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DNA Unwinding Produced by Site-Specific Intrastrand Cross-Links of the Antitumor Drug *cis*-Diamminedichloroplatinum(II)†

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ABSTRACT: The DNA unwinding produced by specific adducts of the antitumor drug *cis*-diamminedichloroplatinum(II) has been quantitatively determined. Synthetic DNA duplex oligonucleotides of varying lengths with two base pair cohesive ends were synthesized and characterized that contained site-specific intrastrand N7-purine/N7-purine cross-links. Included are *cis*-[$Pt(NH_3)_2${d(GpG)}], *cis*-[$Pt(NH_3)_2${d(ApG)}], and *cis*-[$Pt(NH_3)_2${d(GpTpG)}] adducts, respectively referred to as *cis*-GG, *cis*-AG, and *cis*-GTG. Local DNA distortions at the site of platination were amplified by polymerization of these monomers and quantitatively evaluated by using polyacrylamide gel electrophoresis. The extent of DNA unwinding was determined by systematically varying the interplatinum distance, or phasing, in polymers containing the adducts. The multimer that migrates most slowly gives the optimal phasing for cooperative bending, from which the degree of unwinding can be obtained. We find that the *cis*-GG and *cis*-AG adducts both unwind DNA by 13°, while the *cis*-GTG adduct unwinds DNA by 23°. In addition, experiments are presented that support previous studies revealing that a hinge joint forms at the sites of platination in DNA molecules containing *trans*-GTG adducts. On the basis of an analysis of the present and other published studies of site-specifically modified DNA, we propose that local duplex unwinding is a major determinant in the recognition of DNA damage by the *Escherichia coli* (A)BC excinuclease. In addition, local duplex unwinding of 13° and bending by 35° are shown to correlate well with the recognition of platinated DNA by a previously identified damage recognition protein (DRP) in human cells.

cis-Diamminedichloroplatinum (*cis*-DDP[1] or cisplatin) is one of the most effective anticancer drugs. Its probable mode of action involves formation of platinum–DNA adducts capable of blocking DNA replication (Bruhn et al., 1990; Heiger-Bernays et al., 1990; Pinto & Lippard, 1985). The interaction with and processing of cisplatin–DNA adducts by cellular proteins, referred to as damage recognition proteins or DRPs, are important aspects of the molecular mechanism. These proteins may be repair enzymes that excise platinum damage from DNA, or they may bind to the sites of platination, rendering them inaccessible to other cellular components. Both possibilities are potentially relevant to the mechanism of action (Donahue et al., 1990; Ciccarelli et al., 1985; Eastman & Schulte, 1988; Gibbons et al., 1990; Hoeijmakers et al., 1990; Sheibani et al., 1989). It is therefore of considerable importance to understand the structures of the major cisplatin–DNA adducts and to learn how these structures modulate their interaction with DRPs. From such knowledge it may be possible to explain why *cis*-DDP is active while its trans isomer is not and why the drug is effective against only certain types of cancers and eventually to facilitate the rational design of more effective chemotherapeutic agents.

cis-DDP binds bifunctionally to DNA with a high affinity for the purine N7 positions. As a result, a variety of adducts form readily when the platinum complex binds to random sequence DNA (Eastman, 1986; Fichtinger-Schepman et al., 1985, 1987). It is not yet clear which are responsible for the anticancer activity. The relative amounts of the different

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[1] Abbreviations: DDP, diamminedichloroplatinum(II); *cis*-GG, *cis*-[$Pt(NH_3)_2${d(GpG)}] intrastrand cross-link; *cis*-AG, *cis*-[$Pt(NH_3)_2${d(ApG)}] intrastrand cross-link; *cis*-GTG, *cis*-[$Pt(NH_3)_2${d(GpTpG)}] intrastrand cross-link; *cis*-GXG, *cis*-[$Pt(NH_3)_2${d(GpXpG)}] intrastrand cross-link (where X is any nucleoside); *trans*-GXG, *trans*-[$Pt(NH_3)_2${d(GpXpG)}] intrastrand cross-link (where X is any nucleoside); DRP, damage recognition protein; HPLC, high-performance liquid chromatography; en, ethylenediamine (when used in conjunction with any of the abbrevations for *cis*-DDP adducts, the corresponding [$Pt(en)Cl_2$] adduct is implied); dach, 1,2-diaminocyclohexane; AAF, *N*-acetylaminofluorene. GG20, GG21, AG21, GTG20, etc. are terms used to identify duplex oligonucleotide monomers, as defined in Figure 1; *cis*-GTG21, *cis*-AG, etc. are terms used to denote these oligonucleotides containing *cis*-[$Pt(NH_3)_2${d(GpTpG)}], *cis*-[$Pt(NH_3)_2${d(ApG)}], etc. intrastrand cross-links.

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adducts formed upon such global platination of duplex DNA, while somewhat controversial, are 65% *cis*-GG, 25% *cis*-AG, and approximately 10% *cis*-GXG, monofunctional adducts, and interstrand cross-links (Eastman, 1986; Fichtinger-Schepman et al., 1985).

The structures and, more recently, the biological activities of single well-defined adducts have begun to be explored in order to determine those responsible for specific biological activities. Platinated viral genomes have been constructed that contain only a single, well-characterized platinum adduct. These biologically viable molecules have been introduced into cells, and their effects on survival and mutagenesis have been quantitatively monitored (Pinto et al., 1986; Naser et al., 1988; Burnouf et al., 1990). In addition smaller, site-specifically modified DNA templates have been constructed and used to examine the inhibition of DNA replication and transcription as well as DNA repair by the *Escherichia coli* (A)BC excinuclease system (Page et al., 1990; Comess, Naser, Essigmann, and Lippard, manuscript in preparation; Corda et al., 1991; Lemaire et al., 1991). Structural investigations of the *cis*-GG, *cis*-AG, *cis*-GXG, and *trans*-GXG adducts have been performed by a variety of means including NMR spectroscopy and X-ray crystallography. These techniques have made possible exact measurements of sugar conformations and hydrogen-bonding interactions, but a three-dimensional structure of duplex DNA containing these adducts has proved elusive for the most part (Sherman & Lippard, 1987).

Recently, native gel electrophoresis has provided a means of measuring structural features such as DNA bending (Wu & Crothers, 1984; Koo et al., 1986). The principle of this methodology is that bent molecules migrate more slowly in a polyacrylamide gel than do straight molecules of the same mass (Lerman & Frisch, 1982; Lumpkin & Zimm, 1982). This mobility retardation can be quantitated and provides a powerful means of measuring DNA bending caused by specific platinum adducts. In order to obtain sufficient signal to evaluate the degree of bending, DNA monomers containing single platinum adducts are polymerized. The lengths of the monomer units, and hence the interplatinum distance in the multimers, must match the DNA helical repeat so that bends may add constructively. Otherwise, the bends add destructively and the observable gel retardation sums to zero.

We have previously reported the DNA bending produced by several site-specific platinum–DNA adducts (Rice, et al., 1988; Bellon & Lippard, 1990). In particular, *cis*-GG, *cis*-AG, and *cis*-GTG all bend the double helix by 32–35°. The monofunctional platinum compound *cis*-[Pt(NH_3)$_2$(*N3*-cytosine)Cl]Cl does not bend DNA at all. Interestingly, polymers containing the *trans*-GTG adduct bend in more than one direction, forming a hinge joint. Other workers have also examined the DNA bending caused by cisplatin adducts, and in general their results have agreed well with ours (Anin & Leng, 1990; Leng, 1990; Marrot & Leng, 1989).

