A new trinuclear complex of platinum and iron efficiently promotes cleavage of plasmid DNA

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Received July 29, 1992; Revised and Accepted March 5, 1993

ABSTRACT

The compound $[\{\text{Pt}(\text{trpy})\}_2\text{Arg-EDTA}]^+$ is synthesized in five steps, purified, and characterized by ¹H, ¹³C, and ¹⁹⁵Pt NMR spectroscopy, mass spectrometry, UV-vis spectrophotometry, and elemental analysis. The binuclear $[\{\text{Pt}(\text{trpy})\}_2\text{Arg}]^{3+}$ moiety binds to double-stranded DNA, and the chelating EDTA moiety holds metal cations. In the presence of ferrous ions and the reductant dithiothreitol, the new compound cleaves DNA. It cleaves a single strand in the pBR322 plasmid nearly as efficiently as methidiumpropyl-EDTA (MPE), and it cleaves a restriction fragment of the XP10 plasmid nonselectively and more efficiently than $[\text{Fe}(\text{EDTA})]^{2-}$. The mechanism of cleavage was studied in control experiments involving different transition-metal ions, superoxide dismutase, catalase, glucose oxidase with glucose, metal-sequestering agents, and deaeration. These experiments indicate that adventitious iron and copper ions, superoxide anion, and hydrogen peroxide are not involved and that dioxygen is required. The cleavage apparently is done by hydroxyl radicals generated in the vicinity of the DNA molecule. The reagent $[\{\text{Pt}(\text{trpy})\}_2\text{Arg-EDTA}]^+$ differs from methidiumpropyl-EDTA in not containing an intercalator. This difference in binding modes between the binuclear platinum(II) complex and the planar heterocycle may cause useful differences between the two reagents in cleavage of nucleic acids.

INTRODUCTION

Interactions of DNA with small molecules are being vigorously studied today. An important goal in this research is to design compounds that bind to DNA and cleave it. Because of their diverse structures and reactions, transition-metal complexes have proven very useful as chemical nucleases, synthetic reagents for cleavage of nucleic acids (1–6). The first chemical nuclease made contains an intercalator, methidium cation, tethered to a metal chelator, EDTA. This bifunctional reagent, designated MPE, cleaves DNA in the presence of ferrous ions, ascorbic acid or dithiothreitol as a reductant, and hydrogen peroxide or dioxygen

as an oxidant (7,8). The reactive species is the diffusible hydroxyl radical generated near the DNA molecule by the chelated ferrous ion and the oxidant; when the oxidant is hydrogen peroxide, this is the Fenton reaction. Even the untethered $[\text{Fe}(\text{EDTA})]^{2-}$ cleaves DNA in the presence of a reductant and hydrogen peroxide. A disadvantage is that the required concentration of the free complex is higher than that of MPE because many hydroxyl radicals are lost to side reactions. An advantage is that the anionic complex does not bind to DNA and perturb it (6,9,10). Since $[\text{Fe}(\text{MPE})]$ and $[\text{Fe}(\text{EDTA})]^{2-}$ are almost nonselective to DNA sequence, they have been used widely in footprinting of molecules bound to DNA (11–20). Since metal complexes are nonselective and small, they have advantages over DNase I in locating small agents bound to DNA (21,22).

Several other metal complexes that are redox-active in the ground state cleave DNA (2,23–37). A growing number of semisynthetic reagents are formed by linking reactive metal complexes to peptides or oligonucleotides that selectively bind to DNA (38–41).

All of the aforementioned chemical nucleases are mononuclear metal complexes with classical structures. In this study we introduce a trinuclear metal complex, in which different inorganic moieties are responsible for binding and for cleavage.

Kostić and coworkers (42,43) have recently synthesized and characterized a series of binuclear complexes of the type $[\{\text{Pt}(\text{trpy})\}_2\text{gua}]^{3+}$, in which two planar (2,2':6',2''-terpyridine) platinum groups, $\text{Pt}(\text{trpy})^{2+}$, are bridged by various guanidine-containing ligands in the deprotonated state. (When these ligands also contain other ionizable groups, net charge of the complex depends greatly on pH; charges in neutral solution are specified in the formulas below.) The molecular structure (43) of the complex in which the bridging ligand (qua) is the amino acid canavanine (Can) is shown in Fig. 1. Arginine is analogous to canavanine, as the formulas below show, and it acts as the bridging ligand in $[\{\text{Pt}(\text{trpy})\}_2\text{Arg}]^{3+}$. Methylguanidine (MeGua) also can act as the bridging ligand, but it lacks additional functional groups. An unpublished study from this laboratory (44) dealt with binding of $[\{\text{Pt}(\text{trpy})\}_2\text{Can}]^{3+}$ to the following nucleic acids: calf-thymus DNA, self-complementary oligonucleotides d(CGCGAATTCGCG) and d(ATGCGCAT), homooctanucleo-

