the heparin/protein interaction, the structure of the heparin fragments responsible for binding, and the nature of the cell receptor. Application of the methods presented here to placental tissue has already allowed the purification to homogeneity of a human HGFβ.[3] Initial results on the structural and functional characterization of this family of growth factors from both human and bovine sources will be reported shortly.

Acknowledgments

We thank Dr. Bert L. Vallee for continued advice and support and Dr. D. J. Strydom for amino acid analyses. We are grateful to Dr. Michael Klagsbrun for many helpful discussions, as well as access to unpublished observations. The excellent technical assistance of Susan Kane and Judith Lalonde is gratefully acknowledged.

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Interstrand Psoralen Cross-Links Do Not Introduce Appreciable Bends in DNA†

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ABSTRACT: Analysis of the X-ray crystallographic structure of an 8-methoxypsoralen–thymine monoadduct has led to the suggestion that psoralen cross-links would bend DNA by as much as 70° [Peckler, S., Graves, B., Kanne, D., Rapoport, H., Hearst, J. E., & Kim, S.-H. (1982) *J. Mol. Biol. 162*, 157–172]. DNA can exist in a stably bent configuration in solution as recently demonstrated from analysis of polyacrylamide gel electrophoresis and differential decay of birefringence. Using these techniques, we have investigated the structure of DNA cross-linked with 8-methoxypsoralen and 4,5′,8-trimethylpsoralen. The results are not consistent with cross-links introducing any appreciable stable bend in double-stranded DNA molecules. Results suggest that photobound 4,5′,8-trimethylpsoralen molecules lengthen DNA by the equivalent of about one base pair per photobound adduct. We have also determined that 4,5′,8-trimethylpsoralen cross-links are introduced preferentially into 5′-TA compared to 5′-AT DNA sequences.

Recent evidence has demonstrated convincingly that DNA can exist in solution in a stably bent conformation (Wu & Crothers, 1984; Hagerman, 1984a). DNA can bend as a result of the inherent conformation of particular sequence arrangements of DNA as in trypanosome kinetoplast DNA (Wu & Crothers, 1984; Hagerman, 1984a). In addition, DNA may be bent by association with proteins as demonstrated for the CAP–*lac* operator DNA binding interaction (Wu & Crothers, 1984). Bent DNA adds another dimension of complexity, in addition to cruciforms and regions of left-handed Z-DNA, to the structure of DNA. It is possible that these alternate helical conformations of DNA, compared to a linear B-form DNA,

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0006-2960/84/0423-6299$01.50/0

may provide important signals to regulatory or other proteins that must interact with DNA for the regulation of gene expression, for the replication of DNA, or for the repair of damaged DNA.

Psoralens are a class of compounds that are widely used medically in the treatment of vitiligo and psoriasis and as sensitive probes of nucleic acid conformation. Psoralens photobind to DNA, forming monoadducts and interstrand cross-links (Cole, 1970, 1971a; Dall'Acqua et al., 1970). It has been suggested from the X-ray crystallographic analysis of an 8-methoxypsoralen–thymine monoadduct that psoralen cross-links will bend DNA, perhaps by as much as 70° (Peckler et al., 1982; Kim et al., 1983). This is potentially an exciting result since it would provide a relatively simple model DNA substrate for analysis of the biophysical parameters of bent DNA molecules. Moreover, psoralen-induced curvature would be of biological significance since it suggests a structural feature that may be recognized by DNA repair enzymes, such as the uvrABC endonuclease, in the repair of psoralen–DNA monoadducts and cross-links (Cole, 1971b; Cole et al., 1976; Sancar & Rupp, 1983). It has also been suggested by Zolan et al. (1984), assuming a 70° bend is required at cross-links, that the differential rate of cross-link formation in the highly repeated α-DNA of African green monkey cells may be a result of the rigidity of a particular chromatin configuration of α-DNA. Since psoralens are widely used as sensitive probes of both chromatin and DNA conformation and of the state of unrestrained torsional tension in living cells (Sinden et al., 1980, 1982, 1983a,b; Hanson et al., 1976; Cech & Pardue, 1978; Carlson et al., 1982), it is especially important to understand the structure and consequences of the psoralen–DNA interstrand cross-link.

We present evidence that psoralen cross-links do not produce significant bends in the DNA helix axis in contrast to the prediction from the X-ray crystallographic analysis of a psoralen–thymine monoadduct. Psoralen adducts appear to lengthen DNA by the equivalent of about one base pair per bound psoralen molecule.

