Laser-Induced Photo-Cross-Linking of Cisplatin-Modified DNA to HMG-Domain Proteins†

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ABSTRACT: Laser-induced photo-cross-linking was investigated for DNA, modified with cisplatin at specific sites, bound to structure-specific recognition domains of proteins in the high-mobility group (HMG) class. The efficiency of photo-cross-linking depends on the wavelength and power of the laser, the nature of the protein domain, and the oligodeoxyribonucleotide sequences flanking the platinated site. Introduction of 5-iodouridine at thymine sites of the oligodeoxyribonucleotide as an additional photoreactive group did not increase the photo-cross-linking yield. Formation of platinum-mediated DNA−DNA interstrand cross-linking observed previously upon irradiation with 302 nm light [Kane, S. A., and Lippard, S. J. (1996) *Biochemistry 35*, 2180−2188] was significantly reduced with laser irradiation. HMG1 domain B is superior to domain A for platinum-mediated photo-cross-linking, a result attributed to the different positioning of the proteins with respect to the platinum adduct and the greater ability of domain B to access photolabilized platinum in the major groove. Studies with proteins containing specifically mutated amino acids, and with DNA probes in which the sequences flanking the platinum cross-link site were varied, suggest that the most effective photo-cross-linking occurs for protein domains bound symmetrically and flexibly to cisplatin-modified DNA. The thermodynamic equilibrium between the protein−platinated DNA complex and its components, revealed in gel electrophoretic mobility shift assays (EMSAs), is significantly shifted to the right upon irreversible photo-cross-linking. Thus, only upon photo-cross-linking can the interaction of cisplatin−DNA 1,3-intrastrand d(GpTpG) or interstrand cross-links with HMG1 domain B protein be detected. Photo-cross-linking is thus an effective tool for investigating the interaction of cisplatin-modified DNA with damage-recognition proteins under heterogeneous conditions such those in cell extracts or living cells.

The therapeutic mechanism of the potent anticancer drug, *cis*-diamminedichloroplatinum(II) (cisplatin),[1] is of continuing interest. The widespread application of cisplatin and carboplatin in the clinical treatment of cancer has spawned extensive studies to elucidate its mechanism of action (*1*−*3*). Cisplatin reacts with the N7 atoms of adjacent purine residues, preferably two neighboring guanines in DNA, giving rise to 1,2-intrastrand cross-links. These cross-links block several cellular processes including transcription (*4*) and replication (*5*, *6*). The cross-links cause distortions in the DNA duplex structure (*7*−*10*) that are recognized by a class of ubiquitous cellular proteins including HMG1, HMG2, structure-specific recognition protein 1 (SSRP1), sex-determining factor SRY, mitochondrial transcription factor 1 (mtTF1), and other high-mobility group (HMG) domain proteins (*11*−*14*). The HMG domain consists of approximately 80 amino acid residues and recognizes distorted DNA geometries (*15*−*18*). HMG domains comprise three α-helices, which form an L-shaped structure. Intercalating amino acids in helices I and II play an important role in binding to DNA and inducing a bend in the duplex (*19*−*24*).

The nucleotide excision repair (NER) pathway repairs cisplatin-modified DNA (*25*, *26*). Overexpression of NER proteins has been demonstrated in cisplatin-resistant cell lines (*27*) and can be induced by cisplatin treatment (*28*). On the other hand, interaction of HMG-domain proteins with cisplatin-modified DNA can shield the platinum lesion from NER (*29*, *30*). Evidence supporting such a repair-shielding hypothesis includes the enhancement of cell sensitivity to cisplatin by overexpression of HMG1 following treatment with steroid hormones (*31*).

There may be other pathways in the cell by which HMG-domain proteins modulate the anticancer activity of cisplatin. Although the principal roles of many HMG-domain proteins are yet unknown, they appear to be required for controlling chromatin architecture or as signaling molecules in geneti-

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[1] Abbreviations: EMSA, electrophoretic mobility shift assay; HMG, high-mobility group; HMG1a, domain A of high-mobility group protein 1; HMG1b, domain B of high-mobility group protein 1; PAGE, polyacrylamide gel electrophoresis; cisplatin, *cis*-diamminedichloroplatinum(II); bp, base pair; Tris, tris(hydroxymethyl)aminomethane; HPLC, high-performance liquid chromatography; BSA, bovine serum albumin; CE DNA, chicken erythrocyte DNA; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; ESI-MS, electrospray ionization mass spectrometry; IU, 5-iodouridine; G*, platinated guanine; G, 7-deazaguanine; ds, double stranded; ss, single stranded.

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cally regulated repair pathways. In the presence of cisplatin-damaged DNA sites, HMG-domain proteins may be hijacked from their natural binding sites. Gene regulation could be disrupted by this event, resulting in cell death (*1*, *3*).

We previously reported that the photoreactivity of cisplatin bound site-specifically to duplex oligodeoxyribonucleotides is a potentially effective method to survey proteins that recognize cisplatin-modified DNA (*32*). Upon irradiation of HMG1b protein complexed to DNA containing an intrastrand d(GpG) cisplatin-mediated cross-link with UV light (302 nm), the platinum−guanine bond dissociated and a new bond formed between the platinum and a lysine residue of HMG1b. Even in the presence of protein, however, significant amounts of DNA−DNA interstrand cross-links were formed by irradiation. To minimize this undesired background reaction, we examined laser-induced photo-cross-linking of cisplatin-modified oligodeoxyribonucleotides with HMG1 domain proteins. The narrow wavelength dispersion of the irradiation light source allows selective activation of the platinum−guanine bond. This methodology could therefore be extended to examine DNA probes containing several other platinum lesions, such 1,3-intrastrand platinum d(GpTpG) adducts or interstrand cross-links formed by cisplatin or *trans*-DDP. As demonstrated in the present article, the irreversible nature of the photo-cross-linking reaction reveals protein−platinated DNA interactions that have previously gone undetected in electrophoretic gel mobility shift assays.

