# Photoreactivity of Platinum(II) in Cisplatin-Modified DNA Affords Specific Cross-Links to HMG Domain Proteins†

Stefanie A. Kane and Stephen J. Lippard*

*Department of Chemistry, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139*

*Received September 18, 1995; Revised Manuscript Received December 12, 1995*⊗

ABSTRACT: Cisplatin-modified DNA forms specific complexes with proteins that contain the DNA binding motif known as the high-mobility group (HMG) domain. As a tool for investigating the role of these proteins in mediating the cytotoxic effects of cisplatin, a set of cisplatin analogs was prepared in which one of the ammine ligands was replaced with a photoreactive tethered aryl azide ligand. The ability of DNA modified by these platinum complexes to photo-cross-link to HMG1 was investigated. During this study, it was discovered that DNA modified with cisplatin itself can undergo photoinduced cross-linking to HMG1 when irradiated with 300 nm light. The covalent complexes resulting from this latter cross-linking reaction are completely reversed by the addition of sodium cyanide and can be degraded by proteinase K. These results confirm the presence of a protein−DNA cross-link and demonstrate that the platinum atom itself forms the point of attachment. By contrast, DNA modified with *trans*-diamminedichloroplatinum(II), [Pt(dien)Cl]Cl, or $[Pt(NH_3)_3Cl]Cl$ does not cross-link to HMG1 upon irradiation. The photochemistry was exploited to cross-link a 15-base pair oligonucleotide containing a single, site-specific *cis*-$[Pt(NH_3)_2\{d(GpG)$-N7(1),-N7(2)$\}]$ intrastrand adduct to domain B of HMG1. Following proteolytic digestion of the resulting covalent complex, the site of attachment to the protein was determined by Edman degradation of the resulting peptide−DNA complex to be a single residue on HMG domain B, Lys-6. The data further suggest that this amino acid binds to platinum at a site made available by photolabilization of a purine ligand. These results afford the first structural information about the interaction of HMG domain proteins with cisplatin-modified DNA.

*cis*-Diamminedichloroplatinum(II) (cisplatin or *cis*-DDP)[1] is a widely used chemotherapeutic agent employed in the treatment of several types of human cancers (Loehrer & Einhorn, 1984). Although the mechanism by which cisplatin selectively kills cells is not entirely understood, it is generally believed that the therapeutic effects arise from covalent binding of the drug to DNA (Brown et al., 1994). The primary adducts formed by *cis*-DDP, d(GpG) and d(ApG) 1,2-intrastrand cross-links (Fichtinger-Shepman et al., 1985), dramatically alter the DNA structure (Sherman et al., 1988; Takahara et al., 1995) and block replication and transcription (Comess & Lippard, 1993). *trans*-DDP is ineffective as an antitumor agent and, due to geometric constraints, is incapable of forming 1,2-intrastrand cross-links, although its DNA adducts also block replication (Bruhn et al., 1990) and are thus likely to be cytotoxic. This behavior suggested that differential cellular processing of platinum−DNA adducts is likely to contribute to the selective antitumor activity of cisplatin.

Accordingly, significant effort has focused on the isolation and characterization of proteins that interact with cisplatin-modified DNA. Screening of a human cDNA expression library with cisplatin-damaged DNA probes afforded a gene that encodes structure-specific recognition protein 1 (SSRP1) (Toney et al., 1989; Bruhn et al., 1992). Analysis of its predicted amino acid sequence revealed close homology to the high-mobility group 1 protein (HMG1). Both SSRP1 and HMG1 contain a recently identified DNA binding motif, the HMG domain. HMG domain proteins display unusual DNA binding properties, such as the ability to recognize irregular or bent structures and to induce dramatic bends in the helix (Grosschedl et al., 1994). HMG1 and HMG2 bind to DNA containing *cis*-DDP intrastrand d(GpG) and d(ApG) cross-links but not to DNA modified by clinically ineffective platinum compounds (Pil & Lippard, 1992; Hughes et al., 1992). More recently, a yeast SSRP containing two HMG domains, designated Ixr1 for intrastrand cross-link recognition protein, was identified on the basis of its ability to bind to cisplatin damaged DNA (Brown et al., 1993). Deletion of the gene encoding this protein in yeast desensitized the cells to cisplatin because the protein served to shield the adducts from excision repair (Brown et al., 1993; McA'Nulty & Lippard, in press). DNA containing the intrastrand d(GpG) cross-link titrates the ribosomal RNA transcription factor, hUBF, away from its natural DNA binding site (Treiber et al., 1994). Six different HMG domain proteins−HMG1, the mitochondrial transcription-activating factor mtTFA, Ixr1, and the HMG domains from HMG1 (domain B), Lef-1 and mSRY−specifically bind to DNA containing a *cis*-$[Pt(NH_3)_2\{d(GpG)$-N7(1),-N7(2)$\}]$ intrastrand cross-link, inducing bends of 50−90° (Chow et al., 1994). These studies demonstrate that functionally unrelated HMG domain proteins share a common ability to recognize and bend platinated DNA and can mediate the cytotoxicity

† This work was supported by Grant CA34992 from the National Cancer Institute. S.A.K. is the recipient of a National Institutes of Health postdoctoral fellowship.

⊗ Abstract published in *Advance ACS Abstracts,* February 1, 1996.

[1] Abbreviations: bp, base pair; *cis*-DDP or cisplatin, *cis*-diamminedichloroplatinum(II); HMG, high-mobility group; PAGE, polyacrylamide gel electrophoresis; *trans*-DDP, *trans*-diamminedichloroplatinum(II).

0006-2960/96/0435-2180$12.00/0 

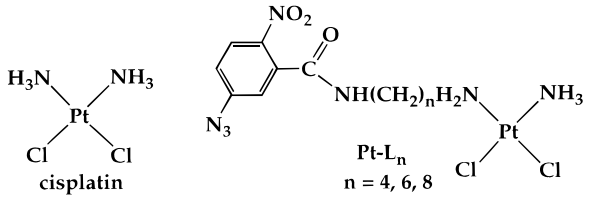


FIGURE 1: Photoreactive platinum complexes employed in the present work as protein cross-linking agents.

of cisplatin by one or more of several mechanisms (Brown et al., 1993; Huang et al., 1994; Treiber et al, 1994; McA'Nulty & Lippard, in press).

To investigate the nature of the interaction of proteins, in particular those containing an HMG domain, with cisplatin-modified DNA we designed the following strategy. A series of cisplatin analogs containing ligands with photoreactive aryl azide moieties was prepared (Figure 1). It was envisioned that the resulting photogenerated nitrene (Bayley, 1983) appended to platinum-bound DNA would form a covalent adduct with a noncovalently bound protein residue. Such a covalent complex would provide a means both for isolating proteins that bind to cisplatin−DNA adducts in vivo and for studying the molecular basis of the interaction. In the present article we describe the results of these investigations, which reveal that DNA modified with *cis*-DDP itself exhibits the highest cross-linking efficiency and provide the first structural information regarding the interaction of HMG domain proteins with cisplatin-modified DNA.