No correlation was found to exist between the extent of DNA bending caused by a particular adduct and its biological activity (Bellon & Lippard, 1990). While all three major cisplatin adducts bend DNA to a similar extent, they are not processed with similar efficiencies by the bacterial (A)BC excinuclease system (Page et al., 1990). This system is responsible for DNA excision repair in *E. coli* cells and selectively processes very different types of DNA damage. In addition, the three platinum adducts are not recognized with equal affinity by a recently discovered cisplatin damage recognition protein isolated from HeLa cells (Donahue et al., 1990). This protein binds avidly only to the *cis*-GG and *cis*-AG adducts, but not to the *cis*- or *trans*-GTG adducts, unplatinated DNA, DNA globally modified with *trans*-DDP, or other types of DNA damage.

Whereas DNA bending does not seem to correlate with the processing of platinum adducts, DNA unwinding may be more important. Several workers have studied the amount of unwinding produced by globally platinated supercoiled plasmid DNA with *cis*- or *trans*-DDP (Cohen et al., 1979; Scovell & Collart, 1985; Scovell & Kroos, 1982; Bowler, 1986; Sherman & Lippard, 1987). This technique measures the amount of bound platinum necessary to remove all of the negative supercoils in a plasmid according to the equation Lk = Tw + Wr (Fuller, 1971; Bauer, 1978). The linking number, Lk, is a topological invariant and does not change unless the plasmid is nicked; Tw, the twist, reflects the duplex unwinding. The writhe, Wr, is determined by the three-dimensional shape of the plasmid, or the degree of supercoiling. It determines the rate of plasmid DNA migration through an electrophoresis gel, which reaches its minimal value when Wr = 0. In a typical experiment, a negatively supercoiled plasmid having Wr < 0 is titrated with increasing amounts of unwinding agent. As Tw gets smaller, Wr increases in order to maintain Lk = constant. The endpoint of the titration occurs when Wr = 0, determined by the minimal gel mobility of the modified plasmid.

Since Wr is the experimental observable, a change in Wr accurately reflects a change in Tw only if the DNA modifying agent affects only Tw and not Wr. If, however, the compound both bends and unwinds DNA, the three-dimensional shape of the plasmid will also be affected and Wr will change. The endpoint condition, Wr = 0, then reflects a change not only in Tw but also in Wr due to DNA bending. Because *cis*- and *trans*-DDP are known to bend as well as unwind duplex DNA, it is difficult to deconvolute the separate effects of unwinding and bending on the change in Wr and rigorously to determine duplex unwinding (White et al., 1988). In addition, because these experiments use globally platinated DNA, the contribution of each type of platinum adduct to the overall change in Wr is impossible to determine. Consequently, the DNA unwinding produced by individual platinum adducts is thus far unknown (Cohen et al., 1979; Sherman & Lippard, 1987).

In the present investigation we have applied the electrophoresis gel shift methodology to measure directly and rigorously the change in helical repeat, or local duplex unwinding, produced by several site-specific adducts of *cis*-DDP. In particular, by systematically varying the platinum–platinum distance in polymers containing specific cisplatin adducts, the sequence repeat that gives the most constructive addition of DNA bends was determined. These experiments reveal the degree of unwinding, which is a sensitive function of the extra nucleotides beyond those of the normal helical repeat of B-DNA required to afford the most constructive addition of DNA bends. This technique directly employs DNA bending to measure duplex unwinding, but the results are in no way affected by the extent of DNA bending involved. Moreover, since the behavior of linear multimers of DNA is being quantitated, topological constraints associated with closed circles do not apply. Furthermore, because the structure that affords the most constructive addition of DNA bends is planar, it has no writhe. Possible correlations between the present structural results and the processing of DNA damage by the (A)BC excinuclease system, as well as recognition by the cisplatin DRP, are also presented.

Materials and Methods

DNA oligonucleotides were synthesized, purified, allowed

GG20	TCTCCTTCTGGTCTCTTCTC AGAGAGGAAGACCAGAGAAG
GG21	TCTCCTTCTTGGTCTCTTCTC AGAGAGGAAGAACCAGAGAAG
GG22	TCTCCTTCTTGGTTCTCTTCTC AGAGAGGAAGAACCAAGAGAAG
GG23	TCTCCTCTCTTGGTTCTCTTCTC AGAGAGGAGAGAACCAAGAGAAG
GG24	TCTCCTCTCTTGGCTTCTCTTCTC AGAGAGGAGAGAACCGAAGAGAAG
AG21	TCTCCTTCTTAGTCTCTTCTC AGAGAGGAAGAATCAGAGAAG
AG22	TCTCCTTCTTAGTTCTCTTCTC AGAGAGGAAGAATCAAGAGAAG
GTG20	TCTCCTTCTGTGCTCTTCTC AGAGAGGAAGACACGAGAAG
GTG21	TCTCCTTCTGTGTCTCTTCTC AGAGAGGAAGACACAGAGAAG
GTG22	TCTCCTTCTTGTGTCTCTTCTC AGAGAGGAAGAACACAGAGAAG
GTG23	TCTCCTCTCTTGTGTCTCTTCTC AGAGAGGAGAGAACACAGAGAAG

FIGURE 1: Synthetic oligonucleotides used in the present study and their abbreviations. The top and bottom strands of each pair are designated top and bottom, respectively, in the text.

to react with platinum reagents, and repurified as described previously (Rice et al., 1988; Bellon & Lippard, 1990). Briefly, oligonucleotides were synthesized on a 1-μmol scale by using phosphoramidite methodology, fully deprotected, and purified by using C18 reversed-phase HPLC. All top strands (Figure 1) were converted to their Na^+ salts on a Dowex cation-exchange resin prior to reaction with platinum. The aqua complex, *cis*-$[Pt(NH_3)_2(H_2O)_2]^{2+}$, was generated from *cis*-DDP by the addition of 1.97 mol equivalents of $AgNO_3$ and allowed to react with the pyrimidine-rich top strands at a ratio of 1.3 Pt atoms per DNA strand. In all reactions a single major product was observed and isolated by using HPLC. Platinated DNA was characterized by carbon rod atomic absorption spectroscopy to determine the amount of bound platinum per strand. The amount of DNA present was estimated by using the calculated extinction coefficients at 260 nm (Fasman, 1975). Enzymatic digestion of the oligonucleotides before and after platination was employed to characterize the nature of the platinum adduct present (Eastman, 1986). DNase I, P1 nuclease, and alkaline phosphatase were used to digest these oligonucleotides into their component nucleosides. The HPLC standards *cis*-$[Pt(NH_3)_2\{d(GpG)\}]^+$, *cis*-$[Pt(NH_3)_2\{d(ApG)\}]^+$, and *cis*-$[Pt(NH_3)_2\{d(Guo)_2\}]^{2+}$ were synthesized independently and used to assign the products of digestion (Bellon & Lippard, 1990). Unplatinated single strands were 5′-end-labeled with [γ-^{32}P]ATP by using polynucleotide kinase, annealed with their phosphorylated complimentary strands, and allowed to react with T4 DNA ligase. The products were separated on 8% polyacrylamide (mono:bis acrylamide ratio = 29:1) gels run in buffer containing 89 mM Tris–borate, 89 mM boric acid, and 2 mM EDTA (Bellon & Lippard, 1990). Platinum was removed from the DNA by treatment with cyanide ion. Such reactions were performed on half of the typical ligation mixture (10 μL) by the addition of 50 μL of a CN^- solution (0.3 M NaCN, 0.3 M Tris base, pH 8.3) for 24 h at 30 °C (Lippard & Hoeschele, 1979). The other half was stored at 4 °C, and the two were subsequently examined on 8% native polyacrylamide electrophoresis gels.

