Homodinuclear (Pt,Pt) and Heterodinuclear (Ru,Pt) Metal Compounds as DNA–Protein Cross-Linking Agents: Potential Suicide DNA Lesions†

Bennett Van Houten,*,‡,§,⊥ Sharon Illenye,‡ Yun Qu,∥,# and Nicholas Farrell*,§,∥,#

Departments of Pathology, Biochemistry, Microbiology and Molcular Genetics, and Chemistry, Vermont Cancer Center, Burlington, Vermont 05405

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ABSTRACT: Homodinuclear (Pt,Pt) and heterodinuclear (Ru,Pt) metal compounds having the generalized formula M(a)$NH_2(CH)_4NH_2$M(b) are shown to form specific DNA lesions which can efficiently cross-link proteins to DNA. In this study, the homodinuclear case is represented by M(a) = M(b) = [*cis*-$Pt(Cl_2)(NH_3)$], and the heterodinuclear case is represented by M(a) = [*cis*-$RuCl_2(DMSO)_3$] and M(b) = [*cis*-$PtCl_2(NH_3)$]. Native and denaturing polyacrylamide gel electrophoresis was used to show the formation of ternary coordination complexes between the metal-treated 49-bp DNA fragment and the *Escherichia coli* UvrA and UvrB DNA repair proteins. Treatment with proteinase K results in loss of the DNA–protein cross-links. DNA–protein cross-links formed between UvrA and DNA previously modified with the dinuclear metal compounds are reversible with the reducing agent β-mercaptoethanol. The DNA lesion responsible for efficient DNA–protein cross-linking is most probably a DNA–DNA interstrand cross-link in which each metal atom is coordinated with one strand of the DNA helix. The formation of DNA repair protein associated DNA cross-links, potential "suicide adducts", suggests a novel action mechanism for these anticancer compounds. In addition, these dinuclear metal compounds should be very useful agents for the investigation of a wide range of protein–DNA interactions.

Dinuclear platinum compounds (Pt,Pt)-2,2,[1] containing two platinum–amine units linked by a variable-length diamine chain ([{*cis*-$PtCl_2(NH_3)$}$_2H_2N(CH_2)_4NH_2$], and see Figure 1), display (1) unique cytotoxicity in both murine and human tumor cell lines rendered resistant to cisplatin and (2) a different spectrum of antitumor activity in comparison to the monomeric cisplatin (Farrell, 1989; Hoeschele et al., 1990; Manzotti et al., 1992; Kraker et al., 1992). These (Pt,Pt) compounds have been found to inhibit DNA synthesis in a manner analogous to cisplatin (Farrell, 1989) and display similar cell cycle effects as cisplatin, implying a role in DNA binding in their mechanism of action.

(Pt,Pt)-2,2 has been found to induce structurally different DNA adducts in comparison to cisplatin. These include interstrand cross-links in which each strand of the helix is linked to one platinum (Roberts et al., 1989; Farrell et al., 1990a,b) and intrastrand cross-links (Bloemink et al., 1992) in which each platinum is attached to adjacent purines on the same strand. For example, (Pt,Pt) compounds produce 250-fold more interstrand cross-links than the parent cisplatin (Roberts et al., 1989). Furthermore, (Pt,Pt) compounds react at other DNA sequences such as GCGC in addition to cisplatin reactive sites such as AG and GG (Farrell et al., 1990b). Finally, (Pt,Pt)-1,1 ([{*trans*-$PtCl(NH_3)_2$}$_2H_2N(CH_2)_4NH_2$]-Cl_2; Figure 1), with monodentate coordination spheres, has been shown to stabilize DNA in the left-handed Z-like conformation (Johnson et al., 1992).

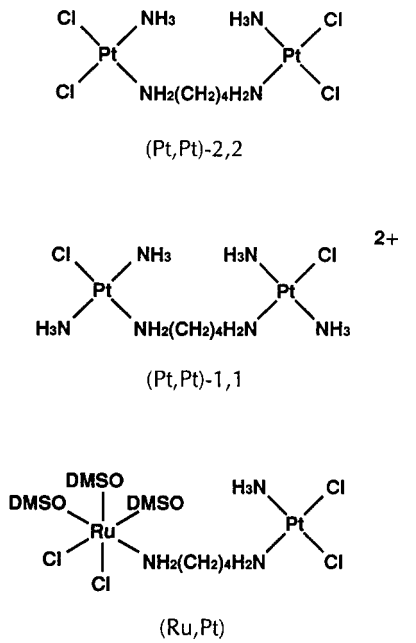

FIGURE 1: Structures of the compounds used in this study. These metal compounds have the generalized formula M(a)NH_2RNH_2M(b), where M(a) and M(b) represent the coordination spheres of the two metals involved and R is a variable chain length linker $-(CH_2)_n-$. In this study the homodinuclear case is represented by M(a) = M(b) = [*cis*-$Pt(Cl_2)(NH_3)$], and the heterodinuclear case is represented by M(a) = [*cis*-$RuCl_2(DMSO)_3$] and M(b) = [*cis*-$PtCl_2(NH_3)$]. R = $-(CH_2)_4-$ in both cases. (Ru,Pt) = [{*cis*-$RuCl_2(Me_2SO)_3$}-$H_2N(CH_2)_4NH_2$}{*cis*-$PtCl_2(NH_3)$}]; (Pt,Pt)-2,2 = [{*cis*-$PtCl_2$-(NH_3)}$_2H_2N(CH_2)_4NH_2$]; (Pt,Pt)-1,1 = [{*trans*-$PtCl(NH_3)_2$}$_2$-$H_2N(CH_2)_4NH_2$]Cl_2.

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‡ Department of Pathology.
§ Department of Biochemistry.
⊥ Department of Microbiology and Molecular Genetics.
∥ Department of Chemistry.
Present address: Department of Chemistry, Virginia Commonwealth University, Box 2006, 1001 West Main St., Richmond, VA 23284.