## MATERIALS AND METHODS

*Materials.* Cisplatin was obtained as a gift from Johnson-Matthey. Recombinant rat HMG1 was expressed and purified in our laboratory as described (Pil & Lippard, 1992). Oligonucleotides were synthesized on a 15 $\mu$mol scale by the phosphoramidite method on a Cruachem PS250 DNA synthesizer. All manipulations involving the platinum compounds with tethered aryl azides were carried out under dim room light or in the dark, when possible. The synthesis and characterization of the Pt−aryl azide complexes and characterization of their DNA-binding properties are described in the supporting information.

*Expression and Purification of HMG Domain B.* Domain B of HMG1 was expressed and purified from pHB1/*Escherichia coli* BL21(DE3) (Chow et al., 1995). Cells were grown in M9 medium (12.8 g of $Na_2HPO_4$, 5 g of $KH_2PO_4$, 1 g of $NH_4Cl$, 0.5 g of NaCl per liter) with 1 mM $MgSO_4$, 0.2% (w/v) glucose and 75 $\mu$g of ampicillin/mL at 37 °C for 5 h ($OD_{600} = 1$). A mixture of the twenty L-amino acids was added to a final concentration of 0.005% (w/v), the cells were incubated for 30 min, and isopropyl $\beta$-D-thiogalactoside (Boehringer Mannheim) was added to a final concentration of 1.5 mM to induce protein synthesis. After 30 min at 37 °C, rifampicin (Sigma) was added to a final concentration of 0.1 mg/mL and the cells were incubated for 1.5 h at 37 °C. The domain was then isolated and purified essentially as described (Chow et al., 1995). The yield was 8−9 mg of protein per liter of culture. The protein concentration was determined by using the extinction coefficient 12 100 $M^{-1}$ $cm^{-1}$ at 280 nm (Chow et al., 1995). The N-terminal sequence, MKKKFKDPN, was confirmed by automated Edman sequencing at the MIT Biopolymers Laboratory; the molecular mass of the protein was confirmed by MALDI mass spectral analysis, 9290 Da $[M + H]^+$.

*Platinated DNA Probes.* A 123-bp DNA fragment was obtained by digestion of a commercially available 123-bp DNA ladder (Gibco BRL) with restriction enzyme *Avi*I and purification of the resulting DNA fragment by native polyacrylamide gel electrophoresis (PAGE). The resulting DNA fragment was 3′-end labeled by a fill-in reaction with the Klenow fragment of DNA polymerase I, [$\alpha$-$^{32}$P]dCTP (Dupont/NEN), and the four deoxynucleotide triphosphates and was purified on a Sephadex G-50 Quick Spin column (Boehringer Mannheim). The diaqua derivatives of the platinum compounds were employed for platination of DNA owing to the low solubility of the compounds in aqueous media (Hartwig & Lippard, 1992). Platination of DNA was carried out at a formal drug-to-nucleotide ratio ($r_f$) of 0.025 by treating a 150 $\mu$M (nucleotide concentration) solution of the 123-bp DNA fragment in 10 mM sodium phosphate, pH 6.8, containing $1 \times 10^6$ cpm of the labeled DNA with the diaqua derivatives of the platinum compounds at 37 °C for 18 h. Unbound platinum was removed by ethanol precipitation. To determine the amount of metal incorporated, calf thymus DNA was modified under similar conditions and the amount of bound platinum was quantitated by flameless atomic absorption spectroscopy (supporting information). An 82-bp DNA fragment was prepared by 5′-end labeling the unplatinated 123-bp probe with T4 polynucleotide kinase and [$\gamma$-$^{32}$P]ATP (Dupont/NEN) followed by digestion with restriction enzyme *Hae*III and purification of the resulting singly end-labeled fragment by native PAGE. The product was platinated at an $r_f$ of 0.036 as described above. A 15-mer, d(CCTCTCTGGTTCTTC), was platinated as described above to afford an oligonucleotide containing a single, site-specific *cis*-[$Pt(NH_3)_2${d(GpG)-N7(1),-N7(2)}] intrastrand cross-link. The product, hereafter referred to as 15Pt, was purified by anion-exchange HPLC on a Dionex Nucleopac PA-100 column (9 × 250 mm) with a solvent system of 10 mM ammonium acetate, pH 6.0, in 10% aqueous acetonitrile and a gradient of 0.2−0.35 M NaCl. The HPLC fractions were desalted by extensive dialysis against deionized water (Spectra/Por 7 tubing, 1000 MW cutoff, from Spectrum). The sites of platination were confirmed by Maxam−Gilbert sequencing analysis (Comess et al., 1990; Brabec & Leng, 1993). The bottom strand 15-mer (15B) was purified by denaturing PAGE. Oligonucleotides were 5′-end labeled with T4 polynucleotide kinase and [$\gamma$-$^{32}$P]ATP and purified on Sephadex G-25 Quick Spin columns.

*Gel Mobility Shift Assays.* To prepare the 15-bp probe, the individual strands were annealed by heating a solution containing 0.2 $\mu$M 15B and 0.18 $\mu$M 15Pt ($1 \times 10^5$ cpm) in buffer containing 10 mM Tris-HCl, pH 7.5, 50 mM NaCl, and 10 mM $MgCl_2$ at 80 °C and cooling to 4 °C over 3 h. The solution was then diluted 10-fold into each of the binding reaction mixtures (10 $\mu$L total volume) containing 10 mM Tris-HCl, pH 7.5, 4% (v/v) glycerol, 10 mM $MgCl_2$, 50 mM KCl, 1 mM EDTA, and 0.05% (v/v) Nonidet P-40. HMG domain B was added, as indicated, and the solution was incubated on ice for 30 min. The mixtures were combined with 0.5 $\mu$L of gel-loading solution [30% (v/v) glycerol, 0.25% (w/v) bromophenol blue and 0.25% (w/v) xylene cyanol] and applied to a pre-run, pre-equilibrated (4 °C) 10% native polyacrylamide gel (29:1 acrylamide:bis). The gel was run in 0.5X TBE (45 mM Tris, 45 mM boric acid,

1.25 mM EDTA, pH 8.3) at 300 V for 3 h and visualized by autoradiography with Kodak X-Omat film at room temperature.

*Photo-Cross-Linking of Platinated DNA Probes to HMG1 and HMG Domain B.* Reaction mixtures (10 $\mu$L total volume) were prepared as described for the gel mobility shift assays and incubated for 30 min on ice to allow protein−DNA binding. For binding of the 123- and 82-bp probes to HMG1, 0.2 $\mu$g of BSA/mL, 2 $\mu$g of competitor chicken erythrocyte DNA, and 300 ng of HMG1 were included in the reaction mixtures. To induce photo-cross-linking of the protein−DNA complexes, the reaction mixtures contained in open microcentrifuge tubes were placed on ice and irradiated from above for 1−2 h at a distance of 4 cm from a transilluminator (Ultra-Violet Products, TM-15) equipped with one of several different lamps, with primary spectral outputs of 254, 302, or 365 nm. The light intensity, 2.6−2.9 J $m^{-2}$ $s^{-1}$, was measured with a UVX radiometer (Ultra-Violet Products). Following irradiation, the reaction mixtures were combined with 5 $\mu$L of gel-loading solution [10 M urea, 1.5 mM EDTA, 0.05% (w/v) bromophenol blue and xylene cyanol]. The DNA was denatured by heating at 90 °C for 4 min and then analyzed by denaturing PAGE. The gels were visualized by autoradiography at −80 °C.

