Identification and Characterization of a Novel Linkage Isomerization in the Reaction of *trans*-Diamminedichloroplatinum(II) with 5′-d(TCTACGCGTTCT)†

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ABSTRACT: The oligonucleotide 5′-d(TCTACGCGTTCT) reacts with *trans*-diamminedichloroplatinum(II) to yield primarily *trans*-[$Pt(NH_3)_2${d(TCTACGCGTTCT)-*N7*-G(6),*N7*-G(8)}], containing the desired *trans*-[$Pt(NH_3)_2${d(GCG)}] 1,3-cross-link. A key element of the platination reaction is the use of low pH to suppress coordination at A(4). The product was fully characterized by pH-dependent NMR titrations, enzymatic degradation analysis, and ^{195}Pt NMR spectroscopy. Interestingly, the 1,3-cross-linked adduct is unstable at neutral pH, rearranging unexpectedly to form the linkage isomer *trans*-[$Pt(NH_3)_2${d-(TCTACGCGTTCT)-*N3*-C(5),*N7*-G(8)}]. This rearrangement product is more stable than the initially formed isomer and could be characterized by pH-dependent NMR titrations, enzymatic degradation analysis, liquid secondary ion mass spectrometric analysis of an enzymatically digested fragment, ^{195}Pt NMR spectroscopy, and modified Maxam–Gilbert footprinting experiments. By contrast, the 1,3-intrastrand cross-linked isomer rearranges during the course of both pH titration and enzymatic degradation experiments to form the 1,4-adduct. The equilibrium constant for this rearrangement is ~3, favoring the 1,4-adduct. Kinetic studies of the linkage isomerization reaction reveal $t_{1/2}$ values for the first-order disappearance of the 1,3-intrastrand cross-linked isomer ranging from 129 (at 30 °C) to 3.6 h (at 62 °C), with activation parameters $\Delta H^{\ddagger} = 91 \pm 2$ kJ/mol and $\Delta S^{\ddagger} = -58 \pm 8$ J/(mol·K). Mechanistic implications of these kinetic results as well as the general relevance of this linkage isomerization reaction to platinum–DNA chemistry are briefly discussed.

The antitumor drug *cis*-diamminedichloroplatinum(II) (*cis*-DDP[1] or cisplatin) and its inactive stereoisomer *trans*-DDP have been extensively investigated [general review, Sherman and Lippard (1987)]. A variety of studies strongly suggest that DNA adducts are responsible for the anticancer activity of cisplatin (Roberts & Thomson, 1979; Pinto & Lippard, 1985b; Reed et al., 1987; Fichtinger-Schepman et al., 1987). Since both isomers form a spectrum of adducts (Sherman & Lippard, 1987; Eastman et al., 1988), it is important to characterize them structurally to understand how platinum binding to DNA brings about antitumor activity. The correlation of adduct structure with cellular events leading to antineoplastic activity may also reveal general features of how small DNA damaging agents alter gene structure to provoke a biological response.

In cells, both *cis*- and *trans*-DDP hydrolyze with loss of chloride ions owing to the low (ca. 4 mM) intracellular chloride ion concentration. The hydrolysis step is a prerequisite for DNA binding (Lim & Martin, 1976; Johnson et al., 1980). Both *cis*- and *trans*-DDP initially bind monofunctionally to DNA, as demonstrated by chemical trapping methods (Fichtinger-Schepman et al., 1985; Eastman, 1985, 1986; Lepre et al., 1990) and recent ^{195}Pt NMR kinetic studies (D. P. Bancroft, C. A. Lepre, and S. J. Lippard, unpublished results). The ^{195}Pt NMR study also showed that monofunctional adducts formed by *cis*- and by *trans*-DDP close to form bifunctional adducts at a similar rate. Numerous structural investigations of *cis*- and *trans*-DDP with synthetic oligonucleotides have led to the conclusion that, once formed, bifunctional adducts are kinetically inert. The only exceptions to date involve platinum complexes with mono- or dinucleotides or nucleobases and their analogues [review, Lippert et al. (1988)]. Examples include metal migration from N1 to N7 of guanine in [Pt(diethylenetriamine)(9-ethylguanine-*N1*)]$^{2+}$ and *cis*-[$Pt(NH_3)_2$(9-ethylguanine-*N1*)(9-ethylguanine-*N7*)]$^{2+}$ (van der Veer et al., 1987), metal migration from N1 to N7 of adenine in the [(1*R*,2*R*)-1,2-cyclohexanediammine]platinum(II) adduct of 5′-d(GpA) (Inagaki et al., 1988), isomerization of binuclear amidate-bridged platinum(II) amine complexes (O'Halloran & Lippard, 1989), and migration of platinum in *trans*,*trans*,*trans*-[$Pt(NH_3)_2$(1-MeC-*N3*)$_2$-$(OH)_2$]$(NO_3)_2$ from N3 to N4 (Lippert et al., 1986).

The present work was undertaken to explore DNA site specifically modified with *trans*-DDP in the biologically viable genome M13mp18, for comparison with similar genomes containing site-specific *cis*-DDP adducts (Pinto et al., 1986; Naser et al., 1988). The first step in such a program is to prepare and characterize oligonucleotides containing a specific platinum adduct. As described here, we discovered during the course of these studies a novel linkage isomerization reaction between *trans*-DDP and the single-stranded deoxydodecanucleotide substrate 5′-d(TCTACGCGTTCT). In particular, the target 1,3-intrastrand cross-link between *trans*-diammineplatinum(II) and two nonadjacent guanosine bases was found to be unstable, rearranging to a more rarely encountered mode of coordination, namely, a 1,4-cross-link between C(5) and G(8). Migration of platinum from one nucleoside to another in an oligonucleotide has not been previously reported. This discovery reveals that unexpected complexities can occur in metal–DNA reactions, even with

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[1] Abbreviations: *cis*-DDP, *cis*-diamminedichloroplatinum(II); *trans*-DDP, *trans*-diamminedichloroplatinum(II); AAS, atomic absorption spectroscopy; HPLC, high-performance liquid chromatography; EDTA, ethylenediaminetetraacetate dianion.

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relatively inert metals such as platinum(II), and has interesting implications about the importance of sequence context in determining target sites on DNA for platinum anticancer drugs.

Experimental Procedures

Oligonucleotide Synthesis. Reagents for DNA synthesis were purchased from American Bionetics (Hayward, CA). The dodecanucleotide 5′-d(TCTACGCGTTCT) was prepared by the solid-phase β-cyanoethyl phosphoramidite method (Sinha, 1983) using a Biosearch 8000 series DNA synthesizer and a 15-μmol scale 500-Å controlled pore glass support from Biosearch Inc. (San Rafael, CA). The resultant dodecanucleotide was manually deprotected with concentrated ammonium hyroxide, desalted by chromatography on Sephadex G-10 (Pharmacia), separated from failure sequences by reversed-phase HPLC, and converted to the Na^+ form on Dowex AG-50W-X8. The final yields after purification from various syntheses averaged 51%, corresponding to an average coupling yield per step of 94%. The concentration was measured by UV absorbance [ϵ_{260} = 103 700 M^{-1} cm^{-1} [based on Fasman (1975)]].

Platination of 5′-d(TCTACGCGTTCT). *trans*-DDP was prepared from K_2PtCl_4 (Engelhard) by the method of Kaufman and Cowan (1963). The initial isomer, a 1,3-intrastrand cross-linked adduct, was prepared by adding 1 equiv of *trans*-DDP to 5′-d(TCTACGCGTTCT) (65 μM) in 86 mL of distilled water at pH 3.8 (adjusted with HNO_3) and 37 °C. The reaction was monitored by reversed-phase high-performance liquid chromatography (Figure 1A). After ca. 24 h, an additional 0.5 equiv of *trans*-DDP was added to drive the reaction to completion. The reaction volume was reduced to 1-2 mL by lyophilization, and the crude reaction mixture was purified by elution from a 45-cm anion-exchange column (DEAE-A25, Pharmacia) with a 400 × 400 mL linear gradient of 0.7–2.0 M ammonium acetate. The largest peak was collected and lyophilized several times to remove excess ammonium acetate, and the product was converted to the sodium form by using Dowex AG-50W-X8. The platinum content of the product was determined by using a Varian AA-1475 atomic absorption spectrophotometer equipped with a GTA-95 graphite furnace, and the purity was checked by HPLC (Figure 1B). The final yields after purification averaged 60–80%.

