Photobleaching of Asymmetric Cyanines Used for Fluorescence Imaging of Single DNA Molecules

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Abstract: The photobleaching of the cyanine dyes YO and YOYO has been investigated for both free and DNA-bound dyes, using absorption and fluorescence spectroscopy coupled with fluorescence microscopy. For the free dyes, the nature of the reactive species involved in the photodegradation process is different for the monomer and the dimer, as shown by scavenger studies. For DNA-bound dyes, photoinduced fading of the visible absorption band occurs by different pathways depending on the drug binding mode and can be attenuated by appropriate scavengers. However, none of these scavengers were found to have any significant effect on the photobleaching of dye fluorescence. It appears that the reduction of fluorescence intensity comes from a quenching of the dye fluorescence by modified DNA bases, possibly 8-oxo-7,8-dihydro-2′-deoxyguanosine.

Introduction

Asymmetric cyanine dyes oxazole yellow YO and its dimer YOYO[1] have been found to have excellent properties for different fluorescence applications, among which detection of DNA on electrophoresis gels[2−4] and visualization of DNA molecules in fluorescence microscopy[5−7] are the most common. These dyes are essentially nonfluorescent when free in solution but strongly fluorescent when bound to DNA.[2] Coupled with their very high binding constants and large molar absorptivities,[8] this results in a large difference in fluorescence intensity between the stained DNA molecules and the background, which provides high sensitivity when imaging DNA.

Previous studies have shown that YO and YOYO bind to DNA in two modes.[9,10] The preferred mode that shows the highest affinity is intercalation (bisintercalation for YOYO), and the immobilization of the cyanine dye between the base pairs results in strong fluorescence. The second mode is external binding to the DNA helix in a geometry that gives a weaker fluorescence. The two modes have significantly different binding constants since external binding seems to occur only below the saturation value of N/C = 5 for YO and N/C = 4 for YOYO (N/C represents the ratio between concentrations of DNA nucleotides and added dye chromophores, one for YO and two for YOYO).

When these dyes are used in fluorescence microscopy imaging, quite high dye loadings (e.g., N/C = 3−8) are commonly employed, which means that the properties of the dyes in both binding modes are of interest from an application point of view. Many serious side effects can arise from high dye loadings, such as an inhomogeneous staining due to a bimodal distribution of dye molecules between the DNA molecules in the sample. However, the dye distribution can be equilibrated by a simple incubation protocol.[11] Another drawback of these dyes is their tendency to photocleave the probed DNA, especially under the intense illumination that is used for imaging of individual molecules.[12] Both YO and YOYO cause single-strand breaks (ssb) as determined from a supercoiled DNA relaxation assay.[12] However, when a molecule is imaged under the microscope, only double-strand breaks (dsb) are observed. In this case, a dsb is obtained when two ssb occur on opposite strands separated by not more than about 15 base pairs. Therefore, it is highly probable that before a break is observed during imaging, the DNA molecule has undergone considerable nicking and probably also other forms of damage that do not result in strand breakage but may affect the conformational and dynamic properties of the stained molecules. In our photocleavage work,[12] we found that the electrophoretic mobility of YO-

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(1) Abbreviations: YO, oxazole yellow (trade name YO-PRO1); YOYO (trade name YOYO-1); CT DNA, calf thymus DNA; DAPI, 4,6-diamino-2-phenylindole; SOD, superoxide dismutase; $^1O_2$, singlet molecular oxygen; 8-oxo-dGuo, 8-oxo-7,8-dihydro-2′-deoxyguanosine; N/C, nucleotide-to-chromophore ratio; LD, linear dichroism; Abs, absorbance; LD^r, reduced linear dichroism (=LD/Abs); β-MeSH, β-mercaptoethanol; TBE, Tris−borate−EDTA.

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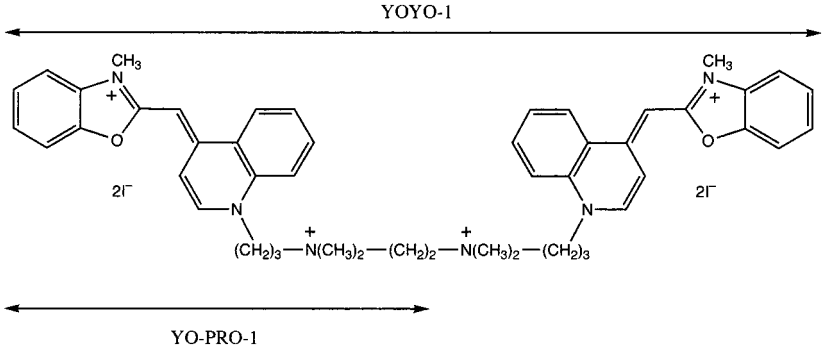
Scheme 1. Chemical Structures of YO and YOYO



stained linear DNA before cleavage was altered by illumination, which may be a manifestation of such an effect on the polymer dynamics. When two YO chromophores are forced into proximity in YOYO, there is an enhancement of dsb cleavage rate because of the nonrandom distribution of ssb from the dimer.[12]

In microscopy work, cleavage is typically suppressed by removal of oxygen and addition of β-mercaptoethanol, but the fluorescence of the stained molecules is nonetheless commonly observed to bleach during prolonged illumination. We have confirmed this observation by performing spectroscopic studies of the dyes in solution, in the absence or in the presence of DNA. In this paper we show that the microenvironment of YO or YOYO bound to DNA influences the photobleaching process: with DNA, the photodegradation of the dyes is not the only cause of photobleaching, although it is for the isolated dyes. Indeed, the process appears more complex in the DNA environment, and we have used a combination of microscopy and spectroscopy to investigate which reactions occur to cause photobleaching of YO and YOYO.

Materials and Methods

A. Chemicals. YO and YOYO were purchased from Molecular Probes (trade names YO-PRO1 and YOYO-1) and supplied as 1 mM solutions in DMSO. Calf thymus (CT) DNA was obtained from Sigma. Supercoiled and nicked circular forms of ΦX174 DNA were from New England Biolabs. The negatively supercoiled sample typically contained 5−10% nicked circles, whereas the nicked sample contained 5−10% linear DNA. The purely linear form was obtained by cleavage of a supercoiled sample with restriction enzyme *Xho*I (Pharmacia), following the protocol from the supplier. T2 (164 kbp) and T7 (38 kbp) DNA were from Sigma. Oligonucleotides were purchased from Eurogentec, desalted, and purified by FPLC.

All experiments were performed at room temperature (ca. 20 °C). DNA concentrations is given in terms of nucleotide concentration. Concentrations were determined spectrophotometrically, using the following extinction coefficients: $\epsilon_{260} = 6600\ M^{-1}\ cm^{-1}$ for DNA bases,[13] $\epsilon_{457} = 96\,100\ M^{-1}\ cm^{-1}$ for YOYO, and $\epsilon_{481} = 66\,000\ M^{-1}\ cm^{-1}$ for YO.[14] Fresh solutions of the dyes were prepared from the DMSO stock solutions prior to use. If not otherwise stated, the dye loading is given as the N/C ratio between the concentrations of DNA nucleotide (N) and added dye chromophores (C; one for the monomer and two for each dimer).

B. Microscopy. Samples. All samples contained 40 μM DNA nucleotide and a dye concentration corresponding to the stated N/C, except for the lowest N/C of 1.5, which contained 20 μM DNA nucleotide. All samples had a final volume of 20 μL in 50 mM TBE buffer (pH = 8.3) and were prepared by adding 5 μL of DNA stock to the dye diluted in the buffer. Subsequent incubation at 50 °C for 2 h was performed to reach an equilibrium in the dye distribution.[11] In the case of deoxygenation, the required number of aliquots were bubbled vigorously with water-saturated argon for 1 min. The efficiency of this degassing procedure has been confirmed by comparison with the results of OhUigin et al.[15] for the effect of oxygen on methylene blue photocleavage. Comparison of the nonilluminated samples demonstrated that no nicking of the supercoiled DNA occurred as a result of the deoxygenation procedure.

Illumination. Samples containing either linear or supercoiled DNA (40 μM) were illuminated in Eppendorff tubes covered with a glass lid, using the epifluorescence optics of a Zeiss Axioplan microscope (50 W mercury lamp; excitation filter, 450−490 nm, dichroic mirror, 510 nm; emission long-pass filter, 520 nm, 5× objective, N.A. = 0.15), equipped with a Hamamatsu photomultiplier used to measure the fluorescence−time profile of the sample in the process of illumination. Some samples were illuminated using the setup with a 150-W xenon lamp and a long-pass filter (320 nm) described in earlier work.[12] The effective light intensity of the microscope setup was a factor of 16.7 times lower than with the conventional setup, as calibrated with the cleavage rate of a supercoiled sample under otherwise identical conditions.