Materials and Methods

Nucleic Acids. Plasmid pBR322 was purified from HB101/pBR322 by using methods described previously (Sinden et al., 1980). pXH9 is a pBR325 derivative that contains a 126 base pair (bp) insert (derived from ϕX174), designated XH9, at the *Eco*RI site (Shore et al., 1981). The plasmid-containing bacterial host, *Escherichia coli* HB101/XH7, was kindly provided by D. Shore. Preparative purification of XH9 followed protocols described elsewhere (Hagerman, 1984a). *Bam*HI and *Kpn*I linkers were obtained from Pharmacia P-L Biochemicals.

Enzymes. *Eco*RI was prepared according to published procedures. *Hae*III was purchased from Bethesda Research Laboratories.

Psoralen Photobinding. 4,5′,8-Trimethylpsoralen (Me_3-psoralen), [^{3}H]Me_3-psoralen, and irradiation protocols have been described previously (Sinden et al., 1980). 8-Methoxypsoralen was purchased from Sigma Chemical Co. Samples were irradiated at 4 °C at incident light intensities of 1.6 or 1.2 kJ·m^{-2}·min^{-1} by using two General Electric F15T8 or F20T8 BLB bulbs, respectively. Additions of 1% (v/v) saturated ethanol solutions of Me_3-psoralen or 8-methoxypsoralen were added to DNA at 12 and 72 kJ·m^{-2} intervals during photobinding reactions, respectively.

Gel Electrophoresis. Polyacrylamide gels (5% and 9%) were run as described by Sinden et al. (1983a) and Hagerman (1984a), respectively. Denaturing 15% polyacrylamide–urea or polyacrylamide–formamide gels were run as described by Maniatis et al. (1975). [^{32}P]ATP end labeling and autoradiography were as described previously (Sinden & Pettijohn, 1984).

Differential Decay of Birefringence (DDB). Instrumentation and methodology have been described elsewhere (Hagerman, 1984a,b).

Results and Discussion

Certain DNA restriction fragments derived from kinetoplast minicircles of the trypanosomatids behave abnormally on polyacrylamide gels (reduced mobility for size) (Simpson, 1979; Challberg & Englund, 1980), leading to the proposal (Marini et al., 1982; Wu & Crothers, 1984) that this electrophoretic anomaly might be due to an unusual, perhaps bent, helical structure. A detailed analysis of one such molecule (242 bp) using the technique of differential decay of birefringence (DDB; Hagerman, 1984a,b) indicated that stable curvature of the helix axis was responsible for the electrophoretic anomaly. Moreover, for this 242 bp molecule, which has a region of curvature symmetrically disposed about its center, the *apparent* angle of curvature (52°) is associated with an increase in the apparent molecular weight of more than 2-fold on 9% polyacrylamide gels. This result suggests that the predicted 70° bend associated with psoralen cross-linked DNA would result in substantial reductions in electrophoretic mobility. A decrease in the mobility of bent DNA during gel electrophoresis is also expected from theoretical considerations (Lumpkin & Zimm, 1982).

Electrophoretic Mobility of Cross-Linked DNA. For a linear DNA molecule possessing a number of cross-link sites (points of potential bend formation), the generation of psoralen cross-link-induced DNA bends would be accompanied by a decrease in the intensity of the non-cross-linked band as numerous bands appeared corresponding to migration positions of the various bent DNA molecules. It is possible that as the molecule became heavily cross-linked the population of bands might converge to a position corresponding to that for a heavily cross-linked and severely bent configuration. In contrast, many published examples of cross-linked DNA on agarose and acrylamide gels show little if any change in lightly cross-linked DNA, followed by a slight decrease in electrophoretic mobility at higher extents of cross-linking [for examples, see Cech (1981), Carlson et al. (1982), and Sinden et al. (1983a,b)].