Linkage Isomerization Reaction. The 1,4-intrastrand cross-linked isomer, *trans*-[$Pt(NH_3)_2${d-(TCTACGCGTTCT)-*N3*-C(5),*N7*-G(8)}], was prepared from aqueous solutions of the 1,3-cross-linked isomer at or above pH ~4.5, and was isolated by HPLC, employing the conditions described in the caption to Figure 1. The appropriate fraction was collected, lyophilized several times from water to remove excess NH_4OAc, and converted to the Na^+ form by using Dowex AG-50W-X8. The platinum concentration was determined by AAS.

pH-Dependent ^1H NMR Studies. Platinum binding sites were determined by pH-dependent ^1H NMR (500 MHz) studies as described previously (Caradonna et al., 1982). Samples were lyophilized several times from 99.8% D_2O and dissolved in 99.996% D_2O containing 0.5 mM Na_2EDTA. The following parameters were employed in pH-dependent studies of the 1,3-intrastrand cross-linked isomer: 4.5K real data points, 12 μs (90°) pulse, water presaturation, and 64 free induction decays per spectrum. A temperature of 50 °C was used to sharpen the signals and prevent oligonucleotide base pairing and aggregation. Exponential line broadening (1 Hz) was applied. The concentration of the oligomer was 1 mM per strand. Tetramethylammonium chloride was used as a temperature- and pH-independent internal chemical shift standard. The pH of the sample was recorded both before and after each spectrum and was not corrected for the deuterium isotope effect (Glasoe & Long, 1960). The pH was altered by addition of small amounts of NaOD or DCl in D_2O. A total of 18 spectra in the pH range 1.6–10 were recorded. NMR parameters used in titration studies of the 1,4-intrastrand cross-linked isomer were the same as above, with the following exceptions: the temperature was 40 °C, 128 transients were summed per spectrum, and the concentration of the oligonucleotide was 225 μM. A total of 17 spectra in the pH range 2.0–10.4 were recorded.

Modified Maxam–Gilbert Footprinting Experiments. The binding sites of platinum in the 1,4-intrastrand cross-linked isomer were mapped by comparing autoradiograms of fragments obtained from platinated and unplatinated dodecamers following modified Maxam–Gilbert sequencing reactions. Base-specific chemical reactions were performed on platinated and unplatinated ^{32}P 5′ end labeled dodecanucleotides according to the procedure of Banaszuk et al. (1983). The oligonucleotide 5′-d(TCTACGCGTTCT) (18 pmol) or the 1,4-intrastrand cross-linked isomer was 5′ end labeled by using 1–5 units of T4 polynucleotide kinase (New England Biolabs) in 25 mM Tris-HCl, pH 8, 10 mM $MgCl_2$, and 10 mM dithiothreitol, containing 2 pmol of [γ-^{32}P]ATP (New England Nuclear, 3000 Ci/mmol). Products of this reaction were purified and desalted by elution from a NENSORB 20 nucleic acid purification cartridge (Du Pont NEN). Aliquots (75 000 cpm) were treated separately in the presence of 1.5 μg of sonicated calf thymus DNA with dimethyl sulfate, diphenylamine in formic acid, hydrazine in water, hydrazine in 3.75 M NaCl, and potassium permanganate to give specific guanine-, purine-, pyrimidine-, cytosine-, and thymine-specific strand-weakening reactions, respectively. Reactions were stopped (Banaszuk et al., 1983), and the solutions were desalted, dried, and treated either with (i) piperidine (1 M, 100 μL, 30 min, 90 °C) or (ii) sodium cyanide (0.3 M, pH 8.4, 30 μL, 15 h, 37 °C) to remove platinum from DNA, followed by desalting and piperidine treatment as in (i). After an alkaline running dye (80% formamide, 10 mM NaOH, xylene cyanol, bromophenol blue) was added, samples were resolved on a 20% polyacrylamide/7 M urea denaturing gel (~2.5 h at 2000 V) and analyzed by autoradiography.

^{195}Pt NMR Spectroscopy. ^{195}Pt NMR spectra were acquired on a Varian VXR-500 spectrometer operating at 107.25 MHz with a 5-mm broad band probe. A total of 2.25–2.85 × 10^6 free induction decays, each comprising 1024 data points, were summed for all spectra over a 100-kHz spectral width by using a 16-μs (90°) pulse and 5-ms acquisition time; the temperature was either ambient or 37 °C, as noted. Chemical shifts were determined by using external 0.1 M K_2PtCl_4 in 0.1 M DCl at −1624 ppm (Na_2PtCl_6 is at 0 ppm on this scale). Line broadening (300 Hz) and a base-line correction function were applied. Two micromoles of the 1,3-intrastrand cross-linked isomer were dissolved in 740 μL of water and examined by ^{195}Pt NMR spectroscopy. The same sample was allowed to rearrange to the more stable 1,4-intrastrand cross-linked isomer by incubation for 105 h at 44 °C (pH 6.87, 0.1 M NH_4OAc). The sample was then lyophilized several times to remove excess NH_4OAc and reconstituted in 740 μL of H_2O for ^{195}Pt NMR spectroscopy.

Enzymatic Digestion Studies. Digestions were carried out by using DNase I (Sigma, from bovine pancreas), nuclease P1 (Boehringer Mannheim, from *Penicillium citrinum*), and

alkaline phosphatase (Boehringer Mannheim, from calf intestine) (Eastman, 1986). Samples (12 nmol of oligonucleotide) were allowed to react with 20 units (10 μg) of DNase I at 37 °C for 4 h. Nuclease P1 (10 μg) was then added, and the reaction was allowed to continue at 37 °C for 16 h. Alkaline phosphatase (5 units) was then added and the incubation continued for an additional 4 h at 37 °C. Products were analyzed by reversed-phase HPLC with a gradient of 5–22.5% acetonitrile in ammonium acetate over 30 min; buffers are described in the caption to Figure 1. Peaks were identified by liquid secondary ion mass spectrometry or by comparison with 2′-deoxynucleosides (Sigma) and *trans*-$[Pt(NH_3)_2\{d(Guo)_2\}]^{2+}$ standards. The latter was constructed by reacting 1 equiv of *trans*-DDP with 2 equiv of 2′-deoxyguanosine monohydrate (99+ %, Sigma) at a nucleoside concentration of 1.2 mM for 72 h at 37 °C. Free nucleoside peak areas were also measured, after correction for extinction coefficient differences (Fasman, 1975), to monitor their concentrations.

Liquid Secondary Ion Mass Spectrometry. The enzymatic digestion product from *trans*-$[Pt(NH_3)_2\{d$-(TCTACGCGTTCT)-*N3*-C(5),*N7*-G(8)$\}]$ was identified by liquid secondary ion mass spectrometry (LSIMS) and tandem mass spectrometry. Spectra were acquired with a JEOL HX110/HX110 tandem mass spectrometer, employing a 25-keV primary beam of Cs^+ ions. The accelerating voltage was +10 kV, and the postacceleration voltage was −18 kV at the detector. Mass range MS-1 m/z 100–1500 was scanned in 1 min at a resolution of 1:1500. $(CsI)_nCs^+$ cluster ions were used to calibrate the mass scale. FAB MS/MS was carried out by using all four sectors of the instrument, with collision-induced fragmentation taking place in the third field free region. Helium was used as the collision gas at pressure sufficient to reduce the precursor ion signal by 80%. The second mass spectrometer was calibrated with a mixture of CsI, NaI, KI, and LiCl (Sato et al., 1987). MS/MS spectra were acquired during linked scans of MS-2 at constant B/E for ions selected by MS-1 at unit resolution. Samples were dissolved to a concentration of ca. 2 mM in distilled water, and a 1-μL aliquot containing ca. 2 nmol of the sample was combined with 0.5 μL of glycerol and concentrated on the probe tip for each LSIMS analysis.

Characterization of the Stereochemistry at Platinum in the 1,4-Intrastrand Cross-Linked Isomer. A modification of the Kurnakow (1894) test was used to determine whether the trans configuration of ammine ligands at platinum was retained following isomerization (Arpalahti & Lippert, 1987). A 15 μM aqueous solution, 20 μM in HNO_3, of the platinated oligonucleotide was incubated with excess (9:1) thiourea (Fluka, puriss p.a.). The mixture was incubated for 200 min at 40 °C, and then analyzed by HPLC, using either the same column, gradient, and detection wavelength described previously for platination reactions or isocratic conditions (95% 0.1 M NH_4OAc/5% methanol) with the detector set at 235 nm. The results were analyzed by comparison with authentic samples of $[Pt(tu)_4]^{2+}$ and *trans*-$[Pt(NH_3)_2(tu)_2]^{2+}$ prepared from *cis*- and *trans*-DDP, respectively (Arpalahti & Lippert, 1987).