Results

Gel Mobility Strategy. The mobility of a linear DNA molecule through a native polyacrylamide gel is directly proportional to its end-to-end distance (Lerman & Frisch, 1982; Lumpkin & Zimm, 1982). DNA multimers of identical length and number of bend units, but with differently phased bends, will have different end-to-end distances. The DNA bends of a polymer must be spaced evenly and phased with the DNA helical repeat in order to add constructively. Such constructively phased bends add in plane yielding short end-to-end distances and the most anomalous gel migration (Wu & Crothers, 1984; Koo et al., 1986). Interestingly, if the ends come close enough together, they may close covalently upon reaction with DNA ligase to form small DNA circles typically 100–200 bp long (Rice et al., 1988). Destructively phased bends yield polymers with longer end-to-end distances and migrate almost normally. The products of ligation reactions typically used to generate these polymers are resolved on native polyacrylamide gels where linear and any circular molecules separate to form slightly overlapping ladders of bands.

Previously we used a sequence repeat of 22 bp to study the DNA bending of a variety of platinum-induced bends (Rice, et al., 1988; Bellon & Lippard, 1990). The 22-bp spacing is almost exactly two helical repeats and so provided a good constructive addition of bends. The 22-bp fragment was employed instead of 2 × 10.5 bp units, the normal helical repeat of B-DNA being 10.5 bp (Rhodes & Klug, 1980; Wang, 1979), because the platinum compounds studied were known to produce a small amount of duplex unwinding (Sherman & Lippard, 1987). Here we systematically varied the lengths of DNA oligomers containing the three major cisplatin adducts in order to determine exactly which sequence repeat allows the most constructive addition of DNA bends. Any difference between this optimal sequence length and the 10.5-bp helical repeat of B-DNA will be caused by platinum-induced helical unwinding. We explored Pt–Pt distances of 20–24 bp in order to define accurately the most ideal platinum phasing separation. Only sequence lengths of 21 and 22 bp were used to measure the DNA unwinding produced by the *cis*-AG adducts because these two oligomers behaved exactly the same as the *cis*-GG21 and *cis*-GG22 oligomers and required no additional characterization. For all three adducts examined, the 22-bp oligomers were the same samples used to determine the extent of DNA bending reported previously (Bellon & Lippard, 1990).

Characterization of Oligonucleotide Monomers. A list of all sequences examined is given in Figure 1; the accompanying names refer to the unplatinated duplex oligomers. The top strands were designed to contain only one high-affinity platinum binding site, either two adjacent or nearby guanine bases or a guanine and an adenine base. Once complexed with *cis*-DDP, the single strand or duplex has been given the prefix cis. All sequences were designed to leave 2-bp overhangs at their 3′-ends in double-stranded form. These overhangs are non-self-complimentary, facilitating polymerization of the synthetic monomers in only one orientation, and maintaining a constant Pt–Pt distance throughout the resulting multimer.

The five *cis*-GG-containing oligomers, 20–24 bp in length, were found by atomic absorption analysis to contain 1.05, 1.09, 1.01, 1.15, and 1.08 Pt atoms/strand, respectively. The two oligomeric *cis*-AG adducts, *cis*-AG21 and *cis*-AG22, had 1.16 and 1.08 Pt atoms/strand whereas *cis*-GTG20, *cis*-GTG21, *cis*-GTG22, and *cis*-GTG23 had 1.27, 1.36, 1.27, and 1.17 Pt

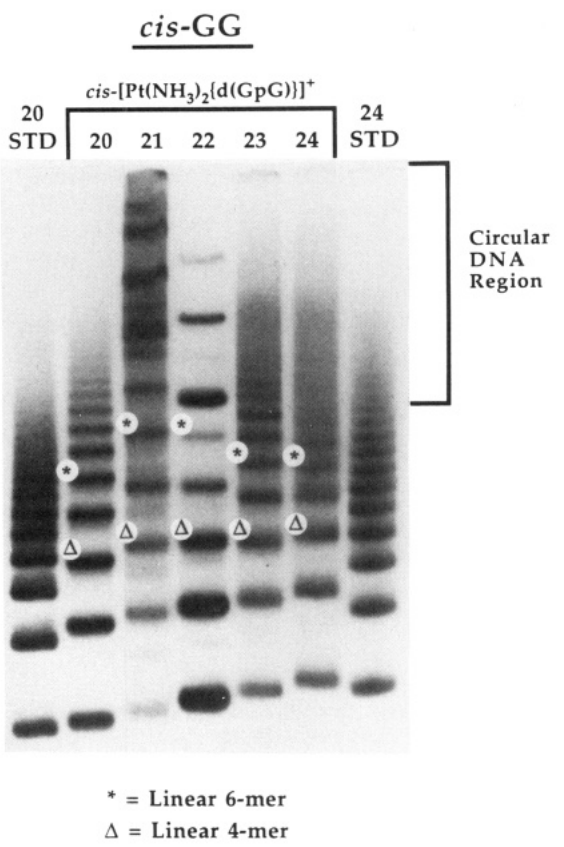

FIGURE 2: Autoradiogram of the ligation products of *cis*-GG-containing oligomers separated on an 8% polyacrylamide gel.

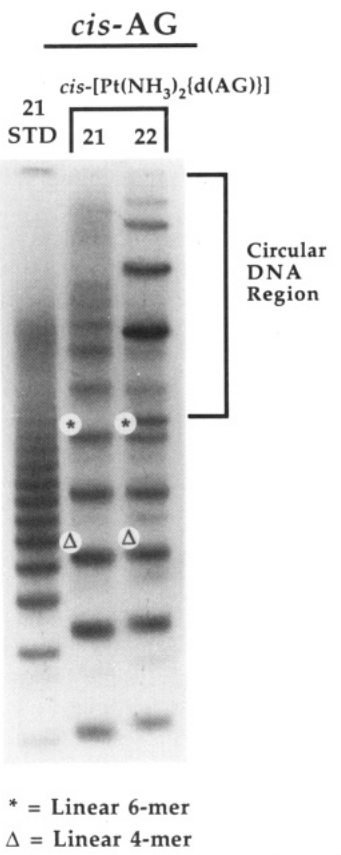

FIGURE 3: Autoradiogram of the ligation products of *cis*-AG-containing oligomers separated on an 8% polyacrylamide gel.

