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Base-Catalyzed Reversal of a Psoralen–DNA Cross-Link†

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ABSTRACT: Base-catalyzed reversal of a psoralen–DNA cross-link has been observed under denaturing alkaline conditions at elevated temperatures. The cross-link was formed between 4′-(hydroxymethyl)-4,5′,8-trimethylpsoralen and the two thymidine residues (T) on opposite strands of the double-stranded DNA formed from the self-complementary oligonucleotide 5′-GGGTACCC-3′. In contrast to the photoreversal of the cross-link, which yields mostly the furan-side monoadducted oligonucleotide [Cimino, G. D., Shi, Y., & Hearst, J. E. (1986) *Biochemistry 25*, 3013-3020], base-catalyzed reversal of the cross-link yields only pyrone-side monoadducted oligonucleotides as identified on the basis of their mobilities on a 20% polyacrylamide–7 M urea gel and their chemical and photochemical properties. A mechanism has been proposed to explain the base-catalyzed reversal reaction. This observation suggests a way to make pyrone-side monoadducted DNA. It also suggests that caution must be taken when psoralen-adducted DNA is treated under denaturing alkaline conditions.

The widespread use of psoralen in the medical and biological fields (Song & Tapley, 1979; Parson, 1980; Fitzpatrick et al., 1982; Parrish et al., 1982; Cimino et al., 1985) has promoted detailed investigation of the photochemistry between psoralen and nucleic acids. Psoralens can intercalate between base pairs of double-stranded nucleic acids. Upon near-UV (320–380-nm) irradiation, the intercalated psoralens form a set of well-characterized adducts with pyrimidine bases (Straub et al., 1981; Kanne et al., 1982a,b; Peckler et al., 1982). The first step of the photoreaction yields either a furan-side monoadduct or a pyrone-side monoadduct, depending upon whether the 4′,5′-double bond of the furan ring or the 3,4-double bond of the pyrone ring of the intercalated psoralen photoreacts with the 5,6-double bond of a pyrimidine residue. The furan-side monoadduct can be driven into an interstrand diadduct upon absorption of a second photon if there is an adjacent pyrimidine residue located on the other strand available for photoreaction. Under this irradiation condition, the pyrone-side monoadduct cannot be converted into a diadduct since it does not absorb light in the 320–380-nm wavelength region.

We have previously reported the wavelength dependencies for the photoreactions of the diadduct and the monoadducts formed between the psoralen derivative (HMT)[1] 4′-(hydroxymethyl)-4,5′,8-trimethylpsoralen and thymidine (T) and these adducts in deoxyoligonucleotides (Cimino et al., 1986; Shi & Hearst, 1987a,b). We found that photoreversal of the cross-link in a double-stranded oligonucleotide yields mostly the furan-side monoadducted oligonucleotide. The pyrone-side monoadducted oligonucleotide is a minor product of the photoreaction. The pyrone-side monoadducted oligonucleotide exists as either the pyrone ring opened form or the pyrone ring closed form as revealed by analysis of the products on a denaturing polyacrylamide gel. While investigating the pH

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[1] Abbreviations: HMT, 4′-(hydroxymethyl)-4,5′,8-trimethylpsoralen; ATP, adenosine 5′-triphosphate; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; XL, HMT–DNA cross-link; T–HMT–T, thymidine–HMT–thymidine diadduct; C-M_{Py}, pyrone ring closed form of the pyrone-side monoadducted DNA; O-M_{Py}, pyrone ring opened form of the pyrone-side monoadducted DNA; DNase, deoxyribonuclease; HPLC, high-performance liquid chromatography.

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dependence of the photoreversal of the cross-link, we found that under certain alkaline conditions the cross-link could undergo reversal without exposure to actinic UV light to produce the unmodified DNA and the pyrone ring opened and closed forms of the pyrone-side monoadducted DNA. We report here a more detailed study of this base-catalyzed reversal reaction of the cross-link. We have found that, in contrast to photoreversal, base-catalyzed reversal of the cross-link yields only the pyrone-side monoadducted and unmodified DNA. No furan-side monoadducted DNA was detected under the base-catalyzed reversal conditions.