MATERIALS AND METHODS

Preparation of Oligodeoxyribonucleotide Probes Containing Platinum Intrastrand Cross-Links. All DNA strands were synthesized by using standard phosphoramidite methods on an Applied Biosystems 392 DNA/RNA synthesizer on a 1.0 μmol scale. Phosphoramidites were obtained from Glen Research. After automated synthesis, deprotection was performed in ammonium hydroxide at 65 °C for 1 h, or at room temperature overnight for 5-iodouridine-containing probes (Table 1, *n*-IUs), according to the manufacturer's directions. Single-stranded oligodeoxyribonucleotides were purified by 20% denaturing PAGE (7.5 M urea, 19:1 acrylamide:bisacrylamide, 90 mM Tris−borate, 2.0 mM EDTA, pH 8.3) for 6 h at 300 V. Oligodeoxyribonucleotide bands were visualized under UV light, cut from the gel, and soaked at 37 °C overnight in buffer containing 10 mM Tris-HCl, pH 7.5, 50 mM NaCl, and 1 mM EDTA. The eluent was cleared by filtration (0.2 μm syringe filter, Pall Corp.) and ethanol precipitated. The purity of material was checked by ion-exchange HPLC and the amount of oligodeoxyribonucleotide quantitated spectrophotometrically.

For the preparation of a 15-bp oligodeoxyribonucleotide cross-linked by cisplatin or *trans*-DDP in an intrastrand manner, single-stranded oligodeoxyribonucleotides having only two guanines in a GG or GTG sequence were platinated with 2 equiv of cisplatin or *trans*-DDP, activated by incubation with 2.0 equiv of silver nitrate in water overnight in the dark. The DNA platination reactions were performed in 10 mM sodium phosphate, pH 6.8, at 37 °C overnight. The material was purified by ion-exchange HPLC (Dionex NucleoPac PA-100, 9 × 250 mm, 10% acetonitrile, 25 mM ammonium acetate, linear gradient of 0.3−0.5 M NaCl over 30 min), dialyzed (1000 molecular weight cutoff, Spectra/Por), and lyophilized. The platinum content per strand was determined by a combination of UV−visible and atomic absorption spectroscopy. The products were further characterized by ESI-MS (Table S1, Supporting Information).

Table 1: Sequence of Oligodeoxyribonucleotide Duplexes Employed for This Study

Probe	Sequence[a]
G*G*	5′-CCTCTCTG*G*TTCTTC-3′ 3′-GGAGAGAC C AAGAAG-5′
3-IU	5′-CCU^{I}CTCTG*G*TTCTTC-3′ 3′-GGA GAGAC C AAGAAG-5′
5-IU	5′-CCTCU^{I}CTG*G*TTCTTC-3′ 3′-GGAGA GAC C AAGAAG-5′
7-IU	5′-CCTCTCU^{I}G*G*TTCTTC-3′ 3′-GGAGAGA C C AAGAAG-5′
10-IU	5′-CCTCTCTG*G*U^{I}TCTTC-3′ 3′-GGAGAGAC C A AGAAG-5′
11-IU	5′-CCTCTCTG*G*TU^{I}CTTC-3′ 3′-GGAGAGAC C AA GAAG-5′
13-IU	5′-CCTCTCTG*G*TTCU^{I}TC-3′ 3′-GGAGAGAC C AAGA AG-5′
14-IU	5′-CCTCTCTG*G*TTCTU^{I}C-3′ 3′-GGAGAGAC C AAGAA G-5′
$N_1G^*G^*N_2$	5′-CCTCTCN$_1$G*G*N$_2$TCTTC-3′ 3′-GGAGAGN$_4$C C N$_3$AGAAG-5′
cis-G*TG*	5′-CCTCTCG*TG*TTCTTC-3′ 3′-GGAGAGC AC AAGAAG-5′
trans-G*TG*	5′-CCTCTCG*TG*TTCTTC-3′ 3′-GGAGAGC AC AAGAAG-5′
cis-ICL	5′-CCTCTCTG*C TTCTTC-3′ 3′-GGAGAGAC G*AAGAAG-5′
trans-ICL	5′-CCTCTCTG*CTTCTTC-3′ 3′-GGAGAGAC*GAAGAAG-5′

[a] U^{I} = 5-iodouracil; N_n= A, T, G, C, or G (7-deazaG). Asterisks denote the sites of platination.

Cisplatin- or *trans*-DDP-modified single-stranded oligodeoxyribonucleotides (0.2 nmol) were radiolabeled with [γ-^{32}P]-dATP (Dupont/NEN) in a solution (50−100 μL) containing 70 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 5 mM DTT, and 10 units of T4 polynucleotide kinase (New England Biolabs) at 37 °C. Another 10 units of T4 polynucleotide kinase was added to the solution after 45 min. After a total of 90 min, the reaction solutions were passed through spin dialysis columns (G-25 Sephadex Quickspin column, Roche). The eluents were diluted to 100 μL with water and twice extracted by 100 μL of 25:24:1 phenol:chloroform:isoamyl alcohol. The water layer was ethanol precipitated to isolate the DNA product.

The platinated, radiolabeled top strands were annealed with 1.1 equiv of bottom strands in an aqueous solution (1 mL) containing 10 mM Tris-HCl, 10 mM $MgCl_2$, and 50 mM NaCl. The incubation was performed at 90 °C for 4 min, followed by slow cooling to room temperature over 5 h and storage at 4 °C overnight. For the flanking sequence selectivity studies, all probes indicated as N_1G*G*N_2 (Table 1) were annealed, HPLC-purified, and then radiolabeled on both strands in a manner described previously (*18*).

Preparation of Oligodeoxyribonucleotide Probes Containing Platinum Interstrand Cross-Links. The interstrand cross-linked oligodeoxyribonucleotide duplexes were prepared according to literature procedures (*26*, *33*, *34*). Briefly, a 15-bp single-stranded oligodeoxyribonucleotide containing only one G was allowed to react with *cis*- or *trans*-$[Pt(NH_3)_2(H_2O)Cl]^+$, derived from the parent chloride compound by incubation with 1.0 equiv of silver nitrate in DMF overnight in the dark. The reaction was quenched by adding NaCl to 0.4 M, and the monofunctional platinum adducts were purified by ion-exchange HPLC under conditions similar to those described above for the bifunctional, intrastrand cross-links. After dialysis against 0.1 M NaCl at 4 °C, the DNA solution was concentrated to one-fifth volume under reduced pressure. The 15-mer was annealed with 1 equiv of the complementary strand in 0.45 M NaCl, 2 mM $MgCl_2$, and 20 mM Tris-HCl, pH 7.4, at room temperature for 2 h and then at 4 °C for 2.5 h. The duplex solution was dialyzed against 0.1 M $NaClO_4$ at 4 °C for 4 h and incubated at 37 °C for 40 h to form interstrand cross-links. The product was purified by ion-exchange HPLC (Dionex NucleoPac PA-100, 9 × 250 mm, 10% acetonitrile, 25 mM ammonium acetate, linear gradient of 0.3−0.5 M NaCl over 30 min followed by 0.5−1 M NaCl over 10 min). The decreased mobility in 15% denaturing PAGE and reversibility after NaCN treatment confirmed the presence of the interstrand cross-link. The products were also characterized by ESI-MS (Table S1, Supporting Information). The position of connectivity of the interstrand cross-link shown in Table 1 was determined by Maxam−Gilbert sequencing (data not shown) (*33*).