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[1] Abbreviations: bisacrylamide, *N,N'*-methylenebisacrylamide; BSA, bovine serum albumin; *cis*-DDP, cisplatin, *cis*-diamminedichloroplatinum-(II); DATD, *N,N'*-diallyltartardiamide; DMSO, dimethyl sulfoxide; EDTA, ethylenediaminetetraacetic acid, FAAS, flameless atomic absorption spectroscopy; PA, polyacrylamide; PAGE, polyacrylamide gel electrophoresis; Pt, platinum; (Pt,Pt)-1,1, [{*trans*-$PtCl(NH_3)_2$}$_2H_2N(CH_2)_4$-NH_2]Cl_2; (Pt,Pt)-2,2, [{*cis*-$PtCl_2(NH_3)$}$_2H_2N(CH_2)_4NH_2$]; (Ru,Pt), [{*cis*-$RuCl_2(Me_2SO)_3$}$H_2N(CH_2)_4NH_2${*cis*-$PtCl_2(NH_3)$}]; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane.

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Both antitumor activity and DNA binding activity vary with alterations of the backbone linker (chain length and steric effects), coordination sphere (one or two leaving groups), and coordination geometry (Farrell, 1993). In a model study, we have shown that dinuclear metal compounds with inequivalent coordination spheres may be designed that are capable of selective reaction at one coordination sphere (Qu et al., 1992). An extension of this concept is to introduce a second, less reactive metal as the other coordination sphere. Indeed, (Pt,-Pt) may be considered as only one specific example of a new class of antitumor agents based on the dinuclear metal structure.

To this end we have synthesized a heteronuclear compound containing both platinum and ruthenium, (Ru,Pt) ([{*cis*-$RuCl_2(Me_2SO)_3$}$H_2N(CH_2)_4NH_2${*cis*-$PtCl_2(NH_3)$}], and see Figure 1). Ruthenium was chosen as the second metal for a number of reasons. First, ruthenium compounds are of interest because of their antitumor activity (Clarke, 1989; Mestroni et al., 1989; Keppler et al., 1989; Farrell, 1989). Secondly, the octahedral coordination sphere of Ru is sterically more demanding than that of square-planar Pt(II), and this property may provide greater sequence specificity as well as enhanced kinetic control over the types of DNA adducts formed. A further consideration is that Ru compounds have been used extensively to site-specifically label proteins involved in electron-transfer reactions (Bowler et al., 1990). Since, in general, Ru compounds are less reactive than Pt compounds, the combination may be used to impart reactivity to particular DNA sequences and to facilitate cross-linking of unique proteins with DNA.

Ternary protein–platinum–DNA complexes represent only a very small proportion of cisplatin adducts and are not considered to contribute significantly to its cytotoxicity (Zwelling et al., 1979). The recent identification of proteins which interact specifically with cisplatin-damaged DNA in both bacterial cells [reviewed in Van Houten (1990) and Van Houten and Snowden (1993)] and mammalian cells (Billings et al., 1992; Donahue et al., 1990; Hughes et al., 1992; Pil & Lippard, 1992) and our studies on (Pt,Pt)–DNA interactions suggested that the tetrafunctional nature of these compounds could lead to a higher degree of DNA-protein ternary complex formation as compared to the parent cisplatin. Accordingly, we set out to examine this possibility with homodinuclear (Pt,Pt) and heterodinuclear (Ru,Pt) compounds, Figure 1.

One candidate class of proteins which has the potential to be cross-linked to metal–DNA adducts are DNA repair enzymes. Previous experiments have shown that the *Escherichia coli* UvrABC nuclease system will incise DNA which has been treated with (Pt,Pt) compounds (Roberts et al., 1989). The UvrABC nuclease system acts to initiate and remove many different types of DNA adducts in the process of nucleotide excision repair (Van Houten, 1990). The UvrA and UvrB proteins form a $UvrA_2B$ complex in solution (Orren & Sancar, 1989, 1990). This trimeric protein complex, in an ATP-requiring reaction, has been shown to travel along DNA with a limited helicase activity (Koo et al., 1991). It is believed that this $UvrA_2B$ complex first pauses upon encountering a DNA lesion and then undergoes a reorganization of the nucleoprotein complex which is dependent upon the structure of the DNA lesion (Van Houten & Snowden, 1993). Only those DNA lesions which significantly distort the conformation of the DNA helix lead to the dissociation of the $UvrA_2$ dimer, resulting in a long-lived UvrB–DNA complex (Orren & Sancar, 1990; Visse et al., 1992; Van Houten & Snowden, 1993). The UvrB–DNA complex acts as a binding signal for the UvrC protein, which facilitates dual incisions of the phosphodiester backbone bracketing the DNA lesion (Lin & Sancar, 1992a,b).

This paper reports on our studies showing that the *E. coli* UvrA– and UvrB–DNA repair proteins are efficiently cross-linked to DNA modified by either dinuclear metal compound. In the homodinuclear (Pt,Pt) case, the effect of the number of overall potential binding sites was studied by comparing the cross-linking efficiency of bifunctional (Pt,Pt)-1,1 or tetrafunctional (Pt,Pt)-2,2 compounds (Figure 1).

MATERIALS AND METHODS

Chemical Synthesis and Characterization. The (Pt,Pt) compounds [{*cis*-$PtCl_2(NH_3)$}$_2H_2N(CH_2)_4NH_2$] and [{*trans*-$PtCl(NH_3)_2$}$_2$($H_2N(CH_2)_4NH_2$)]Cl_2 were prepared according to literature procedures (Farrell, 1989; Farrell et al., 1990a,b). The detailed synthesis and chemical properties of the (Ru,Pt) compound [{*cis*-$RuCl_2(Me_2SO)_3$}$H_2N(CH_2)_4NH_2${*cis*-$PtCl_2$-(NH_3)}] will be reported separately. A dinuclear compound with inequivalent coordination spheres must be prepared by a linking reaction where one metal center, the precursor, is bound to a "dangling" amine and the second metal center acts as target for the free amine functionality of the precursor molecule (Qu et al., 1992, 1993). The linking was achieved by preparing a dangling amine on the Ru atom using a blocked diamine followed by acidification to produce a free RNH_3^+ group. The precursor was synthesized by the following scheme:

[*cis*-$RuCl_2(DMSO)_4$] + $NH_2(CH_2)_4NH$*t*-Boc →
[*cis*-$RuCl_2(DMSO)_3(NH_2(CH_2)_4NH$*t*-Boc)]

[*cis*-$RuCl_2(DMSO)_3(NH_2(CH_2)_4NH$*t*-Boc)] →
[*cis*-$RuCl_2(DMSO)_3(NH_2(CH_2)_4NH_3Cl)$]

The linking was then achieved through reaction with an appropriate Pt target molecule:

[{*cis*-$RuCl_2(DMSO)_3$}$(NH_2(CH_2)_4NH_3Cl)$] +
$K[PtCl_3(NH_3)]$ →
[{*cis*-$RuCl_2(DMSO)_3$}$H_2N(CH_2)_4NH_2${*cis*-$PtCl_2(NH_3)$}]

The compound was characterized by C,H,N elemental analysis, IR, and ^1H and ^{195}Pt NMR spectroscopy (supplementary material). All compounds used in this study (Figure 1) were dissolved in H_2O and stored at −20 °C.

