Low-Level Psoralen–Deoxyribonucleic Acid Cross-Links Induced by Single Laser Pulses†

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ABSTRACT: While many intercalated psoralens require a 1.3-μs relaxation time between absorption of the first and second photons for cross-link formation to occur, some psoralens can form cross-links within the lifetime of a 10-ns laser pulse. This effect is largely or completely oxygen independent. Structural, kinetic, and energetic considerations suggest that the 1.3-μs delay may be due to a conformational change in the deoxyribonucleic acid (DNA) at the intercalation site which could be required for proper alignment of the double bonds which react in the second photoreaction. The cross-links which can form with single pulses of light may result from intercalation complexes which are already in a conformation such that, within 20 ns after absorption of an initial photon, a monoadduct is formed which can absorb a second photon and thence result in a cross-link. These intercalation sites may be distinguished by the type and sequence of base pairs at the site or, alternatively, at the moment of the pulse, random motions of the DNA may have brought those sites into a conformation which allows cross-linking without the 1.3-μs delay. Unlike "ordinary" cross-links, these rapidly forming cross-links appear to be monophotonic; i.e., they increase linearly with laser pulse energy. This suggests that the second photostep for these adducts effectively saturates at much lower laser intensities than is the case for ordinary cross-links.

Psoralens are skin-photosensitizing compounds (furocoumarins), which intercalate between the base pairs of double-helical nucleic acids and, upon absorption of long-wave UV light, can form cyclobutane adducts to adjacent pyrimidines. Capable of photoreacting at both ends of the molecule, a favorably positioned psoralen can cross-link the double helix by reacting with pyrimidines on opposite strands lying above and below the intercalation site.

In earlier work (Johnston et al., 1977), we utilized single pulses from a ruby laser (λ = 347 nm) to produce monoadducts between 4′-(aminomethyl)-4,5′,8-trimethylpsoralen (AMT)[1] and bacteriophage T4 DNA, with no cross-links detectable.

Our interpretation of this result postulated the existence of an intermediate excited state which "decays" into a monoadduct capable of further photoreaction (i.e., forming a cross-link) only after a time interval long compared to the 15-ns duration of the laser pulse. We have measured the $1/e$ time required for formation of a "cross-linkable" monoadduct to be 1.3 μs (Johnston et al., 1980) and suggested that a conformational change is required after formation of a monoadduct before it is capable of forming a cross-link. In the course of this measurement, which required the use of different laser sources, higher pulse energy, and an increased accuracy in cross-link detection over the original work, it was found that some cross-links are formed by two pulses of light spaced closer than 100 ns and indeed by single 15-ns pulses. Spacing two pulses farther apart than 1 μs simply increases the yield of cross-links, dramatically at the ruby wavelength and less dramatically at the wavelength of the Nd-YAG laser (355 nm) used in the two-pulse experiments. In the present work we have analyzed this effect and suggest that a certain subpopulation of intercalated psoralens is capable of forming a cross-link within the lifetime of a single laser pulse because of an alternation in the conformation of the DNA at those intercalation sites, a result of either transient flexing of the DNA or special base sequences at those sites.

Materials and Methods

T4 DNA. DNA isolated from T4 phage grown on normal *Escherichia coli* host bacteia was found to contain too many single-strand nicks to allow an accurate determination of cross-linking by the assay used in this work. Consequently, a ligase-overproducing *E. coli* mutant, N1625 *lop8*, kindly donated by I. R. Lehman, was used. Bacteria were grown up to 7×10^8 cells/mL in bactotryptone–NaCl medium and infected by T4 *os*$^+$ phage (osmotic shock resistant strain, gift of M. F. Maestre) at a multiplicity of 0.1 phage/cell. After 3 h of growth at 37 °C, the medium cleared and chloroform was added to complete cell lysis. After stirring for 15 min, the suspension was centrifuged at 4000*g* for 20 min to bring down cell debris. The supernatant was made 0.5 M in NaCl and again centrifuged at 4000*g* for 20 min. The resulting supernatant was made 8% in poly(ethylene glycol), and after being allowed to stand for 30 min at 4 °C, the phage were pelleted by centrifugation at 16000*g* for 30 min. The pellet was suspended in a minimum volume of 5 mM Tris–0.5 M NaCl–1 mM $MgSO_4$, pH 7, and centrifuged at 400*g* for 20 min. The supernatant was centrifuged at 34000*g* for 45 min to pellet the phage, which were then resuspended in a small volume of 0.01 M sodium phosphate, pH 6.8.

Hydroxylapatite was added until the suspension cleared, indicating that all the phage had been adsorbed. This required ~15 mL of hydroxylapatite slurry for 10 mL of concentrated (~10^{14}/mL) phage suspension. In a glass column with a fritted-glass bottom the hydroxylapatite–phage slurry was washed with 0.02 M sodium phosphate, pH 6.8, until the eluate showed no absorbance at 260 nm. Broken phage were then

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[1] Abbreviations used: AMT, 4′-(aminomethyl)-4,5′,8-trimethylpsoralen; DNA, deoxyribonucleic acid; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; DEAE, diethylaminoethyl; bp, base pair; kbp, kilobase pair.

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eluted with 0.1 M sodium phosphate, and intact phage were eluted with 0.2 M phosphate (both buffers pH 6.8). The phage suspensions were made 0.05 M in NaCl, 1 mM in $MgSO_4$, and 0.5 mM in Tris for storage at 3 °C.