Proteins. HMG1a, HMG1b, and mutant proteins derived therefrom, HMG1a-A16M, HMG1a-A16F, HMG1a-F37A, HMG1a-A16M/F37A, HMG1a-A16F/F37A, HMG1b-F16A, HMG1b-I37A, and HMG1b-F16A/I37A, were prepared in a manner previously reported (*16*, *24*).

Electrophoretic Mobility Shift Assays (EMSAs) with HMG1 Domain Proteins. Radiolabeled probe DNA (40 nM) was incubated on ice for 1 h in the presence or absence of 400 nM HMG1 domain protein. The reaction solution (10 μL total volume) contained 10 mM Tris-HCl, pH 7.5, 4% v/v glycerol, 10 mM $MgCl_2$, 50 mM KCl, 1 mM EDTA, 0.05% v/v Nonidet P-40, and 0.2 μg/mL BSA. The reaction mixtures were resolved on prerun (>1 h) 10% native polyacrylamide gels with 0.5× TBE (45 mM Tris-HCl, 45 mM boric acid, and 1.0 mM EDTA, pH 8.3) at 4 °C and 300 V for 1.5 h. The gels were dried, exposed to a molecular imaging plate, and analyzed on a Bio-Rad GS-525 Molecular Imager using the Multi-Analyst software package (Bio-Rad).

Photo-Cross-Linking with HMG1 Domain Proteins. Reaction solutions for photo-cross-linking experiments were prepared in Thermowell Tubes (Costar) under exactly the same conditions as EMSA reaction mixtures (vide infra). The reaction mixtures were incubated on ice for at least 30 min prior to irradiation. A helium cadmium laser 3230I (Liconix) at 325 nm (2.5 mW) or an argon ion laser Innova 300 (Coherent) at 350 nm (900 mW, unless otherwise described) was employed as the source. Irradiation was initiated at 4 °C in a quartz container filled with ice−water. After irradiation, 5 μL of gel loading buffer (10 M urea, 1.5 mM EDTA, 0.05% w/v bromophenol blue and xylene cyanol) was added, and the mixture was heated to 90 °C for 2 min, followed by quick cooling at ice temperature. The product was separated by denaturing 8% PAGE run in 1× TBE (90 mM Tris-HCl, 90 mM boric acid, and 2.5 mM EDTA, pH 8.3) for 1.5 h at 300 V with cooling. The gels were dried, visualized, and analyzed as described above. Data points in all figures represent the results from three independent experiments, and the error bars represent ±1 estimated standard deviation.

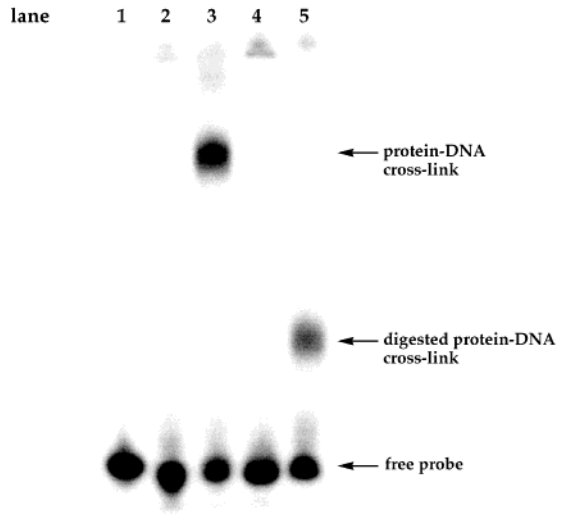


FIGURE 1: Denaturing polyacrylamide gel demonstrating the photo-cross-linking reaction of platinated 15-bp DNA with HMG1b protein induced by laser (325 nm, 30 min) irradiation. Lane 1: with protein, no irradiation. Lane 2: no protein, irradiated. Lane 3: with protein, irradiated. Lane 4: same as lane 3, followed by NaCN treatment (0.2 M, pH 8.5). Lane 5: same as lane 3, followed by proteinase K treatment (0.1 mg/mL).

RESULTS

Laser-Induced Photo-Cross-Linking of Platinated Oligodeoxyribonucleotides to HMG1 Domains A and B. A platinated DNA duplex oligodeoxynucleotide having a site-specific d(GpG) cross-link (G*G*, Table 1) forms a non-covalent complex with HMG1b protein when incubated at 4 °C, as evidenced by EMSA (Figure S1, Supporting Information). Upon laser irradiation of the complex at 325 nm for 30 min, a fraction was detected by denaturing gel electrophoresis with significantly retarded mobility compared with that of the free probe (Figure 1, lane 3). This band disappeared after treatment with sodium cyanide (lane 4) and was replaced by a new band with faster mobility after treatment with proteinase K (lane 5). These results suggest that the species is a protein−DNA cross-link tethered by platinum−guanine and platinum−protein covalent bonds (*32*). The absence of such a band upon irradiation of the probe alone (lane 2) further confirms that protein−DNA photo-cross-linking occurs only for the specific noncovalent complex formed between platinated DNA and protein. The amount of DNA−DNA interstrand cross-linking in these experiments