The change in electrophoretic mobility of *Hae*III restriction fragments from pBR322 cross-linked with 8-methoxypsoralen or Me_3-psoralen is shown in Figure 1. Following photoaddition of increasing numbers of 8-methoxypsoralen or Me_3-psoralen molecules, the mobilities of all *Hae*III restriction fragments decreased. It is also evident that the change in migration was not uniform for all restriction fragments. However, this can be explained in part by the sequence specificity of psoralen binding, as discussed in more detail below. When the cross-link assay described by Cech (1981) was used, which involves analysis of migration patterns on denaturing formamide gels, by the earliest Me_3-psoralen cross-linking dose shown (Figure 1, lane G) >90% of the five larger (587, 540, 504, 458, and 434 bp) fragments were cross-linked. At the highest cross-linking doses (lane J), >90% of the three smaller (64, 57, and 51 bp) fragments were cross-linked. The two sets of photobinding reactions, 8-methoxypsoralen (lanes B–E) and Me_3-psoralen (lanes G–J), produce similar extents of unwinding of supercoiled plasmid DNA when analyzed on agarose gels (data not shown), suggesting similar numbers of total bound psoralens. By the most photoreacted time point, most supercoils were removed from naturally supercoiled

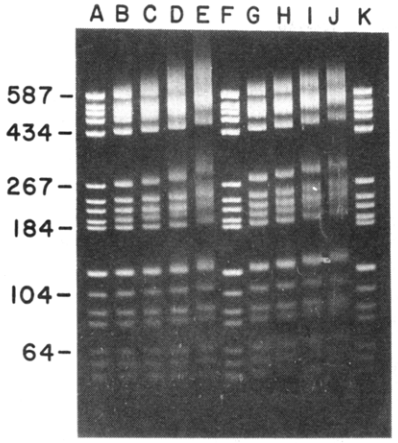

FIGURE 1: Analysis of cross-linked DNA on 5% polyacrylamide gels. Shown are *Hae*III digestion patterns of pBR322 following various extents of psoralen photobinding. Lanes A, F, and K are control, non-cross-linked DNA samples. Lanes B, C, D, and E were treated with 8-methoxypsoralen and 36, 72, 144, and 216 kJ·m^{-2} 360-nm light, respectively. Lanes G, H, I, and J were treated with Me_3-psoralen and 6, 12, 24, and 48 kJ·m^{-2} 360-nm light, respectively. Following photobinding, samples were extracted with chloroform–isoamyl alcohol (24:1), adjusted to 0.3 M sodium acetate, precipitated by the addition of 2 volumes of ethanol, and redissolved in electrophoresis buffer. 5% polyacrylamide gels (18 × 14 × 0.15 cm) in 40 mM tris(hydroxymethyl)aminomethane (Tris)–borate buffer (pH 8.3) were run at 8–10 mA. *Hae*III restriction fragments shown above are 587, 540, 504, 458, 434, 267, 234, 213, 192, 184, 124 + 123, 104, 89, 80, 64, 57, and 51 bp in length (Sutcliffe, 1978; Peden, 1983). The positions of selected bands are indicated.

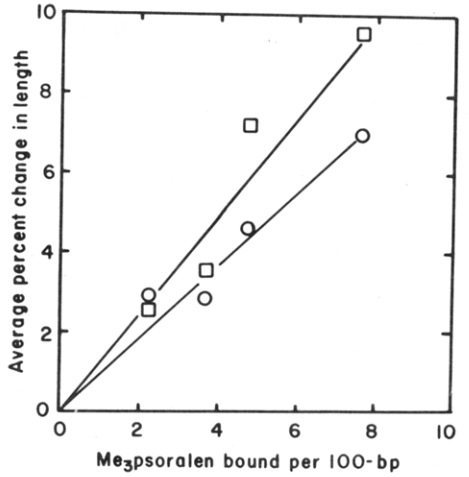

FIGURE 2: Quantitative analysis of the change in electrophoretic mobility as a function of Me_3-psoralen photobinding. Shown is the analysis from 5% and 9% polyacrylamide gels (similar to that shown in Figure 1) of *Hae*III restriction fragments treated with [^{3}H]-Me_3-psoralen and light. The average number of psoralen molecules bound per 100 bp was determined from specific activity measurements of aliquots of the entire *Hae*III digest. The average percent change in length was determined from analysis of gel photographs. For the 5% polyacrylamide gels, the average change was that for the 587, 434, 267, 234, 213, 192, 184, 124 + 123, 104, and 89 bp restriction fragments (□). For the 9% polyacrylamide gels, the average change in mobility represents that for the 267, 234, 213, 192, 184, 124 + 123, 104, 89, and 80 bp fragments (○).

plasmid DNA. Although the patterns shown in Figure 1 are quite similar, it was evident by using the cross-linking assay of Cech (1981) that the 8-methoxypsoralen-treated DNA samples were not as heavily cross-linked as the Me_3-psoralen-treated DNA samples. This is consistent with the observations of Kanne et al. (1982a). It should be pointed out that we are uncertain of the molecular basis for the apparent "smearing" of the higher molecular weight DNA bands observed for the more heavily photoreacted samples. The smearing may represent a migration of a population of heterogeneously photoreacted molecules. It is possible, however, that 8-methoxypsoralen photoproducts introduce a greater distortion in DNA, resulting in a greater heterogeneity in electrophoretic mobility than Me_3-psoralen photoproducts. The smearing does not correlate with cross-linking since the 8-methoxypsoralen-reacted samples are more smeared but less cross-linked than the Me_3-psoralen-treated DNAs.