In some experiments, fresh dye was added after the cleavage/bleaching, to investigate the restoration of fluorescence. In these cases, the time profiles of the fluorescence intensity were measured by applying the excitation light intermittently, to avoid bleaching.

Electrophoretic Analysis and Quantification of Product Concentrations. Products of cleavage or bleaching experiments were analyzed on 1% agarose gels (in 50 mM TBE, pH 8.3) at 3 V/cm for 4.5 h, and the positions and the integrated intensities of the separated products were obtained from scans of the fluorescence intensity along each lane of the gel. We have used both approaches for staining the DNA, using either YO or DAPI, as described in detail earlier.[12]

Calculation of Number of ssb and dsb. The formation of form II DNA by ssb was analyzed in terms of single-exponential kinetics with a time constant τ. To facilitate comparison, rate constants are normalized with respect to the binding ratio N/C (and figures are shown for the data adjusted to take account of this normalization),

$$k_I = (1/\tau)^*(N/C) \qquad (1)$$

The average number of ssb and dsb per DNA molecule was calculated from the equations of Povirk and co-workers,[16,17] and the details of this analysis have been presented earlier.[12]

C. Photobleaching. All samples contained 5 μM dye and a CT-DNA concentration corresponding to the stated N/C ratio. The final volume of the samples was 1.5 mL in 50 mM phosphate buffer (pH = 6.9). The same equilibration protocol as for the microscopy work was followed for the samples containing DNA.[11] For argon and oxygen saturation, the samples were bubbled vigorously with water-saturated gas for 1 h. Scavengers were added as 40 mM except for desferrioxamine (100 μM). Enzymes were added to give a final concentration of 22 U/mL.

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(17) Povirk, L. F.; Houlgrave, C. W. *Biochemistry* **1988**, *27*, 3850−3857.

Irradiations. A 450 W xenon lamp was employed, with a long-pass filter (3 M KNO_3, 5 cm path; $\lambda > 340$ nm). Each sample, placed in a fluorescence cuvette, was irradiated for several periods of 10 min (1 h in total). The cuvette was shaken before and after each irradiation period.

Absorption and Fluorescence Measurements. The absorption spectrum of each sample was measured on a Cary spectrophotometer, before irradiation and then after every 10 min irradiation period. The fluorescence spectrum was measured at the same time intervals on a SPEX Fluorolog-τ2 spectrofluorimeter. Excitation was always at 460 nm. Deconvolution of absorption spectra into monomer and dimer contributions was performed in MATLAB.

Linear Dichroism Measurements. Linear dichroism (LD) spectroscopy of oriented DNA−ligands complexes is a powerful technique for determination of the ligand-binding geometry of the ligands. Linear dichroism is defined as

$$\mathrm{LD}(\lambda) = A_{||}(\lambda) - A_{\perp}(\lambda)$$

where $A_{||}(\lambda)$ and $A_{\perp}(\lambda)$ denote the absorption at wavelength λ of light polarized parallel and perpendicular, respectively, to a reference orientation axis which, for DNA aligned by shear flow generated in a Couette cell, is parallel to the flow direction and hence the helix axis by shear flow with a gradient of 310 s^{-1}. LD was measured on a Jasco J-720 spectropolarimeter equipped with an Oxley prism. LD signals are obtained only from oriented molecules; hence, dyes that are not bound to DNA make no contribution to the LD spectrum. The reduced linear dichroism normalizes for the absorption of different chromophores:

$$\mathrm{LD}^{\mathrm{r}} = \mathrm{LD}/\mathrm{Abs}$$

However, it should be noted that unbound dye will contribute only to the absorption and may confuse interpretation of LD^{r} results.

Results

A. Microscopy. Cleavage and Bleaching Time Profiles. Figure 1a shows a typical band pattern obtained on electrophoretic analysis of supercoiled ΦX174 DNA with bound YOYO (N/C = 6), after illumination in the microscope (λ = 450−490 nm) for increasing periods. Initially, the concentration of nicked circles (II) increases at the expense of the supercoiled. For longer irradiations, II is consumed by accumulation of ssb eventually leading to dsb, which results in the appearance and growth of an intermediately positioned zone of linear molecules (III). Ultimately, these linear molecules are in turn analogously degraded by accumulated ssb to form linear fragments (IV) perceptible as a leading smear of weak intensity, extending from the band of intact linear molecules in the direction of migration. It is also seen that the linear band migrates progressively faster as the time of illumination increases.

The relative amount of each form of DNA was quantified using the fluorescence of bound YOYO which remains bis-intercalated during electrophoresis,[18] and Figure 1b shows the same type of product profiles as those obtained with the conventional light source ($\lambda > 320$ nm) used in an earlier study of photocleavage of DNA by YOYO,[12] although the cleavage rate is slower because of the lower light intensity in the microscope. One notable feature is that the total integrated emission from the gel scan (including fragments) decreases with illumination time. The loss of fluorescence intensity continues after complete conversion of the supercoiled form and is therefore a true chemical or physical bleaching rather than a redistribution of dye between supercoiled and relaxed forms of the DNA, which could result in a lower average quantum yield.

(18) We have confirmed previously that this is a valid quantitative procedure by destaining and restaining with DAPI (see ref 11).

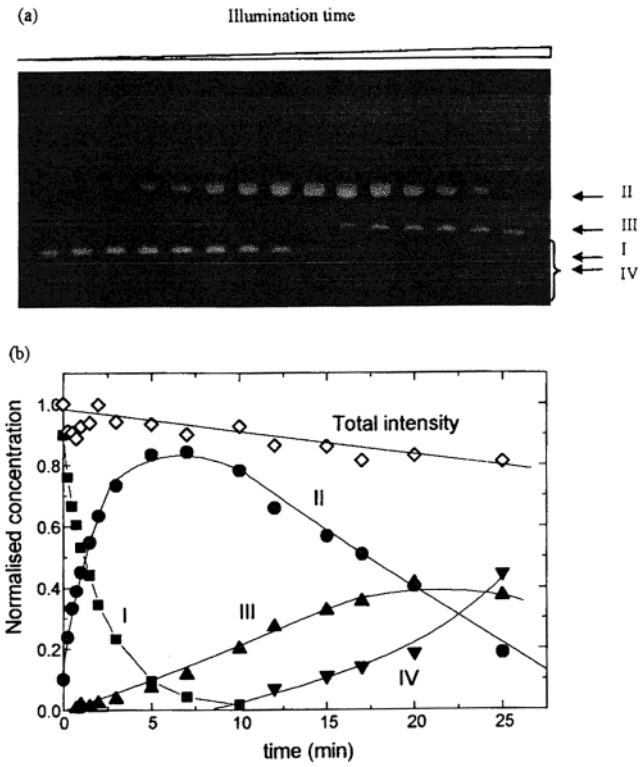

Figure 1. Cleavage of ΦX174 supercoiled DNA by YOYO at N/C = 6. (a) Gel photograph showing separation pattern after illumination for 0, 1, 2, 3, 5, 10, 15, 20, 40, 60, 80, 100, 120, 150, and 200 s. The positions of supercoiled (I), nicked circular (II), and linear (III) forms are indicated. Fragmented DNA (IV) is analyzed as the integrated intensity for any DNA that migrates faster than for III (apart from form I). (b) Cleavage profiles of supercoiled ΦX174 DNA by YOYO (N/C = 3, air-saturated solution). Notice that the cleavage is considerably slower than with the conventional light source under otherwise identical conditions due to lower illumination intensity. The open symbols show how the integrated emission from the gel scan decreases with time.

The bleaching observed in this system is a significant and reproducible effect: ca. 3% of the intensity is lost when half the supercoil has been converted, and ca. 13% is lost when all the supercoil has been converted.

Effect of DNA Topology and Binding Density on the Bleaching. Perhaps surprisingly, the rate of bleaching was affected by the topology of the DNA (Figure 2a). With initially supercoiled DNA, the bleaching contained a slow initial phase which is not apparent in the integrated lane intensities (Figure 1b), whereas prelinearized DNA gave a smoothly decaying intensity. The two curves decrease in parallel only after the supercoiled form is completely converted (ca. 10 min). This topology dependence observed at N/C = 3 was not observed at N/C = 8 (not shown) and is evidently a feature associated with external binding to supercoiled DNA. To avoid complications due to DNA topology, further bleaching studies were performed with prelinearized DNA.