atoms/strand, respectively. These deviations from the expected value of 1.0 Pt atom/strand have been observed for other platinated oligonucleotides (Bellon & Lippard, 1990). The source of the discrepancy is the use of the calculated strand extinction coefficient to quantitate the DNA concentration, which does not take into account contributions from the *cis*-diammineplatinum(II) moiety or its effect on base-stacking interactions. Enzymatic digestion of all top strands was used to characterize the platinum adducts (Eastman, 1986). The enzymes employed do not cleave Pt–N bonds and thus leave the platinum adducts intact (Eastman, 1986). Digestion of 1,2-intrastrand cross-linked adducts does not break the intervening phosphodiester linkages, whereas the enzymes do cleave the intervening nucleotide from 1,3-adducts, leaving free nucleosides bound to platinum. HPLC analysis of the enzymatic digestion reactions of *cis*-GG20, *cis*-GG21, *cis*-GG22, *cis*-GG23, and *cis*-GG24 in all cases revealed three products that coeluted with cytosine, thymine, and *cis*-$[Pt(NH_3)_2\{d(GpG)\}]^+$. HPLC analysis of the enzymatic digestion of *cis*-AG21 and *cis*-AG22 also yielded peaks that coeluted with thymine and cytosine, but the third peak coeluted with *cis*-$[Pt(NH_3)_2\{d(ApG)\}]^+$. Similarly, digestion of *cis*-GTG20, *cis*-GTG21, *cis*-GTG22, and *cis*-GTG23 showed thymine and cytosine as well as a third peak coeluting with *cis*-$[Pt(NH_3)_2\{d(Guo)_2\}]^{2+}$. The adduct composition of all oligomers studied was determined in this manner.

Gel Mobility Studies. All bottom strands were radiolabeled, allowed to anneal with phosphorylated top strands, and then ligated to form multimers. Autoradiograms of the electrophoresis gels revealing resolution of the ligation products of *cis*-GG-, *cis*-AG-, and *cis*-GTG-containing oligomers are shown in Figures 2, 3, and 4, respectively. The linear multimers used to quantitate the amount of DNA bending form a ladder of bands, beginning with dimers that appear at the

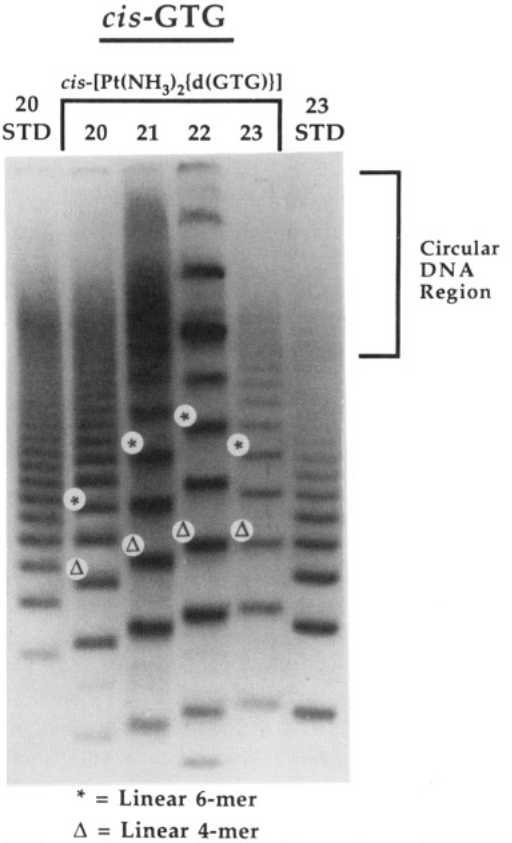

FIGURE 4: Autoradiogram of the ligation products of *cis*-GTG-containing oligomers separated on an 8% polyacrylamide gel.

bottoms of the gels. Linear multimers containing either four or six platinum bends are marked with triangles or asterisks, respectively. Also produced in ligations of 21 or 22 bp mo-

nomers are a separate series of bands arising from small DNA circles that migrate close to the top of the gel. They can be observed only for 21- or 22-bp multimers because only these sequence lengths phase the platinum-induced bends sufficiently well to allow the ends of the polymers to approach one another with the proper orientation to close covalently (Wu & Crothers, 1984; Koo et al., 1986; Rice et al., 1988; Bellon & Lippard, 1990). Unplatinated multimers, labeled STD, were used to calibrate the mobility of the linear platinated species. The relationship between interplatinum distance and phasing is most clearly appreciated by examining the bands denoted with triangles or with asterisks in Figures 2, 3, and 4. These bands form a bell-shaped pattern because the extent of retardation in gel mobility is sensitive to proper positioning of platinum atoms in the multimers. Such phasing allows the bends to add constructively in plane and greatly affects the retardation in gel mobility. Maximum retardation corresponds to the best phasing with the helical repeat. As the interplatinum distance departs from the helical repeat of the DNA, the bends add less constructively, the DNA lies more out of plane, and the multimers are less retarded as they migrate through the gel. The bell-shaped patterns are more pronounced for the 6-mers than for the 4-mers because a larger number of platinum-induced bends have added together (Koo et al., 1986; Rice et al., 1988; Drak & Crothers, 1991). The peak, or maximum, of these curves gives the precise helical repeat of the platinated DNA (Drak & Crothers, 1991).

Examination of Figure 2 shows that the maximum, defined by the asterisks, lies almost exactly in between 21 and 22 bp. Because the 22-bp 6-mer is 132 bp and the 21-bp 6-mer is only 126 bp, the fragment made up of 22 bp units experiences a slight additional mobility retardation simply because of its greater mass. Correcting for this size effect shifts the maximum away from 21.5 toward 21 bp. Figure 3 reveals that the *cis*-AG multimers behave identically with the multimers of *cis*-GG. The positions of both the asterisks and the triangles are exactly the same for the *cis*-GG and *cis*-AG adducts. For this reason, other sequence lengths containing the *cis*-AG adduct were not examined and a full analysis was only performed on the *cis*-GG adduct. Examination of the asterisk positions in Figure 4 demonstrates that the maximum has shifted toward 22 bp. Clearly the *cis*-GTG adduct unwinds the double helix to a greater extent than either the *cis*-GG or *cis*-AG adducts. The analysis that follows will quantitatively define the maxima of these bell-shaped curves in order to measure the DNA unwinding produced by these platinum adducts.

Quantitation of Platinum-Induced DNA Unwinding. DNA having no appreciable bends migrates in electrophoresis gels with a characteristic rate that depends only upon its length (Koo et al., 1986). From the unplatinated (STD) oligomers in Figures 2–4 may be constructed standard curves against which the mobilities of the platinated oligomers can be compared (Wu & Crothers, 1984; Koo et al., 1986). Platinated oligomers are assigned an effective length in this manner only on the basis of their rates of migration. Relative mobility, R_L, is defined as the ratio of the calculated length of a multimer to its real length (Koo et al., 1986). The higher this ratio, the more bent is a given oligomer. A R_L value of 1.00 means that the oligomer migrates as though it had no bends. The R_L vs length curves for the *cis*-GG-, *cis*-AG-, and *cis*-GTG-containing oligomers are plotted in Figures 5, 6, and 7, respectively. The 24-bp oligomers are omitted from the plots because they lie too far from the maximum retardation to be useful in helping to define the best phasing. Since all four curves

