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Modulation of Platinum Antitumor Drug Binding to DNA by Linked and Free Intercalators†

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ABSTRACT: We report the DNA binding site preferences of the novel molecule AO–Pt, in which the anticancer drug dichloro(ethylenediamine)platinum(II) is linked by a hexamethylene chain to acridine orange. The sequence specificity of platinum binding was mapped by exonuclease III digestion of 165 and 335 base pair restriction fragments from pBR322 DNA. Parallel studies were carried out with the unmodified anticancer drugs *cis*-diamminedichloroplatinum(II) (*cis*-DDP) and dichloro(ethylenediamine)platinum(II), [Pt(en)Cl_2]. Oligo(dG) sequences are the most prevalent binding sites for AO–Pt, with secondary binding occurring mainly at d(AG) sites. *cis*-DDP and [Pt(en)Cl_2] bind less readily to the secondary sequences, with *cis*-DDP showing greater binding site selectivity than [Pt(en)Cl_2]. The DNA intercalator ethidium bromide promotes binding of [Pt(en)Cl_2] and *cis*-DDP to many sites containing d(CGG) and, to a lesser extent, d(AG) sequences. AO–Pt exhibits enhanced binding to these sequences without the need for an external intercalator. Unlinked acridine orange, however, does not promote binding of [Pt(en)Cl_2] and *cis*-DDP to d(CGG) and d(AG) sequences. These results are discussed in terms of the sequence preferences, stereochemistry, and relative residence times of the intercalators at their DNA binding sites. By modulating local structure in a sequence-dependent manner, both linked and, in the case of ethidium, free intercalators can influence the regioselectivity of covalent modification of DNA by platinum antitumor drugs.

There is currently much interest in understanding the interaction of platinum complexes with DNA (Pinto & Lippard, 1985; Hacker et al., 1984). This attention stems mainly from the clinical success of *cis*-diamminedichloroplatinum(II), *cis*-DDP,[1] in the treatment of neoplastic disease, particularly testicular cancer (Prestayko et al., 1980; Loehrer & Einhorn, 1984), as well as evidence pointing to differential repair of Pt–DNA adducts as an important aspect of the molecular mechanism of action of the drug (Roberts & Pera, 1983; Ciccarelli et al., 1985). Numerous in vitro and in vivo studies have elucidated both the sequence specificity and structural changes coincident with platinum binding to DNA (Pinto & Lippard, 1985).

In most of this work, platinum–DNA interactions have been studied in the absence of other perturbing agents in the medium. Since *cis*-DDP is usually administered in combination with other drugs, such as actinomycin, vinblastine, bleomycin, and adriamycin (Pizzocaro et al., 1985; Loehrer & Einhorn, 1984; Vugrin et al., 1983; Prestayko et al., 1980), several of which are DNA intercalators [Berman & Young (1981) and references cited therein], we were interested to monitor the mutual effects of DNA intercalators and platinum complexes on DNA binding. Previously, we showed that *cis*-DDP alters the sequence-specific cleavage of DNA by bleomycin (Mascharak et al., 1983) and that the intercalator EthBr changes both the position (Tullius & Lippard, 1982) and the mode (Merkel & Lippard, 1982) of *cis*-DDP binding to DNA. Since these modulations might be related to the clinical synergism between *cis*-DDP and an intercalating antitumor drug, we have extended our investigation to gain further insight into the important parameters involved in the alteration of DNA–platinum binding by DNA intercalators. Specifically, we have synthesized a platinum complex, AO–Pt (Bowler et al., 1984), that covalently links [Pt(en)Cl_2] to the DNA intercalator AO by a polymethylene chain (Figure 1). This compound permits local modifications of DNA structure by an intercalator to be sampled by platinum without the need to add a large excess of external intercalator. Thus, the effect of the intercalator can be monitored at low $(D/N)_0$ ratios of both platinum and intercalator. This approach of coupling metals with DNA intercalators has been used in previous studies involving metallointercalators (Lippard, 1978; Barton, 1983) and intercalator-linked iron complexes (Van Dyke & Dervan, 1984;

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[1] Abbreviations: EthBr, ethidium bromide; AO, acridine orange; CIP, calf intestinal phosphatase; PNK, T4 polynucleotide kinase; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetate; DTT, dithiothreitol; bp, base pair; β-ME, β-mercaptoethanol; $(D/N)_0$, added drug to nucleotide ratio; $(D/N)_b$, bound drug to nucleotide ratio; *cis*-DDP, *cis*-diamminedichloroplatinum(II); en, ethylenediamine; pur, purine; pyr, pyrimidine; AO–Pt, the intercalator-linked (ethylenediamine)platinum(II) complex; AO-en, 3,6-bis(dimethylamino)-10-[6-[(2-aminoethyl)amino]hexyl]acridinium chloride.

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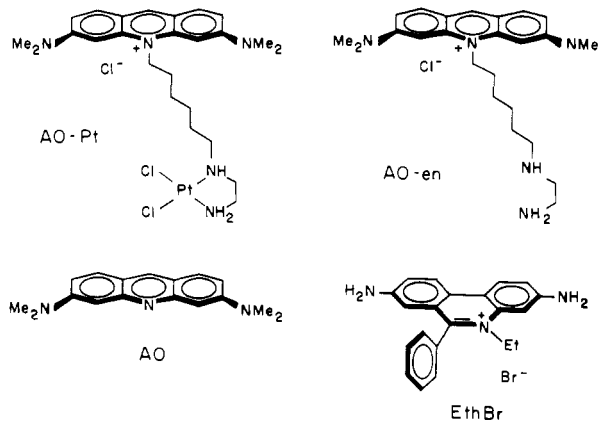

FIGURE 1: Free and linked intercalators used in this study.

Hertzberg & Dervan, 1984) to probe DNA structure and the specificity of drug binding to DNA.

In this study, we have employed the previously described exonuclease III mapping technique (Tullius & Lippard, 1981, 1982; Royer-Pokora et al., 1981) to probe modulations of sequence-specific platinum–DNA binding by the linked acridine orange moiety of AO–Pt. The results are compared to the effects of adding EthBr, unlinked AO, and an N-alkylated AO derivative (AO-en, see Figure 1) on $[Pt(en)Cl_2]$ binding to DNA and to the DNA binding of *cis*-DDP in the presence and absence of external intercalators.