Kinetic Studies. The time dependence of the disappearance of the 1,3-intrastrand cross-linked isomer was measured in 0.1 M NH_4OAc, pH 6.87, solutions, at a starting concentration of 15 μM. At selected time points, aliquots were removed and then frozen and stored at −80 °C for subsequent analysis by HPLC. Analysis was carried out by using conditions similar to those employed for purification of the 1,4-cross-linked isomer. Integration was accomplished by cutting and weighing HPLC peaks. Rates were measured at five different temperatures in the range 29.8–62.0 °C.

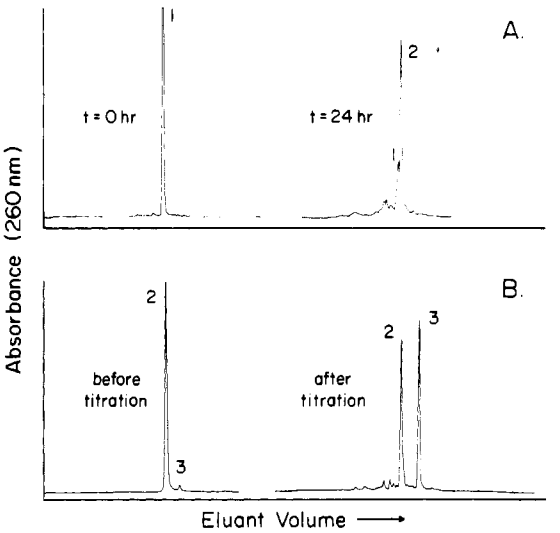

FIGURE 1: Reversed-phase HPLC separation of products and starting materials. (A) Initial platination reaction at start and after 24 h (reaction conditions as described in the text). (B) Purified 1,3-cross-linked isomer before and after pH-dependent NMR titration. A small amount of the 1,4-intrastrand cross-linked isomer is seen to be present before the titration. Data were obtained by using a Perkin-Elmer Series 4 liquid chromatograph equipped with a LCI-100 computing integrator and a Waters μBondapak C_{18} column. Gradient, 14–34% buffer B over 40 min, where buffer A is 0.1 M NH_4OAc (pH 6.5) and buffer B is 1:1 A:CH_3CN; flow rate, 1 mL/min; observation wavelength, 260 nm. Peak 1 is unreacted 5′-d-(TCTACGCGTTCT), peak 2 is *trans*-$[Pt(NH_3)_2\{d$-(TCTACGCGTTCT)-*N7*-G(6),*N7*-G(8)$\}]$, and peak 3 is *trans*-$[Pt(NH_3)_2\{d(TCTACGCGTTCT)$-*N3*-C(5),*N7*-G(8)$\}]$.

RESULTS

Synthesis and Characterization of the 1,3-Intrastrand Cross-Linked Isomer, trans-$[Pt(NH_3)_2\{d$-(TCTACGCGTTCT)-*N7*-G(6),*N7*-G(8)$\}]$. The oligodeoxynucleotide 5′-d(TCTACGCGTTCT) was designed to react with either *cis*- or *trans*-DDP to yield a platinum 1,3-intrastrand adduct formed between the two guanine bases, G(6) and G(8). Reactions were carried out at pH 3.8 to minimize binding at A(4). This low pH value suppresses platinum coordination at the usually favorable N7 and N1 sites on adenine by protonating the N1 position ($pK_a \approx 3.8$) (Izatt et al., 1971), which simultaneously lowers the nucleophilicity of N7. Since the pK_a of cytidine N3 is 4.5, the low pH value also suppresses coordination at this site. Reactions carried out at neutral pH produced a multitude of products, as revealed by HPLC (data not shown), but at pH 3.8 only a single major product was formed (Figure 1A). This adduct was separated from minor products by conventional anion-exchange chromatography and characterized by several methods.

Platinum binding sites were identified by pH-dependent NMR titrations of the nonexchangeable base protons. During the course of this study, a new set of peaks corresponding to the 1,4-intrastrand cross-linked isomer evolved as the pH was raised. This species was readily detected by reversed-phase HPLC analysis (Figure 1B). The new resonances obscured important regions of the spectrum of the 1,3-intrastrand cross-linked isomer. Nevertheless, resonance due to H8 of guanosine and H8 and H2 of adenosine could be observed throughout the titrations (Figure 2A). Assignments were made on the basis of the pH dependence of the nonexchangeable base proton chemical shifts. Guanosine H8 res-

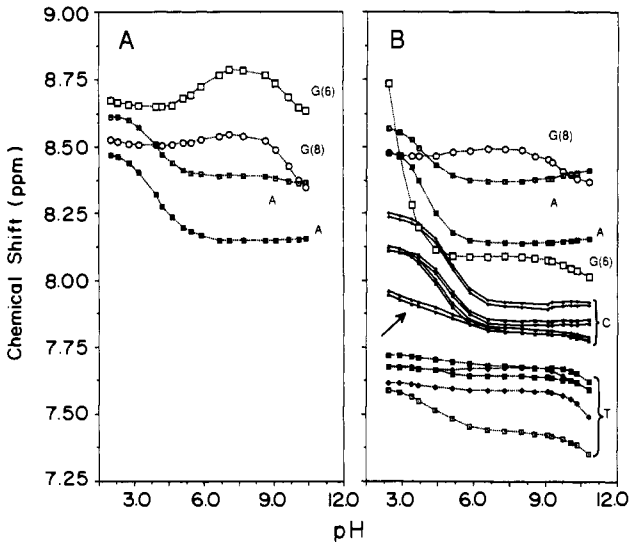

FIGURE 2: pH dependence of the downfield nonexchangeable base proton NMR resonances of (A) *trans*-[$Pt(NH_3)_2${d-(TCTACGCGTTCT)-*N7*-G(6),*N7*-G(8)}] and (B) *trans*-[$Pt(NH_3)_2${d(TCTACGCGTTCT)-*N3*-C(5),*N7*-G(8)}]. Spectra were recorded at 50 (A) and 40 °C (B).

onances for G(6) and G(8) were distinguished as follows. For the 1,4-intrastrand cross-linked isomer, Maxam–Gilbert footprinting experiments (vide infra) reveal that platinum is coordinated to G(8). Thus, the G(8) H8 resonance of the 1,3-intrastrand cross-linked isomer was assigned as the signal having nearly the same chemical shift and pH dependence as for G(8) in the other isomer (cf. parts A and B of Figure 2). In support of this assignment is the pH dependence (Figure 2A) of the H8 resonance of G(6) in the 4.5–5.5 range for the 1,3-isomer, behavior that reflects titration of the two flanking cytosine bases ($pK_a \sim 4.5$). The data for both guanosine nucleosides reveal titration midpoints at pH ~9.5, corresponding to a decrease of the N1 pK_a value from its measured $pK_a \geq 11$ (J. N. Burstyn, K. M. Comess, and S. J. Lippard, unpublished results). This observation, coupled with the lack of any observable chemical shift change in the pH 2.3 region (the pK_a of guanosine N7), proves that platinum is coordinated to the N7 positions of both guanosine bases. In addition, titration curves for the H8 and H2 resonances of the single adenosine exhibit pronounced chemical shift changes at pH ≈4.0, characteristic of the unmodified nucleoside (Figure 2A).

To characterize further the platinum coordination mode, the 1,3-intrastrand cross-linked isomer and unplatinated 5′-d(TCTACGCGTTCT) were enzymatically digested to mononucleosides and studied by reversed-phase HPLC (Figure 3A,B). Examination of peaks in the HPLC traces, identified by coinjection of standards, reveals the loss of dG from the product and the presence of a new species, identified as *trans*-$[Pt(NH_3)_2{d(Guo)_2}]^{2+}$ by coinjection with authentic material. Evidence for the linkage isomerization reaction product is also present in the form of a small peak eluting just before free adenosine, later identified as *trans*-$[Pt(NH_3)_2$-(Guo){d(CpG)}]$^+$ (vide infra).