Figure 2b shows that the rate of bleaching is much faster at N/C = 3 than at N/C = 8. The decay at the higher N/C was monoexponential, with a time constant of 74 min, whereas at N/C = 3 two exponentials were needed to fit the data. The faster (8.1 min) component accounted for 42% of the decay, while the rest of the amplitude decayed with a time constant (77 min) that was approximately the same as for the process at N/C = 8. It may be noted that the directly observed fluorescence intensity (Figure 2b) decreases more strongly than does the integrated intensity from gel scans (Figure 1b). We propose that this is

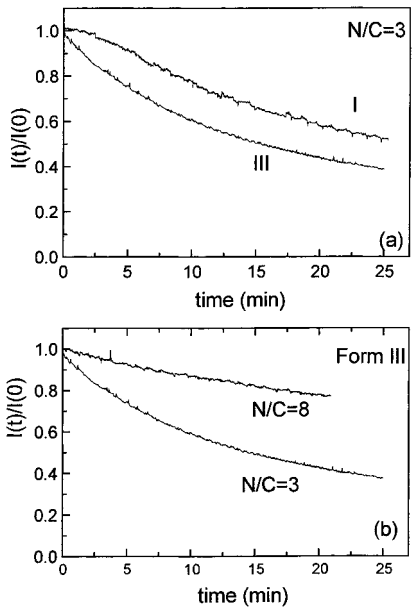

Figure 2. (a) Effect of DNA topology and binding mode on the bleaching of YOYO bound to ΦX174 DNA. YOYO bound to supercoiled (I) or linear (III) form at N/C = 8; [YOYO]= 6.7 μM. (b) Bleaching of YOYO bound to DNA at two different N/C ratios: 3 and 8 (normalized to [YOYO]= 6.7 μM).

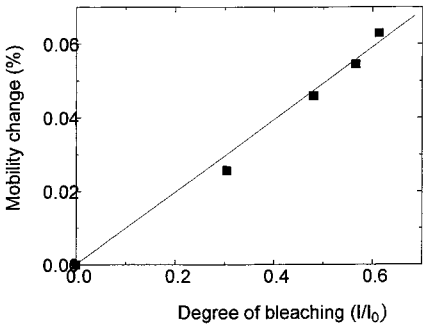

Figure 3. Effect of bleaching of bound YOYO (N/C = 3) on the electrophoretic mobility of linear ΦX174 DNA.

due to loss of externally, and therefore less strongly, bound YOYO during electrophoretic separation. Although these molecules contribute much less to the fluorescence than intercalated dye, they are preferentially destroyed with concomitant fading and bleached (vide infra). Therefore, their bleaching is apparent in the directly observed fluorescence in solution, but their contribution is not registered in the lane scans.

Effects of Illumination on the Electrophoretic Mobility. The velocity of prelinearized DNA stained with YOYO at N/C = 3 after different times of illumination was quantified from the position of the maximum intensity in the corresponding gel scans. As shown in Figure 3, a correlation is found between the increase in mobility and the degree of sample bleaching.

The simplest explanation for this effect would be that bleaching simply reflects a photoinduced loss of bound dye (so that the stained negatively charged DNA molecules lose some positive bound dye charges and migrate faster). To check whether this was the case, fresh YOYO was added to a sample that had been bleached by 43% (Figure 4) under constant illumination. The emission intensity was checked intermittently over a period of 30 min, but no change in dye fluorescence was apparent, apart from an initial small decrease of intensity due to dilution (as evidenced by the same decrease being observed by adding buffer instead). In a control experiment to

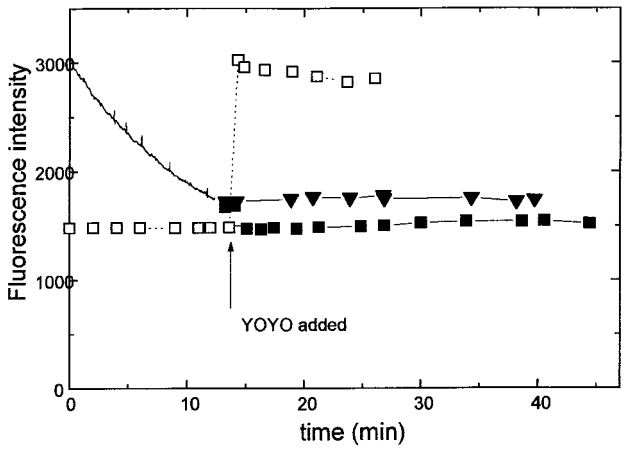

Figure 4. Effect of addition of 10 μL of YOYO solution (12 μM) on the fluorescence of a bleached sample with YOYO bound to DNA at N/C = 3 (filled squares), or on an unbleached sample at N/C = 8 (open squares). Triangles show the fluorescence of the bleached sample if no YOYO is added.

mimic the hypothetical case of dye dissociation, a DNA sample was stained to a lower N/C of 8, giving approximately the same fluorescence intensity as the bleached N/C = 3 sample. The same amount of fresh dye was added to this unilluminated sample as to the bleached N/C=3 sample, and in this case the expected increase in fluorescence intensity due to binding of the added YOYO was observed immediately. Finally, the effect of deoxygenation or β-mercaptoethanol on the bleaching was tested, but neither had a significant effect at N/C = 3 (data not shown).

B. Photobleaching Mechanism. The microscopy work demonstrated marked effects of illumination on the fluorescence of DNA-bound YOYO and allowed a comparison of the time scales for bleaching and photocleavage events. However, it was impossible to propose physical mechanisms for these effects, since the absorption characteristics of the dye could not be simultaneously monitored. Hence, to investigate the mechanisms of fluorescence bleaching, microscopy studies were complemented with a thorough examination of both the absorption and fluorescence characteristics of the dye in solution and when bound to DNA.

Free Dyes in Phosphate Buffer. (i) YO. Figure 5a shows the absorption spectra of 5 μM YO in 50 mM phosphate buffer, before and after different irradiation times. After irradiation, the absorption maximum at 481 nm decreased together with the peak at 457 nm, the ratio $\epsilon_{457}/\epsilon_{481}$ remaining constant. No new absorption bands were observed in the UV (λ > 200 nm) or visible regions of the spectrum. The loss of absorption at 481 nm could be fitted with a monoexponential curve (Figure 5b), suggesting that there is a unique process responsible for the photofading.

Consequently, the effect of different scavengers on the photofading of free YO was investigated (Figure 5b). All substances tested resulted in a slower rate of fading. It appears that materials that can scavenge hydroxyl radicals (e.g., urea) reduce the fading rate dramatically, and the presence of enzymes such as catalase and SOD suppresses the photofading of YO. This result suggests that hydroxyl radicals could be produced by a Fenton reaction catalyzed by Fe^{2+} ions in the solution, and this finding was confirmed by the very strong inhibiting effect of desferrioxamine, which chelates iron very efficiently: the photofading rate in the presence of this substance was reduced by 80%.

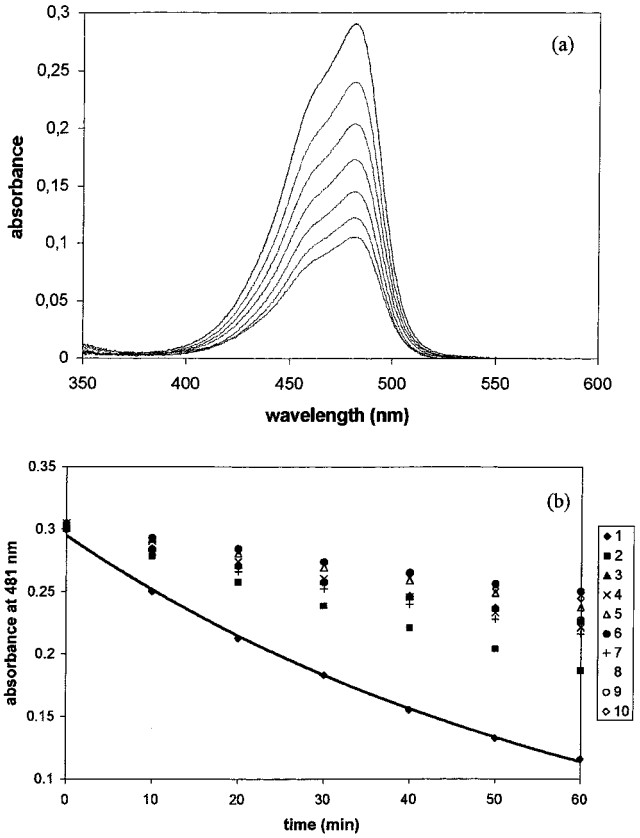

Figure 5. (a) Absorption spectra of free YO (5 μM) in 50 mM phosphate buffer (pH = 6.9) after different irradiation times: 0, 10, 20, 30, 40, 50, and 60 min. (b) Effect of different scavengers on the absorption decay of free YO: 1, without additives; 2, with β-mercaptoethanol; 3, with urea; 4, with catalase; 5, with SOD; 6, with desferrioxamine; 7, with *N*-acetyl histidine; 8, in D_2O; 9, with oxygen bubbling; 10, with argon bubbling. A monoexponential curve has been used to fit series 1 ($k = 0.0159$ min^{-1}).