Materials and Methods

Chemicals. AO–Pt and AO-en were prepared and characterized as previously described (Bowler et al., 1984). $[Pt(en)Cl_2]$ was prepared by a modification of the method of Dhara (1970) and recrystallized 3 times from 0.1 M HCl before use. *cis*-DDP was prepared by P. Mascharak and R. Ciccarelli in our laboratory. Ultrapure agarose was purchased from BRL and electrophoresis-grade acrylamide and bis(acrylamide) from Bio-Rad. 5′-[γ-^{32}P]ATP was obtained from Amersham. EthBr from Sigma was used without further purification. AO from Aldrich was purified by a literature method (Gupta et al., 1967). All other chemicals were reagent-grade.

Enzymes. Restriction endonucleases, *Hpa*II and *Hae*III, were obtained from BRL or Boehringer Manheim Biochemicals (BMB). Exonuclease III, molecular biology grade CIP, and PNK were purchased from BMB.

Buffers. TE is 10 mM Tris-HCl, pH 8, and 0.5 mM Na_2EDTA. TBE used in electrophoresis is 90 mM Tris, 90 mM boric acid, and 2 mM Na_2EDTA, pH 8.3. *Hpa*II buffer is 20 mM Tris-HCl, pH 7.4, 7 mM $MgCl_2$, 6 mM KCl, and 1 mM DTT. CIP buffer is 100 mM Tris-HCl, pH 8.0, 1 mM $MgCl_2$, and 0.1 mM $ZnCl_2$. *Hae*III buffer is 6 mM Tris-HCl, pH 7.4, 50 mM NaCl, 6 mM $MgCl_2$, and 7 mM β-ME. PNK buffer is 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, and 5 mM DTT.

Stock Solutions. Intercalator stock solutions (AO, AO-en, AO–Pt, EthBr) were prepared by dissolution into 10-mL volumes of deionized water to approximately 1 mM concentrations. AO free base was first solubilized in a small volume of approximately 1 equiv of 0.1 M HCl before addition to deionized water. Polyethylene bottles were used to minimize the problem of surface adsorption with these molecules. Stocks were diluted to 10–200 μM for additon to DNA reaction mixtures. Concentrations of the stock solutions were determined spectrophotometrically with the following: AO, ϵ_{470} = 43 000 M^{-1} cm^{-1} (Lamm & Neville, 1965); EthBr, ϵ_{287} = 5.39 $\times$ 10^4 M^{-1} cm^{-1} (Merkel, 1983). The extinction coefficients of AO-en and AO–Pt at the monomer/dimer isosbestic point, determined by the method of Lamm and Neville, are as follows: AO-en, $\epsilon_{475} = (4.75 \pm 0.04) \times 10^4$ cm^{-1}; AO–Pt, $\epsilon_{473} = (4.48 \pm 0.04) \times 10^4$ M^{-1} cm^{-1}. All solutions were prepared immediately before use except for EthBr, which remained spectrophotometrically stable indefinitely if stored refrigerated in the dark.

Solutions of *cis*-DDP and $[Pt(en)Cl_2]$ were prepared immediately before use by dissolution into 0.1 M NaCl to make solutions of approximately 1.0 mM concentration. Owing to its lower solubility, $[Pt(en)Cl_2]$ was assisted in dissolution by heating to approximately 55 °C. Concentrations were determined spectrophotometrically with for $[Pt(en)Cl_2]$ ϵ_{301} = 224 M^{-1} cm^{-1} (Martin et al., 1971) and for *cis*-DDP ϵ_{300} = 132 M^{-1} cm^{-1} (Raudaschl et al., 1983). These stock solutions were diluted 1:25 to 1:50 before addition to DNA reaction mixtures.

DNA. Carrier DNA used in radiolabeled experiments and DNA used in cold samples were calf thymus DNA (Sigma) that was further purified by phenol extraction followed by dialysis against TE buffer. pBR322 plasmid DNA was prepared by H. M. Ushay or C. M. Merkel as previously described (Ushay et al., 1981).

Preparation of 5′-^{32}P-End-Labeled 335- and 165-bp Restriction Fragments. pBR322 DNA was treated with *Hpa*II (37 °C, overnight, 0.3 unit/μg of DNA), and a 527-bp fragment was isolated by electrophoresis through a preparative 1.5% agarose gel, followed by electroelution from the gel slice and purification through a Schleicher & Schuell Elutip-d column. The 527-bp fragment was 5′-end-labeled first by treatment with CIP (Maniatis et al., 1982) followed by treatment with PNK (1 unit/μg of 527-bp DNA, 2 h, 37 °C) in the presence of 250 μCi of 5′-[γ-^{32}P]ATP. The 165- and 335-bp fragments were produced by *Hae*III treatment (2 unit/μg of 527-bp DNA, 37 °C, overnight), resolved by electrophoresis through a 1.5 mm thick 6% polyacrylamide gel, and isolated by electroelution, passage through Elutip-d columns, and ethanol precipitation in the presence of 10 μg of calf thymus carrier DNA. The pieces were stored in TE buffer prior to use. Typically, 75 μg of pBR322 yielded 750 000–2 000 000 cpm per fragment.

Platination Reactions. Platination reactions were carried out in TE buffer containing 2 μg of carrier DNA in 50 μL of solution ([DNA P_i] = 121 μM). This DNA concentration included 20 000 cpm per gel lane of the 165- or 335-bp radiolabeled fragment. Incubations were for 4 h at 37 °C and were performed in the dark to minimize possible photoreactive effects of the intercalators. Intercalator-containing reaction mixtures had a $(D/N)_0$ of 0.15 in intercalator and were preincubated at 37 °C for 15–30 min to equilibrate the intercalator with the DNA. Since intercalators depress the rate of platinum reactions with DNA at high $(D/N)_0$ levels, the $(D/N)_0$ ratios of *cis*-DDP and $[Pt(en)Cl_2]$ were adjusted accordingly. A $(D/N)_0$ ratio of 0.03 was used for $[Pt(en)Cl_2]$, and a value of 0.05 was used for the slower reacting *cis*-DDP in the absence of intercalators. In the presence of AO or EthBr, $(D/N)_0$ ratios of 0.05 and 0.08–0.10 had to be used for $[Pt(en)Cl_2]$ and *cis*-DDP, respectively, to achieve Pt binding levels comparable to the intercalator-free incubations. For AO-en, $(D/N)_0$ levels of 0.2 or higher for $[Pt(en)Cl_2]$ or *cis*-DDP were required. Much lower $(D/N)_0$ levels of 0.005, 0.01, and 0.03 were sufficient for AO–Pt reactions. At the ends of the reactions, 4 M NaOAc was added to a final concentration of 0.22 M, followed by two 50-μL phenol ex-

tractions to remove intercalators, two 75-μL ether washes, and ethanol precipitation to remove unreacted platinum from the DNA. Samples were stored in 50 μL of TE at 4 °C until used for exonuclease III digestions.