Materials and Methods

Materials. HMT and [^{3}H]HMT were gifts from HRI Associates Inc. (Berkeley, CA). The oligonucleotide 5′-GGGTACCC-3′ was synthesized on an automated DNA synthesizer. After synthesis, the oligonucleotide was deprotected in concentrated ammonia solution at 55 °C for 5 h and purified by electrophoresis on a 20% polyacrylamide–7 M urea gel followed by EtOH precipitation. The purification gel (40 cm × 40 cm × 0.12 cm) had a composition of 30:1 acrylamide/bis(acrylamide) and was run at 45 W with an aluminum plate clamped on the gel plate. [γ-^{32}P]ATP was purchased from Amersham. T4 polynucleotide kinase was bought from Bethesda Research Laboratories.

Isolation of DNA from Polyacrylamide Gels. DNA bands on a gel were identified by autoradiography and excised, and the DNA was eluted into a solution of 50 mM NaCl and 1 mM EDTA. The DNA solutions were then adjusted to 0.2 M NaCl and 10 mM $MgCl_2$, followed by precipitation with 2.5 volumes of EtOH.

Preparation of HMT–DNA Cross-Link. (A) 5′-^{32}P-Labeled HMT–DNA Cross-Link. The cross-link (1–20 μg) was prepared as described by Cimino et al. (1986) and stored frozen in 1 mM EDTA.

(B) 5′-^{32}P-Labeled [^{3}H]HMT–DNA Cross-Link. Large amounts of [^{3}H]HMT–DNA cross-link were required for the HPLC analysis of the products of the base-catalyzed reversal. For this purpose, 560 μg of the self-complementary oligonucleotide 5′-GGGTACCC-3′ was 5′-^{32}P-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase followed by a chase of the kinase reaction with unlabeled ATP to phosphorylate all the 5′-ends (Shi & Hearst, 1986). The labeled DNA was then irradiated with 320–380-nm light in the presence of 0.15 mM [^{3}H]HMT (1.2 Ci/mmol) according to the procedures of Shi and Hearst (1986). The cross-linked DNA was separated from the unmodified DNA by electrophoresing the sample on a 20% polyacrylamide–7 M urea gel. This gel and all other analytical gels in this work were identical with the purification gel described earlier except the thickness was 0.05 cm. The cross-link was isolated from the gel . Two hundred thirty micrograms of the ^{3}H-labeled cross-link was thus generated.

Base-Catalyzed Reversal of the HMT–DNA Cross-Link. The base-catalyzed reversal of the cross-link was performed under a variety of conditions in the absence of UV light to avoid the possibility of photochemistry. Appreciable reversal of the cross-link was observed at 37 °C and pH 12 (1 mM Na_3PO_4), at 60 °C and pH 10.5 (10 mM Na_3BO_3), and at 60 °C and pH 10.0 (10 mM Na_3BO_3) in the presence of 3 M urea. Following the incubations at alkaline pH and elevated temperatures, the cross-link samples were adjusted to pH 4–5.5 with 0.2 M succinate (pH 4.0) with or without further incubation at 37 °C. The samples were then adjusted to 0.2 M NaCl, 10 mM NaCl, and 40 μg/mL carrier tRNA followed by precipitation with 2.5 volumes of EtOH. The products were then analyzed by electrophoresis on a denaturing polyacrylamide gel.