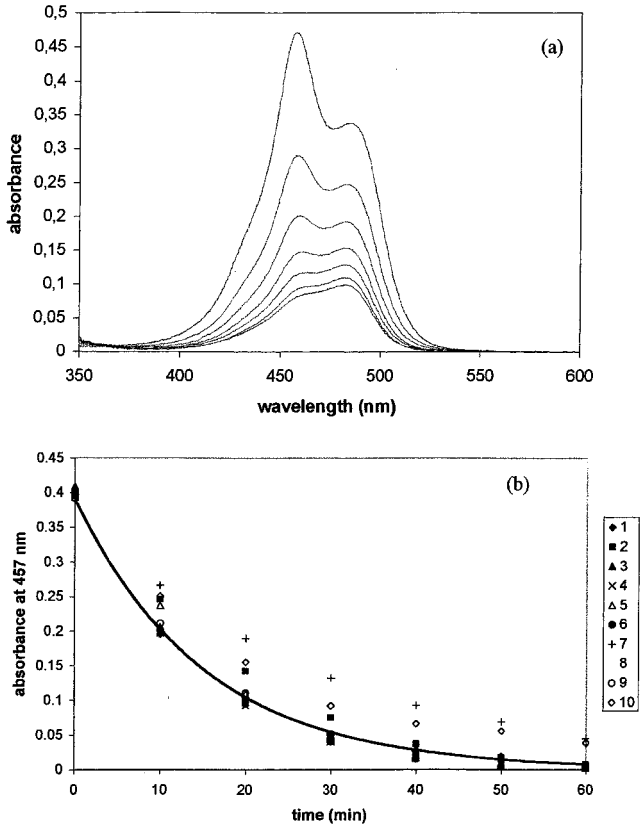

Figure 6. (a)Absorption spectra of free YOYO (5 μM) in 50 mM phosphate buffer (pH = 6.9) after different irradiation times: 0, 10, 20, 30, 40, 50, and 60 min. (b) Effect of different scavengers on the absorption decay of free YOYO: 1, without additives; 2, with β-mercaptoethanol; 3, with urea; 4, with catalase; 5, with SOD; 6, with desferrioxamine; 7, with *N*-acetyl histidine; 8, in D_2O; 9, with oxygen bubbling; 10, with argon bubbling. A monoexponential curve has been used to fit series 1 ($k = 0.0657$ min^{-1}).

Oxygen may be involved in the fading process since argon bubbling was an effective inhibitor, but it is not entirely clear what its role is since bubbling with O_2 gave a similar reduction of fading. However, inhibition of fading by D_2O (which increases the lifetime of $^1O_2$) and by removal of O_2 strongly suggests that $^1O_2$ is not involved.

(ii) YOYO. Comparing spectra (Figure 6a), it is apparent that the profile of the absorption spectrum is different in shape after irradiation, and resembles more that of the monomer. Indeed, an inversion of the ratio $\epsilon_{457}/\epsilon_{481}$ is observed. This indicates either a cleavage of the linker between the two moieties, leading to two free chomophores in solution, or photodestruction of one YO chromophore of the dimer, leaving the other free. For each irradiation time, we have deconvoluted the spectrum to calculate the concentration of the dimer remaining in solution, and Figure 6b shows how the absorption of remaining YOYO dimers in solution varies with illumination time. To fit the decay of residual dimer absorption at 457 nm, one exponential was necessary. As for irradiation of YO, no new absorption bands were detected in the UV or visible regions.

A scavenger study was also performed, and the influence of the same quenchers as for YO was studied (Figure 6b). In contrast to YO, we did not observe any effect of hydroxyl radical scavengers or of catalase, SOD, or desferrioxamine. However, in D_2O the photofading rate was increased, and argon bubbling decreased the fading while O_2 bubbling had no effect.

Absorption and Fluorescence Photobleaching of YO or YOYO Bound to DNA. YO and YOYO can bind to DNA in two distinct modes, the external form appearing below the saturation value of N/C = 5 for YO (where it is predominantly monomeric) and N/C = 4 for YOYO (where it appears to be dimeric).[9] We have therefore investigated the absorption photofading and fluorescence photobleaching of YO and YOYO at N/C = 4 and 40, to fully determine the effect of the binding mode on the photobleaching. In general, the rate of absorption fading is faster at N/C = 4 than at N/C = 40 but still much slower than for the free dyes: DNA provides a microenvironment in which the dyes are protected from reactions that lead to fading. The experimental fading data can be fitted with a single exponential at both N/C ratios (Figure 7). However, the fluorescence bleaching displays different kinetics at the two N/C ratios (Figure 8), being monoexponential at the higher ratio but biexponential at the lower ratio where a fraction of the dye bleaches at a faster rate, consistent with microscopy results. At both N/C ratios the loss of fluorescence is found to be greater than the loss of absorption, an effect that is significantly more marked at N/C = 4 than at N/C = 40, and at N/C = 4 is more pronounced for YOYO than for YO.

The same scavengers as for the free dyes have been used in order to establish which species are responsible for the photofading and -bleaching at the two N/C ratios. At the higher N/C ratio, there was no significant effect of singlet oxygen scavengers on absorption fading. β-Mercaptoethanol had a large inhibiting effect, as did SOD and desferrioxamine, similar to the findings for free YO (Figure 5a). By contrast, at the lower N/C ratio SOD, catalase and desferrioxamine had no significant effect.

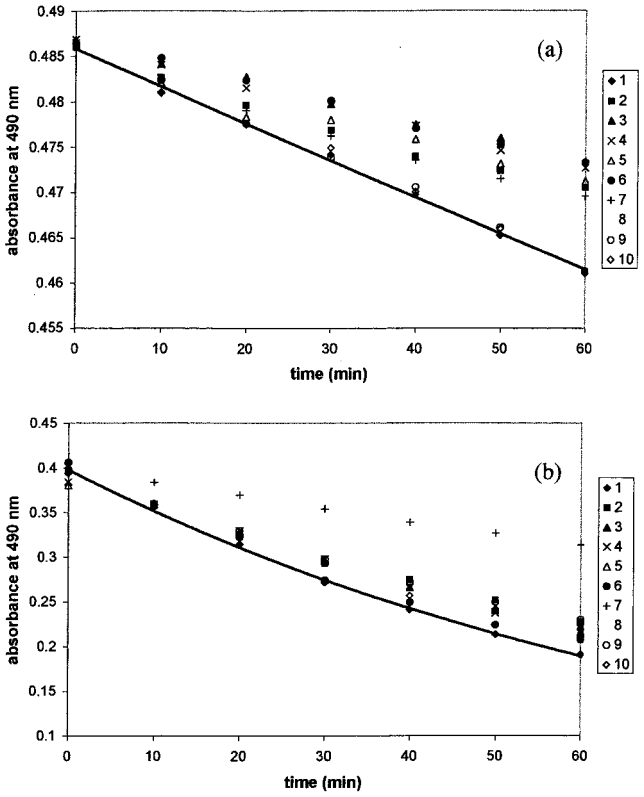

Figure 7. Decay of absorption of YOYO (5 μM) bound to DNA. (a) N/C = 40; (b) N/C = 4. Effect of different scavengers: 1, without additives; 2, with β-mercaptoethanol; 3, with urea; 4, with catalase; 5, with SOD; 6, with desferrioxamine; 7, with *N*-acetyl histidine; 8, in D_2O; 9, with oxygen bubbling; 10, with argon bubbling. A monoexponential curve has been used to fit series 1 at both N/C ratios (N/C=40, $k = 0.0009$ min^{-1}; N/C = 4, $k = 0.0124$ min^{-1}).

