Monofunctional Adducts of Platinum(II) Produce in DNA a Sequence-Dependent Local Denaturation

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ABSTRACT: The effects on the conformation of DNA produced by the monofunctional adducts of chloro(diethylenetriamine)platinum(II) chloride or *cis*-diamminemonoaquamonochloroplatinum(II) have been investigated by means of the single-strand-specific probe chloroacetaldehyde (CAA). The denatured sites to which CAA was bound and that were induced in DNA by the monofunctional adducts of the platinum complexes were characterized by means of three experimental approaches. These include measurement of the fluorescence of a plasmid fragment treated with CAA, analysis of oligonucleotides treated with CAA and cleaved by piperidine, and termination of duplex transcription on a fragment of plasmid DNA treated with CAA. The results indicate that the denaturational change preferentially occurs in the base pair containing the monoadducted deoxyriboguanosine in the trinucleotide sequence Py-deoxyriboguanosine-Py (Py is a pyrimidine deoxyribonucleoside). It was suggested that this conformational alteration facilitates in DNA the formation of minor bifunctional adducts of *cis*-diamminedichloroplatinum(II).

Platinum coordination complexes are used as drugs in cancer chemotherapy (Loehrer & Einhorn, 1984). The mechanism of their antitumor action is uncertain at present but is thought to involve formation of covalent adducts to DNA [for general reviews, see Eastman (1987), Johnson et al. (1989), Lepre and Lippard (1990), and Reedijk (1987)]. *cis*-Diamminedichloroplatinum(II) {*cis*-[$Pt(NH_3)_2Cl_2$], cisplatin}[1] (Figure 1) is the first drug of this kind in widespread use in the clinic. It binds to DNA in a two-step process forming first monofunctional adducts preferentially at the N(7) position of deoxyriboguanosine (dGuo) residues that subsequently evolve to bifunctional lesions (Bancroft et al., 1990; Brabec et al., 1990; Eastman, 1986). The relative amounts of the different adducts formed during a 48-h reaction of *cis*-[$Pt(NH_3)_2Cl_2$] with double-stranded linear DNA are 90% intrastrand cross-links between neighboring purines and approximately 10% intrastrand cross-links between two purine bases separated by a third base, interstrand cross-links, and monofunctional adducts (Eastman, 1986; Fichtinger-Schepmann et al., 1985).

Most of the structural information currently available pertains to the bidentate DNA adducts. Monofunctional adducts have been studied less thoroughly, although information on the first step of platinum binding to DNA may help to better understand how platinum complexes form bifunctional genotoxic lesions. In addition, new platinum(II) antitumor compounds have been synthesized in which the platinum moiety has only one leaving ligand (Farrell et al., 1990; Hollis et al., 1991). Thus, a knowledge of the monofunctional adducts of platinum in DNA may also contribute to the development of new platinum antitumor drugs

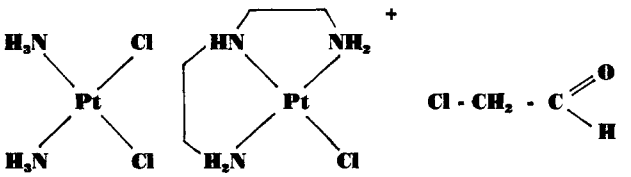

FIGURE 1: Platinum complexes and a chemical probe of DNA structure used in these experiments.

which bind to DNA differently in comparison with *cis*-[$Pt(NH_3)_2Cl_2$].

The results demonstrating that monofunctional adducts of platinum change the conformation of DNA have been summarized in our recent paper (Brabec et al., 1992). Monofunctional adducts of platinum(II) produce in DNA a conformational distortion, which is sequence-dependent, spreads over several base pairs (bp) around the adduct, and is different from that induced by bifunctional adducts of *cis*-[$Pt(NH_3)_2Cl_2$]. Most extensive distortions and resulting destabilization were observed in DNA containing the trinucleotide sequences in which the platinated dGuo was flanked by pyrimidine deoxyribonucleosides. No additional data about the character of the distortion induced in DNA by monofunctional adducts of platinum(II) have been so far presented. It is not even known whether the hydrogen bonds in the platinated base pairs remain intact as a consequence of the formation of monofunctional adducts.

In the present work we examine synthetic oligonucleotide duplexes (19 or 20 bp) containing a unique site-specific monofunctional adduct either of *cis*-diamminemonoaquamonochloroplatinum(II) {*cis*-[$Pt(NH_3)_2(H_2O)Cl]^+$} or of monodentate chloro(diethylenetriamine)platinum(II) chloride {[Pt(dien)Cl)]Cl} (Figure 1). The studies were carried out to demonstrate that these monofunctional adducts induce in DNA conformational changes of denaturational character depending on the base sequence. The studies were also extended to plasmid DNA containing monofunctional adducts of platinum(II) in order to confirm that these lesions also

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[1] Abbreviations: ATP, adenosine 5′-triphosphate; bp, base pair; CAA, chloroacetaldehyde; *cis*-[$Pt(NH_3)_2Cl_2$], *cis*-diamminedichloroplatinum(II); *cis*-[$Pt(NH_3)_2(H_2O)Cl]^+$, *cis*-diamminemonoaquamonochloroplatinum(II); dAdo, deoxyriboadenosine; d(AGT)/d(ACT), d(CGT)/d(ACG), etc., oligonucleotide duplexes (defined in Figure 3); dCyd, deoxyribocytidine; dGuo, deoxyriboguanosine; ELISA, enzyme-linked immunosorbent assay; Pu, purine deoxyribonucleoside; [Pt(dien)Cl]Cl, chloro(diethylenetriamine)platinum(II) chloride; Py, pyrimidine nucleoside; r_b, number of platinum atoms/nucleotide residue; RF, relative fluorescence intensity (defined in text).

