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# Targeted Strand Scission of DNA Substrates by a Tricopper(II) Coordination Complex

Kristi J. Humphreys,† Kenneth D. Karlin,*,† and Steven E. Rokita*,‡

*Contribution from the Department of Chemistry, The Johns Hopkins University, Baltimore, Maryland 21218, and Department of Chemistry and Biochemistry, University of Maryland, College Park, Maryland 20742*

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***Abstract:*** A trinuclear copper complex, $[Cu_3^{II}(\mathbf{L})(H_2O)_3(NO_3)_2](NO_3)_4 \cdot 5H_2O$ (**1**) (**L** = 2,2′,2″-tris(dipicolylamino)triethylamine), with pyridyl and alkylamine coordination exhibits a remarkable ability to promote specific strand scission at junctions between single- and double-stranded DNA. Strand scission occurs on the 3′ overhang at the junction of a hairpin or frayed duplex structure and is not dependent on the identity of the base at which cleavage occurs. Target recognition minimally requires a purine at the first unpaired position and a guanine at the second unpaired position on the 5′ strand. Incorporation of the necessary recognition elements into an otherwise unreactive junction resulted in specific strand scission at that new target and helped to confirm the predictive nature of this complex. Selective strand scission requires both a reductant and dioxygen, suggesting activation of $O_2$ by the reduced form of **1**. The reaction utilizing the trinuclear complex does not appear to involve a diffusible radical species as suggested by its high specificity of target oxidation and its lack of sensitivity to radical quenching agents. Comparisons between the trinuclear copper complex, mononuclear analogues of **1**, and $[Cu(OP)_2]^{2+}$ (OP = 1,10-phenanthroline) indicate that recognition and reactivity described in this report are dependent on the multiple metal ions within the same complex which together support its unique activity.

## Introduction

A number of transition metal complexes have the capacity to differentiate between double- vs single-stranded DNA or B vs Z helical forms of DNA through noncovalent recognition.[1] This selectivity is primarily due to binding of the complex in either the major or minor groove of duplex structures or association with the nucleobases in unpaired strands. The electron-rich character of the nucleobases often serves to make them strong ligands for metals and efficient targets of oxidation.[2] Guanine has the highest affinity for coordination to transition metal ions[3] and is the most easily oxidized followed by adenine, thymine, and cytosine.[4] Although base oxidation can be highly specific and directed to one site, strand scission results only after treatment with subsequent heat and alkaline conditions. In contrast, direct strand scission does not necessarily require any special treatment to detect the sites of reaction. Some complexes that exhibit direct strand cleavage in conjunction with sequence specificity are bleomycin·Fe(II)[5] and the metallointercalator, $[Rh(phen)_2phi]^{3+}$.[6] Although there is both a structural and a sequence requirement in each of these cases, the recognition criteria are not sufficiently unique to limit the number of target sites in DNA. Scission may be targeted specifically to one site by incorporating known DNA recognition elements into the ligand suprastructure of a well-characterized nucleolytic agent such as EDTA·Fe(II),[7] which when underivatized promotes oxidative cleavage of DNA in a random fashion without nucleotide sequence selectivity.[8] While this approach localizes cleavage to a site where the recognition element binds to DNA, reaction is rarely constrained to a single nucleotide. Strand cleavage frequently extends instead over several neighboring residues. A longstanding goal of considerable interest is then to construct transition metal complexes that can mediate direct and specific strand scission targeted to a single base with a significantly high level of recognition such that cleavage occurs at a limited number of sites along a target polynucleotide.

Most investigations focusing on oxidative strand scission of DNA by transition metals typically have relied on mononuclear complexes. Among these complexes, bis(1,10-phenanthroline)-copper, $[Cu(OP)_2]^{2+}$, has been studied extensively due to its high nucleolytic efficiency.[9−11] The cleavage pattern induced by $[Cu(OP)_2]^{2+}$ is predominantly sequence-neutral, although

* To whom correspondence should be addressed. E-mail: rokita@umd.edu, karlin@jhu.edu.
† The Johns Hopkins University.
‡ University of Maryland.