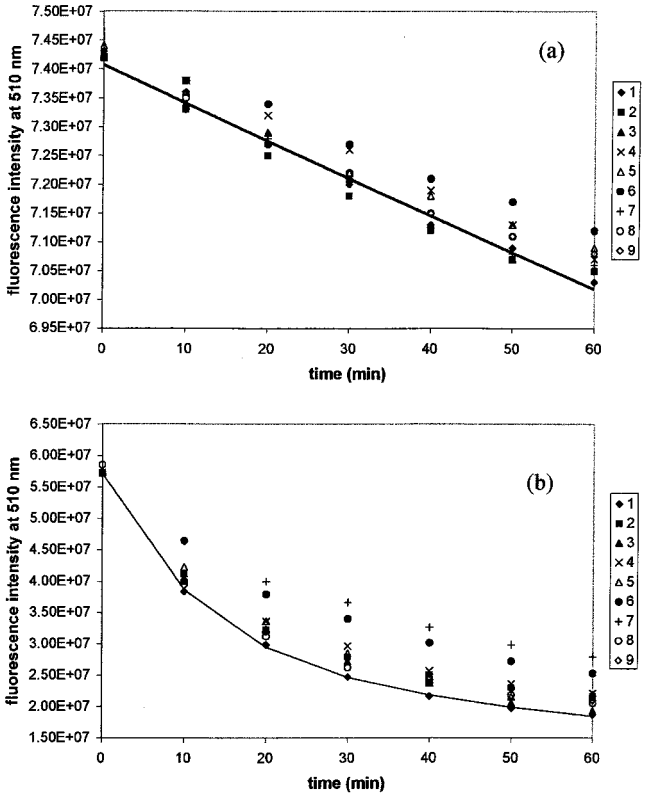

Figure 8. Decay of fluorescence of YOYO (5 μM) bound to DNA. Excitation wavelength: $\lambda = 460$ nm. (a) N/C = 40; (b) N/C = 4. Effect of different scavengers: 1, without additives; 2, with β-mercaptoethanol; 3, with urea; 4, with catalase; 5, with SOD; 6, with desferrioxamine; 7, with *N*-acetyl histidine; 8, with oxygen bubbling; 9, with argon bubbling. A monoexponential curve has been used to fit series 1a (N/C = 40, $k = 0.0010$ min^{-1}), and a biexponential curve has been used to fit series 1b (N/C = 4, $k_1 = 0.0802$ min^{-1}, $k_2 = 0.0062$ min^{-1}).

Instead, DABCO and *N*-acetyl histidine were the most efficient inhibitors of absorption fading, and replacing water by D_2O enhanced the photofading rate (Figure 7b).

Surprisingly, scavengers that inhibited the loss of absorption were all less effective in preventing fluorescence bleaching (Figure 8), which is also consistent with the microscopy work, where only small effects of β-mercaptoethanol or argon bubbling on the fluorescence bleaching were observed.

Restaining of Photolyzed DNA. In another experiment, we addressed the question raised by the microscopy studies of why it was not possible to restore the fluorescence of a YOYO-saturated DNA sample by restaining with YOYO after photolysis and concomitant bleaching. A sample containing 5 μM YOYO and a T2 DNA concentration corresponding to N/C = 4 was photolyzed for 15 min, to give a residual fluorescence emission that was 66% of the initial intensity before irradiation. To this sample was added sufficient YOYO to regenerate the initial absorption, adjusted for dilution effects, and the absorption and fluorescence spectra were recorded (Figure 9). The equilibration protocol was then performed,[11] to ensure that the dye distribution on DNA was homogeneous, and the spectra were recorded again.

After 15 min of irradiation the absorption and emission spectra had the same shape but a lower intensity (note the substantially greater loss of emission than absorption). When an aliquot of dye was then added to compensate for that lost by bleaching, the absorption spectrum showed that YOYO was binding since the absorption profile was restored to that before irradiation (Figure 9a) and is entirely different from that of the free dye. However, we did not recover our initial fluorescence intensity, as only a slight increase of the fluorescence intensity was observed (Figure 9b). The equilibration protocol had no effect on this, since the spectra before and after equilibration had the same shape and intensity (data not shown).

Linear Dichroism of a Photolyzed Sample Containing YOYO-Stained DNA. Linear dichroism was used to determine the binding geometry of YOYO to DNA before and after photolysis. One sample was prepared containing 5 μM YOYO and a T2 DNA concentration corresponding to N/C = 4, in 50 mM phosphate buffer with oxygen but no other additives: T2 DNA (164 kbp) rather than CT DNA was used in this case, since longer DNA is more easily oriented, and therefore produces a better LD signal. After the usual equilibration protocol, the absorption and LD spectra were recorded. The sample was then exposed to light for 15 min identical to the restaining experiment (vide supra), and both spectra were recorded again. While the spectra retained their shapes (Figure 10), they were both strongly reduced in intensity in the dye absorption band, particularly the LD due to photonicking and cleavage of the DNA, leading to more flexible and shorter fragments after photolysis that are more difficult to orient. The monomer-like shape of the dye absorption spectrum and the negative LD band having identical shape are consistent with a bis-intercalation geometry both prior to and following irradiation and restaining. In fact, relative to DNA, the LD^r (=LD/Abs) of the dye becomes even more negative after photolysis [LD^r(490 nm)/LD^r(260 nm) = 1.4] than before [LD^r(490 nm)/LD^r(260 nm) = 0.9], indicating that after restaining more of the dye is

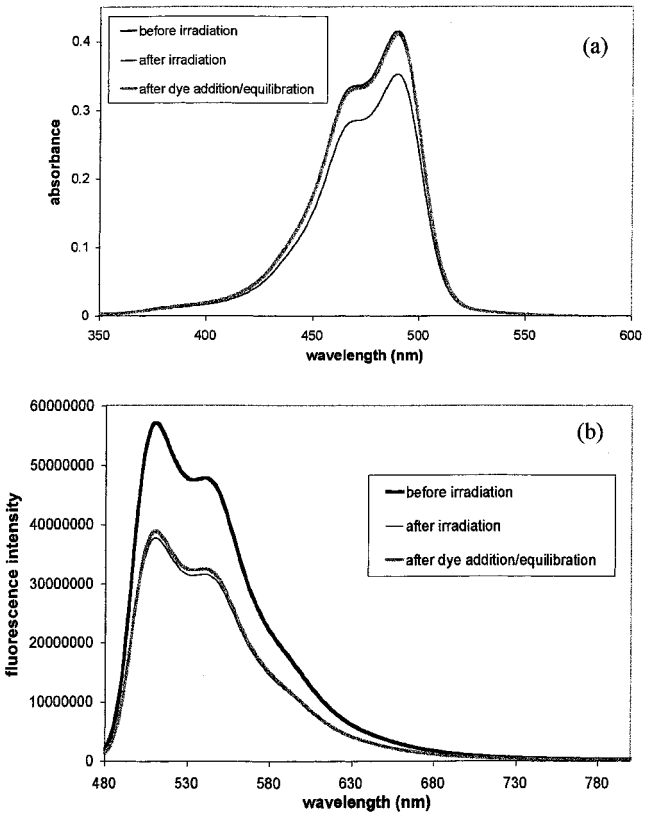

Figure 9. Effect of addition of fresh YOYO on the absorbance (a) and on the fluorescence intensity (b) of a bleached sample (5 μM) (excitation wavelength, $\lambda = 460$ nm).

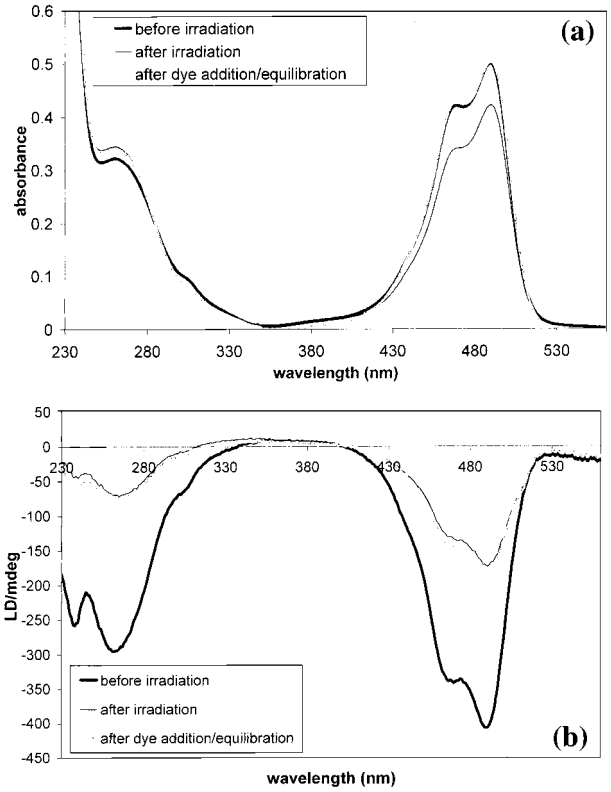

Figure 10. Absorbance (a) and linear dichroism (b) of a sample containing YOYO (5 μM)-stained T2 DNA (N/C = 4), before and after 15 min irradiation.

bound with an orientation perpendicular to the helix axis, a possible indication of less externally bound or unbound dye.