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induce a similar sequence-dependent interruption of hydrogen bonds in base pairs of natural DNA.

MATERIALS AND METHODS

Chemicals. [Pt(dien)Cl]Cl and *cis*-[$Pt(NH_3)_2Cl_2$] were synthesized in Lachema (Brno, Czech Republic). *cis*-[$Pt(NH_3)_2(H_2O)Cl]^+$ cation was generated as described previously (Brabec et al., 1992). Chloroacetaldehyde (CAA) (Figure 1) from Fluka was doubly distilled before use. Restriction enzymes, T4 polynucleotide kinase, and Klenow fragment of DNA polymerase I were from Boehringer-Mannheim and Bethesda Research Laboratory. Nucleoside 5′-triphosphates were from Sigma. SP6 and T7 RNA polymerases, ribonucleotide triphosphates, and RNasin inhibitor were from Promega. 3′-Deoxyribonucleotide triphosphates were purchased from Pharma-Waldhof (Dusseldorf, Germany). All radioactive products were from Amersham. Ultrapure agarose was from Bethesda Research Laboratory, and acrylamide, bis(acrylamide), urea, and NaCN were from Merck.

Synthetic Oligonucleotides and Natural DNA. The oligonucleotides synthesized on an Applied Biosystems solid-phase synthesizer were purified as already described (Brabec et al., 1992). In this paper the concentrations of oligonucleotides are related to the mononucleotide content. Plasmid pSP73KB (2455 bp) was prepared as described (Lemaire et al., 1991).

Platination Reactions. The *NdeI*/*HpaI* restriction fragment (212 bp) from plasmid pSP73KB was modified with [Pt(dien)Cl]Cl in 10 mM $NaClO_4$ at 37 °C for 24 h in the dark. The modification of this fragment with *cis*-[$Pt(NH_3)_2(H_2O)Cl]^+$ was also performed in 10 mM $NaClO_4$ at 37 °C in the dark but only for 15 min. The latter platination reaction was stopped by adjusting the NaCl concentration to 0.1 M and cooling the sample in an ice bath. The ratio of platinum atoms fixed per nucleotide residue (r_b) in this sample was determined by pulse polarographic assay (Brabec et al., 1983; Kim et al., 1990). In some experiments one-half of this sample was precipitated by ethanol, dissolved in 10 mM $NaClO_4$, and incubated for an additional 24 h at 37 °C to evolve bifunctional adducts.

In the experiments in which interstrand cross-links were investigated the *NdeI* restriction fragment from plasmid pSP73KB (2455 bp) was modified with *cis*-[$Pt(NH_3)_2(H_2O)Cl]^+$ in 10 mM $NaClO_4$ at 37 °C for 15 min in the dark. The platination reaction was stopped by adjusting the NaOH concentration to 10 mM and cooling the samples to 0 °C. The platinum content in these samples was estimated by the pulse polarographic assay (Brabec et al., 1983; Kim et al., 1990).

The double-stranded oligodeoxyribonucleotides containing a unique monofunctional adduct of [Pt(dien)Cl]Cl or *cis*-[$Pt(NH_3)_2(H_2O)Cl]^+$ were prepared, purified, and characterized by the procedures described in detail in our recent paper (Brabec et al., 1992). The monofunctional adduct was formed at the central dGuo residue of the top strands of the duplexes listed in Figure 3.

Immunochemical Analysis. Polyclonal antibodies were elicited against double-helical calf thymus DNA modified by *cis*-[$Pt(NH_3)_2Cl_2$] at r_b = 0.08 in 10 mM $NaClO_4$ for 48 h at 37 °C. They were purified and characterized as described in our recent paper (Vrána et al., 1992). It was also shown in this paper that the antibodies bind preferentially to the adducts of *cis*-[$Pt(NH_3)_2Cl_2$] formed in double-helical DNA between adjacent purine residues in one strand. The procedures for their immunoenzymatic analysis and enzyme-linked immunosorbent assay (ELISA) were also recently described (Brabec et al., 1991; Vrána et al., 1992).