Enzymes. All restrictions and DNA-modifying enzymes were purchased from BRL unless otherwise noted. The UvrA and UvrB subunits were purified from *E. coli* overproducing strain CH296 containing plasmids pUNC45 and pUNC211, respectively (obtained from A. Sancar, University of North Carolina). The purification procedure was as described previously (Sancar et al., 1987).

Oligonucleotides and Preparation of Substrates. The sequence of the 49-bp duplex used in this study is shown below:

5'-GACTACTTGGTACACTGACGCGAGCTCGCGGAAGCTCATTCCAGTGCGC-3'

3'-CTGATGAACCATGTGACTGCGCTCGAGCGCCTTCGAGTAAGGTCACGCGG-5'

DNA duplexes were prepared as described previously (Snowden et al., 1990). Briefly, oligonucleotides were labeled at the 5'-end with T4 polynucleotide kinase and ATP-γ-[^{32}P] using standard techniques. DNA duplexes were prepared by mixing 10 μg of either the 5'-end-labeled 49mer or 10 μg of the labeled 50-base complement, respectively, in a buffer containing 10 mM Tris-Cl, pH 7.5, 1.0 mM EDTA, and 10 mM NaCl (TEN buffer). The mixture was then heated to 90 °C for 5 min, followed by slow cooling to room temperature

to allow annealing of the two strands. The duplex DNA was purified by polyacrylamide gel electrophoresis (PAGE).

Dinuclear Metal Binding to DNA. The dinuclear metal compounds were incubated in a reaction (100–500 μL) containing the 5′-labeled 49-bp duplex (20–100 ng) and sodium perchlorate (10 mM) at 37 °C for 1–2 h as indicated in the figure legends. Reactions were stopped by the addition of 0.10 volume of 1 M sodium chloride. Excess unreacted compound was removed by microdialysis against 25 mM sodium perchlorate for 2 h at 4 °C. To determine the amount of metal binding, calf thymus DNA was treated under similar conditions, and the amount of bound platinum was determined by FAAS. Both compounds displayed similar binding profiles, with a 2-h (Ru,Pt) or (Pt,Pt) treatment (1 μM) producing 1–2 adducts/49 bp.

DNA–Protein Cross-Linking Reactions. Two reaction conditions were used for the interaction of the Uvr proteins with the modified 49-bp duplex. One reaction condition (Buffer A) contained sodium perchlorate (25 mM), BSA (1.2 μM), the modified 49-bp duplex (4 nM in duplex), and the indicated concentrations of UvrA or UvrB. The other reaction condition (Buffer B) contained the indicated concentrations of UvrA and UvrB, Tris-Cl (pH 7.5, 50 mM), ATP (2 mM), $MgSO_4$ (10 mM), BSA (1.2 μM), and the modified 49-bp duplex (4 nM in duplex). Both reaction were incubated for 2 h at 37 °C and stopped by making 0.1 M in sodium chloride. The DNA–protein complexes were analyzed by polyacrylamide gel electrophoresis (PAGE) under two different conditions: (1) nondenaturing gel mobility shift assays, (Carey, 1991), which detects proteins that are associated with the DNA, and (2) denaturing PAGE (Laemmli, 1970), which was adapted for the detection of protein–metal–DNA ternary coordination complexes.

Mobility shift assays were performed as described previously (Van Houten & Snowden, 1993). Briefly, protein-binding reactions were diluted with glycerol (10% v:v) and loaded onto a 4% PAG (80:1, acrylamide:bisacrylamide) containing Tris–borate (pH 8.0, 50 mM Tris, 50 mM boric acid), EDTA (1.4 mM), ATP (1 mM), and $MgCl_2$ (10 mM). The samples were electrophoresed at room temperature and constant current (~120 V, 30 mA), for 4–5 h.

The analysis of DNA–protein cross-links was performed as described by Laemmli (1970) with the following modifications. Samples were mixed 1:1 with SDS cracking buffer containing Tris-Cl (pH 6.8, 0.03 M), SDS (1.54%), and glycerol (6%), heated for 5 min at 95 °C, and analyzed using two different PAGE systems. Denaturing PAGE system 1 contained a 3% polyacrylamide (PA), 0.3% DATD (*N,N′*-diallyltartardiamide) stacking gel and a 12% PA, 1.2% DATD running gel in a Tris–glycine–SDS running buffer. Denaturing PAGE system 2 contained a 4% PA, 0.4% bisacrylamine stacking gel and a 7.5% PA, 0.75% bisacrylamide running gel in a Tris–glycine–SDS running buffer. This latter gel system provided enhanced resolution of the high molecular weight DNA–protein complexes. Electrophoresis in both gel systems was performed at 100–200 V at 30 mA for 2.5 h. UvrA, UvrB, and protein standards (BRL, prestained standards) were loaded along with the DNA–protein cross-link reactions.

The analysis of interstrand DNA cross-links was performed on denaturing sequencing gels. Samples were mixed 1:1 (v:v) with formamide containing tracking dyes and heated to 95 °C for 5 min, cooled on ice, and electrophoresed at 25 mA and 1200–1400 V for 2 h on 8% polyacrylamide gels containing 8 M urea in Tris–borate (50 mM, pH 8), EDTA (1.4 mM) running buffer.