To quantitate the change in electrophoretic mobility per photobound psoralen molecule, an experiment similar to that shown in Figure 1 was performed using [^{3}H]Me_3-psoralen. The average number of [^{3}H]Me_3-psoralen molecules bound per 100 bp was calculated from specific activity measurements of a total *Hae*III digest of pBR322 (Sinden et al., 1980). The average percent change in length for each DNA fragment was determined from gels similar to that shown in Figure 1 by analysis of the average apparent molecular weight of the cross-linked DNA fragments relative to the non-cross-linked *Hae*III fragments in the same gel. The results from this analysis for selected series of fragments are shown in Figure 2. With 5% or 9% polyacrylamide gels [used to detect the bent kinetoplast DNA fragments by Hagerman (1984a)], the average percent change in length for the bands listed in the legend to Figure 2 corresponded to a 10.2% and a 7.3% increase in length when an average of eight Me_3-psoralen molecules were bound per 100 bp DNA. The anomalous migration of bent DNA molecules is more pronounced at higher acrylamide gel concentrations (Marini et al., 1982). Thus, the effect on electrophoretic migration of psoralen cross-links in DNA is opposite to that expected for bending. The intercalation and subsequent photobinding of psoralen in DNA may only unwind the DNA and result in an increase in length approximately equivalent to a base pair per photobound psoralen molecule.

These data and those published previously (Cech, 1981; Carlson et al., 1982; Sinden et al., 1983a,b) are not consistent with the interpretation that a significant bend is introduced by an 8-methoxypsoralen or Me_3-psoralen cross-link in DNA.

Identification of the Preferential TA Me_3-psoralen Cross-Link Site. One possible explanation for not observing a cross-link-induced bend in DNA would be if there were no cross-link sites near the centers of any of the *Hae*III restriction fragments of pBR322. It has been well documented that psoralens have a preference for photobinding and cross-linking TA-rich compared to GC-rich DNA sequences (Chandra et al., 1973; Dall'Acqua et al., 1978; Lown & Sim, 1978; Kanne et al., 1982a,b). We have determined the relative initial rates of [^{3}H]Me_3-psoralen photobinding to poly(dG)·poly(dC), poly(dG-dC)·poly(dC-dG), poly(dA)·poly(dT), poly(dA-dC)·poly(dT-dG), calf thymus DNA, and poly(dA-dT)·poly(dT-dA) to be approximately 1, 2, 3.5, 16, 30, and 150, respectively (R. R. Sinden, unpublished results). Since the TA sequences represent the preferential binding and cross-linking site (Kanne et al., 1982a), we wished to determine if there were any preference for Me_3-psoralen cross-linking 5′-AT or 5′-TA sequences since previously we had been unable to cross-link more than a few percent of the 48 bp inverted repeat of pUC7 DNA (R. R. Sinden, unpublished results) which contains a 5′-AT within the *Bam*HI site as the only AT or TA combination.

*Bam*HI (5′-CGGATCCG) and *Kpn*I (5′-CGGTACCG) linkers were end labeled with ^{32}P, treated with Me_3-psoralen and light, and assayed for cross-linking on denaturing 15% polyacrylamide gels. The fraction of the linkers cross-linked