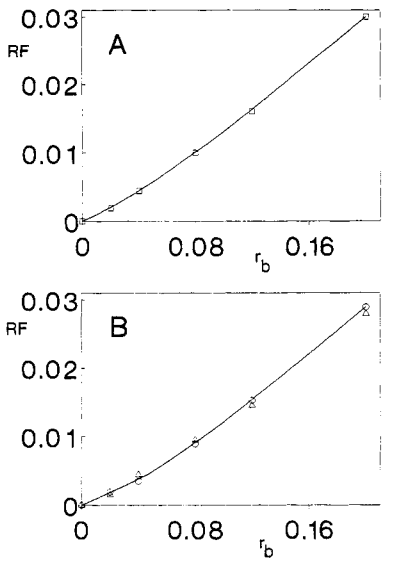

FIGURE 2: Dependence on r_b of the relative fluorescence (RF) yielded by samples of the *NdeI*/*HpaI* fragment of pSP73KB plasmid modified by (A) [Pt(dien)Cl]Cl or (B) *cis*-[$Pt(NH_3)_2(H_2O)Cl]^+$ and subsequently treated with CAA. The time of the modification by [Pt(dien)Cl]Cl was 24 h. The modification by *cis*-[$Pt(NH_3)_2(H_2O)Cl]^+$ was carried out for 15 min at a formal drug-to-nucleotide ratio which gave the r_b values indicated in the graph. The platination reaction was stopped by adjusting the NaCl concentration to 0.1 M and cooling the sample in an ice bath. One-half of each sample was dialyzed against 10 mM $NaClO_4$ and further incubated for an additional 24 h at 37 °C. The platinated samples were treated with CAA, and their fluorescence was measured as described in Materials and Methods. The samples which were not dialyzed against 10 mM $NaClO_4$ gave the results shown in panel B by triangles, whereas those which were dialyzed gave the results shown in panel B by circles.

Modification by CAA. The modifications of oligonucleotides were performed on duplexes 5′-end-labeled with [γ-^{32}P]-ATP by using T4 polynucleotide kinase, whereas the modifications of the *NdeI*/*HpaI* restriction fragment of pSP73KB DNA were carried out without radioactive labeling. The modification reaction was performed in 0.1 M sodium acetate, pH 6.4, for 1 h at 25 °C in a total volume of 0.1 mL; the concentration of the oligonucleotide was 1.6×10^{-5} M, and that of CAA was ca. 0.3 M. In the case of platinated oligonucleotides, platinum was removed after reaction of the oligonucleotide with CAA by incubation with 0.2 M NaCN (at alkaline pH) at 45 °C for 10 h in the dark. Piperidine treatment of the CAA-modified oligonucleotides was conducted in the same way as for the sequencing reaction (Maxam & Gilbert, 1977).

Measurement of Fluorescence. The fluorescence (emission wavelength, 405 nm; excitation wavelength, 280 nm) of the DNA samples (10 μg/mL) treated with CAA was measured with a Shimadzu RF 40 spectrofluorometer. The relative fluorescence intensity (RF) was determined as the ratio of the fluorescence intensity of platinated double-stranded DNA treated with CAA and that of CAA-modified unplatinated DNA, which was, before reaction with CAA, heated to 100 °C for 6 min and then rapidly cooled in an ice bath. The lower detection limit of the determination of denatured DNA in the presence of the surplus of double-helical DNA by this fluorescence assay was ca. 0.1%.

Transcription Mapping. Transcription of the *NdeI*/*HpaI* restriction fragment of pSP73KB DNA with SP6 and T7 RNA polymerases and electrophoretic analysis of transcripts were performed according to the protocols recommended by

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d(AGT)/d(ACT)     5′ CTCTCCTCAGTCTCCTCTCT
                     AGAGGAGTCAGAGGAGAGAG 5′

d(CGT)/d(ACG)     5′ CTTCCTCTCGTCTCCTTCTT
                     AAGGAGAGCAGAGGAAGAAG 5′

d(TGA)/d(TCA)     5′ CTCCTCTCTTGATCTCCTCT
                     GAGGAGAGAACTAGAGGAGA 5′

d(TGC)/d(GCA)     5′ CTTCTCCTTGCTCTCCTTCTCT
                      AGAGGAACGAGAGGAAGAGAGA 5′

d(TGT)/d(ACA)     5′ CTCTCCTCTGTCTCTTCCTC
                     AGAGGAGACAGAGAAGGAGG 5′
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FIGURE 3: Synthetic oligonucleotides used in the present study and their abbreviations. The top and bottom strands of each pair shown are designated top and bottom, respectively, in the text.

Promega (Promega Protocols and Applications, 1989/90, pp 43–46) and in the papers of Brabec and Leng (1993) and Lemaire et al. (1991).

RESULTS

Fluorescence Detection of Unpaired Bases in the Double-Stranded 212-bp Fragment of Natural DNA Containing Monofunctional Platinum(II) Adducts. We used CAA to test whether monofunctional adducts of platinum(II) locally denature the double helix. This probe reacts with N(1) and N(6) of deoxyriboadenosine (dAdo) and N(3) and N(4) of deoxyribocytidine (dCyd) residues (Kohwi-Shigematsu et al., 1987; Lilley, 1983; McLean et al., 1987). In addition, the reaction of CAA with adenine or cytosine bases in DNA at the sites specified above gives fluorescent products (Kohwi-Shigematsu et al., 1978). As the sites in nucleoside residues reactive with CAA are involved in hydrogen bonding in Watson–Crick base pairs, the occurrence of the fluorescent products indicates unpaired base residues.