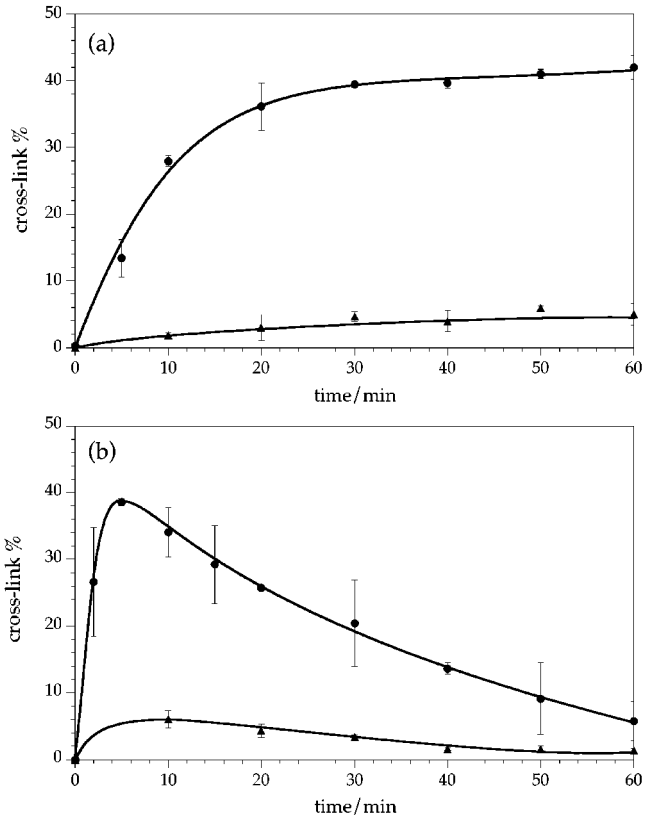


FIGURE 2: Time course of the cross-linking reaction of cisplatin-modified DNA (G*G*, Table 1) with HMG1a (triangles) and HMG1b proteins (circles) using 325 nm at 2.5 mW (a) and 350 nm at 900 mW (b) laser irradiation. Data points in all figures represent the results from three independent experiments, and the error bars represent ±1 estimated standard deviation.

is very small (~5%) as compared to that observed previously upon irradiation by a transilluminator at 302 nm (*32*).

Similar experiments were performed with HMG1a or HMG1b for various laser irradiation times at 325 nm and 2.5 mW or at 350 nm and 900 mW. Figure 2 shows the progression of the laser-induced photo-cross-linking reaction over time. Previous studies revealed that HMG1a binds with higher affinity than HMG1b to cisplatin-modified DNA (*17*, *18*); however, HMG1b is more efficiently photo-cross-linked than HMG1a.

The reaction time−course profile observed upon irradiation of HMG1b with 350 nm, 900 mW laser light is distorted and bell-shaped (Figure 2b). Because no degradation products were observed in the gel during the reaction, the loss of the cross-link with time is considered to be a consequence of cleavage of platinum−protein and/or platinum−guanine bonds formed in the cross-linked product during irradiation. To clarify the effect of laser dose on the photo-cross-linking reaction, different powers were applied for irradiation at 350 nm using HMG1b. In addition to the experiment at 900 mW described above, irradiation was performed at powers of 500, 100, and 3 mW. Figure 3 summarizes the results. Although cross-linking with the 325 nm laser at 2.5 mW is very efficient, similar laser power (3 mW) at 350 nm induced very little cross-linking.

Effect of 5-Iodouridine as an Additional Photoactive Group. A commercially available 5-iodouridine phosphoramidite was employed to prepare platinated duplexes with additional potentially photoreactive groups. Seven thymines

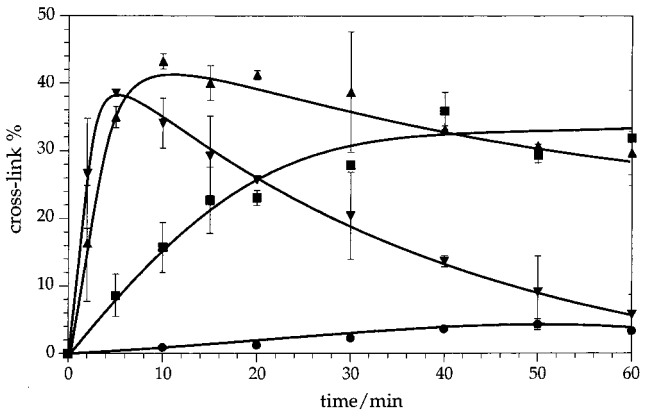


FIGURE 3: Time course of the cross-linking reaction of cisplatin-modified DNA (G*G*, Table 1) with HMG1b proteins using 350 nm laser irradiation at various laser powers. Key: inverse triangles, 900 mW; triangles, 500 mW; squares, 100 mW; circles, 3 mW. Data points in all figures represent the results from three independent experiments, and the error bars represent ±1 estimated standard deviation.

in the G*G* probe [(5′-$CCT_3CT_5CT_7G^*G^*T_{10}T_{11}CT_{13}T_{14}C$)-(3′-GGAGA GACCAAGAAG-5′)] were systematically replaced, one at a time, by 5-iodouridine to produce seven new probes having one 5-iodouridine in each position (*n*-IUs, where $n = 3$, 5, 7, 10, 11, 13, and 14, indicating the position of 5-iodouridine, Table 1). Radiolabeled duplexes containing 5-iodouridine were incubated with HMG1a or HMG1b and analyzed by 10% native gel electrophoresis at 4 °C (Figure S1, Supporting Information). HMG1 domain proteins recognize platinated probes in an efficient and specific manner even in the presence of the artificial 5-iodouridine nucleoside in the platinated strand. 5-Iodouridine does not afford any significant structural or electrostatic change recognized by HMG1 domain proteins.

The following irradiation conditions were employed to estimate the effect induced by 5-iodouridine for photo-cross-linking of HMG1b to platinated DNA: (i) 325 nm, 30 min irradiation; (ii) 325 nm, 5 min irradiation; (iii) 350 nm (500 mW), 10 min irradiation; and (iv) 350 nm (100 mW), 10 min irradiation. Conditions i and iii afford considerable photo-cross-linking by the platinum moiety on the probes, and conditions ii and iv are the conditions where platinum-induced photo-cross-linking is not significant, ~10−15% (see Figures 2a and 3). These two sets of conditions were chosen to evaluate the possible kinetic competition between the two different photoactive groups in platinated, 5-iodouridine-containing probes. Figure 4 summarizes the results, from which we can safely conclude that there is no effect of 5-iodouridine at any of the positions and irradiation conditions examined. The photoreactivity of the platinum moiety in cisplatin-modified duplexes is superior under our conditions to that of 5-iodouridine, which is generally regarded as an efficient cross-linking unit upon laser irradiation at 325 nm (*35*−*37*). No increased photo-cross-linking was observed in similar experiments using HMG1a (data not shown).