T4 DNA Isolation. The purified, suspended phage (broken or intact) were made 10 mM in ethylenediaminetetraacetic acid (EDTA) and 0.5% in sodium dodecyl sulfate (NaDodSO_4) and added to 2 volumes of phenol which had been recently distilled and equilibrated with 0.02 M sodium phosphate, pH 7.5. The mixture, in teflon-capped conical glass centrifuge tubes, was gently mixed by inverting the tubes on a slowly rotating table for 30 min. The phases were separated and the water phase was again extracted with phenol in the same way. The water phases were combined, cooled to 4 °C, and centrifuged at low speed to remove some of the phenol and NaDodSO_4. The supernatant was dialyzed against 0.01 M sodium phosphate–2 mM EDTA (pH 7.1) to complete the removal and then dialyzed against 0.02 M sodium phosphate–1 mM EDTA, pH 7.1. Boundary sedimentation in alkaline sodium chloride (Studier, 1965) showed a DNA peak sedimenting $s_{20,w}$ = 56 S, with a tail of slower sedimenting material. By use of Studier's (1965) relation $s_{20,w} = 0.0528M^{0.4}$, 56 S corresponds to a single-strand molecular weight of 37 × 10^6, indicating the presence of 0.65 nicks per single strand of T4 DNA.

Irradiation. AMT, cold or of specific tritium activities 1.31 × 10^5 and 3.02 × 10^7 cpm/μg, was supplied by S. Isaacs. The lasers used were a Raytheon Model SS-404 Q-switched neodymium-YAG laser (third harmonic = 354.7-nm pulse, duration 15 ns) and a Q-switched ruby laser (frequency doubled, 347.1 nm, pulse duration 10 ns). Irradiation was carried out in a cylindrical flow cell (3-mm diameter × 10-mm light path, Hellma QS-178) having quartz windows. The cell was filled in such a way that the entire sample was exposed to the laser beam.

Assay for Cross-Links. Determination of the level of cross-linking was determined by a variant of the denaturation–renaturation assay previously published (Johnston et al., 1977). A quantity of the irradiated DNA–AMT mixture containing 1 μg of DNA was diluted to 85 μL with 0.01 M sodium phosphate–1 mM EDTA (pH 7.0) and brought to pH 13 by addition of 20 μL of 1 M NaOH. The sample was mixed at room temperature for 2 min by rolling the tube, incubated for 20 s in a 60 °C water bath, and then quickly neutralized to pH 7.3 by the addition of 22.5 μL of 1 M NaH_2PO_4. The tube was reimmersed in the 60 °C bath for 10 s while rolling to mix and was then plunged into ice. To this was added 0.41 mL of a millipore-filtered saturated aqueous solution of CsCl (Merck, Suprapur grade), and 0.48 mL of the resulting solution was placed in a double-sector titanium centerpiece for analytical ultracentrifugation at 42000 rpm in a Beckman Model E equipped with a UV scanner.

After sedimentation to equilibrium, molecules which were not cross-linked and remained single stranded were resolved from those which were cross-linked and renatured rapidly upon neutralization. The degree of cross-linking was characterized by the ratio ds/(ds + ss), where ds and ss are the heights of the double- and single-stranded peaks, respectively. Because some DNA molecules had single-stranded nicks and banded at intermediate buoyant density when cross-linked, the peaks were not Gaussian and the peak ratios are an underestimate of cross-linking; however, they are reliable as measures of relative cross-linking levels.

The 60 °C treatments are critical for reproducibility: lack of a 60 °C incubation while at pH 13 appears to result in incomplete strand separation and an overestimate of cross-linking, even if the sample is left for 30 min at room temperature. However, too long an incubation results in nicking of the single strands of DNA and an underestimate of cross-linking. The brief annealing just after neutralization is also necessary; without it one gets an underestimate of cross-linking due apparently to haphazard base re-pairing at the lower temperature which prevents the cross-linked strands from "zippering up" to re-form the completely base-paired double helix. Renaturation of un-cross-linked molecules occurs only at much longer annealing times [$C_0t_{1/2}$ for T4 DNA = 0.3 M s (Britten, 1968)].

Photoaddition of [^{3}H]AMT to DNA. A suitable amount of the irradiated DNA–AMT mixture was spotted onto a small piece of DEAE filter paper (Whatman DE-81), and AMT which was not covalently bound to the DNA was removed by stirring the paper in five washes of 0.5 M sodium phosphate buffer (pH 7.0) at 37 °C for at least 15 min with each wash or until the wash was nearly free of counts. The paper was rinsed twice with distilled water, once with 95% ethanol, and once with ethyl ether (each rinse 5 min at 37 °C), thoroughly dried, and counted in toluene-based scintillation fluid. Counting efficiency was determined relative to that of the usual cocktails. This procedure (C. Chen and J. E. Hearst, unpublished procedure) is much faster than the previously used technique of extraction and dialysis.

Deoxygenation of AMT–DNA Samples. This was accomplished by making the samples 50 mM in glucose, 0.5 μM (93 μg/mL) in glucose oxidase (Sigma Chemical Co., 15.5 units/mg) (Kusai et al., 1960; Nakamura et al., 1976), 20 nM (4.8 μg/mL) in catalase (Sigma, 11000 units/mg) (Beers & Sizer, 1951), and 10 mM in potassium phosphate, pH 6.8. The samples were immediately sealed in the photolysis cell, which had been previously purged with nitrogen, and allowed to stand at room temperature for at least 9 min before irradiation by the laser. The above reaction conditions were chosen to closely match the salt concentration and pH of aerated samples. This treatment was sufficient to reduce the O_2 concentration to below 0.1% of the air saturation level, i.e., below 0.25 μM. Oxygen concentration was measured with a YSI Model 53 oxygen monitor and a strip-chart recorder (Kip and Zonen BD-8) with a thermostated cell. The zero-oxygen level was ascertained by addition of excess sodium dithionite. It was demonstrated that DNA and AMT at the experimental concentrations had no inhibiting effect on O_2 removal.