*Large-Scale Preparation and Purification of Photo-Cross-linked 15Pt−HMG Domain B Complex.* Reaction mixtures like those described above were scaled up to 100 mL volumes by using 100 nmol of 15-bp duplex and 150 nmol of HMG domain B. Gel mobility shift assays at this DNA concentration (1 $\mu$M) demonstrated that the addition of 1.5 equiv of HMG domain resulted in platinum-specific DNA binding, with ∼50% of the DNA shifting to the bound fraction. Addition of more protein or the use of higher DNA concentrations resulted in mixtures of specific and nonspecific complexes, readily distinguishable by gel electrophoresis, and was therefore avoided. The protein−DNA binding mixture was incubated on ice for 30 min prior to irradiation for 2 h as described above. The solution volume was subsequently reduced to 2 mL by using Centriplus-10 concentrators (Amicon). The complexes were precipitated by addition of 4 vol of ice-cold acetone and collected by centrifugation. The precipitate was redissolved in 10 mM Tris-HCl, pH 8.0, containing 50 mM NaCl and 10 mM $MgCl_2$ and was used in the protease digestions described below.

*Protease Digestions of Photo-Cross-Linked 15Pt−HMG Domain B Complex: (A) Endoproteinase Asp-N Digest.* The mixture of cross-linked and free HMG domain B containing 30 nmol of duplex DNA and 45 nmol (420 $\mu$g) of protein was digested with 4 $\mu$g of endoproteinase Asp-N (Sigma, sequencing grade) in 420 $\mu$L total volume of 50 mM Tris-HCl buffer, pH 8.5. The reaction mixture was incubated at 37 °C for 4 h, and then concentrated to 100 $\mu$L in a Microcon-3 concentrator (Amicon). The mixture was combined with 50 $\mu$L of gel-loading solution [10 M urea, 1.5 mM EDTA, 0.05% (w/v) bromophenol blue and xylene cyanole], heat-denatured, and then purified on a 12% polyacrylamide/7 M urea gel (1.5 mm thick). The gel was visualized by UV shadowing and autoradiography; two closely spaced bands corresponding to protease-digested material were observed. These bands were excised from the gel, and the DNA−peptide complexes were recovered by passive elution into 10 mM Tris-HCl, pH 7.5, containing 50 mM NaCl and 1 mM EDTA. The eluates were filtered (0.2 $\mu$m microfilterfuge tubes, Rainin) to remove gel pieces and then desalted on gel filtration columns (PD-10, Pharmacia) pre-equilibrated with water and then lyophilized to dryness. Approximately 1.5 nmol of each product (corresponding to the "upper" and "lower" migrating bands) were isolated, as determined by the $A_{260}$ for the oligonucleotide.

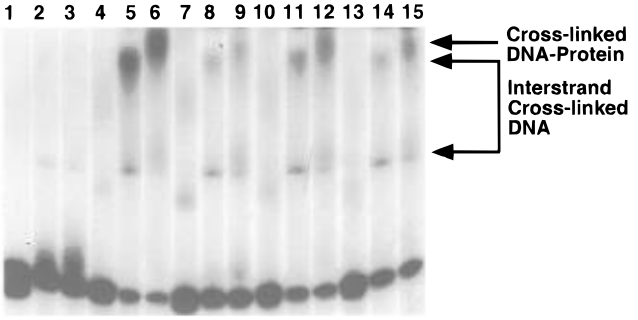


FIGURE 2: Denaturing 8% polyacrylamide gel demonstrating photo-cross-linking of HMG1 to globally platinated 123-bp probes. Lanes 1−3 contain unplatinated probe; lanes 4−6 contain cisplatin-modified probe; lanes 7−9 contain Pt−$L_4$-modified probe; lanes 10−12 contain Pt−$L_6$-modified probe; lanes 13−15 contain Pt−$L_8$-modified probe. Lanes 1, 4, 7, 10, and 13: DNA alone. Lanes 2, 5, 8, 11, and 14: irradiated in the absence of protein. Lanes 3, 6, 9, 12 and 15: incubated with 300 ng of HMG1 and then irradiated.

*(B) Endoproteinase Arg-C Digest.* The mixture of cross-linked and free HMG domain B containing 20 nmol of duplex DNA and 30 nmol (280 $\mu$g) of protein was digested with 2 $\mu$g of endoproteinase Arg-C (Boehringer Mannheim, sequencing grade) in a solution (300 $\mu$L total volume) containing 50 mM Tris-HCl, pH 7.5, 5 mM dithiothreitol, and 5 mM $CaCl_2$. The mixture was incubated at 37 °C for 8 h, after which time an additional 2 $\mu$g aliquot of protease was added, and the solution was incubated overnight at 37 °C. The mixture was concentrated, and the peptide−DNA complex was purified as described above. One band corresponding to protease-digested material was observed. The band was excised from the gel, and the DNA−peptide complex was recovered and desalted as described above to afford approximately 3 nmol of product. N-terminal sequencing analysis was performed by the MIT Biopolymers Laboratory.

## RESULTS

*Photo-Cross-Linking of Platinum-Modified DNAs to HMG1.* Radiolabeled DNA probes globally platinated with Pt−aryl azide complexes were preincubated with HMG1 to promote noncovalent protein−DNA binding. The resulting complex was irradiated with 302 nm light, a wavelength that minimizes DNA damage and corresponds to an absorption maximum of the aryl azide ligands. Following irradiation, the reaction mixtures were analyzed on denaturing polyacrylamide gels (Figure 2). The presence of a slowly migrating species (lanes 9, 12, and 15) signaled the formation of covalently cross-linked protein−DNA complexes. Use of a cisplatin-modified probe (lane 6), initially intended as a control, revealed significantly more efficient photo-cross-linking to HMG1 than any of the DNA probes platinated with the aryl azide complexes. In the absence of protein, other bands appeared having mobilities slower than that of single-stranded DNA (lanes 5, 8, 11, and 14); these bands

are tentatively assigned to DNA containing interstrand cross-links. The presence of more than one such band may represent DNA duplexes containing different numbers and kinds of cross-links, resulting in mixtures of partially and fully denatured species with differing gel mobilities. The use of a medium-pressure mercury lamp emitting over a broad spectral range (300−1000 nm) also promoted protein−DNA cross-linking but less efficiently than light at 302 nm from the transilluminator (data not shown).

Experiments were carried out to demonstrate that the most slowly migrating species corresponds to material containing platinum-induced DNA−protein cross-links. Reaction mixtures containing HMG1 and DNA probe modified either by *cis*-DDP or Pt−$L_n$ complexes were irradiated. Subsequent incubation with NaCN under conditions which reverse platinum−DNA adducts (Comess & Lippard, 1993), or with proteinase K, eliminated bands containing the presumed protein−DNA cross-linked species, converting them to that of the unmodified probe (data not shown). The protein-platinated DNA cross-links could also be reversed with sulfur-containing nucleophiles such as thiourea and $\beta$-mercaptoethanol. These results demonstrate that the protein−DNA cross-links form in a novel reaction of the platinum complex, not from the more commonly encountered direct photochemical linking of a nucleobase to an amino acid (Shetlar, 1980).