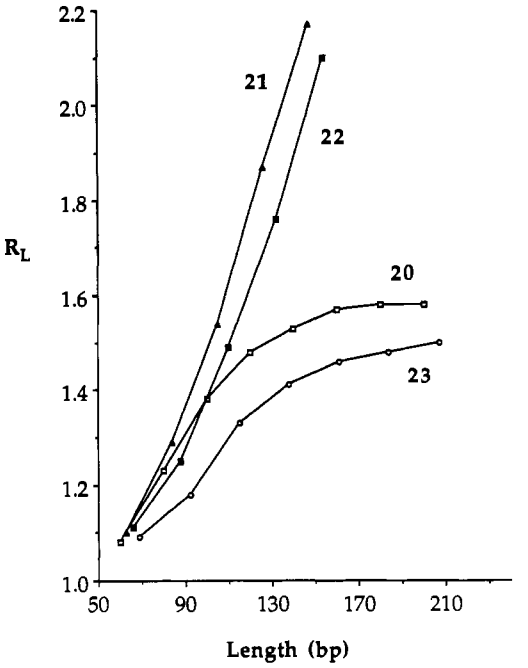

FIGURE 5: Plot showing the relative mobility (R_L) versus actual length curves for the oligomers *cis*-GG20, *cis*-GG21, *cis*-GG22, and *cis*-GG23, denoted respectively as 20, 21, 22, and 23.

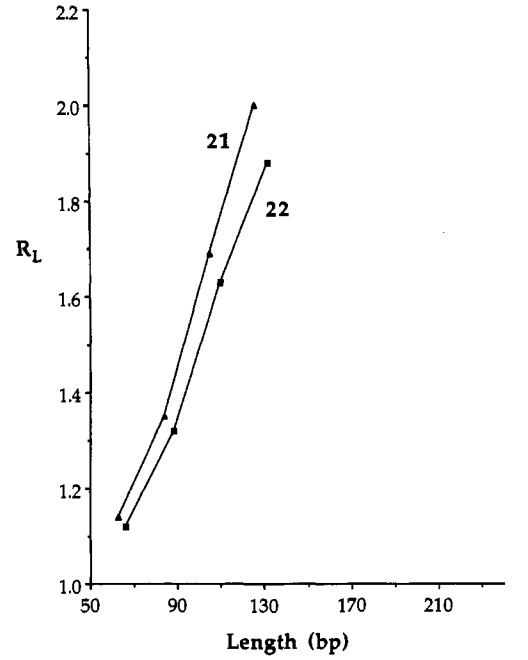

FIGURE 6: Plot showing the relative mobility (R_L) versus actual length curves for the oligomers *cis*-AG21 and *cis*-AG22, denoted respectively as 21 and 22.

in Figure 5 arise from DNA containing exactly the same *cis*-GG adduct, their slopes cannot reflect differences in the absolute magnitudes of the individual components of the bend. The slope of the curve describing multimers with a 21-bp sequence repeat is the steepest because the bends are phased most constructively (Koo et al., 1986). The difference between the natural 10.5-bp helical repeat of B-DNA and that of DNA perturbed by a platinum adduct is a consequence of DNA unwinding. As previously seen in Figure 3, the behavior of the *cis*-AG adduct exactly parallels that of *cis*-GG, a result further underscored by a comparison of the plots of Figures 5 and 6. In both figures, the 21-bp curve has a slightly greater slope than the 22-bp curve. These results demonstrate that

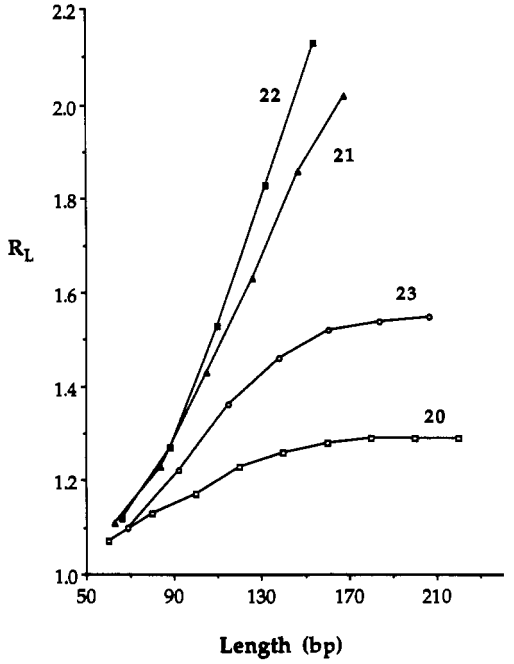

FIGURE 7: Plot showing the relative mobility (R_L) versus actual length curves for the oligomers *cis*-GTG-20, *cis*-GTG21, *cis*-GTG22, and *cis*-GTG23, denoted respectively as 20, 21, 22, and 23.

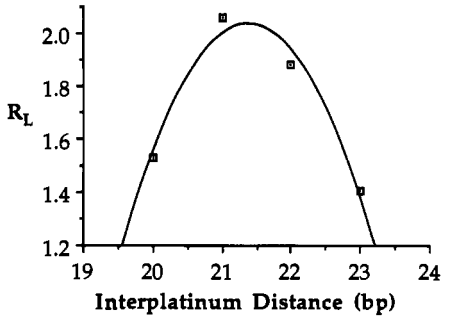

FIGURE 8: Plot showing the relative mobility (R_L) versus interplatinum distance for *cis*-GG oligomers with a total length of 140 bp.

the *cis*-GG and *cis*-AG adducts unwind the double helix to the same extent. For the 1,3-intrastrand *cis*-GTG adduct (Figure 7), the 22-bp curve has a greater slope than the 21-bp curve, which indicates that the DNA is more unwound than for oligomers containing the 1,2-intrastrand cross-linked *cis*-GG and *cis*-AG adducts.

The exact helical repeat, and from it the unwinding angle Φ, can be calculated by interpolation with use of the R_L vs length curves just described (Rice & Crothers, 1989; Drak & Crothers, 1991). In Figures 8 and 9 are plotted the relative mobilities, measured at a constant total sequence length of 140 bp, against the sequence repeat, or interplatinum distance, for *cis*-GG and *cis*-AG. The points on these curves are the average of three independent electrophoresis experiments, providing a more exact and quantitative representation of the triangles and asterisks of Figures 2–4. The maxima of the curves correspond to the interplatinum distances that afford the most constructive addition of DNA bends.

The expressions used to fit the four points in Figures 8 and 9 are simple quadratic equations of the form $ax^2 + bx + c$. The maxima of the curves were determined mathematically to be 21.34 for the *cis*-GG adduct and 21.67 for the *cis*-GTG adduct. On the basis of the spread of values from three independent electrophoretic experiments, an error of ±0.04 bp is assigned to these two values. A cubic equation of the form $ax^3 + bx^2 + cx + d$ was found to fit the data more closely,

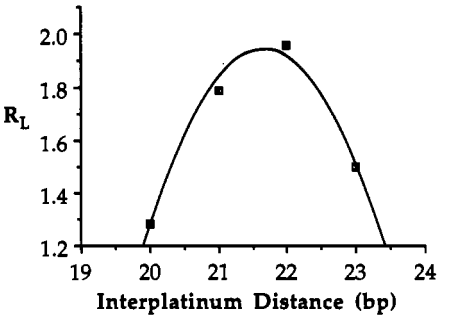

FIGURE 9: Plot showing the relative mobility (R_L) versus interplatinum distance for *cis*-GTG oligomers with a total length of 140 bp.

but the resulting curve was highly asymmetric. Previous examination of the phasing behavior of DNA revealed symmetric relationships (Rice & Crothers, 1989; Drak & Crothers, 1991; Levene & Crothers, 1986; Shore & Baldwin, 1983). Higher order symmetric equations were investigated but gave exactly the same maxima to within one-hundredth of a base pair as did the simple quadratics. In addition, total sequence lengths other than 140 bp were examined and generally gave results within a few hundredths of a base pair of the above values.