Flanking Sequence Effect on Photo-Cross-Linking. Recently, we investigated the effects of the sequences flanking the platination site on HMG-domain protein binding affinity (*17*, *18*). HMG1a prefers flexible A·T base pairs in the flanking positions. Thermodynamic investigations demonstrated the importance of flanking sequence in determining the thermodynamic stability of platinated duplexes (*38*).

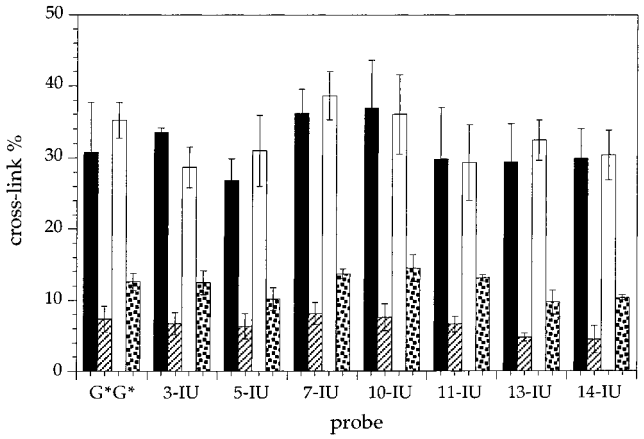


FIGURE 4: Comparison of the photo-cross-linking efficiency of DNA probes containing 5-iodouridine with HMG1b protein. Irradiation condition: filled bars, 325 nm for 30 min; striped bars, 325 nm for 5 min; open bars, 350 nm (500 mW) for 10 min; dotted bars, 350 nm (100 mW) for 10 min. Data points in all figures represent the results from three independent experiments, and the error bars represent ±1 estimated standard deviation.

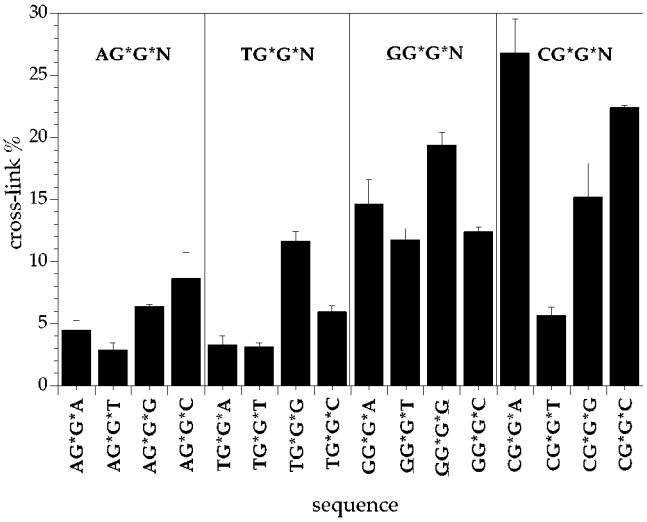


FIGURE 5: Effect of the flanking sequence on the photo-cross-linking efficiency of cisplatin-modified 15-bp probes indicated as $N_1G^*G^*N_2$ (see Table 1 for sequence) with HMG1b protein by irradiation at 325 nm for 30 min. Conditions: [DNA] = 5 nM; [HMG1b] = 285 nM. Data points in all figures represent the results from three independent experiments, and the error bars represent ±1 estimated standard deviation.

Therefore, as a further assessment of the factors that influence the platinum-induced laser photo-cross-linking reaction, we investigated whether the flanking sequence affects the photo-cross-linking efficiency.

The reaction mixtures (10 μL) for examining the dependence of flanking sequence on photo-cross-linking efficiency were identical to those employed in the previous EMSA study (*18*). Irradiation at 325 nm for 30 min revealed that local sequence perturbs the photo-cross-linking reaction in the manner depicted in Figure 5. As a general trend, flanking G·C base pairs results in better photo-cross-linking than A·T base pairs in both N_1 and N_2 positions, although the thermodynamic affinity of HMG-domain proteins for probes with flanking A·T base pairs is greater than for probes with flanking G·C base pairs (*17*, *18*).

Photo-Cross-Linking with Mutant Proteins. HMG1b photo-cross-links to cisplatin-modified DNA through its Lys6

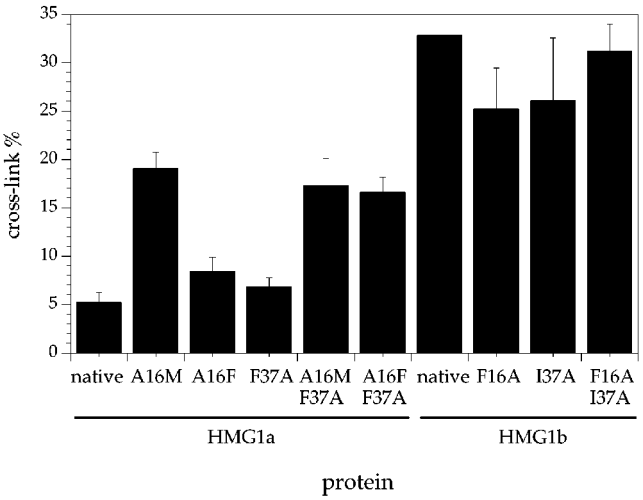


FIGURE 6: Photo-cross-linking experiment with mutant HMG-domain proteins using 325 nm light for 30 min. Data points in all figures represent the results from three independent experiments, and the error bars represent ±1 estimated standard deviation.

residue (*32*). A comparison of the N-terminal regions of HMG1a and HMG1b reveals that domain A lacks a potential intercalating residue in position 16 in helix I, where domain B has a phenylalanine (*23*, *24*). Intercalation by this residue alters the positioning of the HMG1 domain on cisplatin-modified DNA (*24*) and may facilitate movement of amino acid residues at the N-terminus into the major groove and promote photo-cross-linking. HMG-domain proteins have another intercalating amino acid residue in position 37, which is phenylalanine for HMG1a and isoleucine for HMG1b. Intercalation by Phe37 causes the domain to be offset toward the 3′-side of the platinated strand and significantly stabilizes the protein−DNA adduct (*23*, *24*).