The treatment of the thermally denatured *Nde*I/*Hpa*I fragment with CAA gave the fluorescent products. On the other hand, no products were noticed after the treatment of the double-stranded fragment. As shown in Figure 2A, the modification of the double-stranded *Nde*I/*Hpa*I fragment resulting in the formation of the monofunctional adducts of [Pt(dien)Cl]Cl (r_b = 0.02–0.12 for 24 h) also produced the fluorescent products, but in an amount which was markedly lower than that yielded by the CAA-modified unplatinated fragment, which was thermally denatured. The amount of the fluorescent products increased with the growing level of the platination.

The formation of the fluorescent products was also evaluated in the *Nde*I/*Hpa*I fragment modified by *cis*-$[Pt(NH_3)_2(H_2O)Cl]^+$ and subsequently treated with CAA. The fragment was first modified with the platinum complex for only 15 min. The reaction was stopped by adjusting the NaCl concentration to 0.1 M. One-half of each sample was precipitated by ethanol, dissolved in 10 mM $NaClO_4$, and incubated for an additional 24 h. Both 15-min and 24-h samples were characterized by immunochemical analysis using polyclonal antibodies that preferentially bind to DNA intrastrand cross-links of *cis*-$[Pt(NH_3)_2Cl_2]$ between adjacent purine residues. These antibodies can be used to examine conversion of the monofunctional adducts of *cis*-$[Pt(NH_3)_2Cl_2]$ in DNA to the bifunctional lesions (Vrána et al., 1992). In the competitive ELISA experiment, this conversion increases the capability of the modified DNA to competitively inhibit the antibodies. Whereas the antibodies reacted only negligibly with the *Nde*I/*Hpa*I fragment modified by *cis*-$[Pt(NH_3)_2(H_2O)Cl]^+$ (r_b in the range of 0.01–0.1) for 15 min, they reacted markedly more readily with the samples modified for 24 h (DNA-bound Pt concentrations at 30% inhibition estimated by the competitive ELISA were reduced 10 times as a consequence of the additional 24-h post treatment incubation).

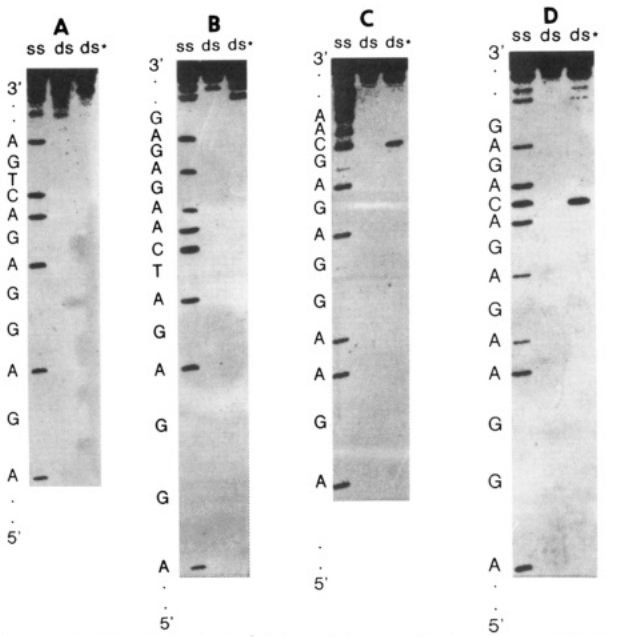
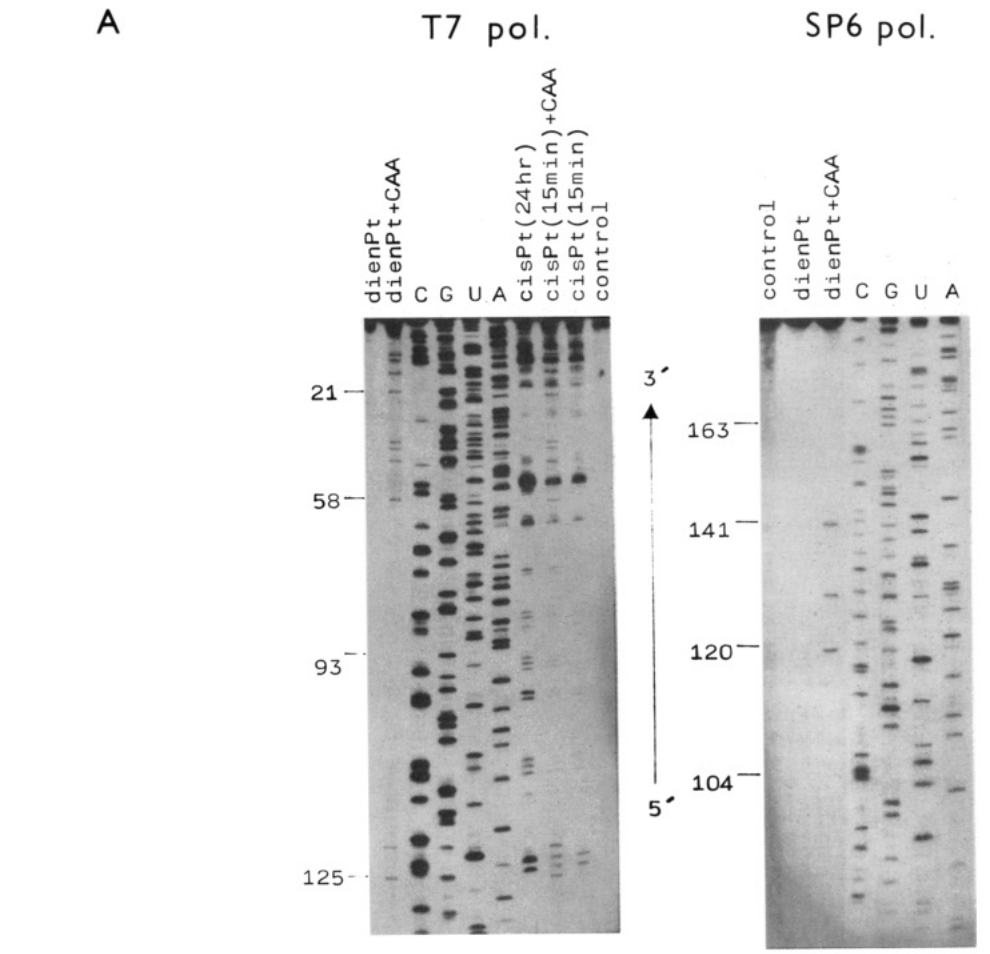