^{195}Pt NMR spectroscopy also was used to characterize ligands bound to platinum in the 1,3-intrastrand cross-linked isomer. A freshly prepared, 2.6 mM solution of this product at ambient temperature exhibits a single ^{195}Pt resonance at −2436 ppm (Figure 4A), diagnostic of platinum coordinated to four nitrogen atoms (Pregosin, 1986). The chemical shift value at 37 °C is −2449 ppm (vide infra). By comparison, *trans*-$[Pt(NH_3)_2{d(GMP)_2}]$ at 37 °C shows a ^{195}Pt resonance at a similar value, −2469 ppm, and *trans*-$[Pt(NH_3)_2Cl]^+$

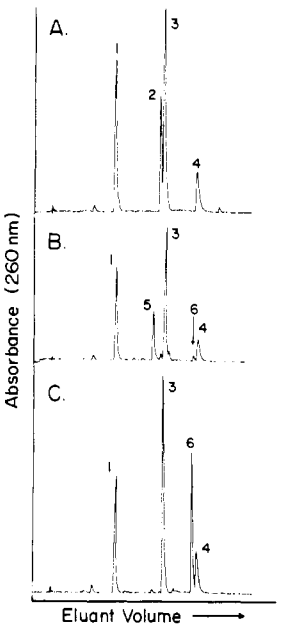

FIGURE 3: Enzymatic digestion analysis of (A) 5′-d(TCTACGCGTTCT), (B) *trans*-$[Pt(NH_3)_2${d-(TCTACGCGTTCT)-*N7*-G(6),*N7*-G(8)}], and (C) *trans*-$[Pt(NH_3)_2${d(TCTACGCGTTCT)-*N3*-C(5),*N7*-G(8)}]. The samples were digested extensively with DNase I, nuclease P1, and alkaline phosphatase and chromatographed as described in the text. Peak 1 is deoxycytidine, peak 2 is deoxyguanosine, peak 3 is deoxythymidine, peak 4 is deoxyadenosine, peak 5 is *trans*-$[Pt(NH_3)_2{d(Guo)_2}]^{2+}$, and peak 6 is *trans*-$[Pt(NH_3)_2{d(Guo)}{d(CpG)}]^+$.

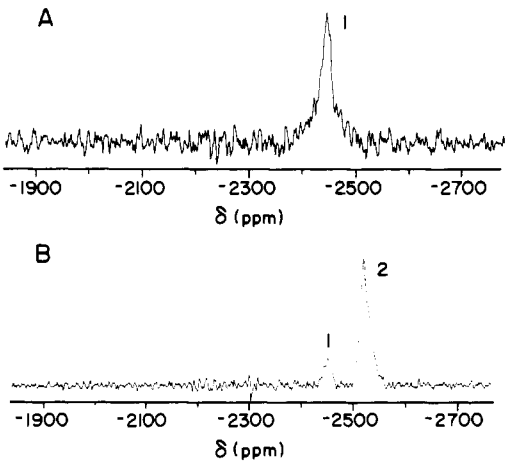

FIGURE 4: 107.25-MHz ^{195}Pt NMR spectra of (A) *trans*-[$Pt(NH_3)_2${d(TCTACGCGTTCT)-*N7*-G(6),*N7*-G(8)}] (peak 1) and (B) an equilibrium mixture of *trans*-$[Pt(NH_3)_2${d-(TCTACGCGTTCT)-*N7*-G(6),*N7*-G(8)}] and *trans*-$[Pt(NH_3)_2${d-(TCTACGCGTTCT)-*N3*-C(5),*N7*-G(8)}] (peak 2). Spectra were recorded at ambient temperature (A) and at 37 °C (B).

monofunctional adducts on DNA at 37 °C have much lower chemical shift values, falling in the range −2326 to −2417 ppm (Lepre et al., 1990).

Identification and Characterization of the 1,4-Intrastrand Cross-Linked Isomer, trans-$[Pt(NH_3)_2${d-(TCTACGCGTTCT)-*N3*-C(5),*N7*-G(8)}]. The 1,4-intrastrand cross-linked isomer is formed from the 1,3-isomer under approximately physiological conditions. This conversion is clearly apparent in HPLC profiles obtained before and after NMR pH titrations of the latter (Figure 1B). The new species, labeled peak 3, was purified and isolated by HPLC. Comparison of platinum AAS and UV spectroscopic results revealed the stoichiometry of both the initial and new products to be one platinum atom per dodecamer strand. When the

new species was ^{32}P 5′ end labeled and studied on 20% denaturing electrophoresis gels, it exhibited the same mobility as the initial product. Both platinated species migrate at the rate of a 14-mer, only slightly more slowly than the unplatinated dodecamer. When the new product was ^{32}P 5′ end labeled and then treated with piperidine according to the Maxam–Gilbert protocol (Maxam & Gilbert, 1980), gel electrophoresis revealed no apurinic or otherwise alkali-labile sites.

A pH-dependent ^1H NMR study was performed on the isolated rearrangement product to characterize the platinum binding sites (Figure 2B). The product was stable throughout the titration, losing less than 7% of its relative integrated area on HPLC traces over the course of the experiment. The titration curves prove that platinum has altered its coordination sites on the dodecamer such that, in the linkage isomer, only one guanosine is bonded to the metal. One of the two titration curves for the nonexchangeable guanosine H8 resonances exhibits an inflection in the alkaline region corresponding to deprotonation of N1 at a pH value at least 0.7 unit lower than for the other, unplatinated, nucleoside. Moreover, this same curve reveals no titration of N7 at acidic pH values, whereas the other guanosine H8 curve titrates nicely with a $pK_a < 2.5$. These results indicate that the N7 atom of one of the guanosine bases is not coordinated to platinum. The adenosine H2 and H8 resonances titrate as expected for an unplatinated nucleoside (cf. Figure 2A). One of the four cytidine H6 doublets, however, titrates differently from the other three sets (arrow, Figure 2B). One possible explanation for this result is that platinum is coordinated at the N3 position of this nucleoside, and the observed minor chemical shift changes in the acidic region are due to the effect of neighboring group titrations. Support for this interpretation is given below. All other nonexchangeable base proton resonances titrate as expected, the cytidines exhibiting inflections at pH 4.9 and the thymidines titrating above pH 10.5.

Modified Maxam–Gilbert Footprinting Experiments. To determine which guanosine is platinated in the 1,4-intrastrand cross-linked isomer and to gain more information about the other platinum coordination site, modified Maxam–Gilbert sequencing reactions were carried out on ^{32}P end labeled oligomers. Since dimethyl sulfate attacks the N7 position of guanosine, coordination of platinum at this site should effectively protect platinated guanosine nucleosides from cleavage. To control for artifactual migration of the DNA cleavage products in the gel, platinum was removed by treatment with sodium cyanide in one set of samples prior to the piperidine cleavage step.

The results of these experiments are shown in Figure 5. Lanes 1–5 refer to the unplatinated oligomer, for which labeled fragments corresponding to all three bases G(6), C(7), and G(8) in the *trans*-DDP target sequence are readily observed. Lanes 6–10 reveal that, in the cyanide reversal experiment, the fragment corresponding to G(8) is missing, from which it can be concluded that platinum is bound to N7 of that nucleoside. In lanes 11–15 are results for platinated DNA that was not subjected to a cyanide reversal step. Lanes 11–14 demonstrate that the three fragments corresponding to cleavage at G(6), C(7), and G(8) are all missing. Cleavage at bases situated between the two platinum binding sites yields fragments migrating slowly in the gel and a blank space (footprint) corresponding to the stretch of DNA spanned by the platinum cross-link. In this case the results indicate that platinum has an additional coordination site upstream (i.e., in the 5′ direction) of G(8). The only reasonable explanation for the footprint spanning G(6)–G(8) is bifunctional coordination of *trans*-$\{Pt(NH_3)_2\}^{2+}$ to N7 of G(8) and one of the following: N3 of C(5), N4 of C(5), or the phosphodiester group linking C(5) and G(6). It is probable that cleavage of an N3- or N4-platinated cytidine can still occur, since hydrazine attacks the C4 and C6 positions (Cashmore & Petersen, 1969, 1978).