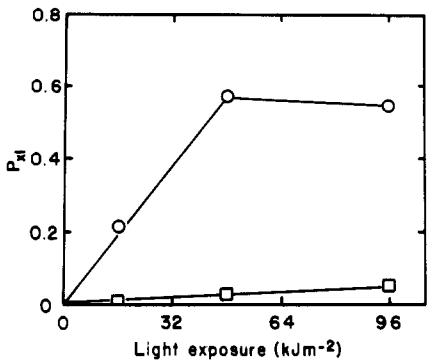

FIGURE 3: Me_3-psoralen preferentially cross-links 5′ TA sequences. Shown are the fractions of *Kpn*I or *Bam*HI 8 bp linker DNA fragments cross-linked by various doses of Me_3-psoralen and 360-nm light. ^{32}P end-labeled linkers were treated with Me_3-psoralen and light, and the extent of cross-linking was determined from densitometric analysis of autoradiograms of denaturing 15% polyacrylamide–urea gels which separates the 8 bp single strands (non-cross-linked) and double-stranded (higher molecular weight) or cross-linked DNA fragments. P_{xl} is the fraction of the total linker that was cross-linked. (○) *Kpn*I linker (5′-CGGTACCG); (□) *Bam*HI linker (5′-CGGATCCG).

at various doses of Me_3-psoralen cross-linking is shown in Figure 3. 5′-TA cross-linked much more readily than 5′-AT sequences when both sequences were in the same neighboring sequence environment of the *Kpn*I or *Bam*HI linker DNA. Assuming that differences in neighboring base pairs do not change the relative rates of cross-linking of 5′-TA vs. 5′-AT, the preferential cross-linking sites are 5′-TA sequences. These results are similar to those of Gamper et al. (1984) for the 5′-TA cross-linking specificity of 4′-(hydroxymethyl)-4,5,8-trimethylpsoralen.

Examination of the distribution of 5′-TA sequences within the *Hae*III restriction fragments shows many examples of TA sequences near the center of DNA fragments (Sutcliffe, 1978; Peden, 1983). It is also evident that there is a significant difference in distribution in TA sequences among various fragments. For example, the 123 and 124 bp fragments (which migrate as a single band) have five and three TA sequences, respectively. In the 123 bp fragment, three of the five possible TA cross-link sites are located within the center 10 bp of the DNA fragment. These fragments run together on 5% or 9% polyacrylamide gels and are not resolved following psoralen photobinding (see Figure 1). The 192 bp *Hae*III restriction fragment has an unusually high concentration of 13 5′-TA sequences compared to 3 and 7 for the 184 and 213 bp fragments, respectively. It can be seen in Figure 1 that this band has a correspondingly greater change in mobility than fragments of similar size for all extents of photobinding. Although there is ample opportunity to introduce bending near the center of numerous *Hae*III restriction fragments, we have not obtained evidence for bending from our analysis using gel electrophoresis.

Analysis of Cross-Linked DNA Using Differential Decay of Birefringence (DDB). A 126 bp DNA restriction fragment, XH9 (Shore et al., 1981), was treated with Me_3-psoralen and light to introduce an average of approximately eight Me_3-psoralen molecules (mono- and diadducts) per fragment (determined from analysis on 5% polyacrylamide gels as described in Figure 2). XH9 contains seven 5′-TA-3′ dinucleotide residues and is therefore a good candidate for cross-linking studies. The TA residues occur at (fractional) positions 0.26, 0.38, 0.43, 0.52, 0.66, 0.81, and 0.84. An analysis of the psoralen-treated DNA on denaturing polyacrylamide gels (see Materials and Methods) indicated that greater than 95% of the molecules had at least one cross-link,

