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Kinetics of Formation of Deoxyribonucleic Acid Cross-Links by 4′-(Aminomethyl)-4,5′,8-trimethylpsoralen†

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ABSTRACT: If a mixture of T4 deoxyribonucleic acid (DNA) and 4′-(aminomethyl)-4,5′,8-trimethylpsoralen is irradiated with two closely spaced pulses of long-wave UV laser light, the resulting cross-linking is dependent on the time delay between the pulses. As the delay lengthens to 1 μs, a rise in the number of cross-links is observed which follows first-order kinetics. This delay, the time required for most monoadducts to be able to absorb a second photon and thereupon form a cross-link, is interpreted in terms of a conformational change in the DNA at the psoralen intercalation site which may occur upon monoadduct formation.

Psoralens are planar heterocyclic molecules (furocoumarins) which photosensitize skin erythema and are used to treat psoriasis and vitiligo. In addition to this clinical utility, they have been used to locate nuclease-protected regions of deoxyribonucleic acid (DNA) in chromatin and regions of secondary structure in single-stranded DNA and ribonucleic acid (RNA), to study DNA repair mechanisms, and in other applications [for recent reviews, see Scott et al. (1976), Song & Tapley (1979), and Hearst (1979)]. The chemical and at least a part of the biological activity of psoralens results from their ability to intercalate between the base pairs of nucleic acids and, upon absorption of long-wave UV light (320–380 nm), form mono- and diadducts to pyrimidine bases.

A good deal of evidence (Musajo et al., 1967a, b; Krauch et al., 1967) suggests that monoaddition involves the formation of a cyclobutane bridge between the 5,6 double bond of a pyrimidine and either the 4′,5′ or the 3,4 double bonds of psoralen. Photoreaction at both ends of the psoralen forms

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an interstrand cross-link. Recently, some photoreaction with purines has been reported: photoaddition to adenine in transfer RNA (tRNA) (Ou & Song, 1978) and cross-linking of poly(inosinic acid)·poly(cytidylic acid) (Hochkeppel & Gordon, 1979).

In addition to the importance of characterizing the psoralen–DNA photoreaction as a clinical and biochemical tool, this system has intrinsic interest because the juxtaposition of the reactants is constrained by the structure of the intercalation complex. Hence, the kinetics of the reaction depend on that structure and its flexibility, which in turn are altered by the reaction.

In previous work (Johnston et al., 1977) we described the use of single, 15-ns pulses of UV light from a frequency-doubled ruby laser to generate psoralen–DNA monoadducts in a mixture of T4 DNA and 4′-(aminomethyl)-4,5′,8-trimethylpsoralen (AMT) without significant production of cross-links. The latter are formed only with additional pulses of light. This result was interpreted in terms of a model in which cross-links can only be formed via the absorption of a photon by an appropriate monoadduct. Such a monoadduct appears only after a delay (long compared to the laser pulse width) following the initial absorption of a photon by AMT, and the delay represents the time required for the excited AMT to form a monoadduct capable of further photoreacting.

In the present paper we report measurements of the required time delay and put forth a possible interpretation in which, after formation of the initial cyclobutane bridge, the DNA must undergo a conformational change prior to the second step of cross-link formation. In a separate paper (Johnston & Hearst, 1981), we report conditions under which some cross-links are formed by a single pulse of laser light as short as 10 ns and suggest that there are two pathways for cross-link formation, one requiring the measured time delay and the other requiring no delay longer than 10 ns. To measure the time delay, we have subjected samples containing T4 DNA and AMT to two laser pulses separated by a variable time interval and monitored cross-linking as a function of the time between pulses.

Materials and Methods

Growth of T4 phage on a ligase-overproducing host, isolation of DNA, measurement of total photoaddition of [^{3}H]AMT to DNA, and assay for cross-links are described separately (Johnston & Hearst, 1981). Briefly, the cross-link assay involves alkali denaturing and renaturing the DNA under conditions which minimize nicking and haphazard base re-pairing while maximizing strand separation in un-cross-linked molecules. Single- and double-stranded molecules are resolved by banding in CsCl in an analytical ultracentrifuge.

Laser Irradiation. The experimental setup for delivering two laser pulses to the sample is shown schematically in Figure 1. A small cylindrical cell (3-mm diameter × 10-mm path length, Hellma 178-QS) was placed in the path of two laser beams entering the quartz windows coaxially from opposite directions. One (the first pulse) was the 354.7-nm third harmonic of a neodymium-YAG laser (Raytheon Model SS-404), delivering ~16 mJ in 15-ns pulses, and the other (the second pulse) was from a focused nitrogen laser (Molectron UV 1000) delivering ~3 mJ of 337.1-nm light in 10-ns pulses, each beam completely filling the cell. By the triggering of one laser from the other using a variable electronic time delay, separations of the two pulses could be achieved from nanoseconds up to 100 ms.