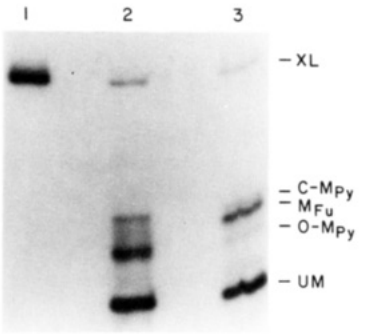

FIGURE 1: Base-catalyzed reversal and photoreversal of the HMT–DNA cross-link. The HMT–DNA cross-link, after EtOH precipitation, was dissolved in 700 μL of 10 mM $NaBO_3$ and 3 M urea, pH 10.1. Lane 1: Cross-link solution (130 μL) kept in the dark at room temperature as the control. Lane 2: Cross-link solution (350 μL) incubated at 60 °C for 2 h. Lane 3: Cross-link solution (220 μL) photoreversed with 254-nm light from a low-pressure germicidal lamp for 5 min at a distance of 2.5 inches from the lamp. After treatment, all samples were adjusted to ca. pH 4.5 with 0.2 M succinate (pH 4), EtOH precipitated in the presence of carrier tRNA, and then analyzed on a polyacrylamide–urea gel. Abbreviations: XL, HMT–DNA crosslink; M_{Fu}, furan-side monoadducted oligonucleotide; C-M_{Py}, pyrone ring closed form of the pyrone-side monoadducted oligonucleotide; O-M_{Py}, pyrone ring opened form of the pyrone-side monoadducted oligonucleotide; UM, unmodified oligonucleotide.

HPLC Analysis of Products of the Base-Catalyzed Reversal. EtOH-precipitated ^{3}H-labeled cross-linked oligonucleotide (230 μg) was dissolved in 1.5 mL of 10 mM Na_3BO_3 and 3 M urea, pH 10.5, split into six 1.5-mL Eppendorf tubes, and incubated at 65 °C for 2 h. One hundred microliters of 0.2 M succinate (pH 4.0) was then added to each tube, and the mixture was incubated at 37 °C for 1.5 h. The DNA was EtOH precipitated and electrophoresed on a polyacrylamide gel. The products were isolated and digested to nucleosides by sequential treatment with DNase II, phosphodiesterase II, and alkaline phosphatase according to published procedures (Kanne et al., 1982a). The digests were adjusted to ca. pH 2.2 with H_3PO_4 and analyzed by HPLC on a 10 mm × 25 cm reverse-phase C_{18} column. The digests were loaded onto the column and eluted with a linear 10 mM (pH 2.2) KH_2PO_4–CH_3OH gradient over a period of 80 min (flow rate = 4 mL/min). The percentage of CH_3OH was gradually changed from 0 to 100 from time 10 min to time 70 min. Standard HMT–T adducts were used to calibrate the retention times of the adducts under these conditions (Cimino et al., 1986).

Results

Base-Catalyzed Reversal of HMT–DNA Cross-Link. The cross-link used in this work was formed by the cycloaddition of HMT to the two thymidine residues in the DNA helix formed by the self-complementary oligonucleotide 5′-GGGTACCC-3′. When this cross-link was incubated in the absence of UV light at 60 °C at pH 10.1 in the presence of 3 M urea (Figure 1, lane 2), it was reversed into the unmodified oligonucleotide and the pyrone-ring opened (O-M_{Py}) and pyrone-ring closed (C-M_{Py}) forms of the pyrone-side monoadducted oligonucleotide (Cimino et al., 1986). No furan-side monoadducted oligonucleotide was generated under these conditions. The amount of the products increased with increasing incubation time (Figure 2, lanes 1–4). No reversal of the cross-link was observed when urea was omitted (Figure 2, lanes 5–8) or the pH of the incubation solution was lowered to pH 8.5 (Figure 2, lanes 9–12). These results suggest that

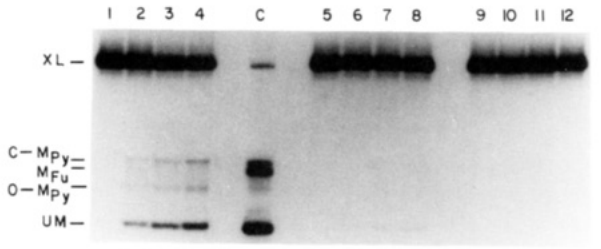

FIGURE 2: Base-catalyzed reversal of the cross-link. Lane C: XL photoreversed with 254-nm light. Lanes 1, 2, 3, and 4: XL in 10 mM $NaBO_3$ and 3 M urea, pH 10, incubated at 60 °C for 0, 0.5, 1, and 2 h, respectively. (Less products were produced here as compared to those in Figure 1. This is most likely due to the pH difference under the two reaction conditions.) Lanes 5, 6, 7, and 8: XL in 10 mM $NaBO_3$, pH 10, incubated at 60 °C for 0, 0.5, 1, and 2 h, respectively. Lanes 9, 10, 11, and 12: XL in 10 mM $NaBO_3$ and 3 M urea, pH 8.5, incubated at 60 °C for 0, 0.5, 1, and 2 h, respectively. After incubation, 75 μL of 0.2 M succinate (pH 4) was added to each sample (350 μL) (final pH, 4–5). The samples were then incubated at 37 °C for 30 min, EtOH precipitated in the presence of carrier tRNA, and analyzed on a polyacrylamide–urea gel.