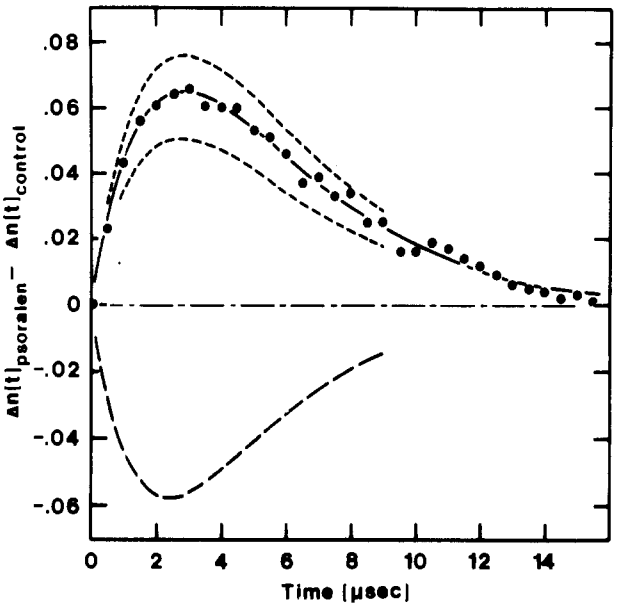

FIGURE 4: Differential decay of birefringence (DDB) for a 126 bp DNA restriction fragment in the presence or absence of psoralen cross-links. In a standard birefringence experiment, the decay of the birefringence signal $[\Delta n(t) = n(t)/n(0)]$, following removal of the orienting electric field, is measured as a function of time, the rate of birefringence decay being a sensitive measure of molecular shape. In the present experiment, the *difference* in birefringence decay curves for the DNA fragment in the presence and absence of psoralen adducts $[\Delta n(t)_{psoralen} - \Delta n(t)_{control}]$ is plotted directly as a function of time. This approach, when used to study DNA structure, is capable of resolving length differences as small as 1% (Hagerman, 1984a,b). A positive difference curve implies that the psoralen-treated DNA fragment is longer than the untreated control. Filled circles represent experimental DDB data [85 μg/mL DNA, 2.0 mM Tris-HCl, and 0.1 mM disodium ethylenediaminetetraacetic acid (Na_2EDTA), pH 7.4, °C]. The solid line represents a best fit to the experimental data for τ(psoralen)/τ(control) = 1.19, where τs represent relaxation times for birefringence decay [for details of the analysis, see Hagerman (1984a,b)]. The relaxation ratio of 1.19 corresponds to a length *increase* of the psoralen-treated fragment of 8%, following correction for residual flexibility of XH9 (Hagerman, 1981; Hagerman & Zimm, 1981). If each psoralen added were to contribute 3.4 Å (1 bp equivalent) to the length of the fragment, the 8% increase would correspond to approximately 10 psoralen molecules per fragment. (---) Effect of a 2 bp addition (top line) or deletion (lower line) to the psoralen-treated fragment (approximately 1.5% change in length). (– – –) Predicted effect for a population of molecules, each of which has one psoralen cross-link (random position; 70° bends) associated with a passive DNA contour length increase of 8%, as above.

suggesting an average extent of cross-linking of at least four cross-links per molecule. If, as predicted by Peckler et al. (1982), psoralen cross-links bend DNA by 70°, the psoralen-treated XH9 should be less extended in solution than its untreated counterpart. A sensitive solution method for the study of such long-range configurational changes in DNA is the technique of differential decay of birefringence (DDB; Hagerman, 1984a,b), which is capable of resolving differences in length as small as 1%. Figure 4 displays the results of the DDB analysis of the psoralen-treated XH9 fragment. The most striking feature of the data is that the psoralen-treated fragment is more extended than the nontreated control. In fact, the degree of extension is close to that predicted if each psoralen bound increased the fragment length by the equivalent of 1 bp. A curve is also displayed which represents the expected DDB plot for a population of molecules, each possessing 1 cross-link-associated 70° bend (at any one of the 7 TA sites) and 10 psoralen adducts (passive axial length increase of 8%). Molecules having an average of four cross-link-associated bends of 70° (all trans bends as a lower bound estimate for

the degree of contraction) would result in a more negative DDB curve than that associated with the single cross-link case.

The length increase per psoralen monoadduct is not known; however, even if the length increase were the equivalent of 2 bp, a 70° bend would still be inconsistent with our DDB results. Although we cannot say from these data that psoralen cross-links (or monoadducts for that matter) do not introduce any axial curvature at the site of the cross-link, the bend angle would have to be less than half of the predicted 70° value. Moreover, if the length increase per psoralen bound is 1 bp equivalent, the bend angle would be less than 20°. Work is currently in progress using molecules having single psoralen cross-links at the center of the molecule in an effort to further refine an estimate of the degree of bending introduced through psoralen cross-linking.

Conclusions

The results presented here suggest that psoralen cross-links do not result in significant bending of the DNA helix axis. It is possible that a slight bending or other distortion of the DNA at the cross-link site (or even at monoadduct sites) exists, but it is not detectable using either polyacrylamide gel analysis or DDB measurements. These experimental results represent a significant departure from the prediction of Peckler et al. (1982) and Kim et al. (1983) which is based on an extension of their X-ray crystallographic analysis of the 8-methoxypsoralen–thymine structure. It is possible that the interplanar angle of approximately 50° observed in their crystals reflects crystal packing constraints. Moreover, it is not clear how much energy would be required to reduce the interplanar angle to the near-zero values presumed to exist for the monoadducts. The failure to see cross-link-associated 70° bends in duplex DNA undoubtedly reflects constraints placed upon the psoralen cross-link geometry by the helix structure itself.

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