Because laser output is higher in a repeating pulse mode, single pulses from each laser were selected from a train of

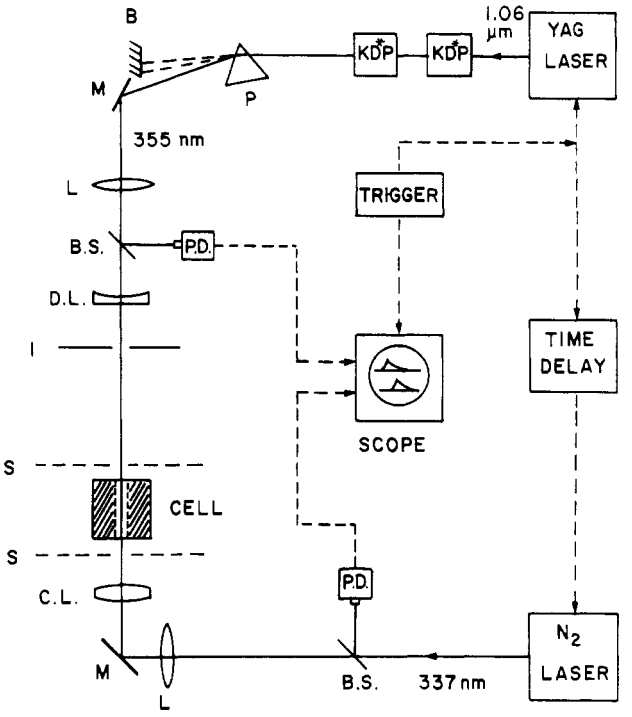

FIGURE 1: Schematic diagram of apparatus for two-pulse experiment. The light path is a solid line and electrical connections are dotted lines. KD*P, frequency-doubling crystals; M, plane mirrors; L, converging lens; BS, beam splitters; DL, diverging lens; I, iris; S, hand-operated shutter for selecting single pulses from each laser; CL, cylindrical lens; PD, photodiodes; P, prism; B, a block to remove 1060- and 530-nm wavelengths. Distances are not to scale.

pulses 0.37 s apart by manually lifting cardboard baffles on either side of the sample cell. Each pair of pulses in the train was separated by the chosen time delay. In cases where the time delay was longer than 0.1 s, the lasers were fired independently. Each beam was focused by using two quartz lenses; for the nitrogen laser one lens was cylindrical, to focus the rectangular beam to a square. The energies of individual pulses were monitored by using photodiodes and a dual-beam oscilloscope, which also served to measure the time delay. All manipulations of the DNA–AMT mixtures before and after irradiation were performed under safelights (Kodak OA and 1A filters), and the samples were carefully shielded from stray laser light during irradiations. It was found that samples left exposed to normal room fluorescent lights for 20 min become cross-linked to a degree comparable to that achieved by two well-spaced laser pulses.

Results

Representative ultracentrifuge banding patterns as a function of the time delay between the two laser pulses are shown in Figure 2. The degree of cross-linking was characterized by the ratio ds/(ds + ss), where ss is the height of the single-stranded peak and ds is the height of the curve measured at the position where double-stranded DNA bands. These values are plotted as a function of time delay in Figure 3. A distinct rise in the level of cross-linking is seen at a delay of ~1 μs. A plot of log [0.423 − ds/(ds + ss)] vs. time delay, where 0.423 is the plateau level of ds/(ds + ss) for long time delays, gives a straight line (Figure 4), indicating a first-order process. The slope of this line yields a rate constant of (7.9 ± 2.2) × 10^5 s^{-1}, or a $1/e$ lifetime of ~1.3 ± 0.3 μs.

The dotted portion of Figure 3 which intercepts the Δt = 0 axis is an extrapolation based on Figure 4. The level of cross-linking due to a single pulse (0.188) is much higher than one-half the extrapolated value for two simultaneous pulses

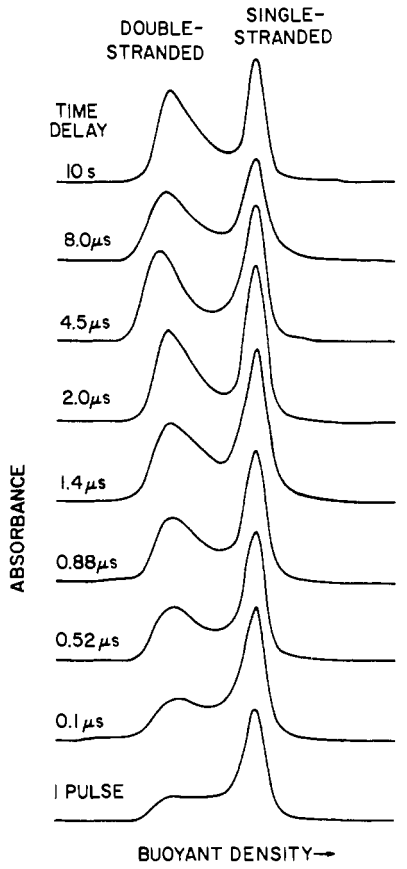

FIGURE 2: Representative ultracentrifuge traces, showing the effect on cross-linking of one pulse (Nd-YAG laser only) and different delays between two pulses. Traces are not normalized to total absorbance nor are they corrected for laser intensity.