The ratio of bound platinum per nucleotide, $(D/N)_b$, was determined by using samples containing carrier DNA only, incubated simultaneously with the radioactive samples. Platinum concentrations were determined by atomic absorption spectrophotometry by using a Varian AA 1475 spectrophotometer, in combination with a CRA 90 carbon rod atomizer. DNA concentrations of the same samples were determined by a fluorescence assay (Thomas & Farquhar, 1978). $(D/N)_b$ ratios determined from these data are given in the figure legends.

Exonuclease III Reactions. In these experiments each sample contained 2 μg of carrier DNA and an initial radioactivity of 20 000 cpm per gel lane. Platinated samples were ethanol-precipitated and resuspended in deionized water. A 10-fold concentrated solution of exonuclease III buffer was added along with 1 μL of 50 mM β-ME to yield a final buffer solution of 66 mM Tris-HCl, pH 8.0, 6.6 mM $MgCl_2$, and 1 mM β-ME in a 50-μL total volume. The enzyme digestion was started by adding 200 units of exonuclease III, and the solution was incubated at 37 °C for 75 min. The reaction was stopped by addition of 200 mM Na_2EDTA to a final concentration of 20 mM and extraction of the enzyme with buffer-saturated phenol. The aqueous layer was washed twice with ether, and for removal of platinum (Tullius & Lippard, 1981), 20 μL of 1.0 M NaCN was added to produce a final concentration of 0.29 M, and the solution was incubated for 3 h at 37 °C. The samples were ethanol-precipitated and resuspended in 3 μL of Maxam–Gilbert sequencing buffer (Maxam & Gilbert, 1980) per initial 20 000 cpm of radioactivity.

Control experiments were performed to ensure that the formation of new exonuclease III detectable Pt binding sites in the presence of EthBr was not an artifact due to the incomplete removal of this intercalator. Exonuclease III digestion of unplatinated DNA that was incubated with EthBr and then phenol extracted produced the same digestion pattern as control DNA. DNA digested with exonuclease III in the presence of EthBr, $(D/N)_0 = 0.07$, produced a slight increase in the number of bands on the gel compared with control DNA digest patterns, but at much lower intensity than digestion of DNA platinated in the presence or absence of EthBr. DNA platinated in the absence of EthBr but digested in the presence of EthBr, $(D/N)_0 = 0.07$, had the same digestion pattern as DNA platinated in the absence of EthBr and digested in the usual manner.

Sequencing Gels. All radioactive samples were resolved on 0.4-mm, 8% polyacrylamide–8.3 M urea gels with TBE running buffer. Gels were preelectrophoresed for at least 1 h at 40-W constant power. Samples were then loaded and electrophoresed at 40-W constant power for a time appropriate to the size of the fragment or the portion of the fragment to be visualized. Gels were exposed on Kodak XAR-5 film for 2–3 days at −70 °C. Maxam–Gilbert base-specific reactions (Maxam & Gilbert, 1980) were electrophoresed in parallel to provide a sequence ladder.

Results

Comparison of [Pt(en)Cl_2] and AO–Pt Binding Sites. In our previous studies of exonuclease III detection of *cis*-DDP binding sites (Tullius & Lippard, 1981, 1982), we examined only the *Hpa*II/*Hae*III-generated 165-bp restriction fragment from pBR322. In order to expand the variety of sequences available for platinum binding to DNA, we now present data from a 335-bp fragment. Moreover, since the Pt-binding portion of AO-Pt (Figure 1) more closely resembles [Pt(en)Cl_2] than *cis*-DDP, we also studied the sequence preferences of the (ethylenediamine)platinum compound for binding to both restriction fragments.

