MV^+, which follow the second-order rate law in the absence of oxygen. If MV^+ was recombining with its EB^+ partner on the same helix where they were formed, then we expect the decay to exhibit first-order kinetics.

We rule out the formation of MV^+ by quenching of EB excited states in the bulk aqueous phase for the following reasons. The fluorescence decay of EB under these conditions, in the absence of MV^{2+}, is strictly monoexponential with a lifetime of 23 ± 1 ns.[2] No component is observed decaying with a lifetime of 1.8 ns characteristic of EB fluorescence in pure water. Thus the majority of EB must be bound to DNA. Even allowing that a small fraction of EB is in the bulk phase, then given a diffusion-controlled rate constant of 10^{10} M^{-1} s^{-1} for quenching of EB excited states, a concentration of 2 × 10^{-4} M MV^{2+} gives a first-order quenching rate of 2 × 10^6 s^{-1}. The fluorescence decay rate (5.6 × 10^8 s^{-1}) will only be slightly increased, resulting in negligible quenching by MV^{2+}. The reduced rate of EB triplet quenching by oxygen measured earlier indicates that triplet EB is bound to DNA and hence MV^{2+} quenching of EB triplet in the bulk phase may also be ruled out. Thus the MV^+ observed in these systems must be that which escapes recombination with its DNA-bound EB^+ partner, and is released from the DNA helix into bulk solution. An obvious consequence of this reasoning is that to escape recombination with its EB^+ partner, MV^+ must escape the DNA helix.

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TABLE I: Quantum Yields and Helix Escape Yields of Reduced Viologen[a]

$10^3[Na_2SO_4]$	F_Q	$10^2\phi_{MV}$	10^2F_{esc}
10	0.43	0.88 ± 0.08	2.0
5	0.64	1.04 ± 0.15	1.6
2.5	0.78	1.13 ± 0.15	1.4
1.25	0.82	1.00 ± 0.02	1.2

[a] Symbols as described in text.

Yields of Reduced Viologen

It is of interest to measure the yield of MV^+ which escapes from the DNA helix in these systems. We have attempted such measurements as a function of ionic strength by varying the concentration of Na_2SO_4. Initially we need to know what fraction of EB excited singlet states are quenched by MV^{2+}. We ignore any small contribution of MV^+ from quenching of triplet EB. The steady-state fluorescence spectra of solutions of 3.6 × 10^{-4} M DNA, 4 × 10^{-5} M EB, and 1.25 × 10^{-3}, 2.5 × 10^{-3}, 5 × 10^{-3}, and 10^{-2} M Na_2SO_4 were measured both in the absence and in the presence of 2 × 10^{-4} M MV^{2+}. The ground-state visible absorption spectra were identical for all solutions, as were the shape and wavelength dependence of the fluorescence spectra. These observations argue against ground-state complexation of EB by MV^{2+}. The ratio of the areas under the fluorescence spectra in the presence and absence of MV^{2+} gives the fraction of EB singlet excited states quenched, F_Q. F_Q values for each salt concentration are given in Table I. Note that simple diffusional quenching in homogeneous solution is far too slow to account for such large fluorescence quenching, and these numbers reflect the increase in efficiency of this process due to the reactants being bound to DNA.

Quantum yields of MV^+ formation in the time-resolved experiments, ϕ_{MV}, were measured by using the charge-transfer excited state of $Ru(bpy)_3^{2+}$ (ϵ_{360} = 2.1 × 10^4 M^{-1} cm^{-1},[19] ϕ = 1[20]) as an actinometer. In the EB/DNA/Na_2SO_4/MV^{2+} systems, absorptions at 395 and 610 nm are a result of both MV^+ and EB^+ absorptions; however, we have measured the relative contributions of each, at these wavelengths, earlier.

A N_2-saturated solution of $Ru(bpy)_3^{2+}$ in water was adjusted to have the same optical density at 532 nm as the EB/DNA/Na_2SO_4/MV^{2+} solutions. Measurements were then made of the optical densities of the $Ru(bpy)_3^{2+}$ charge-transfer excited state at 360 nm, employing a series of laser beam energies (ca. 6 to ca. 0.6 mJ/pulse). Under identical irradiation conditions we then measured the optical densities at 395 and 610 nm for each of the N_2-saturated EB/DNA/Na_2SO_4/MV^{2+} solutions. In all cases the absorptions were extrapolated to time zero by using a first-order kinetic fit for $Ru(bpy)_3^{2+}$, and a second-order kinetic fit for the DNA systems. Care was taken to measure on a sufficiently fast time scale to minimize extrapolation errors. The optical densities for the EB/DNA/Na_2SO_4/MV^{2+} systems were then corrected for the absorption of EB^+. Low laser beam energies (to the limit of our detection sensitivity) were used in order to minimize nonlinear absorption effects due to excessive ground-state