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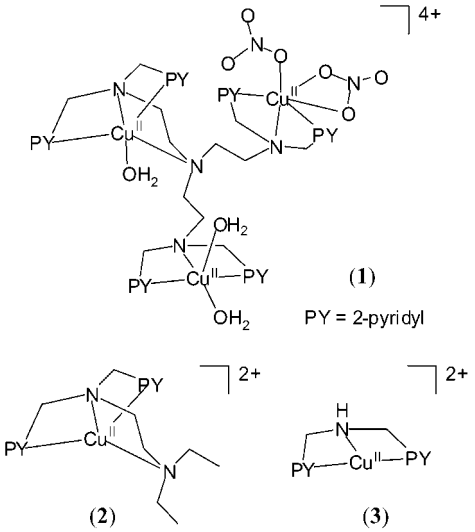
some variability in intensity due to local perturbations of DNA structure affects its efficiency.[12,13] Also a slight, but distinct preference for cleavage at 5′-AT-3′ and 5′-GT-3′ sites has been observed. Otherwise, $[Cu(OP)_2]^{2+}$ like EDTA·Fe(II) may be conjugated to binding elements such as proteins[14] and complementary sequences of RNA or DNA[15,16] that possess affinity for specific sites on DNA.[17] Still, multiple sites adjacent to the locus of recognition are typically oxidized by these complexes even when tethered to a DNA recognition element.

We have previously observed specific strand scission by a trinuclear copper complex that recognizes junctions between single- and double-stranded DNA without conjugation to an independent element of DNA recognition.[18] Cleavage is targeted at a single nucleotide in the single-stranded region that is 3′ to the junction. In addition to this structural requirement, the complex also exhibits a sequence requirement for guanine in the *n+1* (i.e. second unpaired) position of the 5′ overhang. The complex, $[Cu_3^{II}(\mathbf{L})(H_2O)_3(NO_3)_2](NO_3)_4\cdot 5H_2O$ (**1**),[18,19] incorporates three copper ions into a flexible ligand framework with pyridyl and amine nitrogen coordination about the coppers. Mononuclear copper(I) complexes with comparable coordination




are known to form dicopper(II)−peroxo dimers upon exposure to dioxygen.[20−23] Tethering of two of these moieties yields binuclear compounds that form peroxo intermediates, which can be entropically stabilized.[24−26] Addition of a third copper-binding unit can serve to model the blue-copper proteins[27] that effect the four-electron reduction of $O_2$ to water and in the process cleave the O−O bond.[28]

Biomimetic models for the active sites of multicopper proteins have several features that make them interesting for continued investigation of their reactivity with nucleic acids. Incorporation of three divalent copper ions in a single complex produces a highly cationic species that should have a strong electrostatic attraction to the anionic phosphate backbone of DNA. When in the reduced copper(I) state, these same three atoms in close proximity have the potential for three-electron reductive cleavage of $O_2$ producing the equivalent of hydroxyl radical. The X-ray structure of **1** shows that each copper possesses at least one labile coordination site that might allow for concurrent coordination of the substrate and the reactive intermediate.[19]

Multiple roles for metal centers in the binding of DNA and generation of reactive intermediates have been taken into account by researchers studying hydrolysis of nucleic acids,[29,30] but have yet to be fully considered by those investigating oxidation of related substrates. Recently, the trinuclear complex, $[Cu_3^{II}(\mathbf{L})(H_2O)_3(NO_3)_2](NO_3)_4\cdot 5H_2O$ (**1**), was shown to have the potential for uniquely recognizing and mediating oxidative cleavage of DNA in a highly specific manner that is not evident for its mononuclear analogues.[18] The specificity and origins of reaction are now described below in a more comprehensive manner. To test the predictive nature of **1**, an unreactive DNA junction was reengineered to generate a specific reaction within the parameters established in this report to generate a reaction variant. To assess the role of the metal ions and potential cooperative effects between them, two mononuclear compounds, $[Cu^{II}(\mathbf{L}')](NO_3)_2$ (**2**) and $[Cu^{II}(\mathbf{L}'')](NO_3)_2$ (**3**), were also synthesized. These complexes reflect the two possible metal coordination environments evident in the crystal structure of **1** and provide contrast to the activity of the trinuclear species. These results, together with those defining the roles of the reductant and dioxygen, suggest a hitherto unprecedented role for **1** in both recognition of the substrate and generation of the reactive intermediate.