FIGURE 4: Piperidine-induced specific strand cleavage at CAA-modified bases in unplatinated and [Pt(dien)Cl]Cl-modified (A–C) or *cis*-$[Pt(NH_3)_2(H_2O)Cl]^+$-modified (D) oligonucleotides: (A) d(AGT)/d(ACT), (B) d(TGA)/d(TCA), (C) d(TGC)/d(GCA), and (D) d(TGT)/d(ACA). The cleavage patterns of the bottom strands in the duplexes reacted with CAA are shown. Lanes designated ss contain unplatinated single strands, lanes designated ds contain unplatinated duplexes, and lanes designated ds* contain platinated duplexes.

The results of immunochemical analysis indicate that the quantity of the bifunctional DNA lesions of *cis*-$[Pt(NH_3)_2(H_2O)Cl]^+$ markedly increased due to post treatment incubation, apparently at the expense of the monofunctional adducts. Interestingly, in spite of the great difference in the total amount of monofunctional adducts, the 15-min and 24-h DNA samples (modified at r_b = 0.01–0.1) yielded, after treatment with CAA, an almost identical number of the fluorescent products. This number was similar to that yielded by the fragment modified by [Pt(dien)Cl]Cl to the same values of r_b (cf. Figure 2). The results of the fluorescence measurements indicate that only a small fraction of the monofunctional adducts (12–15%) produced the sites in the plasmid fragment that readily react with CAA. In addition, these measurements suggest that the sites reactive with CAA were preferentially formed in DNA due to the monofunctional adducts that are not, in the case of the adducts of *cis*-$[Pt(NH_3)_2Cl_2]$, converted to the DNA intrastrand cross-links between neighboring purine residues during the 24-h post treatment incubation. In order to support this view, the experiments on synthetic oligonucleotides and the transcription mapping studies were performed.

Probing the Oligonucleotide Duplexes Containing a Unique Monofunctional Platinum(II) Adduct by CAA. In order to explore the sequences in which the monofunctional adducts of platinum preferentially induce the sites reactive with CAA, we used synthetic oligodeoxyribonucleotide duplexes (19–20 bp) with defined sequences. The top strands were designed to contain only one high-affinity platinum site, dGuo. Thus,



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     1          11          21          31          41          51
        o o         o           o                   o   o   o           o
5′  AGAACGCGGCT  ACAATTAATA  CATAACCTTA  TGTATCATAC  ACATACGATT  TAGGTGACAC
3′  TCTTGCGCCGA  TGTTAATTAT  GTATTGGAAT  ACATAGTATG  TGTATGCTAA  ATCCACTGTG

    61          71          81          91          101         111
     TATAGAACCA  GATCTGATAT  CATCGATGAA  TTCGAGCTCG  GATCCTCTAG  AGTCGACCTG
     ATATCTTGGT  CTAGACTATA  GTAGCTACTT  AAGCTCGAGC  CTAGGAGATC  TCAGCTGGAC
         SP6                                                             o