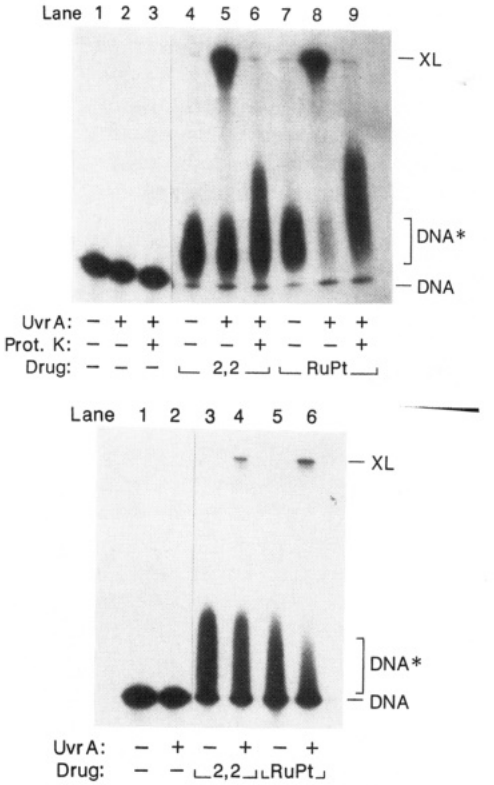

FIGURE 2: Formation and reversibility of DNA–protein cross-links. (Top) DNA duplex (4 nM) which had been reacted with (Pt,Pt)-2,2 (10 μM, 1 h) (lanes 4–6) or (Ru,Pt) (10 μM, 2 h) (lanes 7–9) compounds was mixed with UvrA (100 nM) in Buffer A (see Materials and Methods). Samples were treated with proteinase K (Prot. K) prior to electrophoresis using PAGE system 1. The gels were dried and subjected to autoradiography. DNA* corresponds to the DNA which had been treated with either drug, which has a slightly reduced mobility in this gel system. XL corresonds to slowly migrating DNA species which represents DNA–protein cross-links. (Bottom) Portions of samples were mixed with β-mercaptoethanol and boiled for 5 min prior to electrophoresis. Other symbols are the same as above.

Dried gels were exposed to X-ray film (Kodak XAR-5) overnight at −70 °C using an enhancing screen and, where appropriate, analyzed with a Betascope 603 blot analyzer (Betagen).

RESULTS

Cross-Linking of UvrA to DNA via Dinuclear Metals. The feasibility of metal-promoted DNA–protein cross-link formation was examined using the interaction of the *E. coli* UvrA repair protein with DNA modified by (Pt,Pt)-2,2 and (Ru,-Pt). UvrA was mixed with a radiolabeled 49-bp fragment which had been modified by these metal compounds, and the reaction product were analyzed on 12% polyacrylamide Laemmli gels run in the absence of β-mercaptoethanol (see Materials and Methods; Figure 2, top). To verify that the slowly migrating species was in fact due to a DNA–protein cross-link, the products of UvrA reacting with (Pt,Pt)-2,2- or (Ru,Pt)-modified DNA were treated with proteinase K. As can be seen in Figure 2, top (lanes 6 and 9), proteinase K treatment results in the loss of the slowly migrating species (XL) (lanes 5 and 8). Furthermore, if the cross-linked samples are mixed with β-mercaptoethanol during the heating step prior to loading on the gel, the cross-linked band disappears (Figure 2, bottom).

Dinuclear Metal–DNA Complexes Efficiently Cross-Link UvrA and UvrB to DNA. Damage recognition by the UvrABC system appears to occur by a "specificity cascade" in which UvrA confers a 1000-fold enhanced binding specificity for