## Experimental Section

**Materials.** Oligodeoxynucleotides were purchased from Gibco BRL. T4 kinase and the appropriate buffer were obtained from New England Biolabs and [$\gamma$-$^{32}$P]ATP (3000 Ci/mmol) was obtained from Amersham. The trinucleating ligand **L** (2,2′,2″-tris(dipicolylamino)triethylamine) and its corresponding coordination complex, $[Cu_3^{II}(\mathbf{L})(H_2O)_3(NO_3)_2](NO_3)_4\cdot 5H_2O$, were synthesized according to published procedures.[19] The analogue mononuclear ligand **L′** (**L′** = dipicolylamine) was

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prepared following a literature procedure.[31] Solutions of the metal complexes and other reagents for strand scission were prepared fresh daily in distilled−deionized water (18 MW·cm). Stock solutions of 3-mercaptopropionic acid (MPA, Aldrich), glutathione (GSH), and dithiothreitol (DTT) were titrated with Ellman's reagent to determine the free thiol concentration.[32] All other chemicals were used as supplied by the manufacturer.

**Synthesis of $[Cu^{II}(L')](NO_3)_2$ (2).** A solution of $Cu(NO_3)_2 \cdot 2.5\ H_2O$ (0.25 g, 1.1 mmol) in 10 mL of MeOH was added dropwise to a solution of L′ (0.20 g, 1.0 mmol) in an equivalent volume of MeOH. The mixture was stirred at room temperature until a blue precipitate formed. This solid was isolated by filtration, washed with $Et_2O$, and dried in vacuo to yield 0.28 g of product (72%). Anal. Calcd (Found) for $C_{12}H_{13}N_5CuO_6$: C, 37.26 (37.40); H, 3.39 (3.35); N, 18.10 (17.92).

**Synthesis of L″ (L″ = *N*,*N*-Diethyl-*N*′,*N*′-dipicolylethylenediamine).** 2-Picolyl chloride·hydrochloride (4.74 g, 28.9 mmol) was dissolved in 20 mL of $H_2O$ and the solution was cooled to 4 °C in an ice bath, then 10 mL of 5.7 M NaOH(aq) was added dropwise with stirring. A solution of *N*,*N*-(diethyl)ethylenediamine (2.00 mL, 14.2 mmol) and 10 mL of $H_2O$ was next added dropwise followed by addition of 100 mL of $CH_2Cl_2$. The biphasic reaction mixture was stirred for 5 days at 40 °C. The reaction mixture was then extracted with $CH_2Cl_2$ and washed with $H_2O$. The organic layer was dried over $MgSO_4$, filtered, and concentrated by rotary evaporation to give a brown oil. This crude product was purified by column chromatography on alumina with 99% EtOAc/1% MeOH ($R_f$ = 0.32), yielding 3.86 g of pure product by TLC (68%). $^1$H NMR ($CDCl_3$): 1.0 (t, 6H), 2.5 (q, 4H), 2.7 (m, 4H), 3.9 (s, 4H), 7.1 (t, 2H), 7.5−7.7 (m, 4H), 8.5 (d, 2H).

**Synthesis of $[Cu^{II}(L'')](NO_3)_2$ (3).** L″ (0.304 g, 1.02 mmol) and $Cu(NO_3)_2 \cdot 2.5H_2O$ (0.238 g, 1.02 mmol) were dissolved in 10 mL of MeCN and allowed to stir at room temperature for 1 h, yielding a royal blue solution. The solution was then layered with 15 mL of $Et_2O$, which induced crystallization of royal blue needles after 3 days at room temperature. The crystals were filtered, washed with $Et_2O$, and dried in vacuo to produce the desired product (0.323 g, 65%). Anal. Calcd (Found) for $C_{18}H_{26}N_6CuO_6$: C, 44.49 (44.47); H, 5.39 (5.27); N, 17.29 (17.22).

**Purification and Labeling of DNA.** Oligonucleotides were purified prior to use by denaturing (7 M urea) polyacrylamide gel electrophoresis and elution into 50 mM NaOAc and 1 mM EDTA (pH 5.2). The resulting solution was extracted with phenol/chloroform. DNA was then precipitated by the addition of 3 M NaOAc and 95% ethanol, dried under reduced pressure, and redissolved in water. Concentrations were determined spectrophotometrically at 260 nm with use of calculated extinction coefficients.[33] DNA was radiolabeled by incubation with [$\gamma$-$^{32}$P]ATP and T4 kinase according to the supplier. The 5′-$^{32}$P-labeled DNA was isolated by passage over a MicroBioSpin P-6 column (Bio-Rad). Frayed duplex structures containing a 5′-$^{32}$P-labeled oligonucleotide (100 nM, 90 nCi) and a complementary sequence (200 nM) were annealed in sodium phosphate (10 mM, pH 7.5) by heating to 90 °C followed by slow cooling to room temperature. Hairpin and single-stranded constructs were formed by mixing 5′-$^{32}$P-labeled oligonucleotides (100 nM, 90 nCi) in sodium phosphate (10 mM, pH 7.5).