   121         131         141         151         161
     o   o                                  T7
     CAGGCATGCA  AGCTTCAGCT  GCTCGAGGCC  GGTCTCCCTA  TAGTGAGTCG
     GTCCGTACGT  TCGAAGTCGA  CGAGCTCCGG  CCAGAGGGAT  ATCACTCAGC
           o           o
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FIGURE 5: (A) Autoradiograms of 6% polyacrylamide/8 M urea sequencing gels showing inhibition of RNA synthesis by T7 (left) and SP6 (right) RNA polymerases on the *Nde*I/*Hpa*I fragment containing monofunctional adducts of [Pt(dien)Cl]Cl or *cis*-$[Pt(NH_3)_2(H_2O)Cl]^+$ (r_b = 0.01) and treated with CAA. Lanes: control, unplatinated template; dienPt, DNA modified by [Pt(dien)Cl]Cl; cisPt(15min), DNA modified by *cis*-$[Pt(NH_3)_2(H_2O)Cl]^+$ for 15 min, which was not further incubated to evolve bifunctional adducts; cisPt(24hr), the sample shown in lane cisPt(15min) dialyzed against 10 mM $NaClO_4$ and further incubated for 24 h at 37 °C to evolve bifunctional lesions; dienPt+CAA, the sample shown in lane dienPt treated with CAA; cisPt(15min)+CAA, the sample shown in the lane cisPt(15min) treated with CAA; G, C, U, and A, chain-terminated marker RNAs. The numbers correspond to the nucleotide sequence numbering of panel B. (B) Schematic diagram showing a portion of the sequence used to monitor inhibition of RNA synthesis on the template containing monofunctional adducts of platinum(II) and treated with CAA. The arrows indicate the start sites of the two polymerases; O, stop signals from panel A, lanes dienPt+CAA. Nucleotides 1 and 65 correspond respectively to nucleotides 2492 and 1 on the pSP73KB nucleotide sequence map.

duplexes could be prepared that contained a unique monofunctional adduct of [Pt(dien)Cl]Cl or *cis*-$[Pt(NH_3)_2(H_2O)Cl]^+$ at the dGuo flanked by various deoxyribonucleoside residues. A list of all sequences examined is given in Figure 3; the accompanying names refer to the unplatinated duplexes and were chosen according to the central trinucleotide sequences. The unplatinated and modified duplexes were treated with CAA. Then the platinum was removed from the

modified duplexes by sodium cyanide, the duplexes were cleaved by piperidine and the resulting products were analyzed by electrophoresis in 24% polyacrylamide/8 M urea gels.

All dAdo and dCyd residues within the unplatinated top and bottom single strands readily reacted with CAA (shown for the bottom strands in lanes ss of Figure 4). However, no reactivity of these residues with CAA was observed within unplatinated duplexes used in this work (Figure 4, lanes ds). Reactivity of dAdo and dCyt residues was also not observed within the duplexes d(AGT)/d(ACT) and d(TGA/TCA) containing a unique monofunctional adduct of [Pt(dien)Cl]-Cl or *cis*-$[Pt(NH_3)_2(H_2O)Cl]^+$ {shown for the bottom strands of the duplexes modified with [Pt(dien)Cl]Cl in lanes ds* of Figure 4}. In contrast, within the double-stranded oligonucleotides d(CGT)/d(ACG) (not shown), d(TGT)/d(ACA), and d(TGC)/d(GCA) containing a unique monofunctional adduct of either [Pt(dien)Cl]Cl or *cis*-$[Pt(NH_3)_2(H_2O)Cl]^+$ the single dCyd residue in the bottom strands (complementary to the platinated dGuo residue in the top strand) became reactive (Figure 4C,D, lanes ds*). Thus, the formation of the monofunctional adducts of both [Pt(dien)Cl]Cl and *cis*-$[Pt(NH_3)_2(H_2O)Cl]^+$ at the dGuo residues makes the sites in some dCyd residues complementary to the platinated dGuo accessible to react with CAA. This result indicates that the hydrogen bonds between some platinated dGuo residues and their complementary dCyd residues are interrupted. Our results also indicate that this denaturational alteration only occurs in the duplexes containing the monofunctional adduct at the dGuo in the trinucleotide sequence Py-dGuo-Py (Py designates a pyrimidine deoxyribonucleotide) and is only localized at the platinated base pair.

Mapping of the Unpaired Bases in the 212-bp DNA Fragment Containing Monofunctional Adducts of Platinum-(II). Further investigations were aimed at finding the sites in natural DNA at which monofunctional adducts of platinum-(II) induced an interruption of hydrogen bonds in base pairs. Recent work has shown that the *in vitro* RNA synthesis by RNA polymerases on DNA templates modified by chemical agents can be terminated at the level of the adducts (Brabec & Leng, 1993; Corda et al., 1991, 1992; Lemaire et al., 1991).

Cutting of pSP73KB DNA by *Nde*I and *Hpa*I endonucleases yielded a 212-bp fragment containing SP6 and T7 RNA polymerase promotors directed toward each other from opposite ends of the fragment. Initial experiments were carried out using this DNA fragment modified by [Pt(dien)Cl]Cl for 24 h at $r_b = 0.01$ for RNA synthesis by SP6 or T7 RNA polymerases (Figure 5A, lanes dienPt). RNA synthesis on this template was not prematurely terminated [see also Brabec and Leng (1993) and Lemaire et al. (1991)]. RNA synthesis on the DNA template modified by *cis*-$[Pt(NH_3)_2(H_2O)Cl]^+$ for only 15 min (to reach an r_b of 0.01) yielded only a small amount of RNA fragments which were shorter than would correspond to a full transcription of the *Nde*I/*Hpa*I fragment [see only the faint bands in the lane cisPt(15min) of Figure 5A, left]. If, however, the platinated DNA fragment used in the preceding experiment was further incubated in 10 mM $NaClO_4$ for an additional 24 h to evolve bifunctional lesions, the faint bands yielded by the template modified by *cis*-$[Pt(NH_3)_2(H_2O)Cl]^+$ only for 15 min became markedly more intense [Figure 5A, left, lane cisPt(24hr)]. In this case the electrophoresis pattern was identical to those observed earlier (Brabec & Leng, 1993; Lemaire et al., 1991) if RNA synthesis was carried out on the same DNA template but modified directly by *cis*-$[Pt(NH_3)_2Cl_2]$ at $r_b = 0.01$ for 24 h. These data suggest that the faint bands yielded by the DNA sample