In order to convert the curve maxima in base pairs into duplex unwinding angles in degrees, one must compare the values with that of the normal helical repeat of B-DNA, 10.5 ± 0.05 bp. This value has been determined by many independent means (Wang, 1979; Rhodes & Klug, 1980). The difference between the helical repeat of B-DNA and the cisplatin-modified DNAs *cis*-GG and *cis*-AG, therefore, is [(21.34 ± 0.04) − 2(10.5 ± 0.05)] bp. Propagating errors yields a difference of 0.34 ± 0.08 bp. Given that there are 360 deg/10.5 bp, the DNA unwinding due to one *cis*-GG or *cis*-AG adduct is 13 ± 3°. Similarly, the DNA unwinding caused by the *cis*-GTG adduct is 23 ± 3°.

Cyanide Reversal Studies. The gel electrophoretic mobility of linear multimers containing *cis*- and *trans*-GTG adducts has also been examined by Anin and Leng (1990), who employed the same methodology used here and previously described (Rice, et al., 1988; Bellon & Lippard, 1990). Their results for the *cis*-GTG adduct are qualitatively the same as those reported here, although the unwinding angle was not given. Interestingly, the *trans*-GTG adduct was reported to bend DNA in a directed manner and not to form a flexible hinge joint. These results directly contradict our previous work, which concluded that the *trans*-GTG adduct allows DNA to bend in at least two directions (Bellon & Lippard, 1990).

Previously we found that multimers of 22-bp oligomers containing either *cis*-GTG or *trans*-GTG adducts both produced anomalously migrating linear pieces and small DNA circles (Bellon & Lippard, 1990). When the ligation products of 15-bp monomers were examined, only oligomers containing *trans*-GTG adducts produced slowly migrating linear fragments and small circles. The 15-bp *cis*-GTG-containing linear multimers migrated almost normally, and the ligation reactions contained no circular molecules. The presence of circular multimers in the ligation reactions of 15-bp *trans*-GTG-containing oligomers indicated that the DNA ends could approach one another and covalently close even when their bends were phased destructively. We concluded that *trans*-GTG-modified DNA possessed a hinge joint at the locus of platination and was flexible enough to bend in at least two directions. In these earlier studies, DNA circles were assigned by their diminished rates of migration on polyacrylamide gels and by comparison with the rates of migration of other circular DNA molecules (Rice et al., 1988).

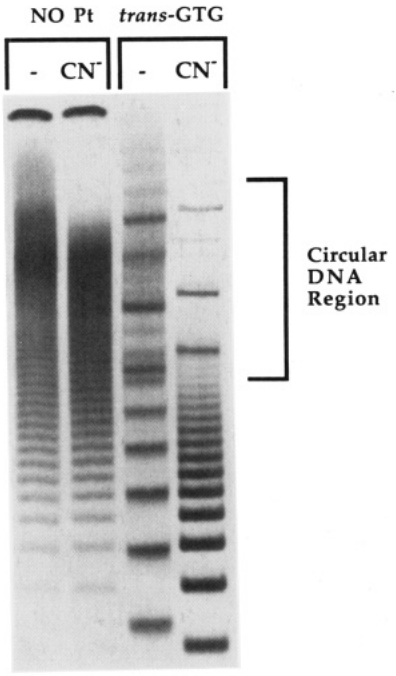

FIGURE 10: Autoradiogram showing the effects of CN^- reversal of ligation reactions of *trans*-GTG15 that demonstrate the presence of DNA circles. Lanes 1 and 2 are the control, unplatinated GTG15 multimers; lanes 3 and 4 are *trans*-GTG15 multimers; lanes 2 and 4 are samples that were treated with cyanide ion as described in the text prior to running the gel.

In order to demonstrate by independent means the presence of small DNA circles among the ligation products of *trans*-GTG15, and thus strengthen our original claim that *trans*-GTG-modified DNA can bend in at least two directions, we performed an additional set of experiments utilizing CN^- ion that is reported here. In a typical ligation reaction with platinated oligonucleotides containing well-phased bends, or one involving the *trans*-GTG adduct, both linear and circular fragments are produced. If cyanide ion is allowed to react with the products of the ligation reaction, platinum will be removed in the form of $[Pt(CN)_4]^{2-}$ (Lippard & Hoeschele, 1979). After reaction with CN^-, the linear multimers are expected to migrate normally in electrophoresis gels since they no longer possess any DNA bends. The shapes of the circular fragments, on the other hand, are not expected to change significantly upon removal of platinum since they are covalently closed, and consequently their gel mobilities will be relatively unchanged.

Polymers of *trans*-GTG15 as well as control polymers of the corresponding unplatinated DNA were constructed as before (Bellon & Lippard, 1990). Each ligation reaction product was divided into two, half being maintained at 4 °C while the other half was allowed to react with CN^- solution. The gel electrophoretic separation of these polymers with and without cyanide treatment is displayed in Figure 10. As can be seen from the first two lanes in the autoradiogram, reaction with cyanide ion produces no degradation or change in the mobility of the ladder of bands in the control, unplatinated DNA. The second set of lanes, corresponding to platinated DNA, reveals the usual overlapping ladders of linear and circular bands. Upon removal of platinum with CN^-, bands corresponding to linear multimers revert to their normal mobility, indistinguishable from the ladder of bands in lanes 1 and 2. The ladder of bands corresponding to circular DNA remains at the top of the gel, however, and exhibits slightly retarded mobility after CN^- treatment. This decrease in migration rate is fully consistent with the effect first described by Cohen et al. (1979), who reported that nicked circular DNA treated with *cis*- or *trans*-DDP migrated faster than unplatinated control DNA of the same length. This phenomenon results from an effective shortening of the circumference of the circles upon platination, as confirmed by electron microscopy (Cohen et al., 1979). The source of the diminished diameter of the circular, platinated DNA can now be appreciated as arising from multiple bends. In the cyanide reversal experiments carried out here, we conclusively demonstrate the presence of DNA circles in ligation reactions of *trans*-GTG oligomers, confirming that the *trans*-GTG adduct introduces a hinge joint in DNA.

Discussion

DNA unwinding produced by platination with a variety of complexes has been extensively studied by titrating the negative supercoils of plasmid DNA (Cohen et al., 1979; Scovell & Collart, 1985; Scovell & Kroos, 1982; Sherman & Lippard, 1987; Bowler, 1986; Keck and Lippard, manuscript in preparation). These experiments measure the change in superhelical density arising from the menu of different adducts formed when a bifunctional platinum compound is allowed to react with random sequence DNA. As discussed in the introduction, this methodology measures the removal of negative supercoils; it cannot separate the effects of DNA bending, which can change Wr, from unwinding, which modifies Tw.