Bands assigned to interstrand cross-linked DNA were also absent following NaCN, but not proteinase K, treatment. This result provides good evidence that these species also resulted from platinum-mediated reactions and not from nonspecific photochemical cross-linking, for example, to the carrier protein bovine serum albumin (BSA) in the reaction medium. Moreover, the fact that carrier BSA did not cross-link to the platinated DNA under any conditions suggests that the cross-link to HMG1 occurred only after formation of a specific noncovalent complex with this protein. Since the DNA probe modified with cisplatin underwent the most efficient photo-cross-linking, further work with the Pt−aryl azide complexes was not undertaken.

*Wavelength Dependence of the Photo-Cross-linking Reaction.* To gain insight into the mechanism of the photo-cross-linking reaction, the dependence of the reaction on the wavelength of irradiation was examined (Figure 3). As seen in lanes 1−6, 254 nm light promotes the formation of DNA interstrand cross-links and protein−DNA cross-links with the cisplatin-modified probe, but unplatinated DNA was also reactive under these conditions. Furthermore, 254 nm light caused significant photodegradation of both unplatinated and platinated probes, as evidenced by the appearance of DNA fragments at the bottom of the gel. Irradiation of the cisplatin-modified probe with 302 nm light (lanes 7−12) caused both DNA interstrand cross-linking and protein−DNA cross-linking in the presence of HMG1. Unplatinated probe did not react under these conditions. Both probes were unreactive when irradiated with 365 nm light. The 302 nm wavelength light was therefore used in all subsequent experiments.

*Photoreactivity of DNA Modified with cis-DDP, trans-DDP, [Pt(dien)Cl]Cl, and [Pt(NH$_3$)$_3$Cl]Cl.* The specificity of the photo-cross-linking reaction was assessed by comparing the photoreactivity of DNA modified with different platinum compounds (Figure 4). DNA containing *cis*-DDP adducts was highly reactive when irradiated with 302 nm

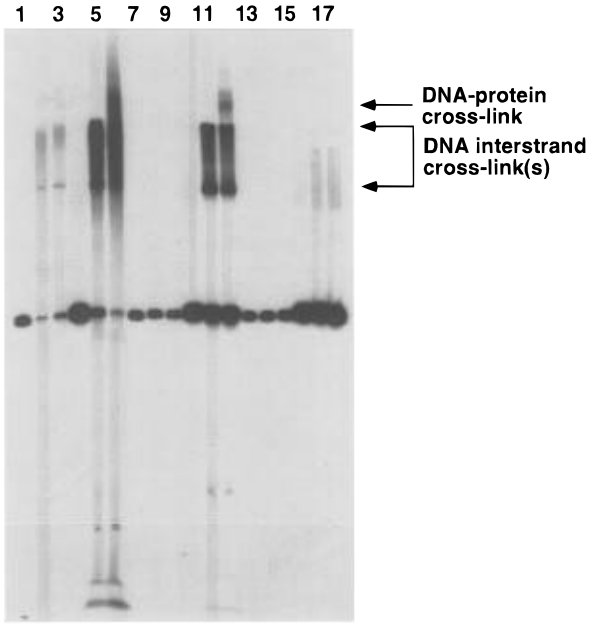


FIGURE 3: Denaturing 6% polyacrylamide gel demonstrating the wavelength dependence of the photoreactivity of cisplatin-modified 82-bp DNA. Lanes 1−3, 7−9, and 13−15 contain unplatinated probe. Lanes 4−6, 10−12, and 16−18 contain cisplatin-modified probe. Lanes 1, 4, 7, 10, 13, and 16: DNA alone. Lanes 2, 3, 5, and 6: irradiated with 254 nm light. Lanes 8, 9, 11, and 12: irradiated with 302 nm light. Lanes 14, 15, 17, and 18: irradiated with 365 nm light. HMG1 (300 ng) was present in lanes 3, 6, 9, 12, 15, and 18.

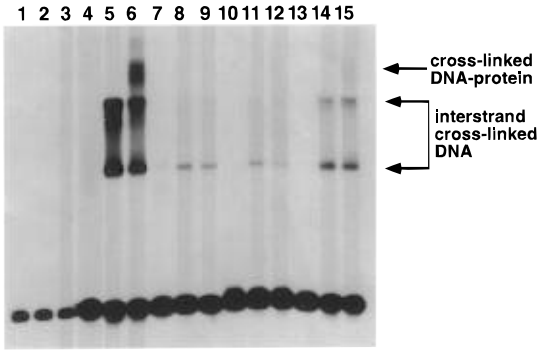


FIGURE 4: Denaturing 6% polyacylamide gel demonstrating the photoreactivity of various 82-bp platinated probes. Lanes 1−3 contain unplatinated DNA. Lanes 4−6 contain cisplatin-modified DNA. Lanes 7−9 contain DNA modified with *trans*-DDP. Lanes 10−12 contain DNA modified with [Pt(dien)Cl]Cl. Lanes 13−15 contain DNA modified with [Pt(NH$_3$)$_3$Cl]Cl. Lanes 1, 4, 7, 10, and 13: DNA alone. Lanes 2, 5, 8, 11, and 14: irradiated in the absence of protein. Lanes 3, 6, 9, 12, and 15: incubated with 300 ng of HMG1 and then irradiated.

light, producing both DNA interstrand cross-links and protein−DNA cross-links in the presence of HMG1 (lanes 5 and 6). DNA containing *trans*-DDP adducts (lanes 7−9) or platinum monoadducts (lanes 10−15) did not photo-cross-link to HMG1 upon irradiation and produced only small amounts of the DNA interstrand cross-links. In a separate experiment, DNA modified with [Pt(en)Cl$_2$] underwent photoinduced DNA interstrand cross-linking and cross-linking to HMG1, although with about half the efficiency as the *cis*-DDP modified probe (data not shown). These results and the fact that DNA modified with the Pt−aryl azide complexes also undergoes photo-cross-linking demonstrate that the light-driven reactions are unique to DNA modified

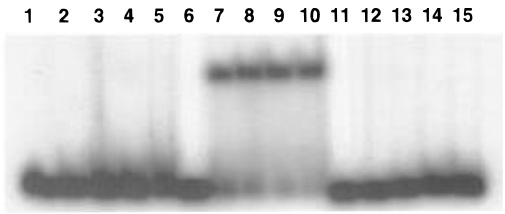


FIGURE 5: Native 10% polyacrylamide gel demonstrating binding of HMG domain B to site-specifically platinated 15-bp DNA. Lanes 1−5 contain unplatinated DNA. Lanes 6−10 contain cisplatin-modified DNA. Lanes 11−15 contain single-stranded cisplatin-modified oligonucleotide. Lanes 1, 6, and 11: no protein. Lanes 2−5, 7−10, and 12−15 contain 10, 20, 30, and 40 equiv of HMG domain B, respectively.