To assess the role of amino acid residues that intercalate into the hydrophobic notch of the platinated DNA base pair, the following mutant proteins were subjected to laser photo-cross-linking: HMG1a-A16M, HMG1a-A16F, HMG1a-F37A, HMG1a-A16M/F37A, HMG1a-A16F/F37A, HMG1b-F16A, HMG1b-I37A, and HMG1b-F16A/I37A. The results (Figure 6) show that mutation of Ala16 to Met in domain A (HMG1a-A16M) increases the photo-cross-linking efficiency by about 4-fold relative to that of native HMG1a. Substitution of phenylalanine in this position (HMG1a-A16F) has a small effect, possibly due to steric crowding, as suggested from EMSA experiments and molecular modeling (*24*). Deletion of phenylalanine at position 37 does not alter the photo-cross-linking efficiency, because the HMG1a-F37A mutant still forms cross-links. In contrast, no interaction is observed in EMSA experiments for this mutant (*23*). The domain B mutants exhibit negligible sensitivity to the identity of the amino acid residues in both intercalating positions for photo-cross-linking.

Photo-Cross-Linking of Other Platinum−DNA Adducts. Having demonstrated the detection threshold of the cisplatin-mediated DNA−protein photo-cross-linking to be different from that of EMSA assays, we were interested in extending our studies to other platinum lesions, such as G*TG* or interstrand cross-links formed by cisplatin or *trans*-DDP. EMSA experiments performed prior to photo-cross-linking indicate that 15-bp duplexes containing cisplatin 1,3-intrastrand d(GpTpG) (*cis*-G*TG*, Table 1), cisplatin interstrand

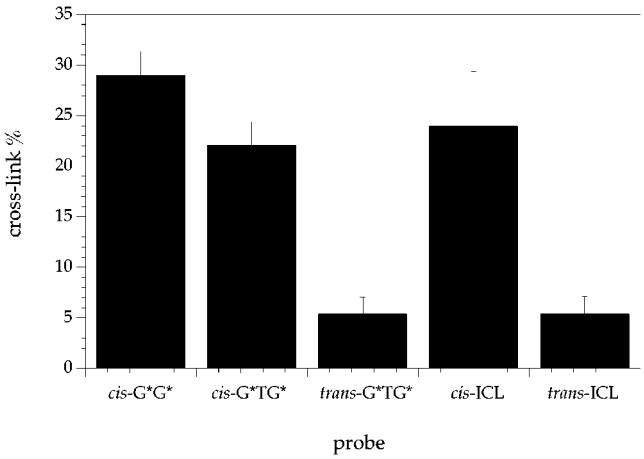


FIGURE 7: Comparison of the photo-cross-linking efficiency of the platinated 15-bp duplex modified by cisplatin or *trans*-DDP with HMG1b protein by laser irradiation at 325 nm for 30 min. Data points in all figures represent the results from three independent experiments, and the error bars represent ±1 estimated standard deviation.

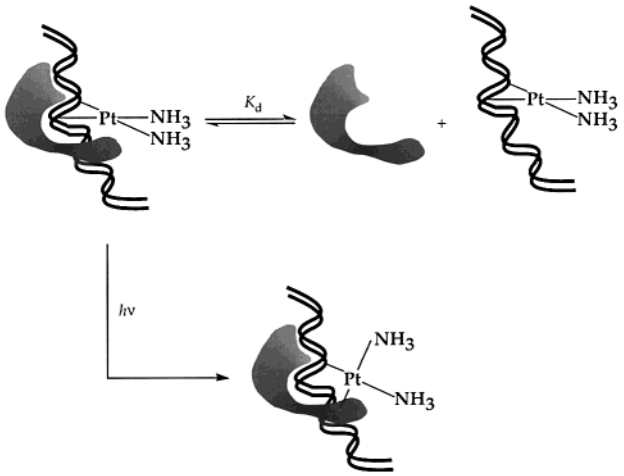
(*cis*-ICL), *trans*-DDP 1,3-intrastrand d(GpTpG) (*trans*-G*TG*), and *trans*-DDP interstrand (*trans*-ICL) cross-links do not form stable complexes with HMG1b as revealed under standard EMSA assay conditions (Figure S2, Supporting Information). In contrast, photo-cross-linking (Figure 7) clearly reveals that *cis*-G*TG* as well as *cis*-ICL forms cross-links with HMG1b upon irradiation, whereas significantly fewer DNA−protein cross-links are formed by the *trans*-DDP derivatives. The platinum moiety in *trans*-DDP-modified duplex DNA is photoreactive, as demonstrated by laser irradiation of the DNA probes in the absence of protein. Loss of platinum from DNA and the formation of DNA−DNA interstrand cross-links upon laser irradiation were identical for *trans*-G*TG* and *cis*-G*TG*. Duplex DNA having the site-specific *cis*-G*G* adduct formed more deplatinated DNA and fewer DNA−DNA interstrand cross-links (data not shown).

DISCUSSION

Laser-Induced Photo-Cross-Linking between HMG1 Domain Proteins and Cisplatin-Modified DNA. The mechanism of the photoreaction of platinum(II) bound to DNA in a protein complex has been discussed previously (*32*). Upon irradiation, a platinum−guanine bond dissociates to give a reactive intermediate (*39*), which then coordinates to a well-positioned amino acid residue in the HMG-domain protein. DNA−DNA interstrand cross-links also form when the irradiation is performed with 302 nm light, possibly because the base in the opposite strand is similarly activated by the broad dispersion of the irradiation wavelength of the transilluminator bulb as a light source. The 325 nm laser irradiation employed in the present study significantly suppressed this undesired side reaction. This finding was very important for our further investigation of laser-induced photo-cross-linking reactions.