the reversal of the cross-link under these conditions is a base-catalyzed reaction. The requirement for urea implies that the reaction occurs only if the cross-linked DNA helix is denatured. We have observed that the HMT–DNA cross-link could be reversed upon incubation at pH 12 at 37 °C in the absence of urea and that a similar cross-link formed between another psoralen derivative, 4′-(aminomethyl)-4,5′,8-trimethylpsoralen, and the oligonucleotide 5′-GGGTACCC-3′ could be reversed upon incubation in concentrated ammonia solution at room temperature or higher temperatures. The cross-linked DNA helix would be expected to be denatured and the pyrone ring of the psoralen moiety in the cross-link would be opened (Cimino et al., 1986) at such high-pH conditions.

The properties of the reversal reaction are characteristic of a light-independent chemical reaction. First, increasing the incubation time in the absence of actinic light results in more product formation. Second, increasing the catalyst (OH^-) (see Discussion) concentration (increasing pH from 10 to 10.5) increases the reaction rate. In fact, the amount of the product formed at pH 10.5 in the absence of urea is comparable to that formed in the presence of 3 M urea at pH 10 (data not shown), even though little reaction was observed at pH 10 in the absence of urea (Figure 2). Finally, raising the reaction temperature from 60 to 65 °C increases the reversal rate (data not shown).

Characterization of Products of the Base-Catalyzed Reversal. The pyrone-side monoadducted DNA can exist in either the pyrone ring opened form ($O\text{-}M_{Py}$) or the pyrone ring closed form ($C\text{-}M_{Py}$). The two forms have different mobilities on a denaturing polyacrylamide gel (Figures 1 and 2) and are interconvertible. Basic conditions (pH 10) open the pyrone ring, and acidic conditions (pH 4) close the ring (Cimino et al., 1986; also, see below). Analysis of the base-catalyzed samples without acidic incubation on a denaturing polyacrylamide gel yielded mostly $O\text{–}M_{Py}$ (Figure 3, lane 3). Acidic incubation of the same sample before gel analysis increased the amount $C\text{-}M_{Py}$ with concurrent loss of $O\text{-}M_{Py}$ (compare lanes 3 and 4 in Figure 3). These results indicate that the initial product of the base-catalyzed reversal is $O\text{-}M_{Py}$ and subsequent acidic incubation converts the $O\text{-}M_{Py}$ into $C\text{-}M_{Py}$.

The interconversion of $C\text{-}M_{Py}$ and $O\text{-}M_{Py}$ is also demonstrated in Figure 3. Incubation of gel-purified $C\text{-}M_{Py}$ in basic solution (pH 10) converted most of the $C\text{-}M_{Py}$ into $O\text{-}M_{Py}$

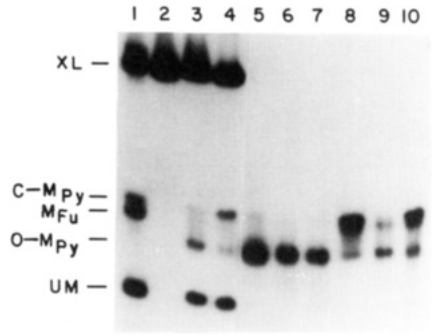

FIGURE 3: Lane 1: XL photoreversed with 254-nm light. Lanes 2–4, 5–7, and 8–10: XL, gel-purified $O\text{-}M_{Py}$, and gel-purified $C\text{-}M_{Py}$, respectively. Lanes 2, 5, and 8: untreated control samples. Lanes 3, 6, and 9: Samples (250 μL each) in 10 mM $NaBO_3$ and 3 M urea (pH 10.5) incubated at 60 °C for 2 h, followed by addition of 100 μL of 0.2 M succinate (pH 4) and EtOH precipitation in the presence of carrier tRNA before gel analysis. Lanes 4, 7, and 10: Same as lanes 3, 6, and 9, respectively, except that they were incubated in the acidified solution at 37 °C for 1 h before EtOH precipitation.