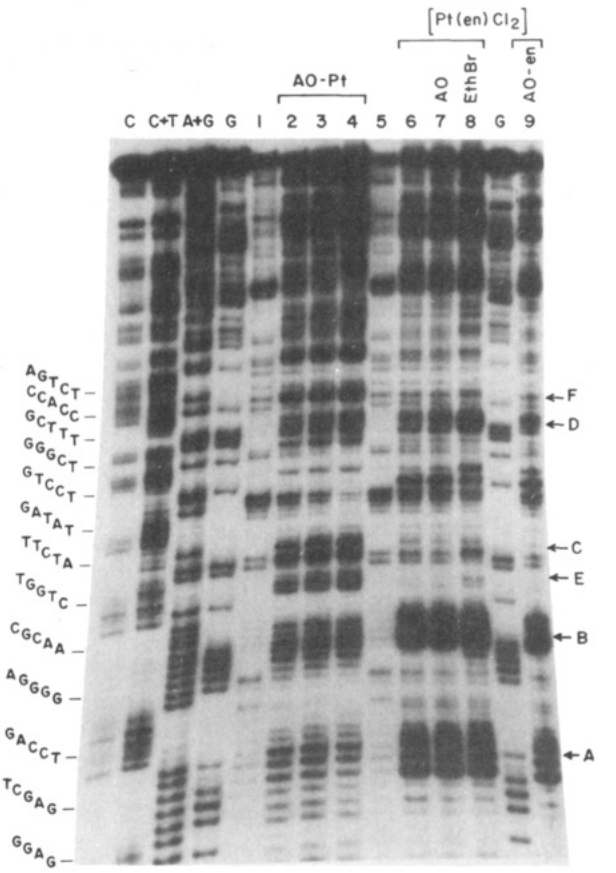

FIGURE 2: Autoradiogram of an 8% polyacrylamide/8.3 M urea electrophoresis gel displaying results for the binding of AO–Pt, [Pt(en)Cl_2], or [Pt(en)Cl_2] in the presence of EthBr, AO, or AO-en on the 165-bp restriction fragment as detected by exonuclease III digestion. Electrophoresis was carried out at 40-W constant power for 3 h. The lanes labeled C, C+T, A+G, and G are Maxam–Gilbert base-specific reactions. Lanes 1–9 correspond to exonuclease III digestion products as follows: (1 and 5) control unplatinated 165-bp fragment; (2–4) AO–Pt at $(D/N)_b$ = 0.001, 0.0045, and 0.009, respectively; (6) [Pt(en)Cl_2], $(D/N)_b$ = 0.011; (7) [Pt(en)Cl_2]/AO, $(D/N)_b$ = 0.010; (8) [Pt(en)Cl_2]/EthBr, $(D/N)_b$ = 0.012; (9) [Pt(en)Cl_2]/AO-en, $(D/N)_b$ = 0.007. Letters A–D indicate oligo(dG) binding sites, and E and F indicate $(dC)_2$ sites. The sequence is given to the left of the gel.

Figure 2 shows a sequencing gel of the 165-bp fragment platinated by [Pt(en)Cl_2] or AO–Pt prior to enzymatic digestion. Exonuclease III stopping sites for [Pt(en)Cl_2] (see lane 6) are essentially identical with those observed previously for *cis*-DDP (Tullius & Lippard, 1981), namely, the oligo(dG) sequences d(GAG_3AG), $(dG)_5$, $(dG)_2$, and $(dG)_3$ labeled A–D respectively in the figure. These same oligo(dG) sites are also observed for AO–Pt-modified DNA (see lanes 2–4), but this substrate also produces stops corresponding to $(dC)_2$ sequences, labeled E and F on the gel, which we interpret as arising from exonuclease III digestion inhibited by AO–Pt bound to $(dG)_2$ sequences on the unlabeled strand. Presumably, the adduct formed by AO–Pt with DNA can be sensed even when situated on the (unlabeled) strand not being directly monitored. The simpler platinum complex [Pt(en)Cl_2], like *cis*-DDP, can be detected by exonuclease III only when bound to the labeled strand.

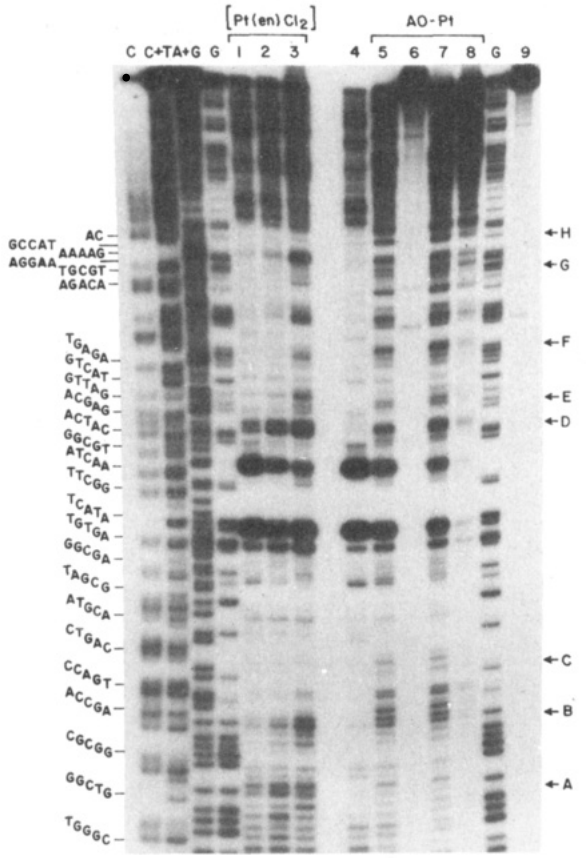

FIGURE 3: Autoradiogram of an 8% polyacrylamide/8.3 M urea electrophoresis gel showing the effect of increasing binding levels of $[Pt(en)Cl_2]$ or AO–Pt on the exonuclease III digestion of the 335-bp restriction fragment. Electrophoresis was carried out at 40-W constant power for 3.5 h. The lanes labeled C, C+T, A+G, and G contain Maxam–Gilbert base-specific reactions. Lanes labeled 1–5 and 7 and 8 contain exonuclease III digested 335-bp fragment modified as follows: (1–3) $[Pt(en)Cl_2]$, $(D/N)_0 = 0.005$, 0.01, and 0.03 respectively; (4) unplatinated control; (5, 7, and 8) AO–Pt, $(D/N)_0 = 0.005$, 0.01, and 0.03, respectively. Lane 6 is the same as lane 7, but the sample was not treated with exonuclease III. Lane 9 is untreated 335-bp restriction fragment. Letters A–H indicate stopping sites described in the text. The sequence is given to the left of the gel.

Figure 3 displays the results of exonuclease III digestion of a 335-bp DNA fragment at three levels of modification with $[Pt(en)Cl_2]$ and AO–Pt (compare lanes 1–3 to lanes 5, 7, 8). Both compounds bind at oligo(dG) sequences as can be seen from the stops in the figure. In the lower part of the gel, stops A and B at $(dG)_3$ and $(dG)_4$ sequences are evident, and a very strong stop (D) is seen at the d(GCGG) sequence near the middle of the gel. The different appearance of the pattern of this stop for DNA modified with AO–Pt vs. $[Pt(en)Cl_2]$ is due to the presence of the intercalator, as we shall see shortly. Other differences between AO–Pt and $[Pt(en)Cl_2]$ binding sites include a greater propensity for AO–Pt to produce stops near d(AG) (stops E–G, Figure 3) and d(GNG) sequences and at $(dC)_2$ sequences (stops C and H, Figure 3) corresponding to $(dG)_2$ on the unlabeled strand, as with the 165-bp fragment. None of the $(dC)_2$ and only some of the d(AG) binding sites observed for AO–Pt are enhanced for DNA modified by $[Pt(en)Cl_2]$ in the presence of free intercalator (see below), demonstrating that the linked intercalator produces some unique effects on platinum binding.

Intercalator Effects on Platinum Binding. Significant increases in the levels of exonuclease III detectable *cis*-DDP binding at a $d(G_6CG_2)$ sequence on the 165-bp fragment were previously observed when increasing amounts of EthBr were present during adduct formation (Tullius & Lippard, 1982).