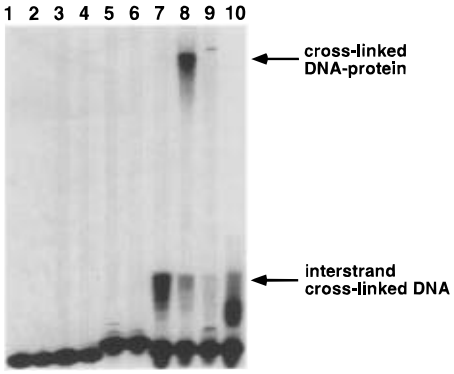


FIGURE 6: Denaturing 8% polyacrylamide gel demonstrating photo-cross-linking of HMG domain B to site-specifically platinated 15-bp DNA. Lanes 1−4 contain unplatinated DNA. Lanes 5−10 contain cisplatin-modified DNA. Lanes 1 and 5: DNA alone. Lanes 2 and 6: 40 equiv of HMG domain B. Lanes 3 and 7: no protein, irradiated. Lanes 4 and 8−10: incubated with 40 equiv of HMG domain B and then irradiated. Lane 9, following irradiation, treated with 0.2 M NaCN, pH 8.5. Lane 10: following irradiation, treated with 0.1 mg of proteinase K/mL.

with platinum compounds containing two cis amine ligands bound in a bifunctional manner.

*Photo-Cross-linking of a Site-Specifically Platinated 15-bp DNA Duplex to HMG Domain B.* Having established the specificity of protein photo-cross-linking by cisplatin-modified DNA, we next carried out experiments to identify the site(s) of cross-linking. To simplify the task, we employed the smallest system capable of modeling the interaction of HMG1 with cisplatin-modified DNA. The isolated B domain of HMG1 binds specifically to oligonucleotides containing a single *cis*-DDP intrastrand d(GpG) cross-link, in the same specific manner as HMG1 binds to longer platinated DNA probes (Chow et al., 1995). Accordingly, a 15-base oligonucleotide containing a single *cis*-[$Pt(NH_3)_2${d(GpG)-N7(1),-N7(2)}] intrastrand adduct (15Pt) was prepared and hybridized to its complement (15B), and the interaction of the corresponding duplex with HMG domain B was characterized in a gel mobility shift assay (Figure 5). Domain B bound specifically and efficiently to the platinated duplex (lanes 7−10), the amount of bound DNA increasing as the protein concentration increased. Binding to unplatinated or single-stranded DNA control substrates was not observed.

The ability of the platinated 15-bp duplex to photo-cross-link to HMG domain B was next investigated. Protein−DNA mixtures were equilibrated, irradiated, and then analyzed by denaturing gel electrophoresis (Figure 6). Irradiation of the platinated DNA in the absence of protein

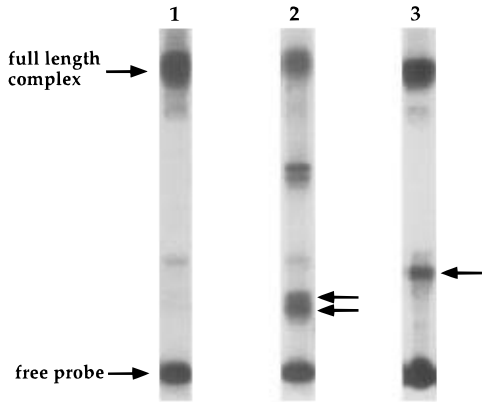


FIGURE 7: Denaturing 12% polyacrylamide gels revealing products of proteolytic digestion of the cross-linked platinated oligonucleotide−HMG domain B complex. An aliquot of each reaction mixture is shown. Lane 1: full-length protein−DNA complex. Lane 2: products of endoproteinase Asp-N digestion. Lane 3: products of endoproteinase Arg-C digestion. The bands in lanes 2 and 3 corresponding to the smallest peptide−DNA complexes, indicated by arrows, were isolated and analyzed by N-terminal amino acid sequencing. The unmarked intermediate bands in lane 2 represent incomplete digestion to the isolated products, as indicated by more extensive endoproteinase treatment (data not shown).

resulted in interstrand cross-linked DNA (lane 7). In the presence of HMG domain B, a slowly migrating band was observed (lane 8) which was reversed following NaCN treatment (lane 9) and digestion with proteinase K (lane 10). Treatment of the cross-linked protein−DNA complex with proteinase K produced a cluster of bands that migrated slightly more slowly than the free oligonucleotide. These new bands presumably contain platinated oligonucleotide attached to short peptide fragments of HMG domain B; it is likely that platinum binding blocked the protease, preventing complete digestion. No cross-linking was seen for unirradiated reaction mixtures. Also of interest is that the uncross-linked starting material remaining in lanes 7−10 migrated slightly faster than platinated oligonucleotide that had not been irradiated (lane 6) but slower than unplatinated oligonucleotide (lane 1). This result suggests perhaps that platinum is no longer bound bifunctionally after irradiation (vide infra).

*Determination of the Photo-Cross-Linked Amino Acid in HMG Domain B.* The site of cross-linking in HMG domain B was determined in the following manner. The volume of the photo-cross-linking reaction mixture was scaled up by several orders of magnitude to facilitate the generation of sufficient material for these experiments. Aliquots of this reaction mixture, containing cross-linked and free HMG domain B, were employed for digestion with different proteases. The resulting proteolyzed complexes were purified on denaturing gels and then analyzed by N-terminal amino acid sequencing. The point of interruption of the corresponding peptide sequence indicates the exact position of attachment. This methodology has been previously employed to identify UV-cross-linked sites of single-stranded DNA binding proteins (Merrill et al., 1984), contacts between integration host factor and its DNA target (Yang & Nash, 1994), and amino acid residues at the DNA binding site of *E. coli* uracil DNA glycosylase (Allen et al., 1991). The products of proteolytic digestion of the full-length protein−DNA complex are shown in Figure 7. Digestion of the cross-linked complex (lane 1) with endoproteinase Asp-N afforded two very closely spaced bands that migrated more slowly

Table 1: Amino Acid Sequences of Proteolyzed Cross-Linked Peptide Complexes of Platinated DNA

| peptide fragment | N-terminal sequence[a] | expected fragment on the basis of protease specificity |
|---|---|---|
| Asp-N, upper | MKKKF(−) | MKKKFK |
| Asp-N, lower | MKKKF(−) | MKKKFK |
| Arg-C | MKKKF(−)DPNAPK | MKKKFKDPNAPKR |
| full HMG domain B | MKKKFKDPN | |

[a] The symbol "(−)" denotes a sequencing cycle in which no amino acid was detected, ostensibly because the cross-linked platinated DNA altered its usual HPLC mobility.

than free probe on the gel (lane 2). Both of these bands, indicated by arrows, were isolated and employed for N-terminal sequence analysis. By contrast, digestion with endoproteinase Arg-C produced only one band that migrated somewhat more slowly than the endoproteinase Asp-N products (lane 3). For each of the digestions, the yield of total products was approximately 10%−15% of the initial amount of oligonucleotide employed.