Table I: Rate and Equilibrium Data for the Linkage Isomerization of *trans*-[$Pt(NH_3)_2$\{d(TCTACGCGTTCT)-*N7*-G(6),*N7*-G(8)\}]

T (°C)	$t_{1/2}$ (h)	$10^6\ k_{obs}$[a] (s^{-1})	$10^6\ k_f$[b] (s^{-1})	$10^6\ k_r$ (s^{-1})	K_{eq}[c,d]
29.8	129.3	1.49 ± 0.02	1.14 ± 0.02	0.350 ± 0.005	3.2 ± 0.2
37.0	46.6	4.2 ± 0.1	3.03 ± 0.07	1.17 ± 0.03	2.6 ± 0.2
44.0	22.7	8.6 ± 0.3	6.9 ± 0.2	1.75 ± 0.06	3.9 ± 0.1
51.3	10.1	19.6 ± 0.6	15.3 ± 0.5	4.3 ± 0.1	3.5 ± 0.1
62.0	3.6	54 ± 1	41.2 ± 0.8	12.8 ± 0.2	3.2 ± 0.2

[a] k_{obs} for disappearance of *trans*-[$Pt(NH_3)_2$\{d(TCTACGCGTTCT)-*N7*-G(6),*N7*-G(8)\}] was obtained from a nonlinear least-squares fit of P_t vs time (eq 5). [b] k_f and k_r were calculated from eqs 1 and 2. [c] $K_{eq} = (1 - P_\infty)/(P_\infty)$ where P_∞ is the fractional peak area of *trans*-[$Pt(NH_3)_2$\{d(TCTACGCGTTCT)-*N7*-G(6),*N7*-G(8)\}] (eq 4) at equilibrium, obtained from the best fit to eq 5. [d] K_{eq} at 29.8 °C was obtained by averaging the P_∞ values for the other temperature points, since the reaction was not followed long enough to yield a reasonable P_∞ at this lowest temperature.

Table II: Selected Peaks in the MS/MS Spectrum of *trans*-[$Pt(NH_3)_2$(Guo)\{d(CpG)\}]$^+$, m/z 1051

m/z	% rel abundance	assignment
1051	precursor	[$Pt(NH_3)_2$(Guo)\{d(CpG)\}]$^+$
1034	100	[$Pt(NH_3)$(Guo)\{d(CpG)\}]$^+$
1017	30	[Pt(Guo)\{d(CpG)\}]$^+$
606	4.4	[$Pt(NH_3)_2$(Guo)(C)]$^+$
589	34	[$Pt(NH_3)$(Guo)(C)]$^+$
473	12	[$Pt(NH_3)$(G)(C)]$^+$
456	37	[Pt(G)(C)]$^+$

Characterization of the 1,4-Intrastrand Cross-Linked Isomer by Enzymatic Digestion, Mass Spectrometric, and Optical Spectroscopic Analyses. Complete enzymatic digestions of the 1,4-intrastrand cross-linked isomer were carried out by using DNase I, nuclease P1, and alkaline phosphatase to generate HPLC-separable pools of platinated and unplatinated nucleosides for analysis. Digests of the two platinated oligonucleotide isomers exhibit very different HPLC profiles (Figure 3). Integration and comparison of nucleoside peaks indicate that, for the 1,4-intrastrand cross-linked isomer, both guanosines as well as one of the cytidines are removed from the pool of free nucleosides. These results indicate that the platinated digestion product peak must contain two guanosines and a cytidine. To test this hypothesis and to determine what type of "$\{Pt(Guo)_2(Cyd)\}$" species might be present, the peak corresponding to the platinated fragment was isolated by HPLC and studied by liquid secondary ion mass spectrometry. The molecular ion exhibited a cluster of peaks at m/z 1050–1052, consistent with [$Pt(NH_3)_2$(Guo)\{d(CpG)\}]$^+$ (calcd M_r, 1051.9). Collision-induced fragmentation of the m/z molecular ion 1051 selected by MS-1 and analysis by a second mass spectrometer yielded a fragment with a m/z value of 456.1, consistent with [^{195}Pt(G)(C)]$^+$ (calcd m/z, 456.3) (Table II). In the collision-induced spectrum of m/z 1050, this peak shifted to m/z 455.0 (^{194}Pt). This result further supports the conclusion that, in the 1,4-intrastrand cross-linked isomer, the platinum coordination sites have shifted from two guanosines to one guanosine and one cytidine nucleoside. Incomplete digestion of *trans*-DDP adducted DNA to yield platinated dinucleoside monophosphates has been previously observed (Eastman et al., 1988). The UV absorption spectrum

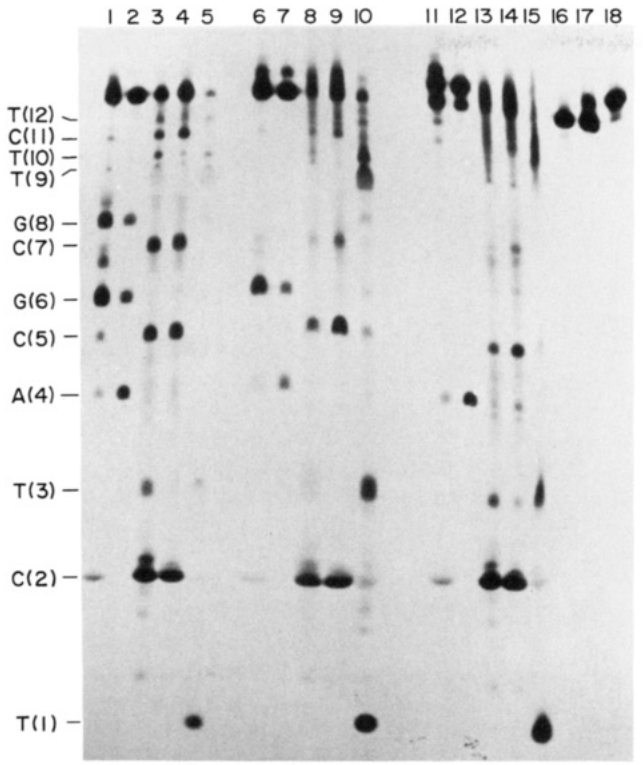

FIGURE 5: Modified Maxam–Gilbert footprinting experiment performed as described in the text. (Lanes 1, 6, and 11) Guanosine-specific sequencing reactions. (Lanes 2, 7, and 12) Purine-specific sequencing reactions. (Lanes 3, 8, and 13) Pyrimidine-specific sequencing reactions. (Lanes 4, 9, and 14) Cytidine-specific sequencing reactions. (Lanes 5, 10, and 15) Thymidine-specific sequencing reactions. (Lanes 1–5) Products of modified Maxam–Gilbert base-specific reactions on 5′-d(TCTACGCGTTCT). (Lanes 6–10) Products of reactions on *trans*-[Pt(NH_3)$_2$\{d(TCTACGCGTTCT)-*N3*-C(5),*N7*-G(8)\}], with platinum removed by NaCN treatment prior to electrophoresis. (Lanes 11–15) Same as lanes 6–10 except that platinum was not removed. (Lane 16) Unreacted 5′-d-(TCTACGCGTTCT). (Lane 17) Unreacted *trans*-[Pt(NH_3)$_2$\{d-(TCTACGCGTTCT)-*N3*-C(5),*N*(7)-G(8)\}], with platinum removed as above. (Lane 18) Unreacted *trans*-[Pt(NH_3)$_2$\{d-(TCTACGCGTTCT)-*N3*-C(5),*N7*-G(8)\}].

of the purified fragment is also consistent with the presence of both nucleosides, having maxima of 270 (cytidine) and 255 nm (guanosine).

^{195}Pt NMR Spectroscopy. The sample used in the ^{195}Pt NMR analysis of the 1,3-intrastrand cross-linked isomer was allowed to isomerize and then reexamined by ^{195}Pt NMR spectroscopy. The spectrum (Figure 4B) contains a peak at −2449 ppm corresponding to residual *trans*-[Pt(NH_3)$_2$\{d-(TCTACGCGTTCT-*N7*-G(6),*N7*-G(8)\}] and a larger resonance for the 1,4-intrastrand cross-linked isomer at −2517 ppm. The small change in the chemical shift of the former resonance (−2449 versus −2436 ppm) arises from different temperatures, 37 °C versus ambient, employed in recording the spectra in parts A and B of Figure 4. The data clearly indicate that, in the 1,4-intrastrand cross-linked isomer, platinum is not coordinated to an oxygen atom. The chemical shift value falls in the range characteristic of Pt(II) bonded to four N-donor ligands (Pregosin, 1986).