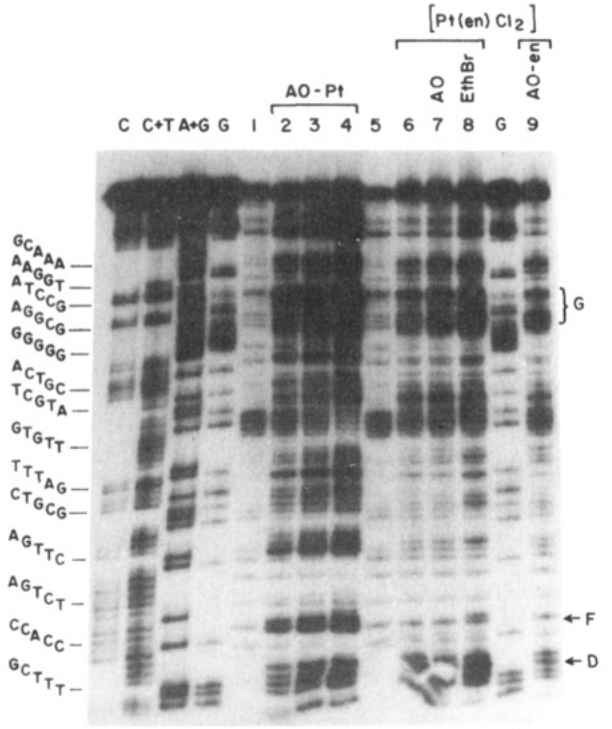

FIGURE 4: Autoradiogram of an 8% polyacrylamide/8.3 M urea electrophoresis gel showing the effects of the binding of AO–Pt, $[Pt(en)Cl_2]$, or $[Pt(en)Cl_2]$ in the presence of AO, AO-en, or EthBr on the exonuclease III digestion of the 165-bp fragment. Electrophoresis was carried out at 40-W constant power for 6 h. The lanes labeled C, C+T, A+G, and G contain Maxam–Gilbert base-specific reactions. Lanes 1–9 are the same as those described in Figure 2. The letter G indicates the $d(G_6CG_2)$ stopping site. Letters D and F are as in Figure 2. The sequence is given to the left of the gel.

These same effects occur for AO–Pt in which an intercalator is covalently linked to a platinum complex. By using the 335-bp fragment, we find many new sites that show enhancement of $[Pt(en)Cl_2]$ binding in the presence of EthBr, thus demonstrating that EthBr-promoted platinum binding to certain DNA sequences is a general phenomenon.

Figure 4 displays at high resolution the $d(G_6CG_2)$ binding region (G) near the 3′ end of the 165-bp fragment. $[Pt(en)Cl_2]$ binding is enhanced in the presence of EthBr at $(D/N)_0 = 0.15$ (compare lanes 6 and 8). Binding of the intercalator-linked compound AO–Pt is even more obviously enhanced relative to $[Pt(en)Cl_2]$ and at even lower $(D/N)_b$ ratios (compare lanes 2 and 6). Interestingly, for AO–Pt this enhancement is almost entirely localized in the $(dG)_2$ region of this sequence (G). Figure 4 also reveals that addition of AO or AO-en to DNA during platination with $[Pt(en)Cl_2]$ does not cause the same enhancement as seen upon addition of EthBr (compare lanes 7 and 9 to lane 8). The failure of AO to enhance the binding of *cis*-DDP to the $d(G_6CG_2)$ region of the 165-bp fragment was noted previously (Merkel, 1983). These results are significant since AO, when covalently linked to $[Pt(en)Cl_2]$ as in AO-Pt, does produce enhancement.

One other oligo(dG) sequence on the 165-bp fragment (D, in Figure 2; compare lane 6 vs. lane 8) shows enhanced exonuclease III stopping when $[Pt(en)Cl_2]$ is bound in the presence of EthBr.

On the 335-bp fragment, a large number of EthBr-enhanced stops occur near the 3′ end of the sequence. In Figure 5, the exonuclease III stops for DNA modified by AO–Pt are compared with results for $[Pt(en)Cl_2]$–DNA adducts formed in the presence of EthBr or AO. Table I summarizes the intercalator-enhanced binding sites revealed in this figure and

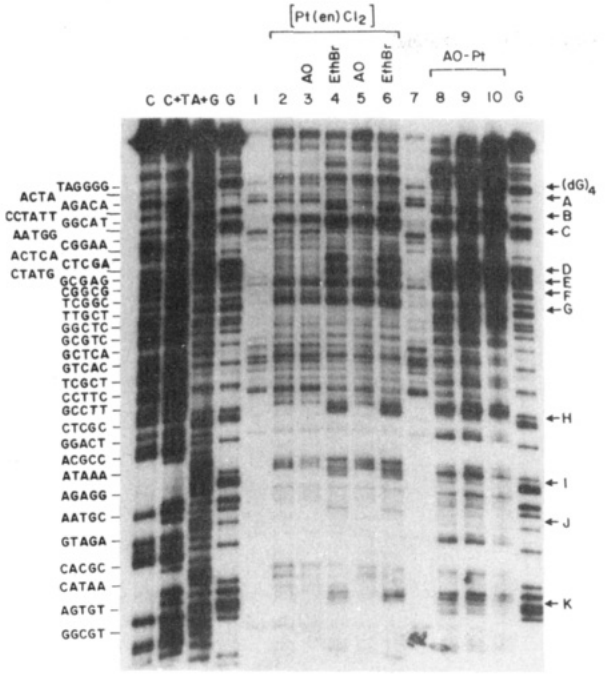

FIGURE 5: Autoradiogram of an 8% polyacrylamide/8.3 M urea electrophoresis gel showing results for the exonuclease III digestion of the 335-bp fragment bound with AO–Pt, [Pt(en)Cl_2], or [Pt(en)Cl_2] in the presence of AO or EthBr. Electrophoresis was carried out at 40-W constant power for 7 h. Lanes labeled C, C+T, A+G, or G contain Maxam–Gilbert base-specific reactions. The DNA in lanes 1–10 is modified as follows for exonuclease III digestion: (1 and 7) control, unmodified DNA; (2) [Pt(en)Cl_2], $(D/N)_b = 0.016$; (3 and 5) [Pt(en)Cl_2]/AO, $(D/N)_b = 0.010$ and 0.013, respectively; (4 and 6) [Pt(en)Cl_2]/EthBr, $(D/N)_b = 0.012$ and 0.018, respectively; (8–10) AO–Pt, $(D/N)_b = 0.004$, 0.011, and 0.023, respectively. The sequence is indicated on the left-hand side of the gel. Letters A–K on the right-hand side of the gel correspond to stopping sites detailed in Table I.

indicates the sequence and the relative intensities of the bands on the gel corresponding to each site. The intensities are uncorrected for statistical effects, which diminish the intensity of smaller DNA fragments on the gel since exonuclease III is a processive enzyme.