Edman degradation of the isolated peptides cross-linked to the platinated oligonucleotide yielded a *single* amino acid sequence which, in each case, matched that of a portion of the known sequence of HMG domain B (Table 1). The yields for each sequencing cycle are included in Table S2 (supporting information). For both of the Asp-N protease fragments, the MKKKF sequence obtained precisely matched that of the first five amino acids from the N-terminus of domain B. Comparison of this sequence with that predicted from the protease specificity revealed that the sixth amino acid, a lysine, did not appear as expected (Table 1). Noteworthy is the fact that, although these two peptide fragments afforded the same exact amino acid sequence, they had slightly different gel mobilities. This result may reflect subtle differences in the two products, such as differential replacement of the platinum ligands following irradiation (vide infra). The endoproteinase Arg-C digest yielded a peptide matching residues 1−12 of domain B with an interruption in the sequence at cycle six. As a control, the full-length unmodified HMG domain B was also subjected to Edman degradation and did not show any interruptions in its amino acid sequence. The absence of any detectable amino acid at cycle six and a decrease in the yields for all subsequent cycles indicate that, by analogy to other types of cross-linked protein−DNA complexes reported previously (Yang & Nash, 1994), the amino acid which becomes cross-linked to the platinated oligonucleotide is Lys-6 of HMG domain B.

## DISCUSSION

The present investigation demonstrates that irradiation of DNA, modified with the anticancer drug cisplatin, with 300 nm UV light generates specific cross-links to HMG1 and its isolated B domain. This result indicates that simple platinum complexes might be generally useful as photo-cross-linking reagents. Moreover, a specific amino acid has been identified which binds covalently to platinum as a consequence of the photochemical reaction. This information must be accommodated in any structural model proposed for the complex formed between cisplatin-modified DNA and HMG domain proteins. Since proteins having one or more HMG domains recognize DNA containing the major adducts of *cis*-DDP (Chow et al., 1994), the photochemical cross-linking reaction discovered here should facilitate new strategies for platinum anticancer drug therapies as well as the study of protein-platinated DNA interactions in vivo.

*Mechanism of the Photoreaction.* The photoreactivity of square-planar platinum(II) complexes is well documented. For example, irradiation of *cis*-[$Ptpy_2Cl_2$] with 313 nm light results in two simultaneous reactions, photoisomerization and photodissociation of a pyridine ligand (Moggi et al., 1971). Loss of pyridine produces a tetracoordinated solvento complex which can recombine with ligand to give a mixture of *cis*- and *trans*-[$Ptpy_2Cl_2$]. By contrast, *trans*-[$Ptpy_2Cl_2$] is insensitive to irradiation at the same wavelength. Cis−trans photoisomerization also occurs in *cis*-[$Pt(glycinato)_2$] upon excitation of the ligand-field band at 313 nm (Balzani & Carassiti, 1968). The products of photochemical reactions of carboplatin have also been characterized (Liu et al., 1994). Excitation of the ligand-field band (313 nm) leads to photosubstitution, producing diaquadiammineplatinum(II) and tetraaquaplatinum(II). By contrast, excitation of the charge-transfer band (254 nm) results in redox chemistry. Cisplatin itself undergoes photosubstitution reactions, losing an ammine ligand when irradiated with 300−350 nm light (Macka et al., 1994). On the basis of this information, we propose that the mechanism of protein photo-cross-linking by cisplatin-modified DNA involves photosubstitution of one of the ligands by a protein residue, Lys-6 in the case of HMG domain B. Furthermore, most of the products seem to result from labilization of a platinum−purine bond.

Support for this proposal comes from two separate experiments. In gel mobility shift assays, HMG1 did not bind to a platinated DNA probe that had been preirradiated, suggesting that the bent DNA structure was no longer intact (data not shown). Photodissociation of a guanine ligand would result in a platinum that was no longer bound bifunctionally to DNA and hence unable to be recognized by HMG domains. Secondly, the chemical reactivity of the guanine bases coordinated to platinum in the 15Pt−HMG domain B photo-cross-linked complex was studied by using Maxam−Gilbert sequencing chemistry. Platinated guanine bases do not react with the guanine- or purine-specific reagents, dimethyl sulfate or formic acid, respectively, because their N-7 atoms are coordinated to the metal ion (Comess et al., 1990; Brabec & Leng, 1993). The following species were subjected to this type of sequencing analysis: the oligonucleotide 15Pt that had been complexed in a non-covalent manner with HMG domain B; the full-length photo-cross-linked 15Pt−HMG domain B complex; and the products resulting from endoproteinase Asp-N digestion of the cross-linked 15Pt−HMG domain B complex (data not shown). The guanine bases in the unirradiated oligonucleotide, as well as in the oligonucleotide that had undergone non-covalent complexation with HMG domain B, did not react with these reagents, as expected. In the cross-linked protein−DNA complex and in the proteolyzed peptide−DNA complex, the guanines were reactive, signaling loss of platinum from these sites.

These experiments suggest that the major pathways by which cisplatin-modified DNA undergoes photoinduced interstrand cross-linking and DNA−protein cross-linking are

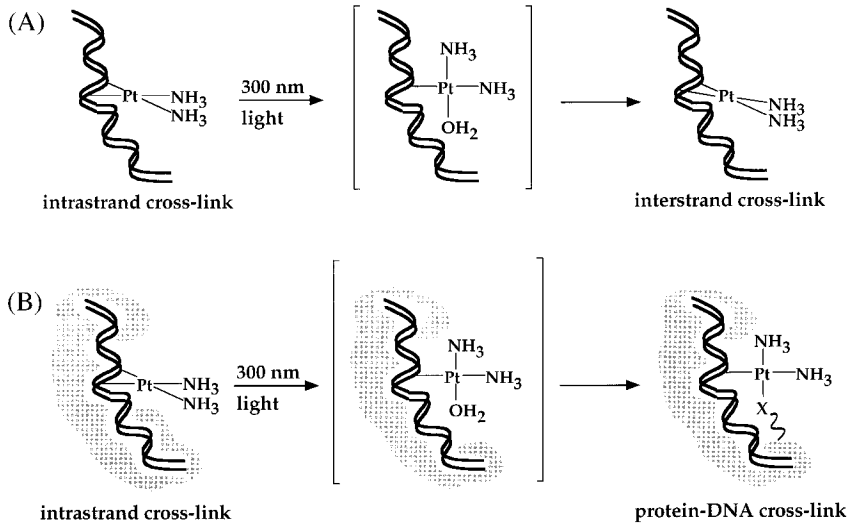


FIGURE 8: Proposed mechanisms of photoinduced DNA interstrand (A) and protein (B) cross-linking by cisplatin-modified DNA.

as illustrated in Figure 8. Starting with the *cis*-[$Pt(NH_3)_2$-{d(GpG)-N7(1),-N7(2)}] intrastrand adduct, irradiation with 300 nm light results in photodissociation of one of the purine ligands, producing a reactive intermediate in which the vacant site is filled with a more labile ligand, presumably a water molecule. This labile ligand can then be displaced by a nucleophile, such as a nucleobase on the opposing DNA strand, producing a DNA interstrand cross-link (Figure 8A). If the same reactive intermediate is formed in the presence of a specifically bound protein, then the labile ligand can be displaced by a proximal nucleophile on the protein, affording a stable protein−DNA cross-link (Figure 8B). It is presumed that these pathway can proceed through labilization of either of the two platinum−purine bonds in order to account for the reactivity of both guanine bases with dimethyl sulfate following irradiation (vide supra). Another likely pathway would involve photosubstitution of one of the ammine ligands of cisplatin, a possibility supported by the observation that DNA containing a $\{Pt(NH_3)_3\}^{2+}$ adduct also forms interstrand cross-links upon irradiation (vide supra).