Results

Absorbance of Laser Light. Because the laser pulse energies were measured after passing through the cell, the transmittance of AMT–DNA mixtures was measured as a function of pulse energy in order to determine what fraction of the light entering the cell was being measured. The results (Figure 1) shows little change in transmittance with pulse energy, indicating that the excitation is not being saturated in the range 0–160 mJ/cm^2 (equivalent to 0–11 MW/cm^2 average power over a 15-ns pulse). Indeed, the slight drop in transmittance with increasing pulse energy suggests that some species, perhaps AMT triplets, are being generated which absorb more strongly than the ground-state AMT.

"Single-Pulse" Cross-Linking: Possible Trivial Sources. Cross-linking as a function of energy density of single, 347-nm laser pulses is shown in Figure 2. Cross-linking is expressed as the relative height of the double-stranded DNA peak and represents roughly the weight fraction of total DNA which had at least one cross-link per molecule (uncorrected for nicking or hyperchromicity of single strands). The apparent

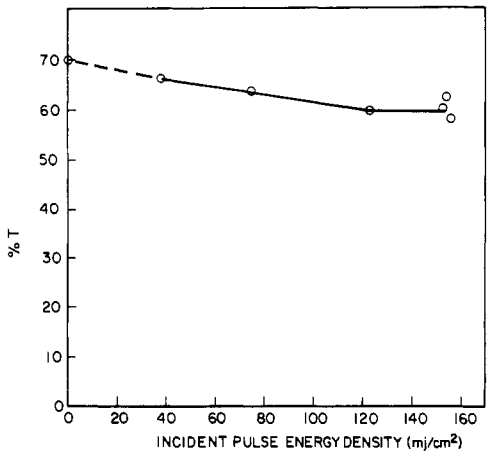

FIGURE 1: Transmittance of a sample containing 11.2 μg/mL AMT and 50 μg/mL DNA to 355-nm laser light pulses, 1-cm path length. The value at 0 pulse energy is measured in a spectrophotometer.

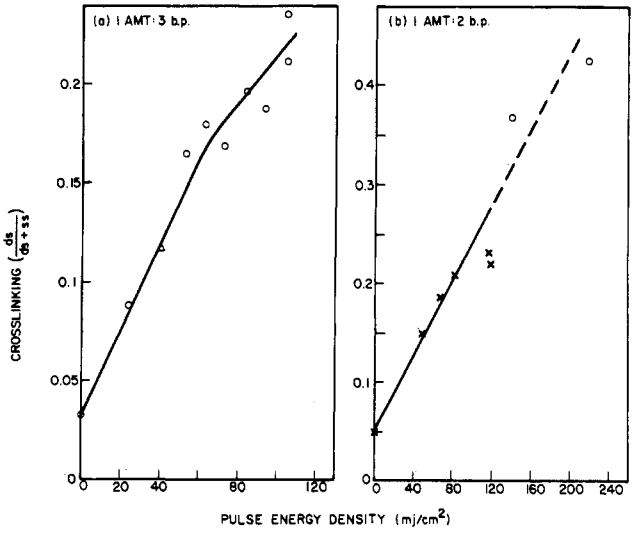

FIGURE 2: Cross-linking by single laser pulses (347-nm light) as a function of pulse energy, which is given as energy exiting from the rear of the cell. (Entering pulse energy can be calculated by using transmittance data in Figure 1.) (a) 11.2 μg/mL AMT and 75 μg/mL DNA (1 AMT/3 bp). (O) Air saturated; (Δ) 100% oxygen saturated. (b) 11.2 μg/mL AMT and 50 μg/mL DNA (1 AMT/2 bp). (×) and (O) are data from two separate runs.

cross-linking at 0 pulse energy is an artifact of the cross-link assay.

We initially suspected that the observed cross-linking with one laser pulse was due to an artifact of the optical system. No cross-linking was observed when the laser flashlamp was firing without any lasing occurring; hence, lingering stray light from the flashlamp was ruled out as a cause. The short-wave UV fourth harmonic of the Nd-YAG laser was of practically undetectable energy and in any case could not have found its way to the sample cell. Cross-linking due to long-lived fluorescence or phosphorescence from excited materials in the walls of the cell was considered unlikely, because all parts of the cell but the windows were masked from the beam, and except for the windows the cell was impregnated with a black material and showed no luminescence to the eye. Absorption by monoadducts of light emitted by AMT as fluorescence or phosphorescence after 1 μs is ruled out by the short singlet lifetime (2 ns; Song & Tapley, 1979; Poppe & Grossweiner, 1975) and the lack of phosphorescence of psoralens in aqueous solution.