Two effects due to the presence of the intercalator are observed. One is a change in the pattern of bands on the gel at the platinum binding site. This effect is illustrated for the d(GCGG) sequence (site N, Table I) in Figure 6 (lanes 2 and 4). The other is a significant enhancement in the intensity of the band. Binding sites D and E are the most dramatic examples of increased binding intensity due to the presence of a linked (AO–Pt) or external ([Pt(en)Cl_2]/EthBr) intercalator (compare lane 2 to lanes 4, 6, and 8–10 in Figure 5).

Table I reveals that over half of the intercalator-modulated oligonucleotide binding sites minimally contain the sequence d(CGG) and many have d(GCGG). The d(CGG) sequence is also present in both the $d(G_6CG_2)$- and $d(CG_3)$-enhanced regions on the 165-bp fragment. Besides these oligo(dG)-containing sites, the data show other enhanced sequences corresponding to d(AG) binding sites (A, J, L, and M in Table I). This result is not surprising in view of the increased selectivity of AO–Pt for d(AG) sequences mentioned above. The intensity of bands corresponding to these d(AG) binding sites, however, is significantly lower than that of bands corresponding to oligo(dG) binding sites.

The data in Figure 5 (compare lane 2 to lanes 3 and 5) further corroborate the finding that AO does not enhance [Pt(en)Cl_2] binding to the sequences listed in Table I. Similarly, no effect on [Pt(en)Cl_2] binding is observed in the

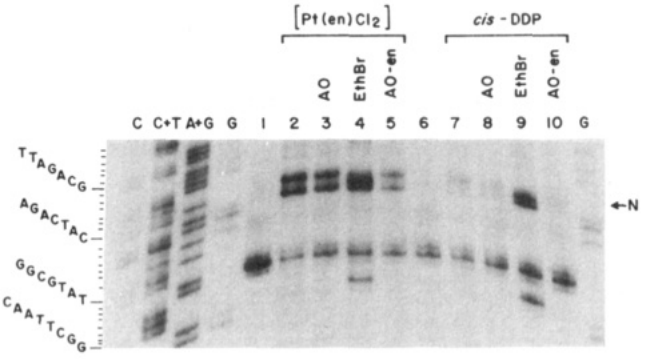

FIGURE 6: Autoradiogram of an 8% polyacrylamide/8.3 M urea electrophoresis gel comparing the exonuclease III digestion of a small region of the 335-bp fragment for DNA modified with *cis*-DDP or [Pt(en)Cl_2] in the presence or absence of AO, AO-en, or EthBr. Electrophoresis was carried out at 40-W constant power for 5 h. Lanes labeled C, C+T, A+G, and G contain Maxam–Gilbert base-specific reactions. Lanes 1–10 contain exonuclease III digestions of DNA modified as follows: (1 and 6) unplatinated, control DNA; (2) [Pt(en)Cl_2], $(D/N)_b = 0.015$; (3) [Pt(en)Cl_2]/AO, $(D/N)_b = 0.012$; (4) [Pt(en)Cl_2]/EthBr, $(D/N)_b = 0.016$; (5) [Pt(en)Cl_2]/AO-en, $(D/N)_b = 0.013$; (7) *cis*-DDP, $(D/N)_b = 0.012$; (8) *cis*-DDP/AO, $(D/N)_b = 0.008$; (9) *cis*-DDP/EthBr, $(D/N)_b = 0.010$; (10) *cis*-DDP/AO-en, $(D/N)_b = 0.005$. The sequence is given to the left of the gel. The letter N corresponds to stopping site N in Table I.

presence of AO-en nor does AO or AO-en enhance the binding of *cis*-DDP to the 335-bp DNA fragment (data not shown).

Relative Effects of EthBr on cis-DDP vs. [Pt(en)Cl_2] Binding to DNA. A comparative study of *cis*-DDP and [Pt(en)Cl_2] binding demonstrated that the latter binds more readily to certain sites on DNA in the absence of intercalators. Figure 6 presents results for exonuclease III digestion of the 335-bp fragment corresponding to binding site N in Table I where the difference between *cis*-DDP and [Pt(en)Cl_2] binding to DNA in the absence of EthBr is especially clear. Almost no binding occurs at site N for *cis*-DDP (lane 7) whereas [Pt(en)Cl_2] binding is very evident (lane 2). Platination in the presence of EthBr produces similar degrees of binding for both compounds (compare lanes 4 and 9). Binding site C and, to a lesser extent, sites F and G in Table I similarly exhibit greater exonuclease III detectable binding of [Pt(en)Cl_2] relative to *cis*-DDP in the absence of EthBr. These observations demonstrate that DNA binding site specificity differences occur between very similar platinum complexes. In the presence of ethidium bromide, however, the subtle structural effects that give rise to these differences are essentially neutralized.