The initial report of *cis*- and *trans*-DDP induced DNA unwinding (Cohen et al., 1979) utilized the superhelical titration method but did not attempt to quantitate unwinding angles. We have therefore analyzed these published data by using superhelical densities and correction factors for ionic strength and temperature (Bauer, 1978). Values of 12.8°/platinum bound were derived for both *cis*- and *trans*-DDP adducts. Similar experiments (Scovell & Kroos, 1982) carried out subsequently also did not report an unwinding number. Analysis of these data without correcting for temperature or ionic strength effects gives a value of 12.8° for *cis*-DDP. Scovell and Collart (1985) have also examined *cis*-DDP unwinding as a function of superhelical stress. At a bound platinum per nucleotide level of 0.08, an unwinding angle of 11° was found. Results for *trans*-DDP adducts (Cohen et al., 1979; Scovell & Kroos, 1982; Scovell & Collart, 1985; Keck and Lippard, manuscript in preparation) are less consistent, ranging from 6 to 13°. The average value of 12° for the unwinding of cisplatin–DNA adducts is in good agreement with that reported here for both the *cis*-AG and *cis*-GG site-specifically modified oligonucleotides, which is perhaps not surprising considering that adducts formed by *cis*-DDP consist primarily of *cis*-GG and *cis*-AG cross-links (Eastman, 1986; Fichtinger-Schepman et al., 1985, 1987). These results also demonstrate that DNA bends as large as 35° (Bellon & Lippard, 1990) do not appreciably affect the determination of unwinding angles obtained by use of the method of titration of negative supercoils.

The anticancer activity of *cis*-DDP is believed to arise, at least in part, through covalent binding to DNA, which inhibits replication (Pinto & Lippard, 1985; Bruhn et al., 1990). It has been postulated that the differential toxicity of *cis*- and *trans*-DDP may result from differential repair of the modified DNA (Sancar & Rupp, 1983; Ciccarelli et al., 1985; Eastman & Schulte, 1988). An initial aspect of repair is recognition of the damage by cellular repair proteins, which are sensitive to the various adducts formed by *cis*- and *trans*-DDP. Comparisons between these structures and the affinity of various proteins for the adducts may elucidate stereochemical features necessary for recognition and binding. The unwinding angles reported here add significantly to the body of information

about platinum-damaged DNA and offer an opportunity to understand the structural basis of protein interactions with damaged DNA. In pursuit of this objective, we have analyzed the binding preferences of two known cellular components that bind platinum and other damaged DNA in the light of the present new and related structural information.

The (A)BC excinuclease complex is responsible for excision repair in *E. coli* (Sancar & Sancar, 1988; Orren & Sancar, 1989; Grossman & Yeung, 1990). While all three subunits are necessary to carry out the complete damage excision and repair process, the UvrA protein has been determined to recognize and bind to damaged DNA, probably as a dimer (Mazur & Grossman, 1991; Myles et al., 1991; Myles & Sancar, 1991). The UvrB subunit cannot bind damaged DNA and is thought to facilitate the formation of a UvrB/C complex that excises the damage as a dodecanucleotide repair patch (Orren & Sancar, 1989; Selby & Sancar, 1990b). The (A)BC excinuclease complex can repair DNA damage caused by a variety of different agents, including *cis*-DDP, $[Pt(en)Cl_2]$, and $[Pt(dach)Cl_2]$ (Beck et al., 1985, Page et al., 1990), as well as AAF, apurinic sites, psoralen cross-links, and thymine dimers (Sancar & Sancar, 1988). While $[Pt(en)Cl_2]$ is not identical with *cis*-DDP, it gives similar adduct distributions and is commonly used in its place for mechanistic studies. The excision rates of several site-specific $[Pt(en)Cl_2]$–DNA adducts have been quantitated. The GTG adduct of this complex is repaired with an efficiency approximately 6 times greater than that of the GG adduct, whereas the rates of repair of the GG and AG adducts generally differ from one another by less than a factor of 2 (Page et al., 1990).

Since DNA containing vastly different types of damage is recognized by the (A)BC excinuclease, it is reasonable to assume that some common distortion of the DNA, and not the adduct itself, is being targeted for repair. Interestingly, the extent of DNA unwinding for the three cisplatin adducts reported here correlates well with the rates of excision repair reported by Page et al. (1990) for the closely related $[Pt(en)Cl_2]$ adducts. We therefore postulate that DNA unwinding is a major determinant of (A)BC repair efficiency. More specifically, we propose that the UvrA subunit recognizes unwound DNA. This hypothesis will be discussed first from an energetic basis and then in terms of the structures of different types of DNA damage known to be repaired by the (A)BC complex.

We assume that the initial rate of excision by the (A)BC excinuclease is determined by the binding affinity of UvrA for damaged DNA. If DNA unwinding is the major signal recognized by the UvrA protein, the energetics of DNA unwinding should relate to the energetics of protein binding and thus to the specificity of binding. Several investigators (Horowitz & Wang, 1984; Levene & Crothers, 1986) have described the energy (G) required to twist DNA about its helix axis. An expression that may be used to calculate this energy is given by

$$G = [(2.7 \times 10^6 \text{ cal})(\Delta Tw)^2]/N \text{ mol} \qquad (1)$$

where ΔTw is a unitless parameter denoting the change in local twist and N is the length in base pairs of the section of DNA being twisted. For example, if a large piece of DNA is gripped in two places separated by 12 bp and twisted locally between these endpoints, then $N = 12$ bp. The choice of 12 bp was made because it corresponds to the size of the UvrABC repair patch (Sancar & Sancar, 1988). Substituting $\Delta Tw = 13°/360° = 0.036$ and $N = 12$ bp into eq 1, we compute a distortion energy of only 0.29 kcal. This amount of energy can readily be supplied thermally at ambient temperature, indicating that unplatinated DNA can easily assume a 13° distortion necessary to bind UvrA. To postulate that UvrA recognizes DNA unwound by 13° allows for little or no binding specificity at damaged sites.

This problem can be resolved by postulating that the $(UvrA)_2$ dimer is configured to bind DNA unwound by some large amount, for example 60°. UvrA, or the $(UvrA)_2$ dimer, may have a conformation designed to make specific hydrogen bonds with the sugar–phosphate backbone on both sides of the damage. The geometry of the complex may resemble two hands gripping the double helix but never making direct contact with the damage locus itself. Unmodified DNA, with $\Delta Tw = 0$, would have its sugar–phosphate backbone improperly configured to interact with $(UvrA)_2$. In order to form a $(UvrA)_2$–DNA complex, either the DNA would have to unwind by 60°, UvrA would have to distort in some way, or some combination of both would have to occur. Since in this model the protein does not make direct contact with the site of damage, the unwinding can occur over the entire region of DNA between the two hands, at a separation of N.

The specificity of UvrA for binding to the *cis*-GG adduct can be calculated by comparing the different distortion energies required for platinated vs unplatinated DNA to unwind by 60°. Unplatinated DNA has to twist by a full 60°, giving $G = (2.7 \times 10^3)(60/360)^2/N$ kcal, or $(75/N)$ kcal. Platinated DNA only has to twist from 13 to 60, or 47°, requiring a free energy of only $(46/N)$ kcal. The difference, $(29/N)$ kcal, or 2.4 kcal for $N = 12$ bp, may be sufficient to give a high specificity of protein binding to platinated vs unplatinated DNA. The energy difference increases if UvrA contacts the DNA at two points separated by less than 12 bp. An initial unwinding of only 13° becomes significant here because of the quadratic form of the potential energy function, eq 1, and significantly diminishes the energy required to achieve the hypothetical 60° unwinding necessary to bind UvrA.