Effect of Oxygen. To determine whether the single-pulse cross-linking was a result of psoralen sensitization of singlet

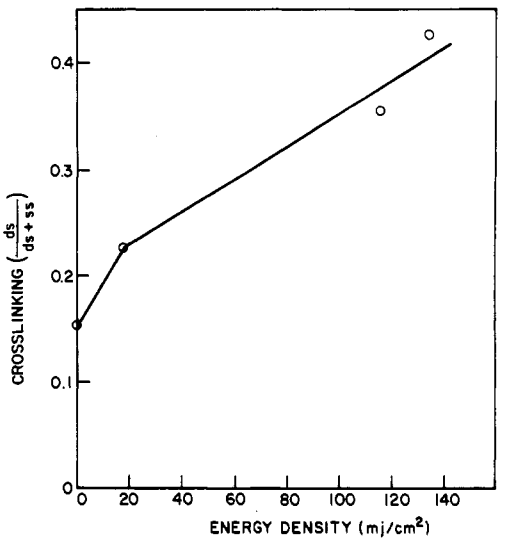

FIGURE 3: Cross-linking by single 355-nm laser pulses in the absence of oxygen. 11.2 μg/mL AMT and 50 μg/mL DNA (1 AMT/2 bp). Because of degradation of the DNA by the glucose oxidase–catalase system used to remove oxygen, the single- and double-stranded peaks from oxygen-free samples were not resolved, the ds peak showing up only as a shoulder. The ds and ss values (see Materials and Methods) used were the absorbances at the buoyant densities at which the two species normally band, and hence the value of ds for 0 pulse energy represents the absorbance due to single-stranded DNA which spread by diffusion to the ds buoyant density. Thus the ds/(ds + ss) values here cannot be compared to those in other figures in which the peaks are clearly resolved. The high apparent cross-linking at 0 intensity (an artifact of the cross-link assay) is unique to the enzyme-treated samples.

oxygen (Poppe & Grossweiner, 1975; Goyal & Grossweiner, 1979) or other oxygen-dependent effects, we deoxygenated samples of AMT and DNA (1 AMT/2 base pairs) by using a glucose oxidase–catalase system. The results, shown in Figure 3, demonstrate that the phenomenon remains, although partial degradation of the DNA during the enzyme treatment prevented a direct comparison with the data in Figure 2 to determine whether there was a component of the single-pulse cross-linking which was oxygen-dependent. The single point representing 100% oxygen saturation in Figure 2a further argues against an oxygen effect. Dependence of total addition (nearly all of it monoaddition) on single-pulse energy is the same at air saturation or 100% O_2 saturation (data not shown).

Effect of AMT to DNA Ratio. The cross-linking curves shown in Figure 2 for the two initial ratios of AMT to DNA base pairs, 1:2 and 1:3, are nearly superimposable. In fact, slight differences in the level of nicking of the DNA from one experiment to another make close comparison of overall cross-linking levels from one experiment to another difficult. To more clearly detect the effect of the AMT/DNA ratio on cross-linking, we compared the ratios 1:2 and 1:6 in a single experiment using the 355-nm light from the YAG laser. The results show little cross-linking enhancement of DNA loaded with drug beyond 1 AMT/6 bp (Figure 4).

Two conclusions can immediately be drawn from Figure 2: cross-linking increases linearly rather than quadratically with light dose, and the effect is not saturated at least up to a pulse energy of 200 mJ/cm². (The cross-linking curves in Figure 4, while not as linear as those in Figure 2, curve in a direction opposite from that expected for a quadratic dependence.) Fresher DNA preparations, having somewhat fewer nicks and double-strand breaks, show no more a quadratic light dependence than do preparations 9 times older. This indicates that the linearity is probably not an artifact arising from heterogeneity in DNA molecular weight, wherein larger fragments

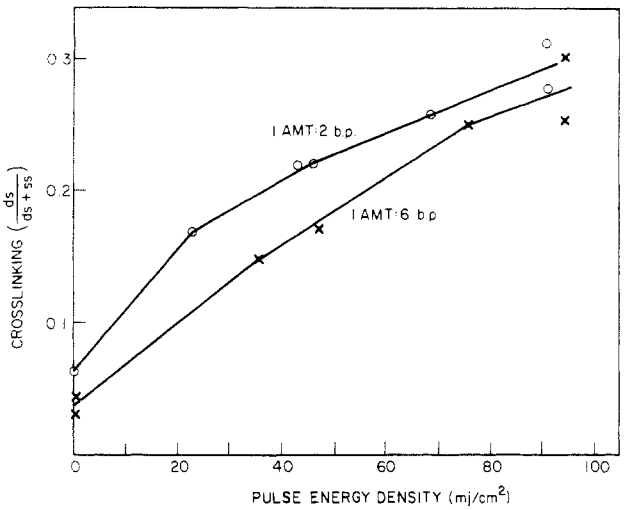

FIGURE 4: Dependence of cross-linking by single 355-nm laser pulses on pulse energy. (O) 11.2 μg/mL AMT and 50 μg/mL DNA (1 AMT/2 bp); (Δ) 11.2 μg/mL AMT and 150 μg/mL DNA (1 AMT/6 bp).

tend to cross-link first, enhancing the apparent cross-linking at low light levels.

Monoaddition. The dependence of total photoaddition on single laser pulse energy is shown in Figure 5. Because cross-links occur at ~1/300 kilobase pairs (kbp) (from Figures 2 and 4) while total addition is several adducts per kilobase pair at 100 mJ/cm², nearly all single-pulse adducts are monoadducts. The level of photoaddition at 1 AMT/2 bp (solution ratio) is about twice that at 1 AMT/6 bp, indicating that additional monoaddition sites become available at loadings above 1 AMT/6 bp. The fact that the monoaddition rate goes up by a factor of ~2 while the AMT/DNA ratio goes up by a factor of 3 suggests one of the following: (a) the additional intercalated sites at loadings beyond 1 AMT/6 bp are less photoefficient or have lower dark-binding constants than those reacting at lower loadings, (b) perhaps helix distortion which reduces photoreactivity is greater at higher loadings, or (c) energy transfer between neighboring psoralens reduces the photoefficiency at high loadings. At higher loadings, as seen in Figure 4, those additional bound sites are even less efficient at cross-linking by single laser pulses than they are at monoaddition.