*Interaction of Cisplatin-Modified DNA with HMG Domain B.* Members of the HMG domain family of proteins have the ability to recognize prebent DNA. The HMG domain is a relatively new DNA binding motif that has been identified in functionally unrelated proteins (Grosschedl et al., 1994). There are two known classes of HMG domain proteins. Members of the sequence specific class, such as SRY and Lef-1, contain only one HMG domain and bind to particular DNA sequences. Members of the other class include HMG1, HMG2, UBF, and mtTFA. They contain more than one HMG domain and bind to DNA with little or no apparent sequence specificity.

The solution structure of domain B from HMG1 has been determined by NMR spectroscopy (Read et al., 1993; Weir et al., 1993). The protein adopts a unique, rather flat L-shaped geometry, comprising three α-helices linked by short turns (Figure 9). No structural information has been available concerning the interaction of this domain with DNA. More recently, the solution structure of the SRY-HMG domain bound to its DNA target sequence was determined by NMR spectroscopy (Werner et al., 1995). In this structure, the protein maintained the L-shape conformation analogous to that observed for HMG domain B. DNA binding occurred on the concave surface formed by helices

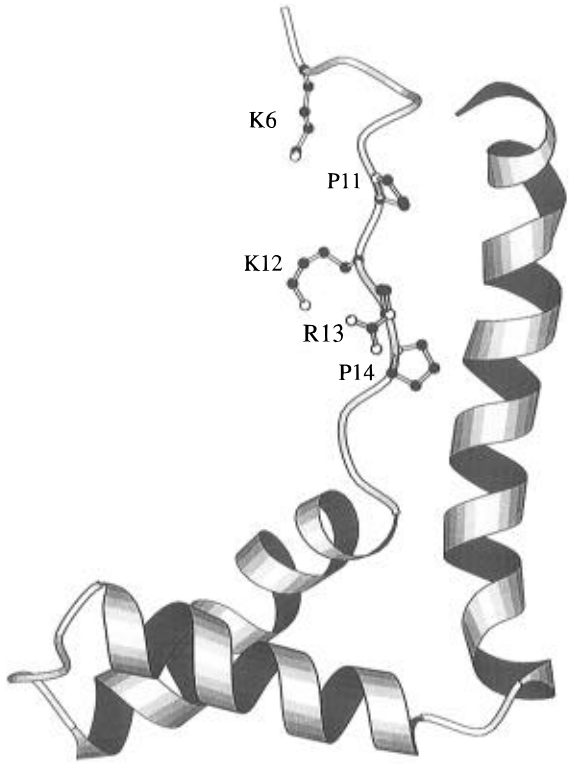


FIGURE 9: Schematic representation of HMG domain B. The coordinates were from the NMR-determined solution structure (Weir et al., 1993). This drawing was generated by using MOLSCRIPT (Kraulis, 1991).

one and three of the L, and the structure was anchored by ridges at one end by helix two and at the other end by the N- and C-terminal strands. All DNA contacts were made within the minor groove. The DNA structure was significantly altered, adopting features intermediate between those of A- and B-DNA. The side chain of Ile-68 was inserted between adjacent DNA base pairs, causing a significant widening of the minor groove. This novel DNA structure, induced upon binding of the SRY domain to its target site, strikingly resembles that of a DNA dodecamer duplex containing the major cisplatin d(GpG) 1,2-intrastrand cross-link, as revealed in a recent X-ray crystal structure determination of platinated d(CCTCTGGTCTCC) (Takahara et al., 1995).

The present work identifies a unique contact made between HMG domain B and cisplatin-modified DNA. The regioselectivity of the reaction is absolute, based on the observation that the sequenced peptides were pure, suggesting a very tight, specific protein−DNA complex. A more detailed model of how the HMG domain interacts with platinated DNA cannot be postulated, however, without further structural information. The SRY domain−DNA and HMG1 domain−platinated DNA structures are not necessarily comparable because the sequences of the domains share only 26% homology. Moreover, these HMG domains clearly display differences in their DNA-binding and -bending properties (Teo et al., 1995). Attempts to overlay the structures of the domains from HMG1 and SRY are complicated by the fact that the N- and C-termini of HMG domain B are disordered, as revealed by the NMR solution structure (Read et al., 1993; Weir et al., 1993), and only the three α-helices of these domains could be superimposed (Werner et al., 1995). The specific cross-link between HMG domain B and cisplatin-modified DNA identified in the present work occurs at a residue located in the extended strand at the amino-terminus of the protein, which lies within the concave surface of the L (Figure 9). The strand contains four proline residues, which may play some role in constraining the conformation in this region (Teo et al., 1995). In the SRY domain, only one of these prolines is conserved, and the role of these residues in modulating the DNA binding properties of the different HMG domains awaits the results of future structural studies.

Although we cannot yet propose a detailed model for the interaction of HMG domains with cisplatin-modified DNA, one important requirement revealed by the present investigation is that the N-terminus of domain B must interact with the *major* groove of the DNA, whereas the α-helices of the domain lie along the minor groove surface. This conclusion is required by the cross-linking of platinum bound to adjacent guanosine residues with Lys-6 of domain B, since the guanine N-7 atoms are located in the major groove of DNA. While this paper was being reviewed, the NMR solution structure of the Lef-1 domain bound to its target DNA sequence was reported (Love et al., 1995). In this structure, the highly basic C-terminal region was found to contact the major groove, crossing over the sugar−phosphate backbone. The N-terminal strand and helix 3 were packed against one other in the minor groove. In HMG domain B, it is the N-terminus that contains a significant number of basic amino acid residues. Our results and these observations suggest that, if a similar helix packing against the DNA were to occur for HMG domain B binding, then the basic N-terminal strand may similarly wrap around the DNA helix, positioning Lys-6 perfectly for photo-cross-linking to the platinum center.

*Cisplatin-Mediated DNA−Protein Cross-links.* The formation of protein−DNA cross-links by *cis*-DDP has been observed previously (Lippard & Hoeschele, 1979; Zwelling et al., 1979). These events occur at such a low frequency compared to the formation of other platinum adducts that their contribution to the biological activity of cisplatin is assumed to be minimal (Comess & Lippard, 1993). Both *cis*- and *trans*-DDP cross-link nuclear DNA and non-histone chromosomal proteins (Banjar et al., 1984; Ciccarelli et al., 1985), and both HMG1 and HMG2 cross-link to micrococcal nuclease-accessible regions of chromatin (Scovell et al., 1987). Although UV irradiation was not used, this result suggested that HMG domain proteins contact the major groove of DNA, as later specifically proposed (Scovell, 1989). Protein−DNA cross-linking has also been observed for a series of bis[platinum(II)] complexes in which a DNA interstrand cross-link is the lesion responsible for protein cross-linking; the *E. coli* repair proteins UvrA and UvrB cross-link to these lesions in vitro (Van Houten et al., 1993).