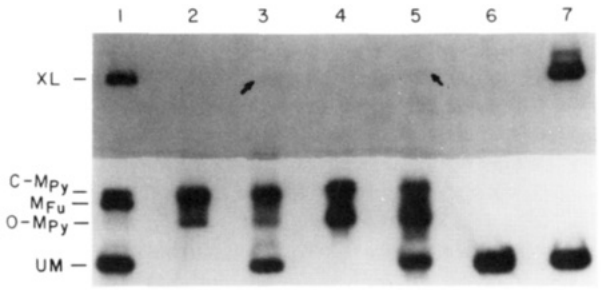

FIGURE 4: Characterization of the products of base-catalyzed reversal. 5′-Labeled UM, $O\text{-}M_{Py}$, and $C\text{-}M_{Py}$ were isolated from the gel shown in Figure 1. Lane 1: XL was photoreversed with 254-nm light from a germicidal lamp for 4 min at a distance of 2.5 in. Lanes, 2, 4, and 6: Dark controls of $C\text{-}M_{Py}$, $O\text{-}M_{Py}$, and UM, respectively. Lanes 3 and 5: $C\text{-}M_{Py}$ and $O\text{-}M_{Py}$, respectively, in 750-μL solution containing 4 μg of unlabeled unmodified oligonucleotide, 10 mM $MgCl_2$, and 100 mM NaCl, was irradiated at 2 °C with 249-nm light (4.6×10^{17} photons), dried in a Speedvac concentrator, redissolved in 300 μL of H_2O, and EtOH precipitated in the presence of carrier tRNA. Lane 7: UM generated from base-catalyzed reversal was irradiated for 3.5 min with 320–380-nm light from a 2.5-kW Hg/Xe lamp (Cimino et al., 1986) in 100 μL of 10 mM $MgCl_2$ and 100 mM NaCl in the presence of 4 μg of unlabeled UM and 0.15 mM HMT, extracted with chloroform and ether, and EtOH precipitated,in the presence of carrier tRNA. All the samples were analyzed on a polyacrylamide gel. Due to the overexposure of the film, the silver grains of the lower part of one side of the X-ray film were removed before making the print.

(lane 9). An acidic incubation of the base-treated $C\text{-}M_{Py}$ sample *before gel analysis* converted all the $O\text{-}M_{Py}$ in the $C\text{-}M_{Py}$ sample back into $C\text{-}M_{Py}$ (lane 10). However, incubation of the gel-purified $O\text{-}M_{Py}$ under acidic conditions (pH 4, 60–65 °C, or pH 2.2, 37 °C) did not convert it back to $C\text{-}M_{Py}$ (Figure 3, lane 7, and data not shown) even though upon storage $O\text{-}M_{Py}$ could be partially converted into $C\text{-}M_{Py}$ (see lane 5 of Figure 3 and lane 4 of Figure 4). These results suggest that a modification of $O\text{-}M_{Py}$ occurred during gel electrophoresis and this modification is irreversible under the acidic conditions described above.

To further characterize the products, [^{3}H]HMT–DNA cross-link was prepared and reversed as described under Materials and Methods, and the products were purified by polyacrylamide gel electrophoresis. The $O\text{-}M_{Py}$ and $C\text{-}M_{Py}$ as well as the cross-link were enzymatically digested to nucleosides and analyzed by HPLC (data not shown). With authentic T–HMT adducts as standards, it was found that the cross-link digest gave T–[^{3}H]HMT–T diadduct. The ^{3}H label