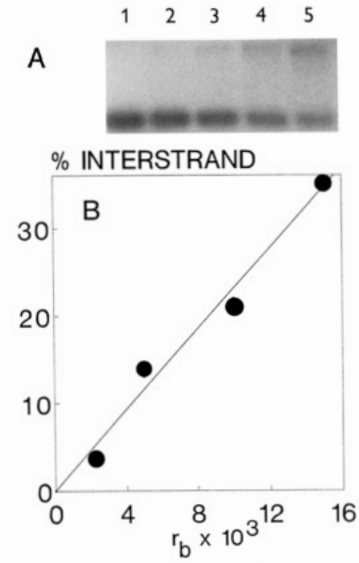

FIGURE 6: Dependence on r_b of interstrand cross-linking in the 2455-bp pSP73KB DNA linearized by the *Nde*I restriction enzyme by *cis*-$[Pt(NH_3)_2(H_2O)Cl]^+$ in 10 mM $NaClO_4$ at 37 °C for 15 min. (A) Autoradiogram of a denaturing 1% agarose gel of DNA which was 3′-end-labeled. Lanes: 1, $r_b = 0$ (control); 2, $r_b = 0.002$; 3, $r_b = 0.005$, 4, $r_b = 0.01$; 5, $r_b = 0.015$. (B) The percentage of interstrand cross-linking was calculated from the ratio of the intensity of the band corresponding to the fragment containing the interstrand cross-link (upper band in panel A) to the sum of the intensities of the two bands corresponding to the non-cross-linked and cross-linked DNA molecules.

modified by *cis*-$[Pt(NH_3)_2(H_2O)Cl]^+$ at $r_b = 0.01$ only for 15 min could be formed due to a small amount of the bidentate DNA adducts. The bidentate but not the monodentate DNA lesions of platinum have been suggested to be capable to terminate RNA transcription (Brabec & Leng, 1993; Lemaire et al., 1991).

The next experiments were conducted to show that bidentate adducts were formed in DNA samples modified by *cis*-$[Pt(NH_3)_2(H_2O)Cl]^+$ only for 15 min. This can be demonstrated by the data on interstrand cross-linking deduced from gel electrophoresis experiments (Figure 6) in the same way as described in our recent paper (Brabec & Leng, 1993). Assuming one interstrand cross-link per DNA molecule (2455 bp), it was found that the interstrand cross-links formed by *cis*-$[Pt(NH_3)_2(H_2O)Cl]^+$ at $r_b = 0.01$ after a 15-min reaction period represented about 0.4% of the total platinum bound. Other bidentate DNA adducts of cis-$[Pt(NH_3)_2Cl_2]$ that could terminate RNA transcription are intrastrand adducts. They are even formed more rapidly than interstrand lesions [the half-time of intrastrand cross-linking is about 2 h (Bancroft et al., 1990), while that of interstrand cross-linking is about 4 h under comparable conditions (Brabec & Leng, 1993)]. Thus, it is reasonable to expect that the total extent of bidentate adducts in DNA modified by *cis*-$[Pt(NH_3)_2(H_2O)Cl]^+$ at $r_b = 0.01$ after a 15-min reaction period which can terminate RNA transcription is at least 0.4% of the total platinum bound. It is, therefore, possible to conclude that SP6 (data not shown) and T7 RNA polymerases are insensitive to monofunctional adducts not only of [Pt(dien)Cl]Cl but also of *cis*-$[Pt(NH_3)_2(H_2O)Cl]^+$.

To determine the location of unpaired bases in the platinated 212-bp DNA fragment, transcription mapping studies were carried out by using the templates containing the monofunctional adducts of [Pt(dien)Cl]Cl or *cis*-$[Pt(NH_3)_2(H_2O)Cl]^+$, which were further treated with CAA. The additional treatment of the platinated DNA templates with CAA was

employed on the supposition that its binding to the unpaired bases in DNA could form adducts, at which RNA synthesis by RNA polymerases is stopped. The treatment with CAA of the DNA fragments modified by [Pt(dien)Cl]Cl resulted in the premature termination of transcription at several sites, as revealed by the bands in the corresponding autoradiogram (Figure 5A, lanes dienPt+CAA). They all occurred at the level of dCyd residues, but exclusively in the trinucleotide sequences Pu-dCyd-Pu (Pu designates a purine deoxyribonucleoside).

If the subsequent treatment with CAA was applied to the DNA template modified by *cis*-$[Pt(NH_3)_2(H_2O)Cl]^+$ for 15 min, in addition to the faint bands corresponding to the bifunctional adducts, new bands occurred [Figure 5A, left, cf. lanes cisPt(15min) and cisPt(15min)+CAA]. The positions of these new bands in the autoradiogram were identical to those of the bands yielded by the RNA transcripts of the DNA templates modified by [Pt(dien)Cl]Cl and subsequently treated with CAA (Figure 5, left, lane dienPt+CAA). It was verified by gel electrophoresis that the treatment with CAA of the unplatinated *NdeI*/*HpaI* fragment resulted in no inhibition of RNA synthesis by RNA polymerases on such templates (not shown).