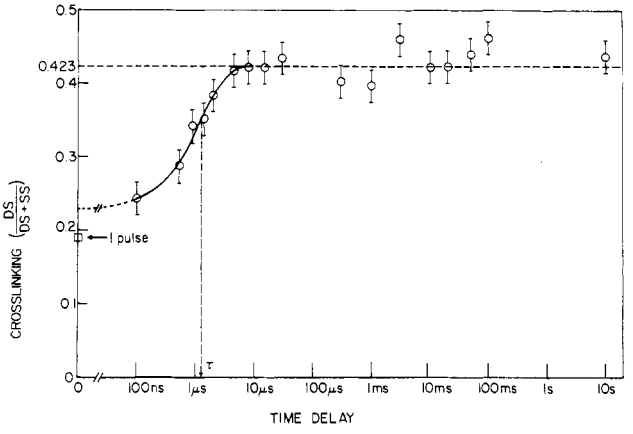

FIGURE 3: Cross-linking as a function of time delay between the two laser pulses, corrected for fluctuations in Nd-YAG laser intensity by using calibration curves showing the dependence of solution transmittance and quantum yield of monoaddition on intensity (M. A. Johnson, B. H. Johnston, and J. E. Hearst, unpublished data; Johnston & Hearst, 1981). Cross-linking is measured as the ratio ds/(ds + ss), where ds and ss are the peak heights of double- and single-stranded DNA peaks in the ultracentrifuge tracing. The dotted extension between 0 and 100 ns is an extrapolation based on the plot in Figure 4. For comparison, the cross-linking due to a single pulse is also shown (□). The value for τ, the time for $1/e$ of the maximum change in cross-linking, is determined from Figure 4. Error bars show the standard deviation of the cross-linking level of two to six separate centrifuge runs for each time interval.

(0.228) mainly because the first pulse contains much more energy than the second.

Discussion

It is clear that there is some first-order relaxation process

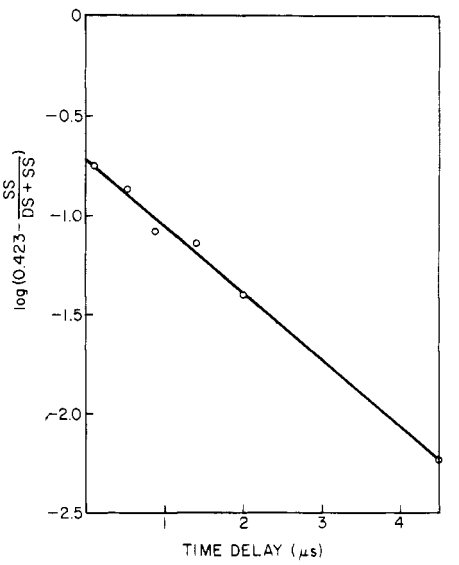

FIGURE 4: Short time delay data from Figure 3, plotting log [0.423 − ds/(ds + ss)] against t on a linear scale, where 0.423 is the plateau level of cross-linking. From the slope of this plot a first-order rate constant of 7.9×10^5 s^{-1} (τ = 1.3 μs) is derived.

following the first laser flash which yields a considerable increase in the number of species that, upon subsequent absorption of light, can form a cross-link. The 1-μs time scale rules out an excited singlet AMT as the intermediate responsible for the delay, as singlet (π, π^*) lifetimes for psoralens are ~2 ns (Song & Tapley, 1979; Poppe & Grossweiner, 1975). It is conceivable that the delay represents the time for reaction from a triplet state; AMT has a triplet lifetime in water of 100 μs (Salet et al., 1980); however, the triplet seems to be quenched when bound to DNA (Beaumont et al., 1979). Indeed, thymine quenches triplet AMT with a rate constant of 2×10^8 M^{-1} s^{-1} (E. J. Land, personal communication), and the effective concentration of thymine for intercalated AMT is likely to be high enough to reduce the triplet lifetime to considerably less than 1 μs. Quenching by cytosine and adenine, while less rapid than by thymine in the case of psoralen and angelicin (Bensasson et al., 1978), is still likely to reduce the lifetime to below 1 μs. Guanosine is as effective a quencher of triplet psoralen as thymine (Bensasson et al., 1978). Moreover, Beaumont et al. (1979) found no transient triplet absorption from intercalated psoralen after photolysis at 347 nm.