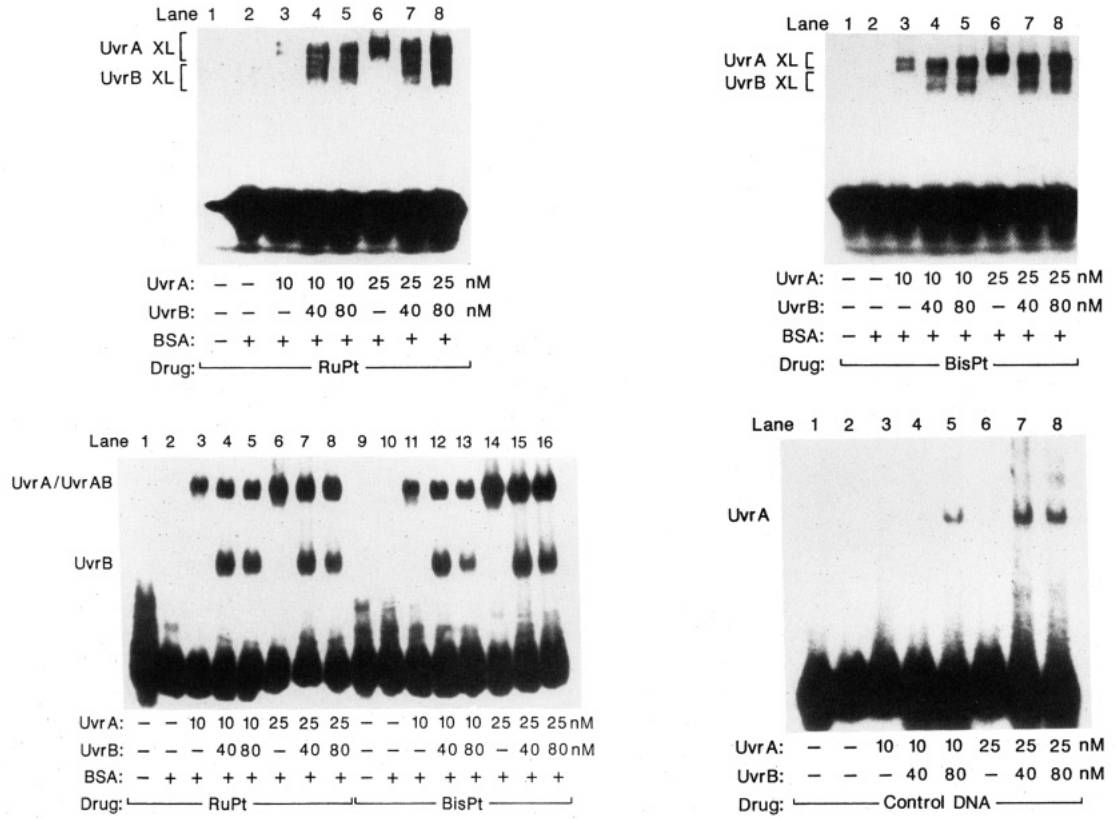

FIGURE 3: (Ru,Pt) and (Pt,Pt)-2,2 metal–DNA complexes used to efficiently cross-link UvrA and UvrB proteins to DNA. . (Top left) Denaturing PAGE analysis (system 2) of Uvr proteins cross-linked to the (Ru,Pt) compound. Indicated concentrations of UvrA and UvrB were mixed with (Ru,Pt)-damaged DNA [1 μM, 2 h (Ru,Pt) treatment] in Buffer B (see Materials and Methods). UvrA XL corresponds to the position where UvrA (104 kDa) attached to a 49-bp DNA (32.3 kDa) migrates. UvrB XL corresponds to the position where UvrB (76.1 kDa) attached to a 49-bp DNA duplex (32.3 kDa) would migrate. Stained protein standards were used to estimate the apparent molecular weights (not shown). (Top right) Denaturing PAGE analysis (system 2) of Uvr proteins cross-linked to the (Pt,Pt) compound. Indicated concentrations of UvrA and UvrB were mixed with (Pt,Pt)-damaged DNA (1 μM, 2 h (Pt,Pt) treatment) in Buffer B. Symbols are as above. (Bottom left) Nondenaturing gel mobility shift assay for Uvr intermediates bound to DNA damaged with the metal compounds. UvrA–, UvrAB–, and UvrB–DNA complexes were assigned on the basis of previous studies (Van Houten & Snowden, 1993). (Bottom right) Gel mobility assay for the interaction of the Uvr proteins with nondamaged DNA (BSA additions were as in the other three panels).

damaged DNA as compared to nondamaged DNA. In the presence of ATP, UvrB by interacting with UvrA appears to increase the binding specificity of the Uvr complex for damaged substrates and decreases binding affinity for nondamaged nucleotides (Van Houten & Snowden, 1993). During this discrimination step there appears to be a concomitant change in the nucleoprotein complex, in which UvrA dissociates from the damaged site, leaving a UvrB–DNA complex (Orren & Sancar, 1990; Visse et al., 1992; Van Houten & Snowden, 1993). Experiments performed in the presence of ATP demonstrated that either UvrA or UvrB can be cross-linked to DNA containing dinuclear metal lesions (Figure 3, top). Analysis of these DNA–protein cross-links by denaturing gel electrophoresis indicated that increasing concentrations of UvrA and UvrB lead to increased amounts of DNA–protein cross-linked material. This experiment also demonstrates the specificity of the reaction in that BSA (1.2 μM) is not cross-linked to the metal-treated DNA (compare lanes 1 and 2 in Figure 3, top left and top right). In addition, when UvrA and UvrB were heat-inactivated at 65 °C for 10 min prior to the addition of metal-treated DNA, no DNA–protein cross-links were observed (data not shown). These experiments imply that the metal-mediated DNA–protein cross-links are specific for active DNA-binding proteins.

Identical reactions as those presented in Figure 3, top, analyzed by nondenaturing gel mobility shift assays (Carey, 1991; Van Houten & Snowden, 1993) (Figure 3, bottom left) revealed that: (1) the UvrAB complex efficiently recognizes the 49-bp DNA complex which had been modified by either (Ru,Pt) or (Pt,Pt)-2,2 (lanes 1–8 and 9–16, respectively) and (2) a significant amounts of UvrB–DNA complexes are formed. We have recenty shown that the formation of UvrB–DNA complexes strongly correlates with efficient damage recognition and incision (Van Houten & Snowden, 1993). The nature of the UvrAB–DNA complexes and UvrB–DNA complexes were confirmed using an identical 49-bp DNA duplex containing a benzylamine-modified abasic site [data not shown, Van Houten and Snowden (1993)]. Under these conditions, little or no binding of UvrA and UvrB is seen with the undamaged 49-bp duplex (Figure 3, bottom right).

Taken together, these results indicate that: (1) the UvrA and UvrB protein make intimate contact as the damaged site and can be efficiently cross-linked to DNA via the metal compound and (2) the metal-mediated cross-linking is specific for DNA-binding proteins, and heat-inactivated DNA-binding proteins do not bind. Having shown that homo- and heterodinuclear metal compounds are DNA–protein cross-linking agents, we next sought to determine the type of DNA adduct which is responsible for the efficient cross-linking.

Polyfunctional Nature of the Homo- and Heterodinuclear Compounds Is Important for DNA–Protein Cross-Linking. Both the (Pt,Pt)-2,2 and the (Ru,Pt) dinuclear metal compounds are polyfunctional since more than one coordination site on each metal can be substituted. To examine the minimum structural requirement for DNA–protein cross-linking, we examined a (Pt,Pt) compound with one reactive site per platinum atom, (Pt,Pt)-1,1, [{*trans*-$PtCl(NH_3)_2$}$_2$-$H_2N(CH_2)_4NH_2$]Cl_2 (Figure 1). It is interesting to note that

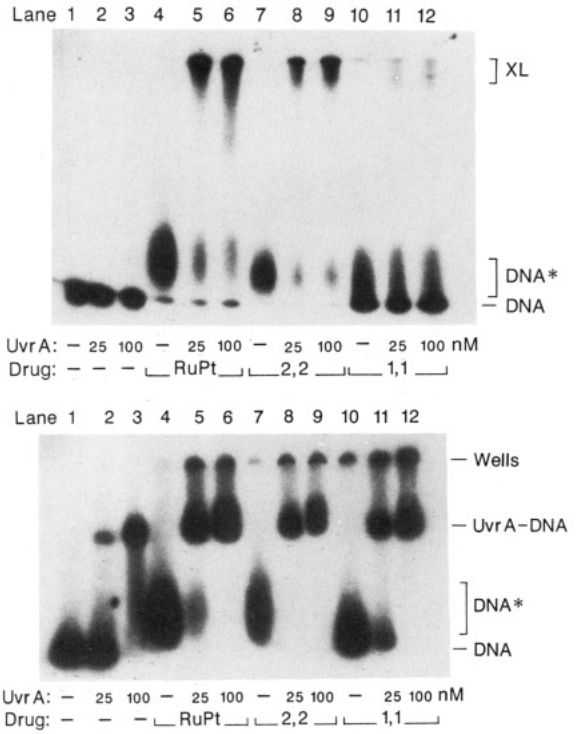

FIGURE 4: Effect of multifunctional dinuclear metal compounds on the cross-linking of UvrA to DNA. (Top) Denaturing PAGE (system 1) analysis of DNA–protein cross-links. Indicated concentrations of UvrA were incubated with DNA which had been treated with (Ru,-Pt) (10 μM, 2 h), (Pt,Pt)-2,2 (10 μM, 1 h), or (Pt,Pt)-1,1 (0.5 μM, 1 h), which yield approximately equal r_B, and mixed with indicated concentrations of UvrA for 2 h in Buffer A. (Bottom) Native gel mobility shift assay. A portion of the reactions described above were simultaneously assayed by native gel electrophoresis. Other symbols are as indicated in Figure 2.