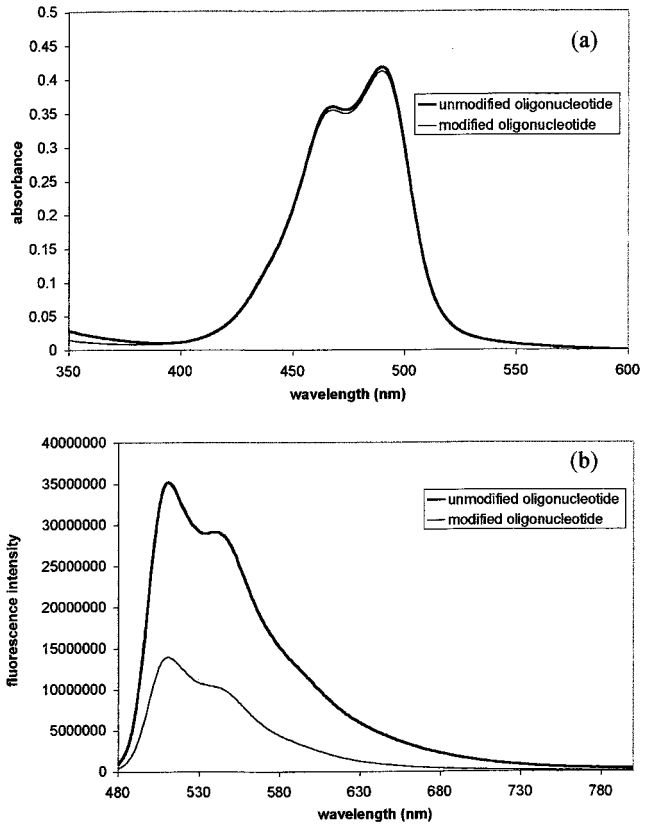

Figure 11. Effect of replacing G by 8-oxo-dGuo on absorbance and fluorescence (excitation wavelength, $\lambda = 460$ nm) of YOYO (5 μM) bound to an oligonucleotide (N/C = 4).

Fluorescence with an Oligonucleotide Containing 8-oxo-dGuo. To investigate whether the fluorescence of YOYO can be quenched by damaged DNA residues, we examined the effect of the most common lesion resulting from oxidative damage, viz. 8-oxo-dGuo. 15-mer oligonucleotides of the following sequences were used: 1, 5′-TAG-TTG-TGA-CGT-ACA-3′; 1′, 5′-TAX-TTX-TXA-CXT-ACA-3′, where X = 8-oxo-dGuo; 2, TGT-ACG-TCA-CAA-CTA-3′. Oligonucleotides 1 and 2, and 1′ and 2, were annealed to form the unmodified and modified double-stranded oligonucleotides, respectively. Two samples containing 5 μM YOYO and an oligonucleotide concentration corresponding to N/C = 4, in 50 mM phosphate buffer, were prepared. After equilibration, the absorption and emission spectra were recorded (Figure 11).

As expected, the absorption spectrum (Figure 11a) of the dye was the same with both the unmodified oligonucleotide and the modified one, showing that the binding of YOYO was not inhibited by the presence of 8-oxo-dGuo. However, there is a striking difference between the emission spectra of the two samples (Figure 11b). Indeed, in the presence of 8-oxo-dGuo, the fluorescence intensity is found to be 60% less than in the presence of unoxidized guanines.

Discussion

High-resolution imaging of single DNA molecules with fluorescence microscopy to examine their physical and dynamic properties and to test theories of polymer statistics[19−24] has been

(19) Perkins, T. T.; Smith, D. E.; Chu, S. *Science* **1994**, *264*, 819−822.
(20) Perkins, T.; Quake, S. R.; Smith, D. E.; Chu, S. *Science* **1994**, *264*, 822−826.
(21) Perkins, T.; Smith, D. E.; Chu, S. *Science* **1997**, *276*, 2016−2021.
(22) Brewer, L. R.; Corzett, M.; Balhorn, R. *Science* **1999**, *286*, 120−123.

facilitated by the development of asymmetric cyanine dyes such as oxazole yellow and thiazole orange derivatives. These dyes have very low fluorescence quantum yields in solution but fluoresce strongly (typically 1000-fold higher) when internal rotation is hindered by interaction with DNA. One of the problems associated with imaging is that these dyes can cause photocleavage of DNA: however, by trial and error it has been found that exclusion of oxygen minimizes any type of DNA damage that manifests itself as double-strand breaks, and it is also common to add β-mercaptoethanol, which is typically used in fluorescence microscopy to reduce dye bleaching.

We have previously reported[12] that YO, YOYO, and TOTO cause photosensitized DNA nicking and, moreover, that both monomer and more particularly the dimers have a tendency to cause higher than statistically predicted yields of double-strand vs single-strand breaks in DNA. The photocleavage mechanism was found to depend on the binding ratio, apparently mediated by $^1O_2$ at high binding densities (where the dye may bind not only by intercalation but also externally) with a greater efficiency than at low dye densities where the mechanism was not clear but was suggested to involve radical species.

To complement our previous work, we initiated an investigation of correlations between the photosensitized cleavage of DNA and the bleaching of dye fluorescence that is usually observed in fluorescence microscopy. Our initial findings under microscopy conditions, particularly that β-MeSH did not inhibit bleaching although it was very effective at arresting photocleavage, prompted us to extend our studies to examine both the absorption and fluorescence properties of the dyes (free in solution and when bound to DNA) as a function of illumination time to determine the basic processes that are responsible for the bleaching.

Different Photodegradation Mechanisms for Free YO and YOYO in Solution. The drop in absorption of free YO or YOYO indicates that both dyes are degraded under irradiation with visible light. The lack of any absorption of the photoproducts in the 200−700 nm spectral region indicates that the dyes are highly fragmented in this process, which is not simply a scission of the interchromophore bridge.[25]

The monoexponential absorption decay of free YO is in agreement with a single process, or at least one dominant process, for the photofading. The photophysics of these dyes in solution have not been extensively investigated, and there are currently no reports on the triplet-state properties in the literature. Results with scavengers show that despite efficient nonradiative deactivation, the excited-state dye can react to produce reactive oxygen species, which in turn attack the dye to cause its degradation. The large inhibiting effects of superoxide dismutase which converts the superoxide anion $O_2^{\bullet-}$ radical to H_2O_2,[26] of catalase which can remove H_2O_2, and of desferrioxamine that complexes Fe^{3+} [27] are consistent with

Scheme 2. Fenton Reaction

$$\left.\begin{array}{l} 2\,O_2^{\bullet-} + 2\,H^+ \longrightarrow O_2 + H_2O_2 \\ O_2^{\bullet-} + Fe^{3+} \longrightarrow O_2 + Fe^{2+} \end{array}\right\} H_2O_2 + Fe^{2+} \longrightarrow Fe^{3+} + OH^- + OH^{\bullet}$$

$^{\bullet}$OH radicals, formed via a Fenton reaction (Scheme 2), attacking and destroying the dye. These reactive oxygen species are produced by reactions of the excited state dye and indicate that it can produce superoxide in solution. Superoxide could be formed by reaction of excited-state dye with molecular oxygen, or with singlet oxygen generated previously by excited-state YO. In fact, other cyanine dyes have been reported to react with O_2 to produce $O_2^{\bullet-}$,[28] and a reaction of the YO singlet state [$E(YO^{\bullet+}/YO) = 1.14$ V vs SCE; $E_{00} = 2.48$ eV][29] with either O_2 [$E(O_2/O_2^{\bullet-}) = -0.57$ V vs SCE] or $^1O_2$ [$E_{00} = 0.976$ eV] to produce superoxide is thermodynamically favorable. The triplet-state energy of YO is unknown, but it is likely that it could undergo electron transfer with singlet oxygen, if not also the ground state. The mechanism is supported by a requirement for O_2 since argon bubbling is inhibiting. However, the inhibition of fading caused by O_2 bubbling and D_2O suggests that the mechanism may be quite complex.