Kinetic Studies. An equilibrium mixture of isomers can be attained from either direction, as demonstrated by observing purified *trans*-[Pt(NH_3)$_2$\{d(TCTACGCGTTCT)-*N3*-C(5),-*N7*-G(8)\}] to isomerize slowly to a mixture of *trans*-[Pt-(NH_3)$_2$\{d(TCTACGCGTTCT)-*N7*-G(6),*N7*-G(8)\}] and *trans*-[Pt(NH_3)$_2$\{d(TCTACGCGTTCT)-*N3*-C(5),*N7*-G(8)\}] (data not shown). The equilibrium lies in the direction of the

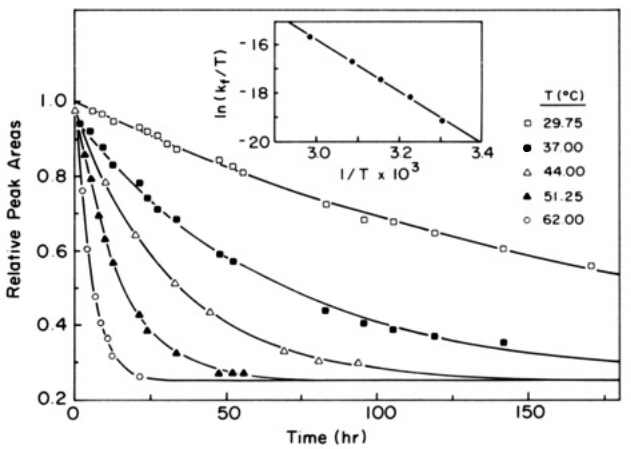

FIGURE 6: Relative peak areas versus time curves for the disappearance of *trans*-[Pt(NH_3)$_2$\{d(TCTACGCGTTCT)-*N7*-G(6),*N7*-G(8)\}], as described in the text. The solid lines are the calculated (eq 5) disappearance of this isomer from a nonlinear least-squares fit of the data. An Eyring plot showing the temperature dependence of the forward rate constant is depicted in the inset.

latter isomer, however, at all temperatures studied (Table I). Rate constants for conversion of the 1,3- to 1,4-intrastrand cross-linked isomer were obtained through time-dependent studies of the HPLC profiles. The method of analysis was similar to that employed in examining the reversible isomerization of binuclear amidate-bridged ethylenediamineplatinum(II) complexes (O'Halloran & Lippard, 1989). The problem was treated as a first-order approach to equilibrium

$$k_{obs} = k_f + k_r \quad (1)$$

where k_f and k_r are defined according to eq 2. The rate

$$\text{1,3-intrastrand cross-linked isomer} \underset{k_r}{\overset{k_f}{\rightleftharpoons}} \text{1,4-intrastrand cross-linked isomer} \qquad (K_{eq} = k_f/k_r) \quad (2)$$

$$\ln (P_t - P_\infty) = -k_{obs}t \quad (3)$$

P = (area 1,3-cross-linked isomer peak)/[(area 1,3-cross-linked isomer peak) + (area 1,4-cross-linked peak)] (4)

constant k_{obs} was obtained from eq 3, where the measured parameter P is defined by eq 4. Samples began to degrade noticeably after 4–5 half-lives, preventing direct measurement of P_∞. These values were therefore identified from nonlinear least-squares analyses of P_t as a function of time according to the expression in eq 5 using the observed P_0 (zero time

$$P_t = P_\infty + (P_0 - P_\infty)[\exp(-k_{obs}t)] \quad (5)$$

point). Plots of P_t versus time, along with curves calculated from eq 5 for best fit values of k_{obs} and P_∞, are shown in Figure 6. The k_{obs} and the K_{eq} calculated from the fitted P_∞ value was used to obtain the forward rate constant k_f at each temperature (Table I). An Eyring plot of temperature dependence of the forward rate constants (Figure 6, inset) was used to obtain kinetic activation parameters, according to the formula given in eq 6, where k is Boltzmann's constant and h is Planck's

$$\ln k_f/T = \ln k/h + \Delta S^\ddagger/R - \Delta H^\ddagger/RT \quad (6)$$

constant. The derived activation parameters are $\Delta H^\ddagger = 91 \pm 2$ kJ/mol and $\Delta S^\ddagger = -58 \pm 8$ J/(mol·K).

Stereochemistry of the Diammineplatinum(II) Moiety. A Kurnakow test was used to confirm that the ammine ligands retain a trans configuration at platinum during the isomerization reaction (Arpalahti & Lippert, 1987). This chemical

test exploits the greater lability of nucleobase nitrogen donor ligands compared to the ammines. Reaction of the 1,4-intrastrand cross-linked isomer with thiourea yielded a product that coeluted with *trans*-$[Pt(NH_3)_2(tu)_2]^{2+}$ in HPLC conducted by using either isocratic or gradient conditions. The product of thiourea reaction with a *cis*-$\{Pt(NH_3)_2\}^{2+}$ moiety would be $[Pt(tu)_4]^{2+}$. The gradient HPLC studies also revealed the presence of the unplatinated oligonucleotide after reaction with thiourea.

Discussion

Analysis of the Reaction of trans-DDP with d-(TCTACGCGTTCT). It has been almost axiomatic that bifunctional adducts formed by *cis*- or *trans*- $\{Pt(NH_3)_2\}^{2+}$ units with DNA are completely inert. The present studies clearly show that the *trans*-[Pt(NH$_3$)$_2${d(GCG)}] 1,3-intrastrand adduct obtained by reaction of *trans*-DDP with the single-stranded dodecanucleotide substrate, d-(TCTACGCGTTCT), isomerizes to afford the thermodynamically more stable *trans*-[Pt(NH$_3$)$_2${d(CGCG)}] 1,4-intrastrand cross-link. The initial product, *trans*-[Pt(NH$_3$)$_2${d-(TCTACGCGTTCT)-*N7*-G(6),*N7*-G(8)}], was identified by means of pH-dependent NMR titrations, enzymatic digestion, and ^{195}Pt NMR spectroscopic analyses. The stoichiometry of one bound platinum atom per dodecamer, determined by platinum AAS and UV spectroscopy, as well as the relative mobilities of the platinated and unplatinated species in electrophoresis gels, rules out the possibility that an interstrand cross-link had formed. Enzymatic digestion analyses and ^{195}Pt NMR studies exclude the possibility that the initial product is a monofunctionally bound intermediate.

Formation and Structure of the Isomerization Reaction Product. The instability of *trans*-[Pt(NH$_3$)$_2${d-(TCTACGCGTTCT)-*N7*-G(6),*N7*-G(8)}] was first noticed during the NMR pH titration studies. The compound converts to another species that could be isolated by reversed-phase HPLC (Figure 1). The new species, subsequently identified as the linkage isomer *trans*-[Pt(NH$_3$)$_2${d-(TCTACGCGTTCT)-*N3*-C(5),*N7*-G(8)}], was first examined to see whether it might have arisen from a chemical degradation of the initial product. Platinum AAS in combination with UV spectroscopy indicated one bound platinum atom per strand. Gel electrophoresis studies revealed both platinated species to migrate only slightly slower than the unplatinated dodecanucleotide, indicating that neither contains an interstrand cross-link. In addition, since the 1,4-intrastrand cross-linked isomer migrates at the same rate in denaturing electrophoresis gels as the 1,3-intrastrand cross-linked isomer, it could not be a truncated form of the latter. Piperidine treatment of the ^{32}P 5′ end labeled strand according to established protocols (Maxam & Gilbert, 1980) followed by denaturing gel electrophoresis revealed the absence of alkali-labile lesions.

A pH-dependent NMR titration study of the linkage isomer confirmed the presence of all 12 nucleosides (Figure 2B). Titration curves for the adenosine H2 and H8 and one of the guanosine H8 resonances were very similar for the two linkage isomers. The other guanosine titration curve differed considerably, however, clearly demonstrating that this nucleoside is not coordinated to platinum in the 1,4-intrastrand cross-linked isomer. In addition, one of the four sets of cytidine H5 doublets failed to shift below pH 5.5 (arrow, Figure 2B), suggesting platinum coordination at N3 or possibly N4 of this nucleoside. Analysis of the 1,4-intrastrand cross-linked isomer by modified Maxam–Gilbert sequencing procedures confirmed that platinum is coordinated to N7 of G(8) (Figure 5, lanes 6–10). Since the footprint (Figure 5, lanes 11–15) spanned G(6)–G(8) and the pH titration indicated A(4) to be unplatinated, the second coordination site must lie between A(4) and G(6). The most likely donor sites are N3 of C(5), N4 of C(5), or the phosphate group between C(5) and G(6).