DNA treated with the (Pt,Pt)-1,1 metal compound, which is formally bifunctional, forms few DNA–protein cross-links (Figure 4, top, lanes 10–12). These data suggest that, for the present compounds studied, potentially tri- or tetrafunctional compounds are most efficient at DNA–protein cross-linking. It was important, however, to show that the UvrA protein efficiently binds to DNA treated with (Pt,Pt)-1,1 compound.

A gel mobility shift assay (Fried & Crothers, 1981; Carey, 1991) was used to show that while the (Pt,Pt)-1,1–DNA adducts were efficiently recognized by the UvrA protein, very few DNA–protein cross-links were observed (Figure 4). For example, while 25 nM UvrA shifts over 90% of the (Pt,Pt)-1,1-modified DNA (Figure 4, bottom, lane 11), little cross-linking is observed Figure 4, top, lane 11). UvrA binds to the nondamaged 49-bp DNA duplex with much lower affinity as compared to the (Pt,Pt)-1,1-modified DNA (compare lanes 2 and 11, Figure 4, bottom; see also Figure 3, bottom right). From these experiments we conclude that a minimum of three coordination sites must be available in these dinuclear compounds for efficient DNA–protein cross-linking. In agreement, UvrA is very poorly cross-linked to DNA modified by either of the monomeric platinum compounds *cis*- and *trans*-DDP (data not shown), containing two leaving chlorides.

DNA–DNA Interstrand Cross-Links Are Efficient Substrates for DNA–Protein Cross-Linking. (Pt,Pt) compounds induce a significant number of DNA–DNA interstrand cross-links (Roberts et al., 1989). It was important to establish whether the (Ru,Pt) compound was also capable of inducing DNA–DNA interstrand cross-links and whether these adducts were responsible for mediating the covalent attachment of the Uvr proteins to DNA (Figure 5). In this experiment, the UvrA concentration was varied over a wide range (0–200

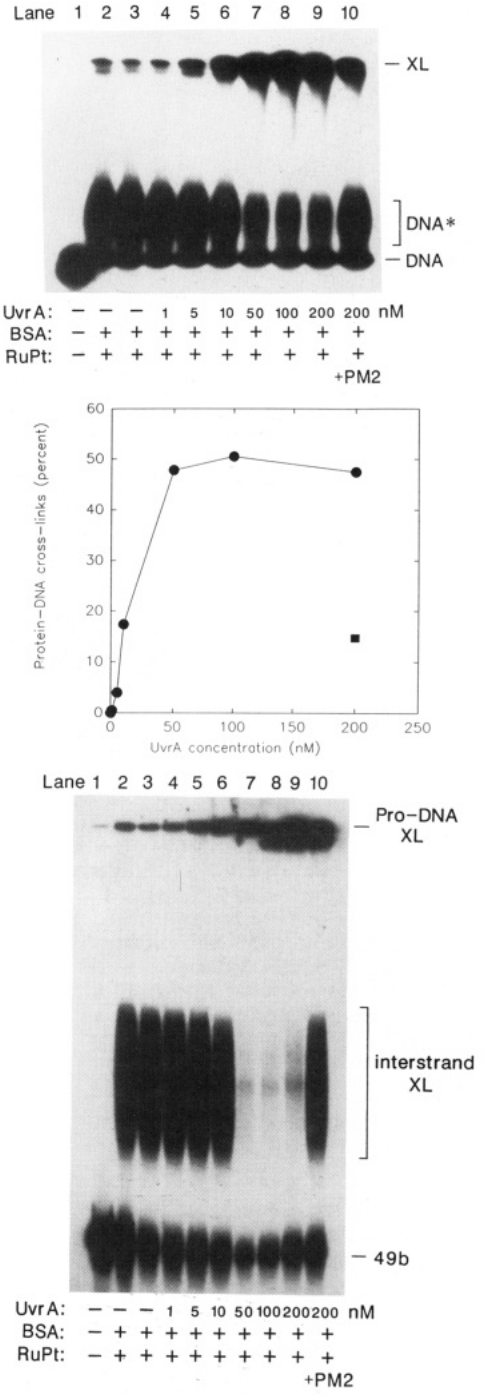

FIGURE 5: DNA–DNA interstrand cross-links capable of efficient DNA–protein cross-links. (Top) Denaturing PAGE (system 1) analysis of DNA–protein cross-links. Reactions containing (Ru,Pt) (10 μM, 2 h)-modified DNA were mixed with indicated amounts of UvrA in Buffer A and analyzed by gel electrophoresis. PM2 indicates the reaction products which contain 40 μM (in base pairs) nondamaged DNA. Lane 2 contains BSA which was added just prior to the addition of SDS-containing dyes, and lane 3 contains BSA which was added in Buffer A and incubated with DNA for 2 h. (Middle) Quantitation of DNA–protein cross-linking. The dried gel from above A was scanned by radioisotope imaging, and the total amount of radioactivity in each band was determined. The amount of DNA which migrated more slowly in the gel indicative of UvrA–DNA cross-links (XL) was used to give the percentage of DNA containing DNA–protein cross-links. DNA–protein cross-links are indicated in the absence (●) or presence (■) of PM2 DNA. (Bottom) DNA sequencing gel showing interstrand DNA–DNA cross-links. Portion of identical reactions were also analyzed on DNA sequencing gels (8% acrylamide, 8 M urea). Interstrand XL indicates DNA duplexes in which both strands are attached by the (Ru,Pt) compound; Pro–DNA XL indicates Uvr–DNA cross-linked material which remains in the wells. Due to precipitation during heating in formamide (prior to loading on the gel), not all of the DNA sample was recovered on the gel.