It appears from Figure 5 that the 355-nm YAG laser light is more efficient at forming monoadducts than is the 347-nm ruby light. This is hard to understand considering the fact that absorption by AMT is somewhat higher at 347, at least at spectrophotometer light levels. The effect may be a result of "hot spots" which occur in the ruby laser beam; because of the lower slope in the photoaddition curve beyond ~40 mJ/cm² (Figure 5b), these hot spots would contribute less than a proportional amount to monoaddition. The curvature in both photoaddition plots below 40 mJ/cm² could make the single-pulse cross-linking curve appear linear in that region even if its light dependence were quadratic; however, this would not explain the linearity of cross-linking beyond 40 mJ/cm².

The YAG laser appears to be approximately as much more efficient than the ruby laser in producing "single-pulse" cross-links as it is in forming monoadducts; hence, the superior cross-linking efficiency may be due solely to superiority in the first photostep. In experiments in which a single, intense pulse was delivered, followed 10 s later by a second pulse of variable intensity, it appeared that ruby laser light was actually *more* efficient than YAG light at forming cross-links from a given number of monoadducts created during the first pulse. Indeed,

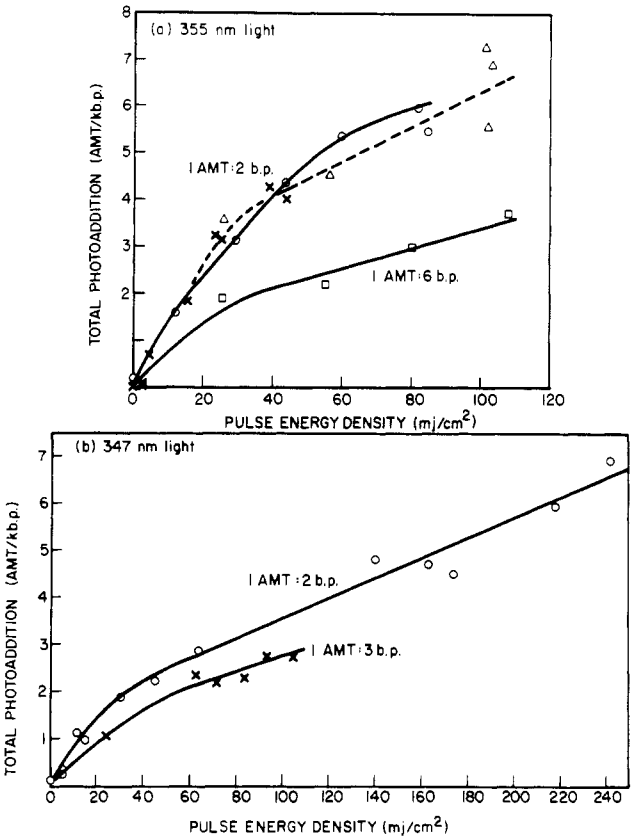

FIGURE 5: Dependence of total photoaddition on single pulse energy. (a) 355-nm light. (O) (Upper solid line) 11.2 μg/mL AMT and 50 μg/mL DNA; (×) (dashed line) 11.2 μg/mL AMT and 50 μg/mL DNA; (Δ) (also dashed line) 10.2 μg/mL AMT and 50 mL DNA; (□) 10.2 μg/mL AMT and 150 μg/mL DNA. (b) 347-nm light. (O) 11.2 μg/mL AMT and 50 μg/mL DNA; (×) 11.2 μg/mL AMT and 75 μg/mL DNA.

published absorption spectra of the 4′,5′ monoadduct show higher absorbance at 347 than at 355 nm (Musajo et al., 1967a).

Finally, we see in Figure 5 that monoaddition does not saturate at pulse energies up to 240 mJ/cm², equivalent to an intensity of 24 MW/cm² during the 10-ns pulse and corresponding to the absorption of ~8.4 photons/AMT molecule.

Discussion

The principal results described above can be summarized as follows: (a) some cross-linking of DNA by AMT occurs with single 10- or 15-ns laser pulses; (b) this cross-linking is not dependent on oxygen; (c) it depends linearly rather than quadratically on pulse energy; (d) neither cross-linking nor monoaddition by single pulses can be saturated at average light intensities up to 24 MW/cm². The last result implies that the singlet lifetime τ of intercalated psoralens must be shorter than ~0.7 ns, from the relation $I_{satd} = h\nu/2\sigma t$, where I_{satd} is the light intensity required for one-fourth of the psoralens to be in the excited state during the pulse, $h\nu$ is the photon energy, and σ is the absorption cross section of AMT. This upper limit on τ is smaller than the fluorescence lifetime of 1.8 ns for psoralen in ethanol (Song & Tapley, 1979; Poppe & Grossweiner, 1975), consistent with fact that the fluorescence yield of AMT drops upon addition of DNA (Salet et al., 1980; Johnston & Hearst, 1981).

Linear dependence of cross-linking on light fluence has been reported for low-intensity continuous irradiation of 4,5′,8-trimethylpsoralen with phage λ DNA in vitro (Cole, 1970), *E. coli* DNA in vivo (Ben-Hur et al., 1979), and Chinese

hamster DNA in vivo (Ben-Hur & Elkind, 1973). While the kinetics of the in vivo reactions could be distorted if the DNA after isolation were of heterogeneous molecular weight (as mentioned above), this would not be the case for λ DNA if shear breakage were avoided. On the other hand, Cassuto et al. (1977) reported psoralen photo-cross-linking of λ DNA, both isolated and in the phage, to increase with the second or third power of the light dose.