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tides d(pA)₈, d(pC)₈, and d(pT)₈, and polymers poly(dG), poly(dA), poly(dC), poly(dT), poly(dG)poly(dC), poly(dA)poly(dT), poly(dG-dC)poly(dG-dC), and poly(dA-dT)poly(dA-dT). Association constants were determined by equilibrium dialysis and ultracentrifugation; this constant is $1.2 \times 10^5 \text{ M}^{-1}$ for calf-thymus DNA in a solution containing 5 mM Tris·HCl and 50 mM NaCl at pH 7.4. The mode of binding to calf-thymus DNA and to the two self-complementary oligonucleotides was deduced from quenching of ethidium luminescence, perturbations of UV-vis spectra, increase in melting temperature, perturbations of ¹H and ³¹P NMR spectra, changes in viscosity, unwinding of DNA (topoisomerase assay), and linear dichroism. The binuclear complex $[\{\text{Pt}(\text{trpy})\}_2\text{Can}]^{3+}$, unlike the mononuclear complexes of the type $[\text{Pt}(\text{trpy})\text{L}]^+$ (45), does not intercalate. It binds by electrostatic and hydrophobic forces, most likely at the minor groove. Indeed, the thickness of the 'double-decker' complex is ca. 6.0 Å and compatible with the width of the minor groove. A similar but thicker 'double-decker' complex does not bind to DNA (46).

In this study we attach a molecule of EDTA to the α -amino group of arginine as it was done in the reagent MPE (7,8). Since, however, the 'double-decker' complex apparently binds at the minor groove and the methidium cation intercalates, the compounds $[\{\text{Pt}(\text{trpy})\}_2\text{Arg-EDTA}]^+$ and MPE may differ in the cleavage of DNA. We apply the new reagent to the plasmids pBR322 and XP10 and demonstrate the efficacy of our molecular design.

MATERIALS AND METHODS

Chemicals

The salt $[\text{Pt}(\text{trpy})\text{Cl}]\text{Cl} \cdot 2\text{H}_2\text{O}$ was prepared by a published procedure (47). The plasmid pBR322 was obtained from Promega Co., and the plasmid XP10 was kindly provided by Dr Alan P. Wolffe. Dithiothreitol (DTT), catalase from bovine liver, superoxide dismutase from bovine erythrocytes, glucose oxidase (type VII) from *Aspergillus niger*, β -D(+)-glucose, bathocuproine disulfonic acid, and $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$ were obtained from Sigma Chemical Co. The iron-sequestering agent $[(\text{HOOCCH}_2)[2-(\text{HO})\text{C}_6\text{H}_4\text{CH}_2]\text{NCH}_2]_2$, designated HBED, was kindly provided by Professor Wesley R. Harris. Water obtained from Aldrich Chemical Co. and all other chemicals were of reagent grade. Our distilled water was further demineralized to a resistance greater than 10 M Ω cm.

Measurements

Ultraviolet-visible spectra were recorded with an IBM 9430 spectrophotometer. Proton and ¹³C NMR spectra of solutions in D₂O were obtained with a Varian VXR300 spectrometer; DSS

Figure 1. Molecular structure of the $[\{\text{Pt}(\text{trpy})\}_2\text{Can}]^{3+}$ complex, which contains two nearly eclipsed and approximately parallel $\text{Pt}(\text{trpy})^{2+}$ groups bridged by the guanidine group of canavanine; the platinum atoms are 2.99 Å apart (Ratilla et al., 1990). A) top view; B) side view.

was an internal reference, and dioxane (at 66.5 ppm) was an external one. Platinum-195 NMR spectra of solutions in D₂O were obtained with a Bruker WM200 spectrometer; a solution of $\text{Na}_2[\text{PtCl}_4]$ that was also 0.20 M in NaCl was an external reference and standard (0 ppm). The pH was measured with a Fischer 805MP instrument and a Phoenix Ag/AgCl reference electrode; the pH* values are uncorrected for deuterium isotope effects in D₂O. Mass spectra were obtained with a Kratos MS50 instrument; chemical ionization was used for the compound $\text{Arg}(\text{OEt})\text{-EDTA}(\text{OEt})_3$, and fast-atom bombardment was used for the compound $[\{\text{Pt}(\text{trpy})\}_2\text{Arg-EDTA}]^+$. Iron and copper impurities in demineralized water and in the compound $[\{\text{Pt}(\text{trpy})\}_2\text{Arg-EDTA}]^+$ were determined by mass spectrometry involving ultrasonic nebulization of the sample into inductively coupled plasma. Elemental analyses were done by Galbraith Laboratories, Inc. All syntheses were done in a well-ventilated hood.