Indeed, there is some evidence that monoaddition at the 4′,5′ position of psoralen may not involve a triplet state at all: (a) 5-methoxypsoralen has a very low intersystem crossing yield in water (Bensasson et al., 1978) yet is photoreactive and a fairly good skin photosensitizer (Rodighiero et al., 1969), and (b) molecular orbital calculations indicate reactivity of the psoralen triplet is higher at the 3,4 position than at the 4′,5′ position (Song et al., 1971). Lack of absorbance above 330 nm by the 3,4 monoadduct (Musajo et al., 1967a; Krauch et al., 1967) suggests that the 4′,5′ monoadduct is the precursor of cross-links, although Chatterjee & Cantor (1978) have argued for the 3,4 adduct.

In view of the difficulties of explaining the delay as due to a long-lived excited psoralen, we suggest that a conformational change in the DNA in the region of the monadduct may be involved. After formation of the initial cyclobutane bridge, some movement could be required to achieve the alignment of bonds between the unreacted end of the psoralen and the second pyrimidine base needed for completion of the cross-link. Indeed, freezing during irradiation suppresses cross-linking

but permits monoaddition (mainly 4',5' adducts; Lown & Sim, 1978). Circular dichroism changes during irradiation of DNA–xanthotoxin mixtures also support the hypothesis of a DNA conformational change upon photoaddition (Kittler & Zimmer, 1976) and, if such a change occurred with a 1.3-μs relaxation time, it would explain the results shown in Figure 3.

From temperature-jump and stopped-flow studies, Pörschke & Eigen (1971) have calculated the time required for formation of a new base pair at the end of a helical region of oligo(A)–oligo(U) to be 0.1 μs, while Craig et al. (1971) calculate a value of between 0.05 and 1 μs. Pörschke (1973) has measured relaxation times of 0.1–1 μs in temperature-jump studies of single-stranded poly(A). Thus the time we measure is on the order required for formation of a base pair or for the single-strand helix-to-coil transition, suggesting that similar motions may be taking place following formation of a monoadduct. This is not unreasonable, since the formation of a cyclobutane bridge removes some of the conjugation and planarity of both reacting species and introduces extra angle strain as long as the psoralen and pyrimidine moieties remain parallel. This strain, in the dihedral psoralen–cyclobutane and cyclobutane–pyrimidine angles, if comparable to the cyclobutane ring strain, would be on the order of 26 kcal/mol (Karapet'yants & Karapet'yants, 1970). The stacking energies for complementary oligoribonucleotides are between 6 and 15 kcal/mol per added base pair (Borer et al., 1974). Stacking interactions would probably be weaker than this for the partially saturated psoralen and pyrimidine moieties; hence, it is likely that a conformational change involving some unstacking will occur in the region of the psoralen immediately after photoreaction, in order to relieve angle strain. We propose that the time delay we observe is due to such a conformational change and that this change is usually necessary to align the other end of the psoralen with a new pyrimidine before a cross-link can form.

Applying the relation from transition-state theory (Moore, 1972)

$$k_m = (kT/\hbar)e^{-\Delta G^{\ddagger}/RT}$$

where k_m is the observed rate constant (7.9×10^5 s^{-1}), $\Delta G^{\ddagger}$ is the free energy of activation for the putative conformational change, k is Boltzmann's constant, $\hbar$ is Planck's constant, R is the gas constant, and T is the temperature, we find $\Delta G^{\ddagger}$ = 9.2 kcal/mol. The enthalpy contributions to $\Delta G^{\ddagger}$ ($=\Delta H^{\ddagger} - T\Delta S^{\ddagger}$) may include stacking energy where stacking is disrupted, rotational barriers around single bonds of the DNA backbone, and possibly changes in hydrogen bonding. If a change in the ribose ring pucker is involved, as occurs in the process of intercalation according to the X-ray structures of a number of complexes between intercalating dyes and dinucleotides (Wang et al., 1978; Sakore et al., 1977; Tsai et al., 1977), then that would also contribute an enthalpy barrier. Röder et al. (1975), using ^{13}C NMR measurements, calculate activation barriers of 4.7 ± 0.5 kcal for purine nucleosides and possibly 6–10 kcal for pyrimidine nucleosides. In a purely theoretical treatment, Levitt & Warshel (1978) have calculated a barrier of only 0.5 kcal for this motion, which can be achieved by pseudorotation.

As can be seen in Figure 3, not all the psoralen molecules capable of cross-linking require the 1-μs time delay. The cross-linking resulting from a single pulse of light we ascribe to intercalation complexes which, because of their nucleotide sequence or other special condition, do not require a large conformational change before cross-linking can occur. This is discussed in a separate paper (Johnston & Hearst, 1981).

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