Ben-Hur & Elkind (1973) derived a relation for cross-link formation which approaches linearity for $k''/k' \geq 4$ by assuming that monoaddition was proportional to $(1 - e^{-k'It})\, e^{-k''It}$, where k' and k'' are the overall rate constants for the first and second photosteps

$$\mathrm{Ps} \xrightarrow[k']{h\nu_1} \mathrm{MA} \xrightarrow[k'']{h\nu_2} \mathrm{XL} \qquad (1)$$

and It is the light fluence. But the assumption about monoadduct kinetics is inaccurate, as shown below, and this explanation does not suffice.

Assuming no photoreversal, no photodestruction of intercalated psoralen, no light-saturation of the electron transitions, no net insertion of nonintercalated psoralen into the helix during irradiation (valid for strongly binding AMT and pulsed-laser irradiation, since intercalation requires ~1 ms), and considering only the proportion of monoadducts which under exhaustive irradiation would form cross-links, we can write

$$\frac{\mathrm{d[MA]}}{\mathrm{d}t} = k'I[\mathrm{Ps}] - k''I[\mathrm{MA}] \qquad (2)$$

and

$$[\mathrm{Ps}] = [\mathrm{Ps}]_0 e^{-k'It}$$

where $[\mathrm{Ps}]_0$ is the initial concentration of intercalated psoralen times the fraction which would form cross-links under exhaustive irradiation. The differential equation (eq 2) yields the solution

$$\frac{[\mathrm{MA}]}{[\mathrm{Ps}]_0} = \left(\frac{k'}{k'' - k'}\right)(e^{-k'It} - e^{-k''It}) \qquad (3)$$

whence

$$\frac{[\mathrm{XL}]}{[\mathrm{Ps}]_0} = 1 - \frac{[\mathrm{MA}] + [\mathrm{Ps}]}{[\mathrm{Ps}]_0} = 1 - \left(\frac{1}{k'' - k'}\right)(k''e^{-k'It} - k'e^{-k''It}) \qquad (4)$$

for $k'' \neq k'$. Expanding in a Taylor series to include the case $k'' = k'$

$$\frac{[\mathrm{XL}]}{[\mathrm{Ps}]_0} = \frac{k'k''}{2}(It)^2 - \frac{k'^2k'' + k'k''^2}{3!}(It)^3 + \ldots \qquad (5)$$

Equation 5 is plotted as a function of $k'It$ for $k'' = k'$ and $k'' = 10^6k'$ in Figure 6a. For $k'' \gg k'$, monoaddition is clearly the rate-limiting step; cross-linking is essentially linear at the outset. The region of Figure 6a which is applicable to the present data, where only a small fraction of the initial cross-linkable psoralens are cross-linked ($[\mathrm{XL}]/[\mathrm{Ps}]_0 \leq 10^{-5}$), is shown in Figure 6b, with the independent variable (here $k''It$) scaled by $(k'/k'')^{1/2}$ so that curves for greatly differing values of k'/k'' can be shown together. In this region of low light dosage, the first (quadratic) term of eq 5 is nearly indistinguishable from a plot of its first term $k'k''(It)^2$ for $10^{-2} < k'/k'' < 10^2$, and even for $k'' = 10^4k'$ the curvature of the

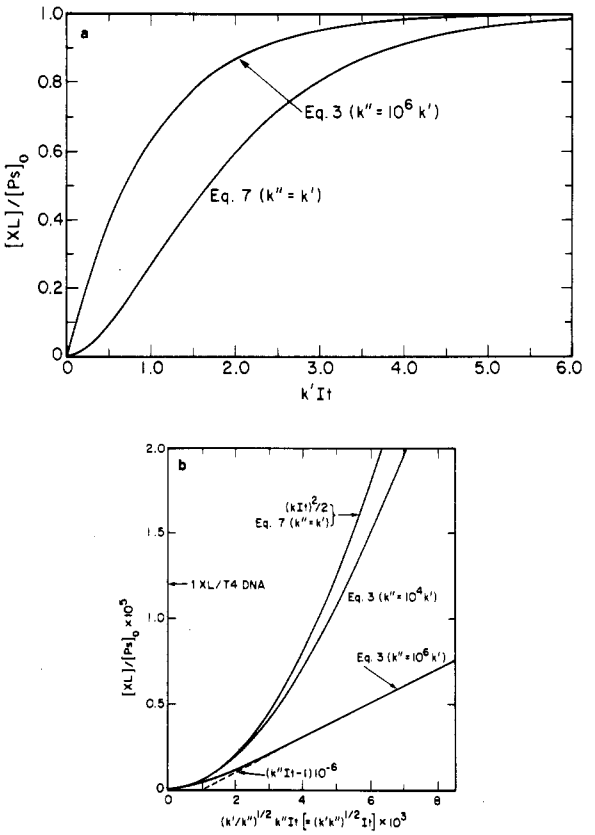

FIGURE 6: Theoretical plots of progress of cross-linking based on eq 5. (a) Total reaction for k' and k'' equal (monoaddition and cross-linking efficiency the same) and for $k'' = 10^6k'$, where the rate is controlled by k'. (b) The region near the origin of (a) expanded by a factor of 5×10^4 to show the shape of the curve at the level of cross-linking used in the presence experiments. Note that the horizontal axis in (b) is chosen so as to allow all three curves to appear on one graph. An arrow indicates the level of cross-linking which corresponds to one cross-link per intact T4 DNA molecule, or ds/(ds + ss) = 0.632, when $[\mathrm{Ps}]_0$ = [DNA bp]/2. The curves for eq 5 ($k'' = k'$) and $(kIt)^2/2$ superimpose. All axes are dimensionless.