Synthesis of EDTA(OEt)₄

To 700 mL of ethanol kept at 0°C were added 45 mL (0.60 mol) of SOCl_2 in drops. After 1 h at 0°C, 30.0 g (0.10 mol) of free

Table I. ^{13}C NMR chemical shifts, in ppm relative to DSS, at pH* 8–9

compound	$\alpha\text{-C}$	$\beta\text{-C}$	$\gamma\text{-C}$	$\delta\text{-C}$	$\epsilon\text{-C}$
Arg ^a	54.9	29.5	24.3	40.8	155.9
$[\{\text{Pt}(\text{trpy})\}_2\text{Arg}]^{3+}$ ^a	55.2	30.0	25.4	42.3	165.2
difference	0.3	0.5	0.9	1.5	9.3
Arg-EDTA	54.7	28.5	24.6	40.6	156.7
$[\{\text{Pt}(\text{trpy})\}_2\text{Arg-EDTA}]^+$	54.8	29.0	25.4	42.0	165.1
difference	0.1	0.5	0.8	1.4	8.4

^a From Ratilla et al., 1990.

EDTA were added. After stirring for 2 h at 0°C and for 5 d at 20°C, the initial suspension became a solution. The excess of SOCl_2 was removed by concentration under reduced pressure. The concentrated solution was diluted by 200 mL of ethanol and made basic by addition of NaHCO_3 . The product was extracted into diethylether. The solution was dried with anhydrous Na_2SO_4 and concentrated to yield 33.0 g (80%) of the tetraethyl ester, $\text{EDTA}(\text{OEt})_4$. The ^1H NMR δ values in D_2O , at pH* 7.0: 1.26, t, 12H; 2.84, s, 4H; 3.57, s, 8H; and 4.19, q, 8H.

Synthesis of $\text{EDTA}(\text{OEt})_3$

To a rapidly stirred solution of 6.00 g (16.2 mmol) of $\text{Cu}(\text{ClO}_4)_2 \cdot 6\text{H}_2\text{O}$ in 250 mL of water were added 6.54 g (16.2 mmol) of $\text{EDTA}(\text{OEt})_4$. The pH value was maintained at 4–5 by gradual addition of a solution containing 0.52 g (13 mmol) of NaOH in 20 mL of water. The precipitate of CuS , formed upon addition of 4.86 g (20 mmol) of $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$, was removed by filtration. The unspent $\text{EDTA}(\text{OEt})_4$ was removed by washing the filtrate with five 50-mL portions of diethylether. The solution was adjusted to pH 1.0 with HCl (caution, H_2S !) and concentrated to ca. 50 mL. The product was extracted with three 50-mL portions of chloroform. The solution was dried with anhydrous Na_2SO_4 and concentrated to yield 2.20 g (36%) of the triethyl ester, $\text{EDTA}(\text{OEt})_3$. The ^1H NMR δ values in D_2O , at pH* 7.0: 1.26, t, 6H; 1.28, t, 3H; 3.03, bt, 2H; 3.22, bt, 2H; 3.61, s, 4H; 3.70, s, 2H; 4.06, s, 2H; 4.19, q, 4H; and 4.27, q, 2H.

Synthesis of $\text{Arg}(\text{OEt})\text{-EDTA}(\text{OEt})_3$

In this step the free carboxylic group in the EDTA triethyl ester and the amino group in arginine ethyl ester were coupled into an amide. A mixture of 2.20 g (5.86 mmol) of $\text{EDTA}(\text{OEt})_3$ and 1.20 g (7.40 mmol) of 1,1'-carbonyldiimidazole in 60 mL of dry N,N -dimethylformamide was stirred for 1 h at room temperature, and 1.61 g (5.86 mmol) of ArgOEt were added to it. After stirring for 24 h at room temperature, the solvent was removed in vacuo. The oily residue was purified on a silica gel column sized 2.5×30 cm, with chloroform containing 15% methanol and 0.2% acetyl chloride as an eluent. The product yield was 2.00 g (57%). The ^1H NMR δ values in D_2O , at pH* 7.0: 1.26, m, 12H, CH_3 ; 1.60–1.70, m, 2H, $\gamma\text{-CH}_2$ Arg; 1.75–2.05, m, 2H, $\beta\text{-CH}_2$ Arg; 2.83, s, 4H, CH_2CH_2 EDTA; 3.23, t, 2H, $\delta\text{-CH}_2$ Arg; 3.41, 3.56, 3.58, all s, $2 \times 2\text{H}$ and 4H, NCH_2CO EDTA; 4.20, m, 8H, CH_2CH_3 ; and 4.43, dd, 1H, $\alpha\text{-CH}$ Arg. Mass spectrum: $\text{C}_{24}\text{H}_{45}\text{N}_6\text{O}_9\text{Cl}$ requires $M=596.4$; found $m/z=561$, M-Cl.

Synthesis of Arg-EDTA

A solution of 0.50 g (0.84 mmol) of $\text{ArgOEt-EDTA}(\text{OEt})_3$ in 100 mL of 0.10 M NaOH was stirred for 30 min, adjusted to

Figure 2. Electrophoretogram in agarose gel, showing cleavage of the supercoiled (form I, uppermost) pBR322 plasmid DNA and formation of nicked circular (form II, lowermost) and linear (form III, intermediate) DNAs. Conditions: 10 μM (in base pairs) pBR322, 0.10 μM $[\{\text{Pt}(\text{trpy})\}_2\text{Arg-EDTA}]\text{Fe}^+$, and 100 μM dithiothreitol in Tris-HCl buffer at pH 7.4 that is 5 mM in NaCl, and 25°C; incubation times are 1, 3, 5, 9, 13, 20, 35, 60, 100, and 150 min from left to right.