nM), and a fixed concentration of (Ru,Pt) (10 μM, 2 h) was used. The reaction products were analyzed by electrophoresis in either Laemmli gels (Figure 5, top) or sequencing gels (Figure 5, bottom). As expected, a dose-dependent increase in the amount of DNA–protein cross-links was seen with increasing concentrations of UvrA (Figure 5, middle). As can be seen in the bottom panel, there is a dose-dependent increase in DNA-protein cross-links which saturates at a UvrA concentration of 100 nM. The reaction in lane 10 (top panel) contained 40 μM (in base pairs) nondamaged PM2 DNA, a 10 000-bp, double-stranded, covalently closed, circular DNA molecule. UvrA binds to nondamaged DNA (as mole of bp) with about 1000-fold less efficiency than damaged DNA (Mazur & Grossman, 1991; Snowden & Van Houten, 1991; Van Houten & Snowden, 1993), and this amount of PM2 efficiently reduced cross-linking of UvrA to the modified 49-bp duplex by ~50%.

The bottom panel of Figure 5 is an autoradiograph of the same reactions analyzed on DNA sequencing gels which shows that a significant amount of the DNA duplex treated with (Ru,Pt) (10 μM) contains interstrand cross-links (lanes 2 and 3). Several species of DNA molecules run together as a fast migrating band (Figure 5, 49 bp); these include a 49-base strand which is either unmodified, attached by one metal only, or only attached by both metals on one strand only (intrastrand adduct) or (Ru,Pt). DNA which contains (Ru,Pt)-mediated interstrand cross-links migrates as a diffuse smear at approximately twice the molecular weight of the 49-base oligonucleotide. This DNA probably runs as a heterogeneous population due to the random positions of the interstrand cross-link (Roberts, et al., 1989; Van Houten, et al., 1986). As can be seen in lanes 4–9, with increasing amounts of UvrA the DNA containing interstrand cross-links disappears and a new band running at the top of the gel appears. It should be pointed out that at higher protein concentrations not all of the labeled DNA enters the sequencing gels. We believe that this is due to precipitation of the DNA–protein complexes which occurs during heating in formamide prior to loading of the gel. Additional experiments (data not shown) in which the concentrations of both UvrA and (Ru,Pt) were varied also indicated that UvrA was efficiently cross-linked to the portion of DNA containing (Ru,Pt) interstrand cross-links.

DISCUSSION

(Pt,Pt) compounds display antitumor activity in a range of murine and human tumor cells both in vitro and in vivo, including those rendered resistant to the parent monomeric compound, cisplatin. Physical chemistry and molecular biology studies have revealed that (Pt,Pt) compounds interact with DNA in ways which are inaccessible to cisplatin. The goal of this study was to examine the ability of potentially tetrafunctional homonuclear and heteronuclear metal compounds to mediate the formation of DNA–protein cross-links. To investigate this objective, the *E. coli* DNA repair proteins, UvrA and UvrB, were mixed with DNA fragments which had been treated with the metal compounds. As shown in Figures 3 and 4, these metal compounds efficiently cross-link the UvrA and UvrB proteins to a defined DNA fragment.

Characterization of DNA–Protein Cross-Links Induced by Platinum–Platinum and Ruthenium–Platinum Compounds. The interaction of the dinuclear compounds with DNA can be envisioned as following a two-step reaction (Figure 6) in which one metal first reacts with guanine or adenine on one strand. Depending on the structure of the compound and the nature of the second metal, the second step may occur quite rapidly (model 3) to give either an intrastrand diadduct

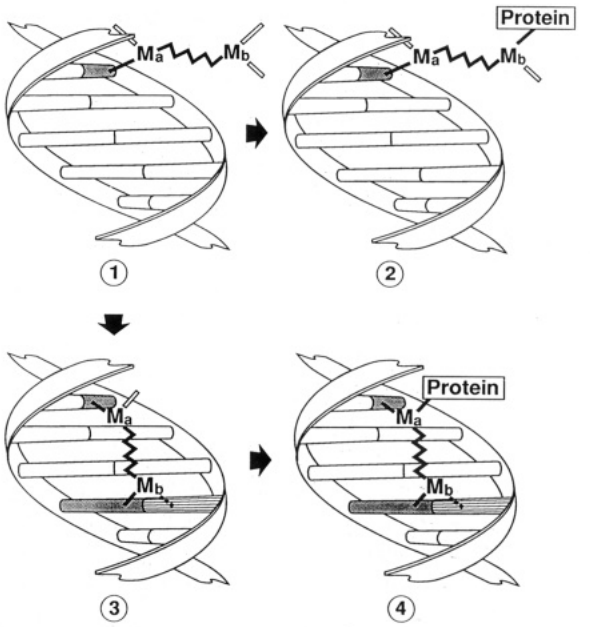

FIGURE 6: Model for the formation of DNA–protein cross-links. In step 1, one of the two metal coordination spheres binds to guanine or adenine of the DNA. Data presented in Figures 5 and 6 do not support the formation of DNA–protein cross-links in which a "dangling" metal binds to the protein (step 2). Rather, it appears that both metals first bind to the DNA, forming a large proportion of DNA–DNA interstrand cross-links (step 3). Protein binding to these lesions leads to the formation of a tertiary adduct in which the protein is attached to a DNA–DNA interstrand cross-link (step 4).

(stippled/lined) or an interstrand cross-link (stippled/stippled). If the coordination of the second metal to the DNA is slow, it is possible that a DNA–protein cross-link could form as shown in model 2. This ternary structure could, in principle, be formed with the (Pt,Pt)-1,1 compound. However, data presented here indicate that a Uvr–metal–DNA ternary complex does not form with this compound, suggesting that the DNA reactivity of the second platinum of this compound is very rapid, preventing the reaction with UvrA (Figure 4, lanes 11 and 12). Likewise, the inefficient cross-linking we have observed with *cis*- and *trans*-DDP (data not shown) may be due to reaction conditions which favor a high proportion of bidentate adducts, precluding ternary complex formation.