(19) There is some discrepancy in the literature concerning the extinction coefficient of the $Ru(bpy)_3^{2+}$ charge-transfer excited state. Values, uncorrected for ground-state absorption, at 360 nm range from ca. 2.2 × 10^4 M^{-1} cm^{-1} (Bensasson, R. V.; Salet, C.; Balzani, V. *J. Am. Chem. Soc.* **1976**, *98*, 3722. Creutz, C.; Chou, M.; Netzel, T. L.; Sutin, N. *J. Am. Chem. Soc.* **1980**, *102*, 1309.) to 1.0 × 10^4 M^{-1} cm^{-1} (Rougee, M.; Ebbesen, T.; Ghetti, F.; Bensasson, R. V. *J. Phys. Chem.* **1982**, *86*, 4404.). In view of this we have remeasured the extinction coefficient by comparison with the steady-state actinometer *meso*-diphenylhelianthrene (MDH) and have obtained a value of (2.1 ± 0.2) × 10^4 M^{-1} cm^{-1} (Hubig, S. M.; Atherton, S. J., to be submitted). For a description of the method of MDH actinometry see Brauer, H.-D.; Schmidt, R.; Gauglitz, G.; Hubig, S. M. *Photochem. Photobiol.* **1983**, *37*, 595. Note that this results in a change in our value of the extinction coefficient of singlet excited EB^4 to 4.6 × 10^3 M^{-1} cm^{-1}.

(20) Kalyanasundaram, K. *Coord. Chem. Rev.* **1982**, *46*, 159.

bleaching. Quantum yields of MV^+ production, ϕ_{MV}, are given by

$$\phi_{MV} = \frac{OD_{MV}}{OD_{RU}} \frac{\epsilon_{RU}}{\epsilon_{MV}}$$

where OD_{MV} and OD_{RU} are the optical densities of MV^+ and $Ru(bpy)_3^{2+}$ charge-transfer excited state, respectively, and ϵ_{MV} and ϵ_{RU} are the extinction coefficients for these species at the appropriate wavelengths. ϕ_{MV} values are given in Table I. Excellent agreement was observed between values measured at 395 and 610 nm and all laser beam energies gave the same ϕ_{MV} values within experimental error. Values in Table I are the average of between 4 and 7 determinations at different laser intensities and either 610 or 395 nm, for each salt concentration. Standard deviations between these values are included. The fraction of MV^+ formed which escapes the DNA helix, F_{esc}, is then given by

$$F_{esc} = \phi_{MV}/F_Q$$

These values are also presented in Table I.

Consideration of the data in Table I leads to three principal conclusions. The fraction of EB excited singlet states quenched, F_Q, exhibits a marked ionic strength dependence, decreasing with increasing salt. The F_{esc} values are very small, maximum 0.02 at 10^{-2} M Na_2SO_4, and also show an ionic strength dependence, increasing with increasing salt. Although the differences between the values are small, they are reproducible and outside experimental error. A simple representation of the reaction scheme, in nitrogen-saturated solution, is given as

$$^1EB^*_{DNA} + MV_{DNA}^{2+} \xrightarrow{k_Q} MV_{DNA}^{+} + EB_{DNA}^{+}$$

$$EB_{DNA} + MV_{DNA}^{2+} \xleftarrow{k_R} MV_{DNA}^{+} + EB_{DNA}^{+} \xrightarrow{k_{esc}} MV_F^{+} + EB_{DNA}^{+}$$

where the subscripts DNA and F refer to species bound to the DNA and free in bulk solution, respectively, $^1EB^*$ represents the EB excited singlet state, and k_Q is the rate constant for fluorescence quenching. k_R is the rate of recombination of MV^+ with its EB^+ partner on the same DNA helix on which it is formed and as such will be a first-order constant. k_{esc} is the first-order rate constant for escape of MV^+ from the DNA helix. Thus F_{esc} values are given by

$$F_{esc} = k_{esc}/(k_{esc} + k_R) \quad (1)$$

and the low values measured for F_{esc} must be because $k_R \gg k_{esc}$. We estimate a lower limit for k_R of 10^9 s^{-1} from observations on nanosecond time scales.[21] A high value of k_R is expected since in addition to the reacting species being formed in close proximity to each other, binding to DNA confines these species in a limited volume thus favoring recombination over escape. The salt effect on F_{esc} is less easily understood since a simple salt effect predicts that k_R would increase with increasing salt, due to a diminishing of the repulsion between two like charged species. If k_{esc} remains constant this would lead to a decrease in F_{esc} with increasing salt, in contradiction to our observations.