Table II. Cleavage of pBR322 DNA^a in the absence of reducing agents

reagent	μM	DNA forms, $\pm 5\%$	
		I	II
none		91	9
Fe^{2+}	10	88	12
$[(\text{EDTA})\text{Fe}]^{2-}$	10	90	10
$[\text{Arg-EDTA}]\text{Fe}^{2-}$	10	89	11
$[\{\text{Pt}(\text{trpy})\}_2\text{Arg-EDTA}]\text{Fe}^+$	0.010	90	10
	0.10	86	14
	1.0	79	21
	10	48	52

^a 10 μM base pairs, in 10 mM Tris-HCl buffer at pH 7.4 that is 5 mM in NaCl, at 25°C, for 1 hr

pH 1.0 with HCl, and concentrated to ca. 3 mL under reduced pressure. Chromatography on a G-10 column sized 2.5×60 cm yielded 0.30 g (68%) of the hydrolyzed product. The ^1H NMR δ values in D_2O , at pH* 7.0: 1.60–1.70, m, 2H, $\gamma\text{-CH}_2$ Arg; 1.80–2.00, m, 2H, $\beta\text{-CH}_2$ Arg; 3.08, 3.41, bt, $2 \times 2\text{H}$, CH_2CH_2 EDTA; 3.21, t, 2H, $\delta\text{-CH}_2$ Arg; 3.32, 3.47, 3.87, all s, $2 \times 2\text{H}$ and 4H, NCH_2CO EDTA; and 4.15, dd, 1H, $\alpha\text{-CH}$. The ^{13}C NMR δ values in D_2O , at pH* 8.0: 24.6, γ ; 28.5, β ; 40.6, δ ; 49.8; 52.6; 54.7, α ; 56.8, probably 2C; 57.4, probably 2C; 156.7, ϵ ; 173.0; and 178.9. Resonances of the three

remaining carbonyl groups coincide with the other resonances or were too weak to be detected.

Synthesis of $[\{\text{Pt}(\text{trpy})\}_2\text{Arg-EDTA}]^+$

A solution containing 0.22 g (0.42 mmol) of Arg-EDTA and 0.58 g (1.08 mmol) of $[\text{Pt}(\text{trpy})\text{Cl}]\text{Cl}\cdot 2\text{H}_2\text{O}$ in 45 mL of 50 mM NaOH was stirred at 70°C in the dark for 24 h. The resulting dark red solution was adjusted to pH 9–10 with HCl. The precipitate obtained upon addition of 135 mL of ethanol was redissolved in the minimum volume of water and chromatographed on a G-10 column sized 2.5×60 cm, which had previously been equilibrated by an aqueous solution of NaOH at pH 9–10. Two rounds of precipitation with a 3-fold excess of ethanol yielded 185 mg (32%) of a dark red solid. The ^1H NMR δ values in D_2O , at pH* 7.0: 1.80–1.90, m, 2H, $\gamma\text{-CH}_2$ Arg; 2.00–2.10, m, 2H, $\beta\text{-CH}_2$ Arg; 3.15 and 3.50, bt, 2×2H, CH_2CH_2 EDTA; 3.50, t, 2H, $\delta\text{-CH}_2$; 3.35, 3.50, 3.94, all s, 2×2H and 4H, NCH_2CO EDTA; 4.30 dd, 1H, $\alpha\text{-CH}$; 7.56, m, 4H, H^5 trpy; 7.93, m, 8H, $\text{H}^{3,3'}$ trpy; 8.16, t, 4H, H^4 trpy; 8.24, d, 4H, H^6 trpy; and 8.30, t, 2H, $\text{H}^{4'}$ trpy. The ^{13}C NMR δ values in D_2O , at pH* 8.0: 25.4, γ ; 29.0, β ; 42.0, δ ; 49.8; 52.6; 54.8, α ; 56.3, probably 2C; 57.9; 58.2; 123.7; 125.3; 129.8; 142.7; 150.9; 152.9; 155.5; 165.1, ϵ ; 170.5; 171.0; and 178.3. Resonances of the two remaining carbonyl groups coincide with the other resonances or were too weak to be detected. The ^{195}Pt NMR δ value in H_2O , –975 ppm. The UV-vis band positions (in nm) and absorptivities (in $\text{M}^{-1}\text{cm}^{-1}$), in H_2O : 242, 53200; 267, 40200; 306, 18000; and 487, 3300. The product was isolated with two sodium cations and three hydroxide anions as counter ions. Mass spectrum: $\text{C}_{46}\text{H}_{50}\text{N}_{12}\text{O}_{12}\text{Na}_2\text{Pt}_2$ requires $M = 1398.4$; found $m/z = 1302$,

M-2Na-3(OH) . Anal. Calcd for $\text{C}_{46}\text{H}_{50}\text{N}_{12}\text{O}_{12}\text{Na}_2\text{Pt}_2$: C, 39.5; H, 3.6; N, 12.0; and Pt, 27.9. Found: C, 40.0; H, 4.0; N, 12.0; and Pt, 27.8.