The free energy of unwinding calculated for damaged DNA is only approximate because eq 1 applies specifically to unmodified DNA. If, however, damaged DNA unwinds more readily than undamaged DNA, then the specificity of UvrA binding will be further enhanced. While it is not necessary to invoke this property in order to explain the results, unwindability may well play a role in determining binding specificity. Protein-induced DNA structural changes have been postulated to contribute to binding specificity in a number of systems (Steitz, 1990). For example, DNA wrapped about the nucleosome core proteins is kinked in several places, and it has been argued that the natural kinkability of DNA determines its ability to bind to the nucleosome (Travers & Klug, 1987; Shrader & Crothers, 1989).

The foregoing model can be tested by measuring the duplex unwinding of damaged DNA before and after UvrA binding. If the model is correct, UvrA binding should increase the unwinding to a considerable extent. By studying negatively supercoiled DNA, the unwinding produced by UvrA binding has been measured to be about 60° (Oh & Grossman, 1986, 1987). This finding gives support to the hypothesis that UvrA has a conformation designed to bind highly unwound DNA.

Intercalators such as ethidium bromide and caffeine do not form covalent linkages with DNA, yet UvrA binds to DNA in the presence of these compounds (Selby & Sancar, 1990a). Ethidium bromide unwinds DNA by 26° with no concomitant bending. The (A)BC excinuclease complex does not excise patches of DNA containing intercalatively bound ethidium, probably because the adducts are not covalently bound to the DNA. The fact that DNA containing these intercalators can

Table I: Unwinding, Bending, and Recognition by (A)BC Excinuclease for Various Platinum and Other DNA Adducts

adduct type	DNA unwinding angle (deg)	DNA bending angle (deg)	(A)BC excinuclease recognition
cis-GG	13[a,b]	32–34[c,d]	(+)[e]
cis-AG	13[a,b]	32–34[d]	(+)[e]
cis-GTG	23[a,b]	32–34[d]	(+)[e]
trans-GTG	6–13[a]	hinge joint[f]	(–)[g]
(N3-Cyt)-G	yes[h]	0[d]	(+)[e]
AAF	22[i]	hinge joint[j,k]	(+)[l]
apurinic site	12[m]	hinge joint[j,k]	(+)[n]
psoralen	30[o]	0[o]	(+)[q]
pyrimidine dimer	14[r]	~50[s,t]	(+)[u]

[a] See text. [b] This work. [c] Rice et al. (1988). [d] Bellon and Lippard (1990). [e] Page et al. (1990). [f] Previous work (Bellon & Lippard, 1990) revealed that the *trans*-GTG adduct makes the double helix more flexible, allowing it to bend in more than one direction. This flexibility makes an exact determination of the bend angle difficult. [g] Beck et al. (1985). [h] The unwinding of supercoiled plasmid DNA was used to estimate duplex unwinding for several monofunctionally bound platinum adducts, including that formed by *cis*-[Pt(NH_3)$_2$(*N3*-cytosine)Cl]$^+$. Values of 6–10° were obtained (M. V. Keck and S. J. L., manuscript in preparation). [i] Drinkwater et al. (1978). [j] Both AAF-modified DNA and depurinated DNA are bent. Schwartz et al. (1989) employed the gel methodology employed here to measure the bend and observed a hinge joint. [k] Schwartz et al. (1989). [l] Sancar et al. (1985). [m] Ciomei et al. (1984). [n] Lin and Sancar (1989). [o] Haran and Crothers (1988). [q] van Houten et al. (1986). [r] Ciarocchi and Pedrini (1982). [s] Husain et al. (1988). [t] Husain et al. (1988) obtained 72° for the pyrimidine dimer bend angle and applied a correction factor of 2.4 to obtain the published value of 30°. Recent studies indicate that bend angle determinations with the ligase closure method used in Husain et al. (1988) should apply a correction factor of 1.5 (Hagerman & Ramadevi, 1990). By using this value one arrives at a bend angle of about 50°. [u] Sancar and Sancar (1988).

serve as a substrate for the DNA recognition subunit of the (A)BC excinuclease further strengthens the arguments that DNA unwinding plays an important role in damage recognition.

The hypothesis that local DNA unwinding is a determinant for UvrA recognition can also be evaluated by examining the structures of DNA modified with various agents, information for which is summarized in Table I. Of all the DNA damaging agents listed, only *trans*-DDP does not produce adducts that are efficiently recognized by the (A)BC excinuclease. The (A)BC studies employed globally platinated DNA, however, whereas the electrophoresis studies used to investigate binding employed site-specific *trans*-GTG adducts. As a general rule, all of the adducts recognized by the excinuclease system unwind DNA. DNA bending does not appear to be as important a determinant, because two adducts that do not bend DNA, psoralen and monofunctionally bound platinum, are in fact recognized by the (A)BC excinuclease. It should be noted that *cis*-[Pt(NH_3)$_2$(*N3*-cytosine)Cl]Cl was the platinum compound used by Bellon and Lippard (1990) to measure DNA bending for monofunctional adducts (Hollis et al., 1991), whereas [Pt(dien)Cl]Cl was the complex used to afford monofunctional adducts in assays with the (A)BC excinuclease. The extent of DNA bending probably does modulate the excision reaction but clearly does not play a primary role in adduct recognition. Unfortunately, quantitative studies of the binding and excision rates have not been performed for most of the adducts listed in Table I, making it impossible to provide more than a qualitative correlation of the degree of unwinding with the strength of UvrA binding and velocity of excision. Monofunctional adducts of *cis*-DDP unwind DNA by a smaller extent than do the *cis*-GG and *cis*-AG adducts yet are repaired 2–3 times more rapidly and with 2–3 times greater efficiency (Page et al., 1990). The monofunctional adducts may simply render DNA more unwindable.

Finally, we explore the possible relationship between the unwinding values reported here and the binding preferences of the cisplatin damage recognition protein in HeLa cell extracts discovered in this laboratory (Toney et al., 1989; Donahue et al., 1990). The DRP specifically binds DNA modified with the antitumor drug *cis*-DDP, but not with *trans*-DDP. With the use of the very same oligomers employed in the present bending and unwinding studies, the specificity of DRP binding to the specific adducts *cis*-GG, *cis*-AG, and *cis*-GTG was evaluated (Donahue et al., 1990). The adducts *cis*-GG and *cis*-AG were recognized with equal affinity by the DRP as judged by band-shift assays. All other types of DNA damage investigated, including *cis*- and *trans*-GTG adducts, monofunctional platinum adducts, and thymine dimers, were not recognized under the conditions used. The *cis*-AG and *cis*-GG adducts both bend DNA by about 35° and unwound the duplex by 13°. All of the other adducts examined bring about different structural perturbations. Thus, the DRP appears to have as its structural recognition element DNA that is bent by 35° and unwound by 13°. These parameters accompany the formation of *cis*-GG and *cis*-AG adducts and most likely occur in natural DNA conformations, as yet undiscovered, to which the DRP normally binds.

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