Discussion

Sequence Preferences for Pt–DNA Binding. In previous exonuclease III mapping experiments using the 165-bp fragment (Tullius & Lippard, 1981, 1982), *cis*-DDP binding was detected only at oligo(dG) sequences. By using the 335-bp fragment, we have detected other, albeit less frequent, binding sites, to the extent that band intensity on the gel corresponds to binding site frequency. In particular, for AO–Pt, high levels of bound [Pt(en)Cl_2], or [Pt(en)Cl_2] bound in the presence of EthBr, binding occurs at d(AG) sites. This result is consistent with data from complete enzymatic digestion studies of DNA platinated with *cis*-DDP [Fichtinger-Schepman et al. (1985) and references sited therein]. We also observe some minor exonuclease III stopping sites that correspond neither to oligo(dG) nor to d(AG) sequences, two of which can be explained as a d(GNG) site (den Hartog et al., 1985), where N is an intervening nucleotide, while others are more difficult

Table I: Analysis of Intercalator-Enhanced Platinum Binding Sites on the 335-bp Fragment[a]

binding sequence[b]	gel label[a]	binding site[c]	intensity of exonuclease III stop[d]		
			[Pt(en)Cl_2] + EthBr	AO–Pt	[Pt(en)Cl_2]
5′-CAGAAT GTCTTA	A	d(AG)	w	w	
5′-ACGGTTA TGCCAAT	B	d(ACGG)	m	m	
5′-AGGCGGTA TCCGCCAT	C	d(AGGCGG)	s	s	m
5′-AGCGGTA TCGCCAT	D	d(GCGG)	s	s	
5′-GCGGCGA CGCCGCT	E	d(GCGG)	s	s	
5′-TTCGGCTG AAGCCGAC	F	d(TCGG)	s	s	m
5′-TCGGTC AGCCAG	G	d(TCGG)	m to s	m to s	w to m
5′-CAGGCGCTC GTCCGCGAG	H	d(AGGCG)	m	m	w
5′-AGGAGAA TCCTCTT	I	d(AGGAG)	m	m	w to m
5′-AGAT TCTA	J	d(AG)	w	w	
5′-ATGCGGTG TACGCCAC	K	d(GCGG)	w to m	w to m	
5′-GAGAGT CTCTCA	L	d(AG)	w	w	vw
5′-AGATT TCTAA	M	d(AG)	w	w	
5′-GCGGCA CGCCGT	N	d(GCGG)	s	s	s

[a] See Figure 5. [b] The upper sequence has the 5′-^{32}P label. [c] Proposed platinum binding site causing exonuclease III stop. [d] s = strong; m = medium; w = weak; vw = very weak.

to classify. These minor binding sites indicate the potential for platinum, especially the intercalator-linked AO–Pt molecule, to bind at regions other than those containing oligo(dG) sequences.

It is interesting that AO–Pt, but not *cis*-DDP (Tullius & Lippard 1981, 1982) or [Pt(en)Cl_2], binding to $(dG)_2$ sites on the unlabeled strand can be detected by exonuclease III digestion. Not only does this result give us information about platinum binding to the unlabeled strand, but it also suggests that there is something different about the adduct formed by AO–Pt, such that it can interfere with exonuclease III digestion of the labeled strand when platinum is bound to the unlabeled strand. We do not yet know whether this difference is due simply to steric bulk or to a specific structural change accompanying the intercalative binding of AO–Pt to DNA.

[Pt(en)Cl_2] vs. cis-DDP Binding. In the absence of EthBr, *cis*-DDP is more selective than [Pt(en)Cl_2] in binding to DNA. Although both compounds bind equally well to several oligo(dG) sequences as detected by exonuclease III digestion, there is significantly less *cis*-DDP than [Pt(en)Cl_2] bound, particularly to sequences that are enhanced by EthBr. Although we do not understand the molecular basis for this greater propensity of [Pt(en)Cl_2] vs. *cis*-DDP to bind DNA, the fact that it does so at some but not all EthBr-enhanced sites suggests that considerable structural variation occurs among these sites taken as a class.

Effects of Linked and Free Intercalators on Platinum Binding Site Selectivity. For both the 165- and the 335-bp restriction fragments, an AO–Pt (D/N) ratio of only 0.005 produces intense exonuclease III stops corresponding to sequences unfavorable for binding [Pt(en)Cl_2] or *cis*-DDP. The intensities of the corresponding bands are equivalent to or greater than those produced at the same sites when DNA is modified by [Pt(en)Cl_2] or *cis*-DDP in the presence of an EthBr $(D/N)_0$ of 0.15. The AO–Pt results prove that intercalator-mediated platinum binding derives from local DNA structure modulations and is not due to global structural effects caused by high levels of intercalators.

We now consider the effect of intercalators on platinum binding to the $d(G_6CG_2)$ sequence on the 165-bp fragment. For [Pt(en)Cl_2], the enhancement produced by EthBr at a $(D/N)_0$ ratio of 0.15 is fairly uniform throughout the $d(G_6CG_2)$ sequence. For AO–Pt, however, the enhancement is localized at the $(dG)_2$ region. Earlier exonuclease III mapping experiments with *cis*-DDP showed that, as the level of EthBr was increased, first the $(dG)_2$ region was enhanced and then the enhancement spread to the $(dG)_6$ region (Tullius & Lippard, 1982). Evidently, at low levels of intercalator, whether linked or unlinked, binding occurs preferentially at the $(dG)_2$ region. This result can be explained in terms of the known sequence preference, pyr(3′–5′)pur > pur(3′–5′)pur ≫ pur(3′–5′)pyr, for intercalation to dinucleoside phosphates (Krugh et al., 1975; Krugh & Reinhardt, 1975; Reinhardt & Krugh, 1978; Kastrup et al., 1978). NMR studies suggested that this pyr(3′–5′)pur sequence would also be favored by the acridines, proflavin (Patel, 1977; Patel & Canuel, 1977), and 9-aminoacridine (Young & Kallenbach, 1981). Several theoretical analyses have indicated such a sequence preference to be an intrinsic property of the lower energy required to open up a pyr(3′–5′)pur base pair to form an intercalator binding site, and that it is not significantly dependent on a particular intercalator (Ornstein & Rein, 1979; Miller et al., 1980; Nuss et al., 1979). It is therefore reasonable to expect a similar preference for AO binding to DNA. Thus, EthBr and the intercalating portion of AO–Pt binding preferentially at the 3′ side of the cytidine nucleoside in $d(AG_6CG_2)$, altering the DNA structure near the adjacent $(dG)_2$ sequence. At low (D/N) ratios of free or linked intercalator, the $(dG)_2$ sequence becomes exposed as a preferential Pt binding site. The $(dG)_6$ region, being immediately preceded by adenosine, is not enhanced for Pt binding at low intercalator levels. It is interesting that, in accord with this analysis, the majority of enhanced

oligo(dG) binding sites in Table I contain the d(CGG) sequence.