A summary of the stop sites relative to the monofunctional adducts of [Pt(dien)Cl]Cl and *cis*-$[Pt(NH_3)_2(H_2O)Cl]^+$ is given in Figure 5B. Interestingly, trascription mapping studies also indicate that the formation of monofunctional adducts of platinum(II) complexes induces even in natural DNA an interruption of hydrogen bonds in the dGuo·dCyd pairs.

DISCUSSION

In this work we have determined the sites in DNA in which monofunctional adducts generated by [Pt(dien)Cl]Cl or *cis*-$[Pt(NH_3)_2(H_2O)Cl]^+$ induce local denaturational alterations. These sites were identified by means of the single-strand-specific probe of DNA conformation, CAA. The binding of CAA to the denatured sites in DNA was determined with the aid of three experimental approaches: (i) measurement of fluorescence of plasmid fragment treated with CAA, (ii) analysis of oligonucleotides treated with CAA and subsequently cleaved by hot piperidine, and (iii) termination of duplex transcription on a CAA-treated fragment of plasmid DNA. The three approaches reveal that the denaturational alteration preferentially occurs in the base pairs containing monoadducted dGuo in the trinucleotide sequence Py-dGuo-Py and that this change is localized at the platinated base pairs. Properties of the monofunctional adducts which are formed by [Pt(dien)Cl]Cl or *cis*-$[Pt(NH_3)_2(H_2O)Cl]^+$ do not differ, which suggests that the effects of [Pt(dien)Cl]Cl and monofunctional adducts of *cis*-$[Pt(NH_3)_2Cl_2]$ on DNA conformation are similar. A similar sequence dependence is observed if the effect on thermal stability of oligonucleotide duplexes produced by the monofunctional adducts of platinum(II) complexes is investigated (Brabec et al., 1992). Thus, these results establish a connection between the abilities of the monofunctional adducts of platinum to destabilize DNA and to induce denaturational alterations in the duplex.

The results of the fluorescence measurements (Figure 2) indicate that the denaturational alterations in natural DNA are only induced by a small fraction of the monofunctional adducts of platinum. These measurements also suggest that the denaturational changes preferentially occur in the base pairs containing the monoadducted dGuo in the sequences, in which the monofunctional adducts of *cis*-$[Pt(NH_3)_2Cl_2]$ are not converted to the DNA intrastrand cross-links between adjacent purine residues (Eastman, 1986; Fichtinger-Schepman et al., 1985). Mapping of the unpaired bases in the fragment of plasmid DNA containing the monofunctional adducts of platinum(II) by means of termination of duplex transcription (Figure 5) supports this conclusion. The intrastrand adducts between adjacent bases occur in the d(5′-AG-3′) or d(GG) dinucleotide sequences. No such sequence contained a denatured base pair as a consequence of the modification by the monofunctional platinum(II) binding (Figure 5B). In contrast, the denaturational change preferentially occurred in the sequences d(5′-GCG*-3′) and d(5′-ATG*-3′) (G* designates an unpaired dGuo residue) in which the monofunctional adduct of *cis*-$[Pt(NH_3)_2Cl_2]$ at the G* could close to the intrastrand cross-link between two purine residues separated by a third base [(1,3) intrastrand cross-link] (Eastman, 1986). It was demonstrated (Anin & Leng, 1990) that a dCyd residue complementary to the 3′ dGuo in the (1,3) intrastrand cross-link of *cis*- and *trans*-$[Pt(NH_3)_2Cl_2]$ is reactive with CAA.

In addition, the denaturational change in DNA due to the monofunctional adduct of platinum(II) also occurs at the sequences d(5′-G*C-3′), at which *cis*-$[Pt(NH_3)_2Cl_2]$ preferentially forms interstrand cross-links between dGuo residues (Sip et al., 1992). It has been shown (Sip et al., 1992) that dCyd residues complementary to the platinated dGuo in these interstrand lesion are heavily distorted and that this distortion is only localized to these residues. Thus, dGuo residues in the sequences in which *cis*-$[Pt(NH_3)_2Cl_2]$ preferentially forms minor bifunctional adducts could be preferred for the formation of the monofunctional adducts that induce denaturation in DNA.

On the other hand, a denaturational change in DNA due to the monofunctional adduct of platinum is not observed in the d(GG) or d(5′-AG-3′) sequences, in which the major intrastrand cross-links of *cis*-$[Pt(NH_3)_2Cl_2]$ between neighboring purine nucleosides are formed. Interestingly, this type of intrastrand lesions does not induce in DNA an interruption of hydrogen bonds in the complementary base residues (Den Hartog, 1984; Schwartz et al., 1989; Vrána et al., 1985). Thus, the denaturational alterations occurring in DNA already on the level of the monofunctional adducts of platinum(II) complexes could facilitate the formation of the bidentate DNA adducts, whose formation is associated with a permanent or transient local denaturation.

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