Cleavage of the plasmid pBR322 by $[\{\text{Pt}(\text{trpy})\}_2\text{Arg-EDTA}]\text{Fe}^+$

The experiments were done at 25°C. The solvent was a 10 mM Tris/HCl buffer at pH 7.4, which was also 5 mM in NaCl. The final concentration of the plasmid DNA was always 10 μM in base pairs. A 50 μM solution of the cleavage reagent in water, freshly prepared by mixing equal amounts of 100 μM solutions of $[\{\text{Pt}(\text{trpy})\}_2\text{Arg-EDTA}]^+$ and of $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2\cdot 6\text{H}_2\text{O}$, was added to the reaction mixture up to a desired concentration in the range 0.010–10 μM . Additional ingredients that were present in some experiments are noted in figure captions and tables. The reaction was started by adding dithiothreitol up to the final concentration of 100 μM . The incubation times varied from 1 to 150 min; in most cases this time was 1 h. The reactions were stopped by addition of 2 μL of a 50 mM solution of EDTA, and mixtures were kept at –40°C until they were analyzed by gel electrophoresis. The gel contained 1% agarose, and a 40 mM Tris-acetate buffer was made 1.0 mM in EDTA. The supercoiled (form I), nicked circular (form II), and linear (form III) DNAs were separated by a current of 50 V for 3 h. The gel was stained with ethidium bromide (caution, carcinogen!) and photographed on a UV transilluminator with a Polaroid camera equipped with the positive/negative film of type 55. The negative was scanned with a Hoefer GS300 densitometer, and the bands were quantitated by a gel-scanning program. The results were corrected by a standard procedure for the lesser stainability of the form I (7).

Table III. Cleavage of pBR322 DNA^a in the presence of 100 μM dithiothreitol

reagent	μM	DNA forms, $\pm 5\%$		
		I	II	III
none		83	17	
$[(\text{EDTA})\text{Fe}]^{2-}$	1.0	77	23	
$[\text{Arg-EDTA}]\text{Fe}^{2-}$	1.0	79	21	
$[\{\text{Pt}(\text{trpy})\}_2\text{Megua}]^{3+} + \text{Fe}^{2+}$	1.0	80	20	
$[\{\text{Pt}(\text{trpy})\}_2\text{Arg-EDTA}]^+$	1.0		98	2
	0.10	33	67	
	0.010	74	26	
$[\{\text{Pt}(\text{trpy})\}_2\text{Arg-EDTA}]\text{Fe}^+$	1.0		79	21
	0.10	5	88	7
	0.010	59	41	

^a As in Table II, footnote a.

Table IV. Effects of Inhibitors on the cleavage of pBR322 DNA^a

inhibitor	$\log K_f^b$	μM	DNA form, $\pm 5\%$		
			I	II	III
none			5	88	7
Mg^{2+}	8.7	100	11	84	5
Co^{2+}	16.3	100	77	23	
Zn^{2+}	16.5	100	77	23	
Ni^{2+}	18.6	100	65	35	
EDTA		1×10^4	69	31	

^a 10 μM base pairs, 100 μM $[\{\text{Pt}(\text{trpy})\}_2\text{Arg-EDTA}]\text{Fe}^+$, 100 μM dithiothreitol, and inhibitor in 10 mM Tris·HCl buffer at pH 7.4 that is 5 mM in NaCl, at 25°C, for 1 h.

^b K_f is the stability constant for binding of the metal ions to EDTA. For Fe^{2+} , $\log K_f = 14.3$.

Cleavage of a restriction fragment of the plasmid XP10 by $[\{\text{Pt}(\text{trpy})\}_2 \text{Arg}-(\text{EDTA})\text{Fe}]^+$ and $[\text{Fe}(\text{EDTA})]^{2-}$