Recently a report has appeared concerning salt effects on the charge separation yields on quenching of the excited singlet state of phenothiazine by MV^{2+} in homogeneous solution. The authors find that whereas the addition of "simple" salts such as sodium chloride reduce the charge separation yield as expected, salts with long alkyl chains increase the charge separation yield.[22] Mechanisms are presented to explain these effects but since Na_2SO_4 is certainly a "simple" salt we do not consider these mechanisms pertinent to the present system.

In order to understand the salt effects on F_Q and F_{esc} we refer to polyelectrolyte theory.[23] According to this theory the concentration of counterions associated with a linear polyelectrolyte is independent of the ionic strength of the solution up to a concentration of ca. 1 M ions. In a polyelectrolyte solution containing several different counterions the composition of counterions surrounding the polyelectrolyte will be represented by the equilibrium constants for binding of each counterion and their concentration in bulk solution, whilst the concentration of bound counterions remains constant. With respect to our system this means that, as the concentration of Na_2SO_4 rises, the equilibrium constant for binding of both MV^{2+} and MV^+ decreases and there is less MV^{2+} and MV^+ bound for a given concentration of these ions. This explains the reduction in F_Q as the Na_2SO_4 concentration is increased and was the basis of our explanation of the salt effects on the quenching of DNA-intercalated EB fluorescence by transition-metal ions.[2]

An explanation for the increase of F_{esc} with increasing salt is obtained by considering the equilibrium constant for MV^+ binding to the DNA helix. Binding may be represented by

$$MV_F \underset{k_b}{\overset{k_f}{\rightleftharpoons}} MV_{DNA}$$

where k_f is the rate of MV^+ attachment to DNA and $k_b = k_{esc}$ is the rate of escape of MV^+ from the helix. The equilibrium constant for binding, K, is equal to k_f/k_{esc}. An increase in salt concentration will be expected to reduce k_f by reducing the attraction between MV^+ and the negatively charged DNA helix. Increasing the bulk Na_2SO_4 concentration will also increase the concentration of Na^+ bound to the DNA helix, and we believe a further reduction in the equilibrium constant for MV^+ binding is necessary to maintain a constant counterion concentration at the helix. Thus we suggest that k_{esc} is increased with increasing salt concentration. From eq 1, since $k_R \gg k_{esc}$, an increase in k_{esc} results in an increase in F_{esc}. We consider that, on increasing the salt concentration in our system, the increase in k_{esc} must dominate over the decrease in k_R due to the simple salt effect. Thus the salt effect on F_{esc} is due to a more rapid release of MV^+ from the DNA helix as the ionic strength increases.

In summary then we have confirmed the increase in efficiency of fluorescence quenching of EB excited singlet state by MV^{2+} when the reactants are bound to DNA. We have shown that this quenching reaction occurs via charge transfer to yield reduced viologen and oxidized ethidium bromide. Our results indicate that in order to escape rapid ($>10^{-9}$ s) recombination with its EB^+ partner, MV^+ must escape from the DNA helix into the bulk aqueous phase. Although yields of escaped MV^+ are low, ca. 2×10^{-2}, we have measured these values as a function of ionic strength. The increase in these yields as the ionic strength rises is explained in terms of a resulting increase in the rate of escape of MV^+ from the DNA helix. Finally the bimolecular rate constants for the reaction of O_2^- with EB^+ and separated MV^+ with EB^+ have been measured and presented.

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(21) We have tried to observe the fast decay of MV^+ which recombines with EB^+ on the DNA helix. With our fastest time resolution of ca. 0.5 ns, a 200-ps pulse, a MV^{2+} concentration of 10^{-2} M, and 2.5×10^{-3} M Na_2SO_4, we observe only the absorption of EB singlet excited state in the 350–450-nm range and fluorescence emission in the 600-nm region. The singlet absorption and emission both decay within ca. 1 ns; thus MV^+ must recombine faster than this.

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