plot is incompatible with the data of Figures 2 and 4. Only when the rate constants differ by a factor of 10^6 does the plot approach linearity for $(k'k'')^{1/2}It \geq 1.5 \times 10^{-3}$. In general

$$\frac{[\mathrm{XL}]}{[\mathrm{Ps}]_0} \cong k'It - \frac{k'}{k''} \qquad \left(\frac{k'}{k''} \lesssim 10^{-6}\right) \qquad (6a)$$

$$\frac{[\mathrm{XL}]}{[\mathrm{Ps}]_0} \cong k''It - \frac{k''}{k'} \qquad \left(\frac{k'}{k''} \gtrsim 10^{6}\right) \qquad (6b)$$

Thus, for cross-linking of high molecular weight DNA to appear linear with light fluence requires that the rate constants differ by many orders of magnitude rather than a factor of 4 as proposed by Ben-Hur & Elkind (1973).

Because a single pulse of laser light may yield six monoadducts but only ~0.003 cross-links per kbp, clearly for most cross-links the condition of eq 6a ($k'/k'' \leq 10^{-6}$) does not hold. In fact, Dall'Acqua et al. (1979) have calculated rate constants which give $k'/k'' = 0.0082$ for psoralen and 365-nm light. However, it may be that a small fraction of monoadducts can form cross-links very efficiently within the lifetime of the laser pulse; i.e., for those monoadducts $k'' \gg k'$ and the quantum yield for the second step is close to unity. Indeed, the initial quantum yield for monoaddition of 8-methoxypsoralen to DNA is much higher than the overall quantum yield (0.77 vs. 0.19, based on intercalated 8-MOP only; Ou et al., 1978), suggesting that some intercalated molecules can photoreact much more efficiently than others. If this were true for the

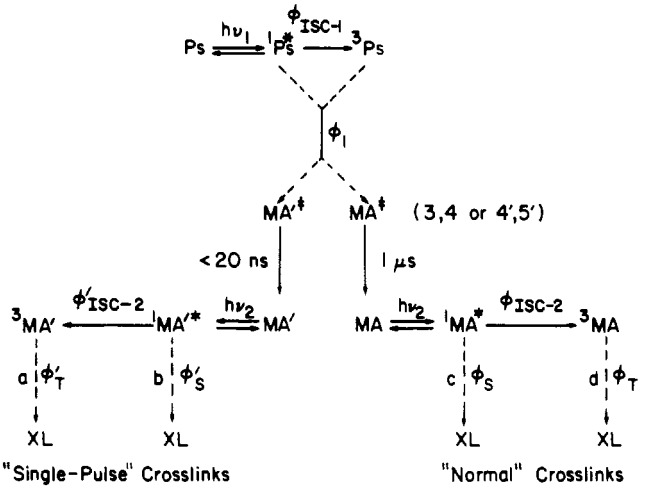

FIGURE 7: Proposed scheme showing dual photochemical pathways for generating single-pulse and normal cross-links. See text for details. Abbreviations: Ps, psoralen; MA, monoadduct; XL, cross-link.

second photoevent as well, it could explain both the linearity of the cross-linking plot and the fact that these monoadducts do not require a 1-μs time delay before they are capable of forming a cross-link.

Figure 7 shows such a scheme, with the question of whether each reaction proceeds via singlet or triplet intermediates left open (dashed lines). Here, two types of monoadducts can be formed from an excited (singlet or triplet) intercalated psoralen: one type (MA‡) must undergo some relaxation process, presumably a conformational change (to MA), before it is able to absorb a second photon and proceed to form a cross-link. This relaxation process requires ~1 μs and is discussed elsewhere (Johnston et al., 1980). The other type of monoadduct (MA′‡) is capable of further photoreaction to become a cross-link very soon after its formation; i.e., MA′‡ → MA′ requires <20–30 ns if indeed there is any difference between MA′‡ and MA′ at all.

An alternative explanation for the linearity of single-pulse cross-linking arises if one of the conditions assumed in writing eq 2, that the electronic transitions are not light saturated, does not hold. If either photostep is light-saturated, then the rate of cross-linking will be determined by the other step, and overall cross-linking will be linear in light dose. It cannot be the first step which is saturated, otherwise the monoaddition curves (Figure 5) would have leveled out at relatively low intensities. Moreover, we have observed a linear dependence of cross-linking by two pulses on the energy of the second pulse (up to at least 125 mJ/cm²) for both 355- and 347-nm light where the energy of the first pulse is kept constant. This implies that, for "normal" (not "single pulse") cross-linking, the second photoreaction is not saturated at pulse energies up to at least 125 mJ/cm². However, the excited singlet lifetime of those monoadducts which can be cross-linked with a single laser pulse (MA′) might be long-lived enough that the MA′ → ^{1}MA′* transition is being saturated by the pulse. Indeed, the 4′,5′ monoadduct analogue 5,7-dimethoxycoumarin has a fluorescence lifetime of 7.2 ns, nearly as long as the laser pulse width (Ou et al., 1978).

At 29 mJ/cm² every AMT absorbs on the average one photon and if the monoadduct extinction coefficient is the same as for AMT, saturation is only possible above 29 mJ/cm². However, curvature in the cross-linking curves (Figures 2 and 4) below 30 mJ/cm² might not be apparent, because of the dearth of data points in that region.

If single-pulse cross-linking proceeded via the triplet state (path a in Figure 7) and if the intersystem crossing yield ϕ_{isc-2} were high enough and the lifetime of ^{3}MA′ long enough that all the MA′-type monoadducts were in the triplet state by the end of pulse, even for pulse energies as low as 30 mJ/cm², then cross-linking would also be linear in intensity, the rate being controlled by the first photoreaction.