Identification of N3 of C(5) as the upstream binding site was established by ^{195}Pt NMR spectroscopy (Figure 4B). The chemical shift values observed for both linkage isomers are indicative of platinum coordinated to four nitrogen atoms and not to chloride, hydroxide, or water ligands (Pregosin, 1986). The ^{195}Pt resonance of the 1,4-intrastrand cross-linked isomer occurs ~68 ppm upfield of the initial adduct, inconsistent with platinum binding to an oxygen atom such as that of a phosphate ester group. Coordination to oxygen would result in a ca. 500 ppm downfield shift (Pregosin, 1986). The −2517 ppm value is also inconsistent with platinum binding to the deprotonated exocyclic amine (N4) of cytidine, for coordination to such a position would also lead to a substantial downfield shift. For example, the ^{195}Pt chemical shift of a similar species, *cis*-[Pt(NH$_3$)$_2$(*N9*-9-aminoacridine)Cl]$^+$, bound to platinum through a deprotonated exocyclic amine, occurs downfield from *cis*-[Pt(NH$_3$)$_2${*N7*-d(G)}Cl]Cl by 81 ppm (D. P. Bancroft, W. I. Sundquist, and S. J. Lippard, unpublished results). Platinum would not be expected to bind to the nondeprotonated exocyclic amine of cytidine. The observed chemical shift is consistent with coordination to N3 of C(5), however. The related complex *cis*-[Pt(NH$_3$)$_2$(*N3*-C){*N7*-d(G)}]$^{2+}$ exhibits a ^{195}Pt resonance at nearly the same position, −2529 ppm (S. F. Bellon, D. P. Bancroft, and S. J. Lippard, unpublished results). Moreover, a ^{195}Pt NMR study of a series of *cis*-[Pt(NH$_3$)$_2$-(N-donor)Cl]$^+$ complexes revealed the chemical shift of *cis*-[Pt(NH$_3$)$_2${*N3*-d(C)}Cl]Cl to be 77 ppm upfield from that of *cis*-[Pt(NH$_3$)$_2${*N7*-d(G)}Cl]Cl (Hollis et al., 1989), a value comparable to the 68 ppm upfield shift observed here.

Complete enzymatic digestions of both linkage isomers provided independent confirmation of several of the preceding results (Figure 3). Upon exhaustive digestion of the 1,3-intrastrand cross-linked isomer with DNase I, nuclease P1, and alkaline phosphatase, a peak appeared in the HPLC trace that coeluted with *trans*-$[Pt(NH_3)_2\{d(Guo)_2\}]^{2+}$. The 1,4-intrastrand cross-linked isomer, when similarly digested, gave a new HPLC peak found by liquid secondary ion mass spectrometry to have a molecular weight consistent with *trans*-[Pt(NH$_3$)$_2${d(Guo)}{d(CpG)}]$^+$. The molecular ion of this fragment was further analyzed by tandem mass spectrometry (Lepre et al., 1990), confirming that platinum is coordinated to cytidine.

Kinetic Studies. To study the mechanism of the isomerization reaction, the time dependence of the disappearance of *trans*-[Pt(NH$_3$)$_2${d(TCTACGCGTTCT)-*N7*-G(6),*N7*-G(8)}] was examined. The results [$\Delta H^{\ddagger} = 91 \pm 2$ kJ/mol, $\Delta S^{\ddagger} = -58 \pm 8$ J/(mol·K)] are similar to those observed for a typical square planar d^8 metal center substitution reaction, such as solvolysis of *trans*-DDP (Basolo & Pearson, 1967). We tentatively conclude that the isomerization occurs through an associative hydrolysis step. If the reaction occurred through direct nucleophilic attack of C(5) on platinum, the entropy of activation would presumably be much more negative. If the transition state had a significant dissociative component, then the entropy of activation would have a large positive value (O'Halloran & Lippard, 1989). The reaction is therefore expected to proceed with retention of configuration about platinum, consistent with results obtained from a Kurnakow test.

Concluding Remarks

The isomerization of *trans*-[$Pt(NH_3)_2${d(TCTACGCGTTCT)-*N7*-G(6),*N7*-G(8)}] to form *trans*-[$Pt(NH_3)_2${d(TCTACGCGTTCT)-*N3*-C(5),*N7*-G(8)}] under approximately physiological conditions is of significance for several reasons. Although the initial 1,3-intrastrand cross-linked isomer is one of the more common *trans*-DDP adducts (Sherman & Lippard, 1987; Eastman et al., 1988), the product of this linkage isomerization reaction, a 1,4-diadduct, has not been reported previously. Coordination of *trans*-$\{Pt(NH_3)_2\}^{2+}$ to N3 of cytidine is consistent with a recent enzymatic digestion study of DNA platinated with *trans*-DDP (Eastman et al., 1988) in which cytidine adducts accounted for 5% and 50% of the total adducts found in single- and double-stranded DNA, respectively. Several model compounds containing platinum bound to cytosine have also been described (Lippert et al., 1988). Studies using anti-nucleoside antibodies to identify and quantitate nucleosides opposite *trans*-$\{Pt(NH_3)_2\}^{2+}$ lesions on DNA revealed the presence of antibody reactive guanosine residues (Sundquist et al., 1986). This result could arise if platinum were bound to cytidine. Replication mapping studies carried out on *trans*-DDP-platinated DNA indicated several polymerase stop sites that could be accounted for by platinum–cytidine coordination (Pinto & Lippard, 1985a; J. N. Burstyn, W. J. Heiger-Bernays, J. M. Essigmann, and S. J. Lippard, unpublished results).

The present study also demonstrates the potential for Maxam–Gilbert sequencing reactions to characterize chemically modified DNA. The ability of cyanide to reverse the platination reactions is a novel feature that facilitated analysis of the results. The approach is applicable to DNA containing any number of platinum atoms, provided the population is homogeneous; all DNA molecules must contain the same adducts. A mixed population of adducts on the same oligomer or DNA fragment could also be analyzed if first resolved chromatographically or by gel electrophoresis. One of the Maxam–Gilbert reagents, dimethyl sulfate, has been previously employed for other types of footprinting [recent examples, Sen and Gilbert (1988) and Voloshin et al. (1988)].

The fact that rearrangements such as that described in this paper have not been previously reported underscores the importance of sequence context in mediating the chemistry of platinum bound to oligonucleotides. A closely related dodecamer, 5′-d(CCTCGAGTCTCC), reacts with *trans*-DDP, forming a stable 1,3-{d(GAG)-*N7*,*N7*} adduct (Lepre et al., 1990). Moreover, *cis*-DDP reacts with 5′-d(TCTACGCGTTCT) to form a 1,3-{d(GpCpG)-*N7*,*N7*} intrastrand cross-link in a manner analogous to the reaction of *trans*-DDP, but the product is completely inert (J. N. Burstyn and S. J. Lippard, unpublished results). Both the specific targeting of platinum to certain regions on native DNA and resistance of other regions containing multiple guanosines to platination have been noted in various studies (Tullius & Lippard, 1981, 1982; Bowler & Lippard, 1986; Malinge et al., 1987; Pinto & Lippard, 1985a; Gralla et al., 1987; Hemminki & Thilly, 1988; Villani et al., 1988; J. N. Burstyn, W. J. Heiger-Bernays, J. M. Essigmann and S. J. Lippard, unpublished results). These sequence context phenomena are not well understood and are worthy of further investigation.

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References

Arpalahti, J., & Lippert, B. (1987) *Inorg. Chim. Acta 138*, 171–173.

Banaszuk, A. M., Deugau, K. V., Sherwood, J., Michalak, M., & Glick, B. R. (1983) *Anal. Biochem. 128*, 281–286.

Basolo, F., & Pearson, R. G. (1967) *Mechanisms of Inorganic Reactions*, Wiley, New York.

Bowler, B. E., & Lippard, S. J. (1986) *Biochemistry 25*, 3031–3038.

Caradonna, J. P., Lippard, S. J., Gait, M. J., & Singh, M. (1982) *J. Am. Chem. Soc. 104*, 5793–5795.

Cashmore, A. R., & Peterson, G. B. (1969) *Biochim. Biophys. Acta 174*, 591–603.