The 303-bp fragment of the plasmid XP10, obtained by cleavage with *Eco*R1 and *Hind* III restriction enzymes, was radiolabeled at the 3' end (48,49). The cleavage experiments were done according to published procedures (50,51). A 100 μM solution of $[\{\text{Pt}(\text{trpy})\}_2 \text{Arg}-(\text{EDTA})\text{Fe}]^+$ was prepared by mixing equal volumes of 200 μM solutions of $[\{\text{Pt}(\text{trpy})\}_2 \text{Arg}-(\text{EDTA})]^{2+}$ and $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$. Labeled DNA (10 μL , 10000 counts/ μL) was diluted to 70–90 μL with a 10mM Tris·HCl buffer at pH 8.0 in an Eppendorf tube. A 10- μL aliquot of each reagent was added to the tube side. The reaction was started by simultaneously dropping all of them into the DNA solution. The final volume was always 100 μL , and final concentrations were as follows: 10 μM in $[\text{Fe}(\text{EDTA})]^{2-}$, 1.0 mM in ascorbate, 10 μM in $[\{\text{Pt}(\text{trpy})\}_2 \text{Arg}-(\text{EDTA})]^{2+}$, 10 μM in $[\{\text{Pt}(\text{trpy})\}_2 \text{Arg}-(\text{EDTA})\text{Fe}]^+$, and 0.03% by weight in H_2O_2 . Reagents present in each reaction mixture are specified in the autoradiograph figure. The reaction was quenched after 5 or 15 min by addition of 43 μL of a solution in the Tris·HCl buffer that was 0.20 M in EDTA and 0.10 M in thiourea. The solids were precipitated twice, rinsed with 70% ethanol, and dried in a SpeedVac concentrator from Savant Instruments, Inc. Cleavage products were resolved on a denaturing 6% polyacrylamide gel, with 60-W current. Images of the dried gel were obtained with two X-ray films: Molecular Dynamics 400E Phosphorimager and Kodak XAR5. The images were analyzed with a Molecular Dynamics 300E computing densitometer.

Control experiments

Some experiments for cleavage of the plasmid pBR322 were done in the presence of the iron-sequestering agent HBED and of the copper-sequestering agent bathocuproin, alone and in combination. Their individual concentrations (50 μM) far exceeded the concentrations of $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$ (0.10 μM), $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (0.10 μM), and CuCl (0.15 μM , to compensate for possible disproportionation). Other experiments were done in the presence of 30 units of catalase or of superoxide dismutase. Still other experiments were done anaerobically, at pH 7.8, in the presence of glucose oxidase (75 $\mu\text{g} \cdot \text{mL}^{-1}$), catalase (12.5 $\mu\text{g} \cdot \text{mL}^{-1}$), and glucose (0.30% by weight), according to a published procedure (52). All these solutions were deaerated before mixing, and their mixture was covered by deaerated mineral oil. The cleaving reagent, plasmid, and DTT in standard amounts were added through the oil layer, and the tubes were capped and sealed with parafilm.

RESULTS AND DISCUSSION

The cleaving reagent

The synthesis of the diplatinum complex is outlined in Scheme I. Actual (de)protonation states of amino and carboxylic groups and net charges are shown only for the final molecule. The dissolved compounds exist in multiple forms, depending on the pH value. The charge shown for $[\{\text{Pt}(\text{trpy})\}_2 \text{Arg}-\text{EDTA}]^{2+}$ is the one that best fits the elemental analysis. The charge shown for $[\{\text{Pt}(\text{trpy})\}_2 \text{Arg}-(\text{EDTA})\text{Fe}]^+$ corresponds to assumed full coordination of the EDTA moiety and pH ca. 7. The tetraethyl and triethyl esters of EDTA were synthesized by a modification of the published procedure (53). Arginine and EDTA were coupled by the procedure previously used for

methidiumpropylamine and EDTA (8). The guanidine group was attached to two $\text{Pt}(\text{trpy})^{2+}$ groups by our published method (43).

Complexes of the type $[\{\text{Pt}(\text{trpy})\}_2 \text{gua}]^{3+}$ containing arginine, canavanine, and methylguanidine as the bridging ligand gua have similar spectroscopic and other properties (43). As Table I shows, the diplatinum moiety in $[\{\text{Pt}(\text{trpy})\}_2 \text{Arg}-\text{EDTA}]^{2+}$ closely resembles the complex $[\{\text{Pt}(\text{trpy})\}_2 \text{Arg}]^{3+}$. The downfield shift of the arginine ^{13}C NMR resonances upon platination monotonically increases from the α -C atom, which is only slightly affected, to the ϵ -C atom, which is greatly affected. Coordination evidently occurs via the guanidine group. Since each complex shows a single set of terpyridine ^{13}C resonances, the two $\text{Pt}(\text{trpy})^{2+}$ groups in each complex are equivalent in solution. Since the ^{13}C resonances of the EDTA moiety have nearly the same chemical shifts in Arg-EDTA and in $[\{\text{Pt}(\text{trpy})\}_2 \text{Arg}-\text{EDTA}]^{2+}$, this moiety does not bind to the $\text{Pt}(\text{trpy})^{2+}$ groups. Similar conclusions were reached by comparing the UV-vis and ^1H and ^{195}Pt NMR spectra of the new complex and of its congeners. The canavanine complex (Figure 1) has the two