The tetrafunctional nature of the (Pt,Pt)-2,2 compounds dictates that further reaction with either DNA or protein is possible even after bifunctional intrastrand (in which each Pt is attached to the same strand) or interstrand cross-linking (in which each metal is attached to the complementary strands). The synthesis of heteronuclear metal compounds containing ruthenium was undertaken to examine the possibility that rapid binding of platinum to the DNA could be followed by a protein-specific interacion with ruthenium. We have previously shown that the (Pt,Pt)-2,2 compound (Roberts et al., 1989) and, in Figure 5, top and middle, the (Ru,Pt) compound cross-link the Watson strand to the Crick strand in a DNA helix. Data presented in this report suggest that the dimetallic interstrand metal cross-link is the precursor to the protein–DNA cross-link (model 4), and very little, if any, of model 2-type binding, in which the protein is cross-linking to the dangling metal, occurs. While we cannot completely rule out the possibility of model 2-type binding inside a cell, the conditions in which the experiments were performed (DNA treatment with the metal compounds, followed by protein binding) greatly favored model 4 binding.

The experiments outlined in this paper confirm the high efficiency of DNA–protein cross-link formation with dinuclear compounds, but the detailed structure of the ternary complexes

awaits further study. The present experiments cannot distinguish between Ru–protein and possible Pt–protein binding, since both coordination spheres contain more than one substitution site. The limiting DNA–DNA interstrand cross-link requires only one coordination site from each metal and thus allows for further attack at either metal. Attempts to prepare the Ru(III) analog of the heterodinuclear compound were not successful due to the instability of the monomeric Ru(III) precursor.

DNA–protein cross-linking has been observed for monomeric platinum compounds. It is interesting to note that *trans*-diamminedichloroplatinum(II) has been used previously in the study of protein–nucleic acid interactions (Tukalo et al., 1987; Baudin et al., 1989). While this work was in progress, Chu and Orgel showed, using a complementary approach, that specific transcription factors such as REB and JUN could be efficiently cross-linked to phosphorothioate-substituted DNA containing their cognate recognition sequences with K_2PtCl_4 (Chu & Orgel, 1992). Our current method does not necessitate the synthesis of uniquely substituted oligonucleotides. Cross-linking of *cis*-DDP to proteins both in vitro and in vivo has been observed (Hayes & Scovell, 1991; Olinski et al., 1987; Zwelling et al., 1981). Cross-linking of HMG 1 and 2 proteins to DNA by *cis*-DDP has been observed upon treatment of *cis*-DDP with chicken erythrocyte nuclei (Scovell et al., 1987). This latter result is of particular interest in view of the recent demonstrations of the specific recognition of *cis*-DDP-damaged DNA by the HMG 1 protein (Billings et al., 1992; Donahue et al., 1990; Hughes et al., 1992; Pil & Lippard, 1992).

Compared to other methods of forming DNA–protein cross-links, such as UV cross-linking (Hockensmith et al., 1992), the use of dinuclear metal compounds has several advantages. First, no special DNA modification such as substitution with bromodeoxyuridine is necessary. Thus any DNA fragment can be simply treated with these metal compounds and used directly. Second, no special light source such as an intense UV laser is necessary to facilitate cross-linking. A third property is the efficiency of cross-linking. As compared to other methods, these dinuclear metal compounds are very efficient at cross-linking UvrA to DNA. By comparing mobility shift assays, which quantifies the amount of Uvr proteins bound to the DNA substrate, with denaturing gels, which measures the amount of protein covalently bound to the DNA, we estimate that 50–75% of all bound protein is actually covalently attached (see Figures 2 and 3). Using excess UvrA protein, all the DNA substrate could be driven into a DNA–protein cross-link (see Figure 4, lanes 6 and 9). Finally, once formed, the DNA–protein cross-link is reversible with reducing agents such as β-mercaptoethanol (Figure 2B).

Biological Implications of Metal Compound-Mediated DNA–Protein Cross-Links. Platinum- and ruthenium–amine compounds form coordination complexes with DNA through the interaction of the N7 position of guanines and adenines. The three major amino acids which are potentially reactive with platinum or ruthenium are methionine, histidine, and cysteine. The ability to cross-link UvrA and UvrB to DNA through these metal compounds strongly suggest that UvrA and UvrB can contact the major groove of the DNA helix within 4–4.5 Å (the length of a N7–metal–X bond, where X is Met, His, or Cys) of the N7 position of G or A. Furthermore, these data suggest that the UvrA and UvrB proteins are making close contact with their amino acid side chains and the metal compound. UvrA contains 18 Met, 25 His, and 14 Cys. Three putative DNA-binding domains have been identified in UvrA: two zinc-finger motifs are located at amino acids 253–280 and 740–766. A putative α-helix–turn–α-helix motif has been suggested to exist at amino acids 494–514 (Visse et al., 1992). Site-directed mutagenesis experiments have suggested that the zinc fingers are important for DNA binding (Myles & Sancar, 1991). Grossman and co-workers have suggested that the helix–turn–helix domain might also be important for damage recognition (Claassen & Grossman, 1991; Wang & Grossman, 1993). Experiments are in progress to identify the specific amino acid side chains in UvrA and UvrB which are making contact with the metal–DNA complexes. That UvrB is capable of making intimate contact with a damaged site was recently shown by Orren et al. (1992). They demonstrated that UvrB can be efficiently cross-linked to a psoralen monoadducted thymine.

In summary, this study shows that *E. coli* DNA repair proteins UvrA and UvrB are efficiently cross-linked to DNA damaged with dinuclear metal–DNA complexes. In principle, these dinuclear metal–DNA complexes could rapidly deplete the cell of a critical protein involved in DNA repair and thus have the novel activity of being potential "suicide DNA adducts". The dinuclear metal–DNA adducts may be capable of trapping other proteins including those involved in repair to the DNA and be useful in the isolation of specific DNA proteins from crude cell extracts. Future studies with other DNA-binding proteins will demonstrate the general utility of this approach.

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SUPPLEMENTARY MATERIAL AVAILABLE

Characterization data for the Ru and Pt complexes described herein (1 page). Ordering information is given on any current masthead page.

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