Cashmore, A. R., & Petersen, G. B. (1978) *Nucleic Acids Res. 5*, 2485–2491.

Eastman, A. (1985) *Biochemistry 24*, 5027–5032.

Eastman, A. (1986) *Biochemistry 25*, 3912–3915.

Eastman, A., Jennerwein, M. M., & Nagel, D. L. (1988) *Chem.-Biol. Interact. 67*, 71–80.

Fasman, G. D. (1975) in *Handbook of Biochemistry and Molecular Biology*, 3rd ed., Vol. 1, p 589, CRC Press, Cleveland, OH.

Fichtinger-Schepman, A. M. J., van der Meer, J. L., den Hartog, J. H. J., Lohman, P. H. M., & Reedijk, J. (1985) *Biochemistry 24*, 707–713.

Fichtinger-Schepman, A. M. J., van Oosterom, A. T., Lohman, P. H. M., & Berends, F. (1987) *Cancer Res. 47*, 3000–3004.

Glasoe, P. K., & Long, F. A. (1960) *J. Phys. Chem. 64*, 188–190.

Gralla, J. D., Sasse-Dwight, S., & Poljak, L. G. (1987) *Cancer Res. 47*, 5092–5096.

Hemminki, K., & Thilly, W. G. (1988) *Mutat. Res. 202*, 133–138.

Hollis, L. S., Amundsen, A. R., & Stern, E. W. (1989) *J. Med. Chem. 32*, 128–136.

Inagaki, K., Tomita, A., & Kidani, Y. (1988) *Bull. Chem. Soc. Jpn. 61*, 2825–2831.

Izatt, R. M., Christensen, J. J., & Rytting, J. H. (1971) *Chem. Rev. 71*, 439–481.

Johnson, N. P., Hoeschele, J. D., & Rahn, R. O. (1980) *Chem.-Biol. Interact. 30*, 151–169.

Kaufman, G. B., & Cowan, D. O. (1963) *Inorg. Synth. 7*, 239–245.

Kurnakow, N. (1894) *J. Prakt. Chem. 50*, 481–507.

Lepre, C. A., Costello, C. E., & Lippard, S. J. (1990) *Biochemistry* (in press).

Lim, M. C., & Martin, R. B. (1976) *J. Inorg. Nucl. Chem. 38*, 1911–1918.

Lippert, B., Schöllhorn, H., & Thewalt, U. (1986) *J. Am. Chem. Soc. 108*, 6616–6621.

Lippert, B., Arpalahti, J., Krizanovic, O., Micklitz, W., Schwarz, F., & Trötscher, G. (1988) in *Platinum and Other Metal Coordination Compounds in Cancer Chemotherapy*

(Nicolini, M., Ed.) Nijhoff, Boston.
Malinge, J.-M., Schwartz, A., & Leng, M. (1987) *Nucleic Acids Res. 15*, 1779-1797.
Maxam, A. H., & Gilbert, W. (1980) *Methods Enzymol. 65*, 499-560.
Naser, L. J., Pinto, A. L., Lippard, S. J., & Essigmann, J. M. (1988) *Biochemistry 27*, 4357-4367.
O'Halloran, T. V., & Lippard, S. J. (1989) *Inorg. Chem. 28*, 1289-1295.
Pinto, A. L., & Lippard, S. J. (1985a) *Proc. Natl. Acad. Sci. U.S.A. 82*, 4616-4619.
Pinto, A. L., & Lippard, S. J. (1985b) *Biochim. Biophys. Acta 780*, 167-180.
Pinto, A. L., Naser, L. J., Essigmann, J. M., & Lippard, S. J. (1986) *J. Am. Chem. Soc. 108*, 7405-7407.
Pregosin, P. S. (1986) *Annu. Rep. NMR Spectrosc. 17*, 285-349.
Reed, E., Ozols, R. F., Tarone, R., Yuspa, S. H., & Poirier, M. C. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 5024-5028.
Roberts, J. J., & Thomson, A. J. (1979) *Prog. Nucleic Acid Res. Mol. Biol. 22*, 71-133.
Sato, K., Asada, T., Ishihara, M., Kunihiro, F., Kammei, Y., Kubota, E., Costello, C. E., Martin, S. A., Scoble, H. A., & Biemann, K. (1987) *Anal. Chem. 59*, 1652-1659.
Sen, D., & Gilbert, W. (1988) *Nature 334*, 364-366.
Sherman, S. E., & Lippard, S. J. (1987) *Chem. Rev. 87*, 1153-1181.
Sinha, N. D., Biernat, J., & Köster, H. (1983) *Tetrahedron Lett. 24*, 5843-5846.
Sundquist, W. I., Lippard, S. J., & Stollar, B. D. (1986) *Biochemistry 25*, 1520-1524.
Tullius, T. D., & Lippard, S. J. (1981) *J. Am. Chem. Soc. 103*, 4620-4622.
Tullius, T. D., & Lippard, S. J. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 3489-3492.
van der Veer, J. L., van den Elst, H., & Reedijk, J. (1987) *Inorg. Chem. 26*, 1536-1540.
Villani, G., Hübscher, U., & Butour, J. L. (1988) *Nucleic Acids Res. 16*, 4407-4418.
Voloshin, O. N., Mirkin, S. M., Lyamichev, V. I., Belotserkovskii, Frank-Kamenetskii, M. D. (1988) *Nature 333*, 475-476.

Mercury-Induced DNA Polymorphism: Probing the Conformation of Hg(II)-DNA via Staphylococcal Nuclease Digestion and Circular Dichroism Measurements†

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ABSTRACT: Exposing native calf thymus DNA to increasing concentrations of $Hg(ClO_4)_2$ not only produces dramatic changes in its circular dichroism (CD) but results also in the decrease, and ultimate cessation, of endonucleolytic DNA cleavage by staphylococcal nuclease. Let r = [moles of added Hg(II)]/[mole of DNA base]: the conservative CD spectrum of the DNA B-form becomes nonconservative in appearance at $0.01 < r < 0.12$ (resembling DNA in C-form geometry) and assumes the spectral characteristics of a left-handed DNA double helix at $0.12 < r \leq 1.0$. DNA cleavage proceeds at or near the rates exhibited by untreated DNA at $0 < r < 0.08$. At Hg(II) levels of $0.08 < r < 0.5$, the rate of DNA hydrolysis decreases monotonically with increasing Hg(II) concentrations, and at $r > 0.4$, DNA cleavage ceases. Both the CD changes and the changes in the rate of DNA digestion are totally reversible upon the removal of Hg(II), at least up to $r = 1.0$, demonstrating that Hg(II) keeps all base pairs in register. For comparison purposes, native calf thymus DNA was also treated with methylmercury [$CH_3Hg(II)$], an agent known to disrupt the secondary structure of DNA. The treatment yielded single-stranded methylmercurated DNA with preserved right-handed helix screwness. In addition, this DNA is digested by staphylococcal nuclease much more rapidly than double-stranded control DNA. Lastly, neither the CD nor the cleavage rate changes are reversible upon the removal of methylmercury. We interpret the Hg(II)-induced alterations in the CD of native calf thymus DNA, and the hydrolysis rate changes observed with staphylococcal nuclease, to indicate that Hg(II) either produces in DNA reversible B ↔ Z transitions, passing transiently through C-like conformations, or generates non-B-conformational structures of presumably left-handed geometry.

Hg(II)[1] is known to interact strongly and yet reversibly with the nitrogen binding sites of purines and pyrimidines (Katz, 1952; Thomas, 1954; Yamane & Davidson, 1961; Simpson, 1964; Nandi et al., 1965). It is believed that with native DNA the metal is chelated between the Watson-Crick base pairs, forming strong bonds to the σ electron pairs of nitrogen atoms in a linear =NHgN= configuration (sp hybridization) (Yamane & Davidson, 1961; Katz, 1963; Nandi et al., 1965).

The concept of a mercury-induced cross-linking of the Watson and Crick strands of DNA, without disturbing the

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[1] Hg(II) is to refer to divalent inorganic mercury, sufficiently ionized so as to bind to the bases of the DNA. Since the precise nature (as well as concentration) of all mercuric species potentially present in the buffer systems of this study is unknown, they are also collectively denoted Hg(II). Similar considerations apply to $CH_3Hg(II)$, the one difference being that methylmercury, albeit divalent, is monofunctional.

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