Figure 3. Electrophoretogram in agarose gel, showing cleavage of 10 μM (in base pairs) pBR322 plasmid DNA in Tris·HCl buffer that is 5 mM in NaCl, at 25°C, for 1 h in the presence of different substances. Lanes, from left to right: (1) no additional substances; (2) 100 μM dithiothreitol; (3) 100 μM dithiothreitol and 0.10 μM [Arg-(EDTA)Fe]²⁻; (4) 100 μM dithiothreitol and 0.10 μM [Pt(trpy)₂Arg-(EDTA)Fe]⁺; (5) 0.10 μM [Pt(trpy)₂Arg-(EDTA)Fe]⁺; (6) 100 μM dithiothreitol and 0.010 μM [Pt(trpy)₂Arg-(EDTA)Fe]⁺; (7) 0.010 μM [Pt(trpy)₂Arg-(EDTA)Fe]⁺; (8) 100 μM dithiothreitol, 0.10 μM [Pt(trpy)₂Arg-(EDTA)Fe]⁺, and 100 μM Co²⁺; and (9) 100 μM dithiothreitol, 0.10 μM [Pt(trpy)₂Arg-(EDTA)Fe]⁺, and 100 μM Mg²⁺.

Pt(trpy)²⁺ groups nearly eclipsed and approximately parallel, with the Pt atoms 3.0 Å apart. The closely similar arginine complex [Pt(trpy)₂Arg-EDTA]⁺ probably has this structure. The mass spectrum and elemental analysis of the arginine complex prove its composition and purity. The ¹H NMR spectrum of the complex in aqueous solution showed that it is stable at pH > 7.0. Although no decomposition was observed by any spectroscopic or other method, all solutions for DNA cleavage were prepared fresh.

Cleavage of the plasmid pBR322 by the new reagent

Figure 2 shows a typical time course of interconversion among the three forms of DNA. Simultaneous disappearance of the supercoiled form I and appearance of the nicked circular form II indicate single-strand cleavage of the former in the presence of the trinuclear complex [Pt(trpy)₂Arg-(EDTA)Fe]⁺. As the concentration of form II increases with time, so does the probability of its further nicking. When the second strand is cleaved near the break in the first one, the linear form III is obtained. Since form III does not appear as form I disappears, form III does not arise from a double-strand cleavage but from two sequential single-strand cleavages.

Since this study concerns nicking of supercoiled DNA, incubation time in subsequent experiments was 1 hr. In this time the form I is fully converted into form II, and form II is only slightly converted into form III.

Table II shows little cleavage of the plasmid DNA even in the presence of a high concentration of Fe²⁺, [Fe(EDTA)]²⁻, and [Arg-(EDTA)Fe]²⁻ because these iron reagents are not brought near the substrate and kept in the ferrous (reduced) state. Of the reagents tested, only [Pt(trpy)₂Arg-(EDTA)Fe]⁺ binds to

Figure 4. Autoradiograph of a polyacrylamide gel showing relative efficiencies with which various reagents cleave the EcoRI, Hind III restriction fragment of the XP10 plasmid DNA in Tris·HCl buffer. Concentrations are given in the Materials and Methods. Lane 2 contained untreated DNA as a control. Conditions for other lanes are shown in the heading.

DNA. Only this complex (even in the absence of a reducing agent) causes considerable cleavage, but the required concentration of it (10 μM in these experiments) is high. For the procedure to be practical, a reducing agent is necessary.

Table III shows much improved cleavage in the presence of a reductant, in this case dithiothreitol. The complex concentration as low as 0.010 μM , which corresponds to 4.4 base pairs per reagent molecule, is sufficient.

Background cleavage of the plasmid pBR322

The results in the first lines in Tables II and III confirm previous reports by others that pBR322 DNA from even the best commercial source contains a slight amount of the nicked circular form II and that dithiothreitol alone causes ca. 8% cleavage. In our experiments sodium ascorbate caused intolerably high background cleavage, while NADH neither caused background cleavage nor appreciably enhanced the cleavage in the presence of [Pt(trpy)₂Arg-(EDTA)Fe]⁺. Dithiothreitol proved to be a suitable reducing agent, and whenever it was used its concentration was 100 μM .

As Table III shows, the diplatinum complex [Pt(trpy)₂Arg-EDTA]⁺ promotes some DNA cleavage even without added iron. Similar finding with the reagent MPE was attributed to an

assumed presence of iron or copper ions as impurities in the reaction mixture (7,8). We examined this possibility by experiments. Mass spectrometric analysis of $[\{\text{Pt}(\text{trpy})\}_2\text{Arg-EDTA}]^+$ showed 0.1–0.2% iron and less than 0.1% (undetected) copper. Since this amount of iron is too low to be responsible for the observed background cleavage, additional iron probably comes from the buffer. The solids Tris and NaCl, not the distilled and demineralized water, are the likely sources of this adventitious iron. Background cleavage becomes comparatively insignificant under the optimal reaction conditions, in the presence of the complete trinuclear reagent $[\{\text{Pt}(\text{trpy})\}_2\text{Arg-(EDTA)Fe}]^+$.