It is conceivable that "single-pulse" cross-linking is actually a one-photon reaction, in which case the linear dependence on laser energy is exactly what would be expected. A single excited psoralen might assume a biradical configuration in which the two ends of the molecule could react independently, without absorption of a second photon [cf. discussion of biradicaloid geometries in photochemistry by Michl (1977)].

Given the different characteristics of normal and single-pulse cross-linking, it is tempting to speculate that one pathway involves the monoadduct triplet state and the other the singlet. It may also be that the two types of monoadducts differ by which end of the psoralen is photoreacted. However, the absorption spectrum of the putative 3,4 monoadduct, which shows practically no absorbance beyond 330 nm (Krauch et al., 1967; Musajo et al., 1967b), suggests that only the 4′,5′ monoadduct can be a precursor to a cross-link for 347–355-nm light, and it has recently been shown that for 4′-(hydroxymethyl)-4,5′,8-trimethylpsoralen under specified conditions some 95% of the monoadducts produced in DNA are of the 4′,5′ type (Straub et al., 1981).

In view of these difficulties with the 3,4 adduct as precursor, we suggest that there is a difference between psoralens which become "single-pulse cross-links" and those which become "normal cross-links" and that the difference lies in the geometry of the intercalation site. Intercalation complexes which are in a conformation that permits a cross-link to form without a 1-μs delay may be distinguished by characteristics of their base sequence or because random motions of the DNA have placed certain of them in that conformation at the time the light pulse comes.

A number of recent findings support the picture that psoralen photoreactivity is affected by base composition and sequence: (a) Total photoreactivity as well as cross-linking of at least some derivatives depends upon DNA base composition and is inhibited by netropsin, which binds specifically to A,-T-rich regions (Dall'Acqua et al., 1978; Lown & Sim, 1978). (b) Comparing Figures 4 and 5a, we see that intercalation sites for single-pulse cross-linking are nearly saturated at 1 AMT/6 bp, while monoaddition increases by a factor of ~2 in going from 1 AMT/6 bp to 1 AMT/2 bp. This suggests that the strongest dark-binding sites are those mainly responsible for single-pulse cross-linking (and perhaps ordinary cross-linking as well). (c) The fact that angelicin reportedly forms cross-links with low efficienty (Lown & Sim, 1978) suggests the possibility that certain angelicin–DNA intercalation sites are in an unusual conformation which brings both reactive double bonds of the angelicin close enough to photoreact with pyrimidines from opposite DNA strands, while at normal sites the angular shape of the molecule would prevent such close approach. Netropsin increases cross-linking of angelicin (Lown & Sim, 1978), perhaps by helping stabilize the unusual conformation. (d) Finally, the discovery that $d(C\text{-}G)_3 \cdot d(C\text{-}G)_3$ can form the novel "Z" structure (Wang et al., 1979) raises the possibility that, without gross perturbation from bound molecules, DNA itself can form stable alternative structures dependent on base sequence. Because all these experiments were done on T4 DNA, we have not excluded the possibility that one of the two putative types of cross-linking sites is characterized by the presence of glucosylated hydroxymethylcytosine near the site or by the type of glucosylation.

Conclusion

We conclude that there are two pathways for forming cross-links. In one, a monoadduct intermediate must experience a conformational change in its surrounding DNA before it can productively absorb a second photon and form a cross-link. In the other pathway, no such conformational change is necessary, or if one is required, it takes place within the lifetime of a 10-ns laser pulse, instead of requiring the 1.3 μs characteristic of the first pathway. The monophotonic behavior of cross-linking via the second pathway may result from very efficient cross-linking of a few monoadducts ($k'' \gg k'$), from light saturation of the MA′ → XL photoreaction, or from the reaction actually requiring only a single photon. The two pathways probably correspond to different populations of intercalated psoralens, distinguished by local base sequence or perhaps by random flexing of the DNA at the site.

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Geometry of the Phosphodiester Backbone in the A Form of Deoxyribonucleic Acid Determined by Phosphorus-31 Nuclear Magnetic Resonance Spectroscopy†

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ABSTRACT: ^{31}P NMR spectra were obtained from poly(dA-dT)·poly(dA-dT) fibers which gave an X-ray diffraction pattern similar to that of the A form of natural deoxyribonucleic acid (DNA). The analysis of the line shape indicated that the A form of poly(dA-dT)·poly(dA-dT) has a single uniform backbone conformation; the orientation of the phosphodiester group relative to the helical axis was determined to be $\beta = 70°$ and $\gamma = 52°$. The ^{31}P NMR spectra of poly(dA-dT)·poly(dA-dT) were in remarkable contrast to the ^{31}P NMR spectra of the A form of natural DNA, which exhibited an unusual line shape. The origins of the abnormalities in the line shape for the A form of natural DNA are discussed in terms of phosphodiester orientations.

It has been shown that the ^{31}P NMR characteristics of deoxyribonucleic acid (DNA) in solution (Shindo et al., 1979; Simpson & Shindo, 1980) are essentially identical with those of B form DNA fibers at high relative humidity (98%) (Shindo et al., 1980; Shindo & Zimmerman, 1980) and that both can be consistently interpreted in terms of a nonuniform backbone conformation. The nonuniformity of the backbone conformation has several important implications. For example, any single conformation used to characterize natural DNA must be an average conformation, and, further, that irregularity in conformation may well be base-sequence dependent.

We have extended these studies to the A form of DNA, which can generally be induced in fibers containing appropriate

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