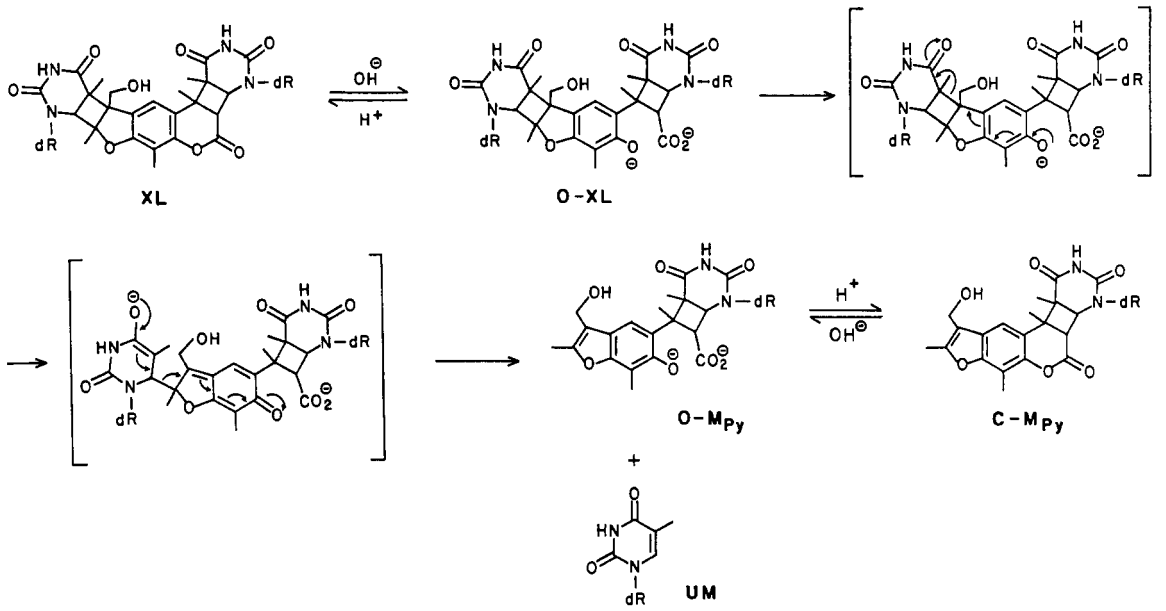

FIGURE 5: A possible mechanism for the base-catalyzed reversal of an HMT–DNA cross-link. Abbreviations: O-XL and C-XL, pyrone ring opened and closed form, respectively, of the HMT–DNA cross-link; see Figure 1 for the rest.

in the digest of C-M_{Py} coeluted with that of authentic T–HMT pyrone-side monoadduct. In addition, the absorption spectrum of this HPLC-purified ^{3}H-labeled product is within experimental error identical with that of the authentic T–HMT pyrone-side monoadduct (Shi & Hearst, 1987a), indicating that C-M_{Py} contains T–HMT pyrone-side monoadduct. The HPLC profile of the digested O-M_{Py} gave two major peaks, with one corresponding to T–HMT pyrone-side monoadduct, generated by the conversion of O-M_{Py} to C-M_{Py} during storage and/or enzyme digestion, and the other corresponding to none of the known T–HMT adducts. Due to our inability to obtain a large quantity of the adduct, it cannot be further characterized at the present time.

The photochemical properties of the products were also investigated. The gel-purified C-M_{Py} and O-M_{Py} from the base-catalyzed reversal were irradiated with 249-nm monochromatic light from a 2.5k-kW Hg/Xe lamp (Cimino et al., 1986) at 2 °C in the presence of the unlabeled oligonucleotide 5′-GGGTACCC-3′. The unlabeled DNA was expected to form double-stranded DNA with itself as well as with the pyrone-side monoadducted oligonucleotide since the stability of pyrone-side monoadducted double helix is similar to that of the unmodified helix (Shi & Hearst, 1986). The results in Figure 4 show the characteristic photochemistry of a pyrone-side monoadducted oligonucleotide (Shi & Hearst, 1987a,b). The irradiation resulted predominantly in photoreversal of the monoadducted oligonucleotide with formation of very little cross-link (a longer exposure of the gel definitely showed the presence of the cross-link band in lanes 3 and 5; data not shown).

The unmodified oligonucleotide generated from the base-catalyzed reversal was irradiated at 2 °C with the broad-band near-UV light (320–380 nm; Cimino et al., 1986) in the presence of a large excess of unlabeled 5′-GGGTACCC-3′ and HMT. The labeled DNA generated from base-catalyzed reversal would be expected to form double-stranded DNA with the unlabeled oligonucleotide and be cross-linked by HMT if the thymidine residue on each strand is intact. The formation of the cross-link (Figure 3, lane 7) shows that the DNA generated from the base-catalyzed reversal reaction is indeed the unmodified 5′-[^{32}P]GGGTACCC-3′. Three additions of HMT (35 μg/mL) each of which was followed by near-UV irradiation could cross-link essentially all the labeled DNA (data not shown).