Accepting the idea that the structure of the d(CGG) sequence inhibits platinum binding until exposed by selective intercalation at that site, we have attempted to understand why some d(CGG) sequences bind [Pt(en)Cl_2] in the absence of EthBr while others do not. Sequences B, D, E, and K (Table I), which exhibit no binding in the absence of EthBr, all share the common sequence d(purCGG). For all three examples of a d(pyrCGG) sequence, F, G, and the d(TCGGG) sequence on the 165-bp fragment, [Pt(en)Cl_2] binding occurs in the absence of EthBr. This correlation is not absolute, however, since sequences such as C and N show significant evidence for [Pt(en)Cl_2] binding in the absence of EthBr, despite the fact that they have d(purCGG) sequences. We postulate, therefore, that d(purCGG) sequences will have a greater tendency than d(pyrCGG) to exclude platinum binding, owing to their local duplex structure. Evidently, even for d(purCGG) sequences, longer range sequence effects can dictate whether and to what degree the local structure excludes platinum binding. X-ray structural studies reveal that synthetic deoxyoligonucleotide duplexes having runs of oligo(dG)·oligo(dC) sequences adopt the A-DNA structure type (Wang et al., 1982). The narrow major groove of this structure might inhibit access of platinum to its preferred binding site on DNA, namely, N(7) of the guanine base. Modulation of A-DNA-type structures by intercalators could permit platinum to bind to N(7) of dG.

Basis for Intercalator Modulation of Platinum Binding. The present results and those of a previous investigation using *cis*-DDP (Merkel, 1983) indicate that EthBr, but not AO, enhances platinum binding. This difference was originally believed to reflect the different stereochemistry of intercalator–DNA adducts as revealed by X-ray crystallographic analyses of intercalator dinucleoside complexes [Berman & Young (1981) and references cited therein]. In particular, the phenyl and ethyl groups of EthBr project toward the minor groove of the double helix, whereas the dimethylamino substituents of acridine orange lie in the major groove. The latter orientation might sterically block access to the N(7) positions of guanosine nucleosides, the favored platinum binding site. Our experiments with AO–Pt, however, reveal that AO, when linked to a platinum complex, produces the same effect as free EthBr on the binding of platinum. This observation appears to be inconsistent with a steric explanation for the inability of unlinked AO to enhance platinum binding. It is possible, however, that the proximity of Pt to AO in the intercalator-linked molecule allows it to bind to N(7) of guanosine despite the presence of the sterically bulky dimethylamino substituents of AO.

There is another explanation for the effects of free and linked intercalators on the sequence-specific binding of platinum complexes to DNA, however. An investigation of actinomycin–DNA binding kinetics (Müller & Crothers, 1968) revealed a strong correlation between the kinetic off-rate of the actinomycin derivative and its biological activity. Slow off-rates correlated with high biological activity and fast-off rates with lack of biological activity. NMR studies (Feigon et al., 1982, 1984; Assa-Munt et al., 1985) indicate that dissociation rates for intercalated acridine derivatives are faster than those for ethidium derivatives, possibly by as much as a factor of 10. This value is in reasonable accord with intercalation dissociation rates measured by temperature jump and stopped-flow kinetic studies [Ryan & Crothers (1984) and references cited therein; Li & Crothers, 1969]. The shorter residence time of AO at an intercalation site may explain the inability of free AO to modulate platinum binding. The lifetime of the AO-modified duplex might simply be too short for an untethered platinum molecule to encounter and react with the altered DNA structure. This effect might be less important for AO–Pt, owing to the high local concentration of the [Pt(en)Cl_2] moiety near the intercalation site in the linked molecule.

Kinetic off-rates alone are insufficient to account for modulation of platinum binding sites on DNA by intercalators. An investigation of the effect of adriamycin on *cis*-DDP binding (Merkel, 1983) showed that it did not enhance *cis*-DDP binding to the d(G_6CG_2) sequence on the 165-bp fragment. The closely related anthracycline antibiotic daunomycin exhibits an intercalative dissociation rate even slower than that of EthBr (Feigon et al., 1984). It was noted previously (Merkel, 1983) that, as indicated by crystallographic studies [Berman & Young (1981) and references cited therein], daunomycin produces a significantly different structural change when it binds to DNA than does AO or EthBr. This structural modulation may not enhance exonuclease III detectable platinum binding.

We propose the following hypothesis to explain the sequence dependence of platinum binding to DNA and its modulation by intercalators. The dependence of DNA tertiary structure upon local sequence (Dickerson & Drew, 1981) causes certain oligo(dG) regions to be less accessible to platinum binding. Addition of an intercalator may cause local changes in DNA structure so as to allow platinum access to a previously restricted site. The ability of an intercalator to accomplish this structure modulation will depend on its own sequence preferences and on the specific DNA structural change it induces. For a series of intercalators that induce similar structural changes, the residence time of the intercalator will determine whether platinum binding will occur. This rationale is experimentally testable, by measuring the off-rates of intercalators used in this study in our buffer system and by studying the modulation of platinum binding to DNA by a broader range of intercalators.

Summary. The present study of exonuclease III detected binding of AO–Pt has provided significant new insight into the problem of how intercalators alter the covalent binding of platinum to DNA. The results for AO–Pt demonstrate that local DNA structural changes due to intercalation are responsible for alterations in platinum–DNA binding and that certain sequences, particularly d(CGG), are hot spots for intercalator-enhanced platinum binding. The importance of sequence-specific intercalator binding is most clearly delineated in the case of the selective enhancement of the d$(G)_2$ region of d(G_6CG_2) by AO–Pt. Finally, the observation that tethered, but not free, acridine orange enhances platinum binding to sequences also enhanced by EthBr suggests a new interpretation of the mechanism of intercalator effects on platinum binding, one in which the kinetic off-rate of the intercalator is an important parameter. We are presently involved in NMR and exonuclease III mapping studies that will further clarify our postulated intercalator-modulated platinum binding mechanism and provide a more detailed understanding of the importance of DNA structure to the interaction of drug molecules with DNA.

Registry No. *cis*-DDP, 15663-27-1; EthBr, 1239-45-8; AO, 65-61-2; AO-en, 92220-84-3; AO–Pt, 92241-08-2; [Pt(en)Cl_2], 14096-51-6.

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