Laser irradiation at 350 nm also affects specific platinum-mediated photo-cross-linking with HMG1b, although increased power is necessary for high efficiency. The time course of photo-cross-linking with the 350 nm laser at various laser powers (Figure 3) reveals that the yield of the cross-linked products depends on both laser power and irradiation

Scheme 1



time. High laser power significantly cleaves the cross-link, and it might be possible that such would occur even with 100 mW laser irradiation for longer time periods. Considering that the photo-cross-linking efficiency of irradiation at 350 nm for 100 mW power is comparable to that at 325 nm for 2.5 mW irradiation, a 25 nm wavelength difference in the irradiation source corresponds to an approximately 40-fold difference in photo-cross-linking efficiency. The temperature in the reaction tube during irradiation was monitored for both 325 and 350 nm irradiation (Figure S3, Supporting Information). The temperature of the reaction mixture after 30 min irradiation by 350 nm laser at 900 mW was around 70 °C, which may contribute to the dissociation of the DNA−protein cross-link.

It has previously been considered that HMG-domain protein-bound oligodeoxyribonucleotides may exist as a complex aggregate (*24*). One possible consequence of such aggregation is that the mechanism of photo-cross-linking might involve intercomplex cross-link formation, in which the photoreaction occurs between adjacent complexes. Such reactions should be affected by changes in the concentration of the complex. We therefore examined the photo-cross-linking reaction in the 10−160 nM duplex concentration range in the presence of 10 equiv of HMG1b. EMSA showed no detectable change in percent complex formation in this concentration range (data not shown). After irradiation at 325 nm for 30 min, no detectable concentration-dependent difference in photo-cross-linking efficiency was observed (data not shown). Although more extensive investigations are required to elucidate the precise reaction pathway, the absence of any dependence of the photo-cross-linking efficiency upon concentration strongly argues against an intercomplex mechanism.

Figure 2 shows that HMG1b is more effective at photo-cross-linking than HMG1a. Since this result does not reflect the affinity of protein binding to cisplatin-modified DNA, photo-cross-linking efficiency is less sensitive to the thermodynamic equilibrium constant than is an EMSA. Such a result may be understood by considering that, even if DNA−protein binding is weak (large K_d), the formation of a DNA−protein covalent bond by photoirradiation is irreversible, driving the equilibrium toward complex formation (Scheme 1). Thus, photo-cross-linking effectively detects weak and transient DNA−protein interactions. The more efficient

photo-cross-linking by HMG1b also reflects the greater flexibility of its noncovalent complex with platinated DNA and more symmetric positioning in the minor groove opposite to the platinum adduct (*24*). The latter is located in the major groove of the duplex, whereas the protein binds largely in the minor groove. Dynamic motion is required for the N- (or C-) terminal region of the protein to react with the photoactivated platinum species, a property of the more weakly and centrally positioned HMG1b. The poor photo-cross-linking ability of HMG1a may also be related to the lack of a suitable amino acid residue (Lys6 for HMG1b) at the appropriate location.

Reactivity of the Cisplatin-Derived Moiety Compared to 5-Iodouridine. 5-Iodouridine is an efficient reagent for photo-cross-linking of nucleic acids to associated proteins (*35*). Homolysis of the carbon−halogen bond is induced by laser irradiation at 325 nm (*36*, *37*), producing the uridin-5-yl radical in the vicinity of aromatic residues of a DNA-bound protein (*35*). Recently, 5-iodouracil was employed to map the binding site of repair proteins to a cisplatin 1,3-intrastrand cross-link (*40*). In the present study, each thymine base in the G*G* oligodeoxyribonucleotide probe was replaced by 5-iodouridine to afford seven IU probes with different 5-iodouridine locations (Table 1). The reactivity of the IU probes with HMG1b (Figure 4) indicates that 5-iodouridine does not increase the photo-cross-linking efficiency of platinated DNA for HMG-domain proteins. The absence of a 5-iodouridine effect under the present conditions illustrates the greater reactivity and generality of cisplatin as a DNA−protein photo-cross-linking reagent. A uridin-5-yl radical buried in the major groove may be less accessible to the protein than the cisplatin moiety linked to the guanine N7 sites.

Factors That Govern the Photo-Cross-Linking Efficiency. Figures 5 and 6 demonstrate that weakly bound and centrally positioned protein complexes of platinated DNA are preferred substrates for efficient photo-cross-linking in related systems. The data indicate that photo-cross-linking can capture even loose or transient interactions between the platinated DNA duplex and protein (Scheme 1). The presence of A·T base pairs flanking the platinated d(GpG) affords tight binding of the protein to DNA (*18*). The intercalating phenylalanine at position 37 fixes the protein to the DNA in an offset manner that does not facilitate protein−DNA covalent bond formation as mediated by the platinum atom upon photolysis (Figure 8). Because the reaction site is located in the major groove of the duplex and photo-cross-linking is afforded by Lys6 in HMG1b, wrapping of the N-terminus (or C-terminus) around the sugar−phosphate backbone would appear to be a prerequisite for photo-cross-linking. Tight, offset binding of the protein in the minor groove, as found by structural and mutagenesis studies (*24*), might prevent the movement necessary for HMG1a to access the major groove and bond to the photoactivated platinum atom. The single-stranded, cisplatin-modified 1,2-intrastrand d(GpG) oligodeoxyribonucleotide does not form protein−DNA cross-links (data not shown). The lack of photo-cross-linking for this single-stranded G*G* oligodeoxyribonucleotide strongly implies that platinum-mediated photo-cross-linking under the present conditions is due to cisplatin-specific interactions between the platinated duplex and the minor groove-binding HMG-domain proteins.