The present work suggests a novel method for promoting protein−DNA cross-linking with the parent compound, cisplatin. This methodology should enable the isolation of proteins from cells that interact with cisplatin-modified DNA in vivo. Furthermore, a platinum cross-linked protein−DNA complex has the potential to enhance the cytotoxic effects of *cis*-DDP by several mechanisms. Work is in progress to use this new methodology for structural studies and to identify proteins that interact with cisplatin-modified DNA. This information should help to unravel the relationship between HMG domain proteins and the biological activity of cisplatin.

## ACKNOWLEDGMENT

We thank Dr. R. Cook for analysis of the N-terminal sequencing data. Mass spectral data were provided by Dr. C. Costello at the MIT Mass Spectrometry Facility, which is supported by NIH Grant No. RR00317 (to K. Biemann). S.A.K. thanks D. E. Coufal for assistance in generating Figure 9.

## SUPPORTING INFORMATION AVAILABLE

Schemes and experimental procedures for the synthesis of the Pt−aryl azide complexes; atomic absorption data for determining the $r_b$ values for DNA modified by cisplatin and Pt−$L_n$ compounds; a gel showing binding of cisplatin-modified and Pt−$L_n$-modified DNA by HMG1; and amino acid sequencing yields for the cross-linked peptide−DNA complexes and HMG domain B (11 pages). Ordering information is given on any current masthead page.

## REFERENCES

Allen, T. D., Wick, K. L., & Matthews, K. S. (1991) *J. Biol. Chem. 266*, 6113−6119.
Balzani, V., & Carassiti, V. (1968) *J. Phys. Chem. 72*, 383−388.
Banjar, Z. M., Hnilica, L. S., Briggs, R. C., Stein, J., & Stein, G. (1984) *Biochemistry 23*, 1921−1926.
Bayley, H. (1983) *Photogenerated Reagents in Biochemistry and Molecular Biology*, Elsevier Science Publishers, Amsterdam.
Brabec, V., & Leng, M. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 5345−5349.
Brown, S. J., Kellett, P. J., & Lippard, S. J. (1993) *Science 261*, 603−605.
Brown, S. J., Chow, C. S., & Lippard, S. J. (1994) in *Encyclopedia of Inorganic Chemistry* (King, R. B., Ed.) pp 3305−3315, John Wiley & Sons, Ltd., West Sussex, England.
Bruhn, S. L., Toney, J. H., & Lippard, S. J. (1990) *Prog. Inorg. Chem. 38*, 477−516.
Bruhn, S. L., Pil, P. M., Essigmann, J. M., Housman, D. E., & Lippard, S. J. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 2307−2311.
Chow, C. S., Whitehead, J. P., & Lippard, S. J. (1994) *Biochemistry 33*, 15124−15130.
Chow, C. S., Barnes, C. M., & Lippard, S. J. (1995) *Biochemistry 34*, 2956−2964.
Ciccarelli, R. B., Solomon, M. J., Varshavsky, A., & Lippard, S. J. (1985) *Biochemistry 24*, 7533−7540.
Comess, K. M., Costello, C. E., & Lippard, S. J. (1990) *Biochemistry 29*, 2102−2110.

Comess, K. M., & Lippard, S. J. (1993) in *Molecular Aspects of Anticancer Drug−DNA Interactions* (Neidle, S., & Waring, M., Eds.) pp 134, Macmillan, London.
Fichtinger-Shepman, A. M. J., van der Veer, J. L., den Hartog, J. H. J., Lohman, P. H. M., & Reedijk, J. (1985) *Biochemistry 24*, 707−713.
Grosschedl, R., Giese, K., & Pagel, J. (1994) *Trends Genet. 10*, 94−100.
Hartwig, J. F., & Lippard, S. J. (1992) *J. Am. Chem. Soc. 114*, 5646−5654.
Huang, J.-C., Zamble, D. B., Reardon, J. T., Lippard, S. J., & Sancar, A. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 10394−10398.
Hughes, E. N., Engelsberg, B. N., & Billings, P. C. (1992) *J. Biol. Chem. 267*, 13520−13527.
Kraulis, P. J. (1991) *J. Appl. Crystallogr. 24*, 946−950.
Lippard, S. J., & Hoeschele, J. D. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 6091−6095.
Liu, W.-P., Yang, Y.-K., Que, Z.-H., & Xiong, H.-Z. (1994) *Sci. China, Ser. B 37*, 799−806.
Loehrer, P. J., & Einhorn, L. H. (1984) *Ann. Intern. Med. 100*, 704−713.
Love, J. J., Li, X., Case, D. A., Giese, K., Grosschedl, R., & Wright, P. E. (1995) *Nature 376*, 791−795.
Macka, M., Borák, J., Seménkova, L., & Kiss, F. (1994) *J. Pharm. Sci. 83*, 815−818.
McA'Nulty, M., & Lippard, S. J. *Mutat. Res.* (in press).
Merrill, B. M., Williams, K. R., Chase, J. W., & Konigsberg, W. H. (1984) *J. Biol. Chem. 259*, 10850−10856.
Moggi, L., Varani, G., Sabbatini, N., & Balzani, V. (1971) *Mol. Photochem. 3*, 141−154.
Pil, P. M., & Lippard, S. J. (1992) *Science 256*, 234−237.
Read, C. M., Cary, P. D., Crane-Robinson, C., Driscoll, P. C., & Norman, D. G. (1993) *Nucleic Acids Res. 21*, 3427−3436.
Scovell, W. M., Muirhead, N., & Kroos, L. R. (1987) *Biochem. Biophy. Res. Commun. 142*, 826−835.
Scovell, W. M. (1989) *J. Macromol. Sci.-Chem. A26*, 455−480.
Sherman, S. E., Gibson, D., Wang, A. H.-J., & Lippard, S. J. (1988) *J. Am. Chem. Soc. 110*, 7368−7381.
Shetlar, M. D. (1980) *Photochem. Photobiol. Rev. 5*, 105.
Takahara, P. M., Rosenzweig, A. C., Frederick, C. A., & Lippard, S. J. (1995) *Nature 377*, 649−652.
Teo, S.-H., Grasser, K. D., & Thomas, J. O. (1995) *Eur. J. Biochem. 230*, 943−950.
Toney, J. H., Donahue, B. A., Kellett, P. J., Bruhn, S. L., Essigmann, J. M., & Lippard, S. J. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 8328−8332.
Treiber, D. K., Zhai, X., Jantzen, H.-M., & Essigmann, J. M. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 5672−5676.
Van Houten, B., Illenye, S., Qu, Y., & Farrell, N. (1993) *Biochemistry 32*, 11794−11801.
Weir, H. M., Kraulis, P. J., Hill, C. S., Raine, A. R. C., Laue, E. D., & Thomas, J. O. (1993) *EMBO J. 12*, 1311−1319.
Werner, M. H., Huth, J. R., Gronenborn, A. M., & Clore, G. M. (1995) *Cell 81*, 705−714.
Yang, S.-W., & Nash, H. A. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 12183−12187.
Zwelling, L. A., Anderson, T., & Kohn, K. W. (1979) *Cancer Res. 39*, 365−369.

BI952240A