Identity of the cleaving reagent

The control experiments in Tables II and III were done with fragments of the trinuclear reagent that were present at higher concentrations than the complete reagent. These conditions favored the undesirable outcome (DNA cleavage), and yet this cleavage was slight. The complex $[\{\text{Pt}(\text{trpy})\}_2\text{Megua}]^{3+}$ can bind to DNA but cannot hold iron ions. The free ligands EDTA and Arg-EDTA can hold iron ions but cannot bind to DNA. None of them caused significant cleavage either without or with added iron ions. The noneffect of added iron and copper ions, even more than the noneffects of metal-sequestering agents (results not shown), indicates that cleavage presented in Table III cannot be caused by metal impurities in the reaction mixture. Efficient cleavage requires both the diplatinum moiety for binding to DNA and the EDTA moiety for complexation of ferrous ions.

Inhibition of the plasmid pBR322 cleavage

As Table IV shows, divalent metal cations whose thermodynamic affinity for EDTA is greater than the affinity of Fe^{2+} do, but an ion whose affinity is less than that of Fe^{2+} does not, inhibit the cleavage reaction when added after the ferrous ion. Inhibition was even more complete when the other metal cations were added before the ferrous ion; these results are not shown. In all of these experiments sufficient time was allowed for the mixture of metal cations and the EDTA moiety of $[\{\text{Pt}(\text{trpy})\}_2\text{Arg-EDTA}]^+$ to reach thermodynamic equilibrium. Methional, a commonly used scavenger for radicals, caused only a small inhibition of DNA cleavage. When dioxygen was carefully excluded in control experiments, cleavage was inhibited; these results are now shown.

Cleavage of a restriction fragment of the plasmid XP10 by the new reagent

Figure 4 shows the results of cleaving a radiolabeled restriction fragment with either $[\{\text{Pt}(\text{trpy})\}_2\text{Arg-(EDTA)Fe}]^+$ or $[\text{Fe(EDTA)}]^{2-}$. Significant cleavage of DNA with platinum-containing compounds is observed only when all of the following are present: $[\{\text{Pt}(\text{trpy})\}_2\text{Arg-(EDTA)}]^+$ and Fe^{2+} ions (premixed to form the complete reagent) and ascorbate. Densitometric analysis showed the new reagent to be about ten times more effective than $[\text{Fe(EDTA)}]^{2-}$ alone under identical conditions. Incubation for 5 min is sufficient. Lane 12 shows that 15 min is too long because it allows for substantial over-cutting of DNA. There are two pieces of evidence for more than 'single hits'. First, the band at the top, due to the complete DNA, is much smaller in this than in other lanes. Second, the signal intensity increases as the fragment size decreases from top to bottom. This pattern is characteristic of multiple cleavages of each DNA fragment, which favor small fragments in the product distribution (50,51). The regions of enhanced cleavage in all the lanes are not due

to sequence-specificity of the new reagent; the same regions show also with $[\text{Fe(EDTA)}]^{2-}$. The enhancements are due to formation of DNA hairpins or to band compression in the gel, or both. These features have been noted before with the plasmid XP10. They do not arise from any sequence-specific interaction between the cleavage reagents and the DNA (49,54).

Mechanism of DNA cleavage

Because neither catalase nor superoxide dismutase inhibits cleavage of plasmid pBR322 (results not shown), neither superoxide nor hydrogen peroxide seems to be involved. The evidence indicates that the ferrous ion in $[\{\text{Pt}(\text{trpy})\}_2\text{Arg-(EDTA)Fe}]^+$ reduces dissolved dioxygen molecules into hydroxyl radicals, which then cleave DNA. The redox reactions that ultimately yield these radicals are only partially understood (55). The weak suppression of the cleavage by methional is not inconsistent with the involvement of hydroxyl radicals. Since the radicals are generated near the DNA, they react with it faster than with the scavengers in solution. Failure of concentrated ethylene glycol to inhibit DNA cleavage by the $[\text{Fe(EDTA)}]^{2-}$ complex linked to an oligonucleotide was explained in the same way (38).

CONCLUSION AND PROSPECTS

This study provides further evidence that inorganic reagents are useful for the cleavage of DNA. We tethered two transition-metal complexes via an amino acid. The redox-inactive, binuclear platinum complex has a charge of +3 and a rigid structure that apparently fits into the minor groove of double-stranded DNA. The redox-active, mononuclear iron complex generates hydroxyl radicals near the DNA. The composite trinuclear complex in the presence of dithiothreitol cleaves supercoiled and linear DNA. This cleavage is not selective to sequence. The new reagent, which appears not to intercalate, may prove useful in structural studies of DNA and RNA and of their adducts with other molecules.

ACKNOWLEDGMENTS

This work was supported by the National Science Foundation through a PYI Award to N.M.K. (grant CHE-8858387). J.S.B. thanks the IBSMA at Johns Hopkins University for a fellowship. We thank Dr Alan P. Wolffe for the plasmid XP10, Professor Wesley R. Harris for the metal-sequestering agents, Lynette Cunningham for some control experiments, and Dr Sam C.K. Shum for analytical determination of iron and copper impurities.

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