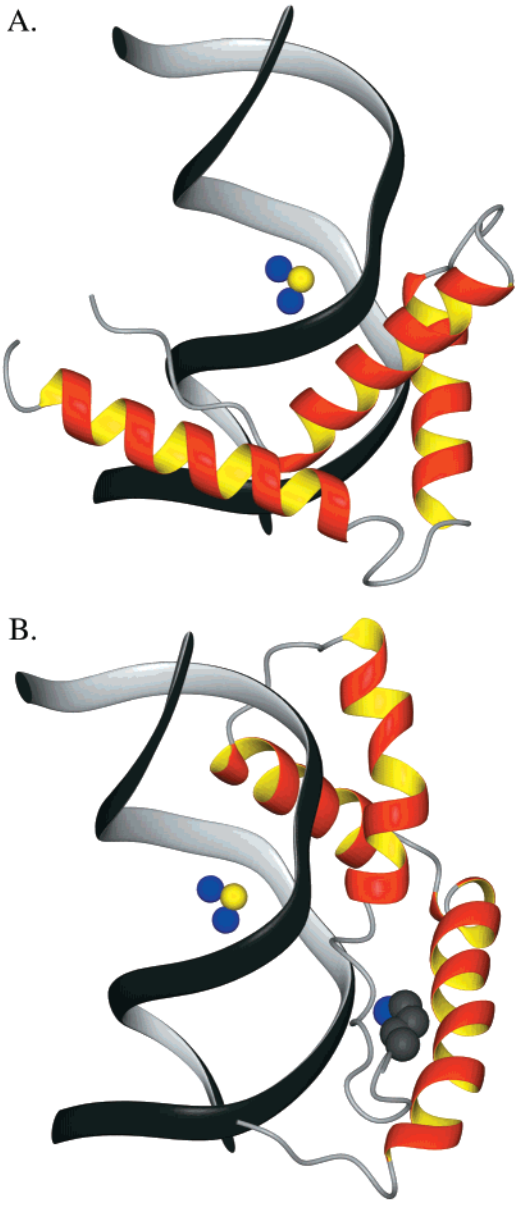


FIGURE 8: Comparison of HMG-domain binding modes. (A) Portrayal of HMG1a bound to cisplatin-modified (yellow sphere, Pt; blue spheres, NH_3) DNA drawn from the crystal structure coordinates (*23*). (B) Model for HMG1b bound to cisplatin-modified DNA (*24*). The side chain of Lys6, which forms a photo-cross-link to platinum, is depicted. Figures were prepared with the program MOLMOL (*43*).

The data presented here are consistent with the notion that loosely, centrally bound protein is most efficiently photo-cross-linked to platinated DNA. The difference in photo-cross-linking efficiency between HMG1a and HMG1b may also be attributed to the lack of a suitable amino acid at the proper location in HMG1a (e.g., Lys6), however. It is interesting to note that this residue plays a crucial role for DNA bending by HMG1b, as revealed in a ligase-mediated circularization assay (*41*).

Photo-Cross-Linking versus EMSA: The Value of a Kinetic Trap. In the previous section we reported significant DNA−protein photo-cross-link formation even for mutant proteins that did not exhibit distinct complexes with cisplatin-modified DNA in EMSAs. Although the EMSA is widely used to investigate noncovalent interactions between proteins

and DNA, it is in some cases sensitive to temperature, the presence of competitors, and other environmental conditions. We therefore examined the complexation of cisplatin-modified DNA with HMG-domain proteins at room temperature and in the presence of competitor DNA by means of both photo-cross-linking and the EMSA. Protein−platinated DNA complex formation was readily revealed by photo-cross-linking at room temperature or in the presence of chicken erythrocyte competitor DNA under conditions where no complex could be observed by EMSA (data not shown). Also, photo-cross-linking was less sensitive to high salt concentrations (∼400 mM NaCl) than EMSA (data not shown). Whereas EMSA visualizes thermodynamically stable complexes, cisplatin-mediated photo-cross-linking can extract DNA−protein complexes continuously from the pool of material in thermodynamic equilibrium (Scheme 1). Accordingly, only photo-cross-linking can reveal weak, loose, dynamic, or transient interactions of HMG-domain proteins with cisplatin-modified duplexes such as those bearing a *cis*-G*TG* or *cis*-ICL adduct (Figure 7). The interstrand cross-link formed by cisplatin has been reported previously to be recognized by HMG1 with affinities similar to that of the cisplatin-modified 1,2-intrastrand d(GpG) cross-link (*34*). A recent paper reported that HMG1 and HMG1a do not bind to interstrand cross-links (*42*). In the present study, HMG1b also does not exhibit a distinct gel shift in the presence of interstrand cross-linked DNA in a conventional EMSA (Figure S2). Upon photo-cross-linking, however, interstrand cross-linked DNA does form an adduct with the HMG-domain protein. Photo-cross-linking is thus a useful tool, which is complementary to conventional EMSA.

Photo-cross-linking can provide information about reactive amino acid residues that are in close contact with the platinum atom in a protein−DNA complex, obviating the need to prepare protein mutants (*32*). By contrast, EMSA requires comparison of the results between wild type and at least one mutant protein to provide information about the interacting residues. Given that cisplatin bound to DNA has considerable reactivity in protein−DNA photo-cross-linking, the laser-induced methodology described here provides a new approach for the investigation of protein−platinated DNA complexes under wide variety of conditions.

CONCLUSION

Laser-induced photo-cross-linking of cisplatin-modified DNA affords an efficient method for investigating DNA−protein interactions in isolated systems. The photo-cross-linking efficiency profile derived by different protein domains, protein mutants, and DNA probes reveals the characteristics of the photo-cross-linking reaction. Binding interactions between HMG-domain proteins and platinated DNA that are symmetrically positioned and flexible are preferentially detected because the photoreaction occurs in the major groove of DNA, whereas the proteins are positioned mainly in the minor groove. The cross-linking occurs even at room temperature, as well as under heterogeneous conditions in the presence of a DNA competitor or under high salt conditions. This property allowed us to visualize the interaction between HMG-domain proteins and cisplatin-modified duplexes containing the minor *cis*-G*TG* or *cis*-ICL adducts, which otherwise remain undetected. Although photo-cross-linking is relatively insensitive to protein−DNA affinity, the methodology should be applicable for identifying factors in the cell that respond to and bind cisplatin−DNA adducts, providing a new tool for investigating the anticancer mechanism of cisplatin. The differential ability of cisplatin- and *trans*-DDP-modified duplexes to be recognized by HMG1b, discovered by the new "photochemical trap", is a new finding that may be related to effectiveness of cisplatin as an anticancer drug. The application of this method to identify new proteins that interact with cisplatin-modified DNA in cell extracts is currently in progress.

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SUPPORTING INFORMATION AVAILABLE

A table of ESI-MS data for platinated oligodeoxyribonucleotides and three figures showing EMSAs for platinated, 5-iodouridine-containing duplexes with HMG1b and temperature changes during the laser irradiation. This material is available free of charge via the Internet at http://pubs.acs.org.

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