The degradation of YOYO is clearly initiated by a different reactive species since the scavenger results strongly suggest that in this case it is singlet oxygen that is responsible for degrading the dye. The reason for the difference may lie in the different solution states of the dimer and monomer, which likely leads to different excited-state properties. For example, it has been reported that the singlet state of a symmetric cyanine dimer has twice the lifetime of the monomer.[30] In YOYO the two linked chromophores stack to form a dimer that is clearly identifiable from its absorption profile.[31] For monomeric YO at the concentrations and ionic strengths used in our studies, there is no strong driving force for stacking,[31] so the chromophores remain isolated. The fluorescence spectra of the monomer and dimer in solution are very different, although both are of low quantum yield, so we anticipate that there will be some differences in excited-state behavior and reactivity. Time-resolved spectroscopic methods are currently being employed in an attempt to characterize the differences.

Externally Bound Dye Induces Efficient Fluorescence Bleaching Independent of Photofading. At high dye binding densities, there is a fast component in the bleaching kinetics which is not observed in the fading kinetics. Nor is it observed at higher loadings, indicating that it is a process associated with nonintercalated dye.

In previous work[12] we reported that externally bound dye produces more ssb than intercalated dye and that it is mediated by $^1O_2$. Singlet oxygen is also implicated in fading and bleaching of the externally bound dye by scavenger studies (Figures 7b and 8b). Consistent with greater reactivity of externally bound dye, fading is more efficient at N/C = 4 than at N/C = 40, implying that externally bound dye is preferentially destroyed. However, since externally bound dye contributes only weakly to the fluorescence, the bleaching reflects predominantly the fluorescence of intercalated dye.

(23) Yanagawa, H.; Ogawa, Y.; Ueno, M. *J. Biol. Chem.* **1992**, *267*, 13320−13326.
(24) Larsson, A.; Akerman, B. *Macromolecules* **1995**, *28*, 4441−4454.
(25) We have found that some asymmetric cyanine dyes (particularly those lacking the cationic side chain) in aqueous solution have a tendency to decompose in the dark, and in those cases the products exhibit UV absorption consistent with a scission of the interchromophore bridge.
(26) Inhibition by superoxide dismutase alone may seem surprising because this enzyme scavenges the superoxide radical to produce H_2O_2, which is the source of hydroxide radicals via the Fenton reaction. However, superoxide has a second role in the Fenton cycle, which is reduction of Fe(III) to Fe(II), which is also required. If Fe(III) reduction is inhibited by scavenging of superoxide, then the Fenton reaction cannot proceed.
(27) Halliwell, B.; Gutteridge, J. M. C. *Free Radicals in Biology and Medicine*; Oxford University Press: New York, 1989.
(28) Clapp, P. J.; Armitage, B. A.; O'Brien, D. F. *Macromolecules* **1997**, *30*, 32−41.
(29) Netzel, T. L.; Nafisi, K.; Zhao, M.; Lenhard, J. R.; Johnson, I. *J. Phys. Chem.* **1995**, *99*, 17936−17947.
(30) Khairutdinov, R. F.; Serpone, N. *J. Phys. Chem. B* **1997**, *101*, 2602−2610.
(31) Carlsson, C.; Larsson, A.; Jonsson, M.; Albinsson, B.; Norden, B. *J. Phys. Chem.* **1994**, *98*, 10313−10321.

We propose that, in addition to efficiently destroying externally bound dye, $^1O_2$ generated at N/C = 4 tends to react more efficiently with DNA than with intercalated dye and hence produces damaged DNA sites that physically quench the intercalated dye fluorescence (vide infra) without affecting its absorption. As externally bound dye is destroyed, the production of reactive species (their nature as well as yield) changes, and consequently the rate of damage is attenuated, becoming more characteristic of intercalated dye. Therefore, the rate of bleaching by physical quenching is also reduced. Ultimately, when all the externally bound dye has decomposed, the kinetics of fading and bleaching should assume those typical for the intercalated dye.

Fluorescence Photobleaching Is Not Solely Due to the Photodegradation of DNA-Bound Dye. The decrease of bound dye absorption is an indication that, like the free dyes, DNA-bound YO or YOYO decomposes when photolyzed with visible light. Externally bound dye photofades faster than the intercalated form, in agreement with the observation that externally bound dye is generally more photoactive than intercalated dye since it also causes more photocleavage. Externally bound YOYO is found to fade faster than externally bound YO, which may be related to the form of the dye: YOYO is predominantly dimeric when externally bound while YO remains mainly monomeric under the conditions we have employed.[9] The dimer and monomer likely have different excited-state properties (e.g., dimeric cyanines have a longer-lived singlet state than the monomer[30]) and therefore different reactivities.

We have determined which active species are involved in the absorbance photofading for both binding modes. It appears that $^{\bullet}$OH radicals are the damaging species when the dye is intercalated (bis-intercalated), whereas singlet oxygen is involved when the dye is externally bound. These same species are implicated in photocleavage of DNA by these dyes under the similar conditions, so the dye and DNA must compete for the reactive oxygen species that are generated by the dye excited state.

A loss of bound dye due to photodecomposition into fragments could trivially explain both the fluorescence bleaching (due to a drop in excitation cross section) and the increase of electrophoretic mobility (which correlated well with the degree of bleaching), as a loss of bound cationic dye would lead to an increase of DNA net negative charge. However, this simple picture cannot account for all the experimental observations—it does not explain why the fluorescence intensity drops faster than the absorption or why adding free dye to a bleached YOYO−DNA complex does not restore the lost fluorescence intensity, even after an incubation of 30 min. Moreover, we have shown that while appropriate scavengers can inhibit the absorption fading, they are all less effective in preventing fluorescence photobleaching.

It is possible that the decrease of fluorescence is due to a change in the dye binding mode rather than to a loss of bound dye, since it has been reported that externally bound dye fluoresces less strongly than intercalated dye.[10] This could occur if the DNA conformation were altered by dye photosensitization in such a manner that intercalation is prevented, e.g., some form of cross-linking. However, this explanation is irreconcilable with the results of the LD experiments. Externally bound dye is expected to have a less negative or even positive LD compared to intercalated dye, while unbound dye makes no contribution to the LD.[10] Instead we observe that the LD^r of YOYO is unchanged or even more negative relative to the LD^r of DNA after photolysis and retains the shape of the monomer spectrum, which seems to rule out any shift from intercalative to external binding in which YOYO is dimeric.[10] Since the dye can be completely accommodated by DNA in an intercalative binding mode, this seems to exclude the possibility that the DNA conformation is modified by cross-linking. Furthermore, if cross-linking between two DNA molecules or even intramolecular cross-linking occurred, during electrophoresis we would expect a large distribution of conformations and therefore mobilities, producing a smear rather than the observed well-defined band with a lower velocity.

Another possibility is that irradiation causes a shift in the sequence distribution of binding sites, since it has been reported that the YOYO fluorescence quantum yield is higher in GC-rich than AT-rich sequences.[10,29] In principle, if damage were to occur predominantly at guanine (as observed, unpublished data), a switch to binding of dyes in a more AT-like environment could occur. However, the bleaching is not greatly inhibited by scavengers that arrest cleavage, so we believe that this is not the main cause of this effect, although it may be a contributing factor.

The Loss of Fluorescence Is Due to Electron-Transfer Quenching by Damaged DNA. Thus, the hypothesis that we advance as most likely responsible for the bleaching is that the dye singlet state is physically quenched. This postulate seems reasonable since we have shown that adding fresh YOYO to photolyzed DNA (Figure 9) does not restore the fluorescence even though by examining absorption and LD^r spectra for YOYO we can see that the dye intercalates with DNA because of the dramatic change of spectral shape. Therefore, YOYO fluorescence could be quenched either by DNA bases that have been modified (by oxidation for instance), by photodegradation products of the dye itself, or even by photoadducts of the dyes with DNA. However, the last possibility seems unlikely, since sequencing experiments of a radiolabeled oligonucleotide photosensitized by YO or YOYO show no evidence for the formation of photoproducts (unpublished results). To test whether dye photoproducts could cause quenching of DNA-bound dye, we photodegraded the dye in solution and added this mixture to a sample of YO-bound DNA, but observed no effect on fluorescence intensity. Additionally, a sample of YOYO bound to DNA was photodegraded by 75%, and when it was subsequently mixed with an unirradiated sample no quenching was observed. Therefore, it appears that dye photoproducts do not quench the remaining bound dye. Hence, the most probable explanation is the first one suggested, i.e., the quenching of dye fluorescence by damaged DNA, perhaps oxidized bases. It is well known that guanine is the most easily oxidized DNA base, and one of its major oxidation products is 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxo-dGuo), which is even more easily oxidized than the intact base.[32] The absorption and emission spectra of YOYO were compared for the dye bound (N/C = 4 with equilibration, corresponding to what is used in microscopy work) to a double-stranded oligonucleotide or to the same ds oligonucleotide in which all guanines were replaced by 8-oxo-dGuo. The absorbance spectra (Figure 11a) were similar for both samples, showing that the dye can bind to the modified ds oligonucleotide as well as to the unmodified one. However, the fluorescence intensity of YOYO bound to the modified oligonucleotide was clearly much lower than with the unmodified oligonucleotide (Figure 11b). Hence, the presence of damaged DNA bases such as 8-oxo-dGuo can lead to a drastic decrease of fluorescence intensity of this dye. Other modified bases may also contribute to quenching, but this has not yet been extensively investigated.