Discussion

We have shown here that a psoralen–DNA interstrand cross-link can be reversed in the absence of UV irradiation under denaturing alkaline conditions at elevated temperatures. The initial products of the reversal reaction are the unmodified DNA and the pyrone ring opened form (O-M_{Py}) of the pyrone-side monoadducted DNA. The O-M_{Py} can be subsequently converted into its pyrone ring closed form (C-M_{Py}) with an acidic treatment. Upon gel electrophoresis on a denaturing polyacrylamide gel, O-M_{Py} is converted into a form that cannot be converted back into C-M_{Py} under the acidic conditions tested. Although the identity of the HMT–thymidine adduct in O-M_{Py} purified from a polyacrylamide–urea gel cannot be proven at the present time, the base-catalyzed reversal yields the same pyrone-side monoadducted DNA as the photochemical reversal reaction. The unknown reaction that occurred to O-M_{Py} during gel electrophoresis can be avoided by incubating the mixture under acidic conditions before gel electrophoresis to convert all O-M_{Py} into C-M_{Py}.

We have previously shown (Cimino et al., 1986) that the pyrone ring of a T–HMT–T diadduct can be opened at pH ≥7.5 and that the photoreversal of the pyrone ring opened form of the diadduct yields only the pyrone-side monoadduct and thymidine. The pyrone ring becomes more resistant to ring opening upon incorporation of the T–HMT–T diadduct into a double-stranded DNA helix formed by 5′-GGGTACCC-3′. At pH 10 in the absence of the denaturant urea only a small fraction of the cross-link is in the pyrone ring opened form. At pH 12, the majority of the cross-link is in the pyrone ring opened form. The requirements of high pH or moderately alkaline solution in the presence of urea suggest that base-catalyzed reversal proceeds through the pyrone ring opened form of the cross-link. We propose the following mechanism for the base-catalyzed reversal reaction (Figure 5). The first step of the reaction is the basic hydrolysis of the pyrone ring of the cross-link. The phenolate anion can then undergo a through-phenyl-ring retro-aldol condensation, breaking the bond between C(5) of the thymidine and C(4′) of the psoralen. This intermediate enolate can then undergo a retro-Michael

reaction, yielding the unmodified DNA and the ring-opened form of the pyrone-side monoadducted DNA. Upon acidification, the pyrone ring can close. This mechanism predicts that a furan-side monoadducted oligonucleotide would undergo similar base-catalyzed reversal to yield the pyrone ring opened psoralen and unmodified oligonucleotide if treated under strong alkaline conditions.

Psoralens have been mostly used as drugs in the treatment of human skin diseases (Fitzpatrick et al., 1982; Parrish et al., 1982) and as probes of nucleic acid structures and interactions (Sinden et al., 1980; Calvet et al., 1982; Thompson & Hearst, 1983; Setyono & Pederson, 1984; Rinke et al., 1985; Inman & Schnos, 1987; Matsuo & Ross, 1987). More recently, it has been shown that psoralen-monoadducted oligonucleotides can be used as probes to detect specific nucleic acid sequences and as tools to study hybridization kinetics and thermodynamics (Gamper et al., 1986, 1987). Our results reported here show that a faulty result may be produced if psoralen-adducted DNAs are treated under denaturing alkaline conditions such as alkaline agarose gel electrophoresis. A failure to detect psoralen cross-linkage may be simply due to the base-catalyzed reversal of the psoralen–DNA adducts formed. Similarly, results obtained from processes involving alkaline treatment could be underestimates of the actual psoralen adducts formed.

While base-catalyzed reversal of a psoralen–DNA cross-link prevents it from being handled under denaturing alkaline conditions, it provides an efficient way to make psoralen pyrone side monoadducted DNA. This is potentially useful for probing nucleic acid secondary and tertiary structures according to the psoralen-transfer method described by Cimino et al. (1986).

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