(32) Burrows, C. J.; Muller, J. G. *Chem. Rev.* **1998**, *98*, 1109−1151.

The quenching of YO fluorescence by 8-oxo-dGuo is probably due to an electron transfer from the modified base to the dye singlet state. 8-oxo-dGuo produces only low levels of strand breaks after treatment with hot piperidine[33] but is highly susceptible to secondary oxidation.[34] We surmise that 8-oxo-dGuo is initially produced in the DNA by YO photosensitization either via intermediate $^{\bullet}OH$ radicals or $^1O_2$, and that the modified base then reacts further with the dye excited state, leading to damage that is manifest as strand breaks. It appears that this may be the main route by which these dyes cause strand lesions in DNA and can explain some apparently inconsistent observations. Preliminary findings from sequencing experiments (unpublished results) have shown direct cleavage as well as piperidine-labile sites almost exclusively at guanine, and the 5′-G in G multiplets is targeted more efficiently at all binding ratios, even under conditions where $^1O_2$ and $^{\bullet}OH$ quenchers/enhancers suggest that this active species is involved.[12] Neither direct stand breaks nor such a cleavage pattern is consistent with a $^1O_2$- or $^{\bullet}OH$-mediated mechanism[35] but rather with an electron-transfer mechanism. Singlet oxygen produces piperidine-labile damage equally at all guanines, whereas hydroxyl radicals usually attack the backbone at any site, causing direct damage. However, the latter also react at the bases, and base specificity could be induced by the binding preference of the sensitizer. Indeed, on the basis of sequencing experiments, others have suggested that direct electron transfer between *TO and guanine is responsible for cleavage.[36] However, even though favorable energetics have been determined for photoinduced electron-transfer reactions of these dyes with guanine itself,[29] this reaction was ruled out on other grounds. On the other hand, it is likely that the 600 mV less energy required for oxidation of 8-oxo-dGuo[34] would be sufficient to drive an electron-transfer reaction. Our proposed two-step mechanism provides an explanation for the inconsistency between scavenger and cleavage pattern results, and studies are continuing to clarify the details.

Relevance of Bleaching for Single-Molecule DNA Imaging. One aim of these studies was to evaluate whether the photochemistry of the dye could produce hidden effects when these dyes are used to image DNA molecules, since we have already emphasized that a great deal of damage in the form of ssb may have accumulated in DNA before damage becomes apparent in the microscope in the form of a double-strand break.[12] One question, therefore, is whether the light intensities used in our studies are relevant for microscopy. By comparing the times required to produce the first double-strand breaks in both experimental setups, it is clear that they are. In our studies of individual T2 molecules (170 kbp) in aerated solution stained with YOYO at N/C = 5 (100× objective; NA = 1.3),[24] the first double-strand break occurs within 1−5 s. Cleavage rates cannot be measured in the microscope with ΦX174 DNA (5386 bp), but the time needed to produce on average 1 dsb per 5386 bp under the same conditions can be estimated as 25−125 s.[37] Using the xenon lamp and a slightly higher dye loading (N/C = 3), we observe an average of 1 dsb per ΦX174 molecule after about 140 s (data not shown), which demonstrates that the effective light intensity during single-molecule imaging at high magnification is comparable to that used in our cleavage and bleaching studies. The microscopy studies reported in this paper are at lower magnification (5× objective; NA = 0.15), with an illumination intensity 16.7 times lower than the xenon lamp using rates of supercoil cleavage for calibration. Since the illuminating intensity varies as the square of the numerical aperture, this implies that in single-molecule studies the intensity is about 4.5 times that with the xenon lamp, consistent with our estimate above. Our results are therefore relevant for discussing possible effects of the photochemical activity of the YO chromophore on the behavior of the imaged DNA.

An important observation is that with the level of staining typically used in DNA imaging (N/C = 3−5), a DNA molecule is likely to have accumulated as many as 5−10 ssb before a dsb is observed in the microscope, and this may have an effect on the properties of the DNA. Indeed, we observe an increased mobility of YOYO-stained linear DNA after irradiation, which correlates well with the degree of bleaching and could, in principle, be due simply to the loss of positively charged dye that has been degraded and no longer binds. However, we rule out this explanation since adding free dye to a bleached DNA−YOYO complex did not restore the lost fluorescence. Instead, we propose that the increased mobility is due to the presence of ssb which are known to affect electrophoretic mobility under certain conditions.[38] Use of lower dye loading would help to minimize the number of ssb formed before a dsb is produced, and also bleaching and cleavage would be less severe effects in general because there would be less externally bound dye. However, this would be at the expense of lower brightness, and the value N/C = 5 commonly employed in DNA imaging may in fact offer the best compromise between optimizing brightness and minimizing hidden photochemical damage.

Conclusions

The asymmetric cyanine dyes YO and YOYO undergo visible light-induced fading of absorption in aqueous solution, due to decomposition caused by reactive oxygen species generated by reactions of the excited-state dye. The degradation processes are different for monomer and dimer with Fenton-generated hydroxyl radicals involved in the photofading of free YO, whereas singlet oxygen is involved for free YOYO. In the presence of DNA, absorbance photofading *and* fluorescence photobleaching occur, although the processes are much slower for the bound dyes than for the free dyes. The active species involved in absorbance fading depends on the dye binding mode. For intercalated dye Fenton-generated hydroxyl radicals are responsible, and for the externally bound dye singlet oxygen is involved. Fading of DNA-bound dye is not solely responsible for fluorescence photobleaching, since bleaching occurs even in the presence of efficient inhibitors of absorbance fading. We have shown that this loss of fluorescence is due not to a change in the dye binding mode or a loss of bound dye, but to physical quenching of fluorescence. We have shown that the oxidized base 8-oxo-7,8-dihydro-2′-deoxyguanosine can quench the

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(34) Hickerson, R. P.; Prat, F.; Muller, J. G.; Foote, C. S.; Burrows, C. J. *J. Am. Chem. Soc.* **1999**, *121*, 9423−9428.

(35) Singlet oxygen produces piperidine-labile damage approximately equally at all guanines, whereas hydroxyl radicals usually attack the backbone at any site, causing direct damage. However, the latter also react at the bases, which may be favored if they are produced in close proximity to the bases, e.g., by intercalated dye. Base specificity could be then be induced by the binding preference of the sensitizer.

(36) Thompson, M.; Woodbury, N. W. *Biochemistry* **2000**, *39*, 4327−4338.

(37) From ref 12: using eq 2b, the number of ssb induced in a molecule of length L after time t varies as $n_1 \sim t^1L^1$ for a constant number of cleavage events per unit length at constant N/C. Using eq 3, the number of double-strand breaks is then given by $n_2 = KL^1t^2$, where K is determined by the dye binding density (N/C) and the light intensity. Hence, the average time to create one dsb in a ΦX174 molecule at 100× under the microscope would be $[(1-5\ s) \equiv (170/5.4)^{1/2}]$.

(38) Mills, J. B.; Cooper, J. P.; Hagerman, P. J. *Biochemistry* **1994**, *33*, 1797−1803.

fluorescence of intercalated YOYO and suggest that this lesion or perhaps other lesions on the damaged DNA give rise to the observed quenching. The postulated involvement of an intermediate 8-oxo-dGuo which is further oxidized by electron transfer with the dye excited state is compelling since it also explains observations such as direct strand breaks and preferential cleavage of 5′-G's in G multiplets in cases where $^1O_2$ is implicated as a reactive species.

Acknowledgment. This research was initially funded by the Swedish Natural Science Research Council (NFR) and the Carl Trygger Foundation. C.K. thanks the French government for a grant to carry out half her Ph.D. studies in Sweden. Fiona Dickinson is thanked for supplementary experimental contributions as part of her MChem project.

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