**Copper-Dependent Strand Scission.** Various concentrations of the trinuclear complex, $[Cu_3^{II}(\mathbf{L})(H_2O)(NO_3)_2](NO_3)_2 \cdot 5H_2O$ (**1**), were combined with labeled DNA samples (100 nM, 90 nCi) in sodium phosphate (10 mM, pH 6.8) and strand scission was initiated by addition of 5 $\mu$L of reductant (5 mM) to yield a total volume of 50 $\mu$L. Following a 15 min incubation at ambient temperature, the reaction was quenched by addition of 10 mM diethyl dithiocarbamic acid (5 $\mu$L) and the DNA was isolated by ethanol precipitation and dried under high vacuum. As indicated, certain samples were further treated with 20 $\mu$L of piperidine (0.2 M) for 30 min at 90 °C. These samples were dried under reduced pressure, twice redissolved with 20 $\mu$L of water, and subsequently dried to remove trace quantities of piperidine. The isolated DNA was resuspended in 10 $\mu$L of water, normalized to 45 nCi per sample, and mixed with 3 $\mu$L of loading buffer (0.25% bromphenol blue, 0.25% xylene cyanole, 3% sucrose, and 7 M urea). The resulting samples were then separated by denaturing (7 M urea) polyacrylamide (20%) gel electrophoresis and visualized by autoradiography and PhosphorImagery (Molecular Dynamics). Quantitation of the products relied on ImageQuant software.

Solutions of $[Cu(OP)_2]^{2+}$ were prepared according to the literature[34] and reacted with 5′-labeled oligonucleotides with the standard reaction conditions for **1** and 5 mM MPA as the reductant.

**$O_2$ Dependence of Strand Scission.** To test the dependence of strand scission on $O_2$, the standard reaction conditions were modified to either limit or increase the amount of dioxygen in the reaction mixture. To limit the $O_2$ concentration, a solution containing the frayed duplex **Ia** (100 nM) and 2.5 $\mu$M $[Cu_3^{II}(\mathbf{L})(H_2O)_3(NO_3)_2](NO_3)_4 \cdot 5H_2O$ (**1**) was degassed by bubbling with prepurified nitrogen using a syringe needle for 15 min prior to initiation of strand scission with 5 $\mu$L of MPA (5 mM, undegassed). A nitrogen atmosphere was maintained by blanketing the reaction mixture with gas over the 15 min incubation. To increase the $O_2$ concentration, a solution of 100 nM **Ia** and 2.5 $\mu$M **1** was saturated with $O_2$ by bubbling with the gas through the solution. Following addition of 5 $\mu$L of MPA (5 mM) to the oxygenated solution, the reaction mixture was kept under an $O_2$ atmosphere. Product analysis then followed the standard procedures described above.

**Strand Scission in the Presence of Standard Radical Scavengers.** To test for participation of diffusible radical intermediates during strand scission, 10 mM ethanol, D-mannitol, and *tert*-butyl alcohol were alternatively added to standard reaction mixtures. Initiation of strand scission with MPA, quenching, and analysis followed the procedure for copper-dependent strand scission outlined above.

**Determination of $T_m$ Values.** To assess the presence of noncanonical base-pairing in the frayed duplexes, **IIa**, **IIb**, and **IIc**, the oligonucleotides (2.5 $\mu$M) were annealed at 90 °C in 10 mM sodium phosphate (pH 7.5) then slowly cooled to ambient temperature. Thermal denaturation was measured by the increase in absorbance at 260 nm, using a Perkin-Elmer spectrophotometer and a temperature controller (2 °C increments from 24 to 62 °C). $T_m$ values were estimated by the average of two independent determinations of the temperature at $^1/_2$ $\Delta$OD from a plot of temperature vs $OD_{260}$.

## Results and Discussion

**Elements of Recognition.** Selective strand scission by $[Cu_3^{II}(\mathbf{L})(H_2O)(NO_3)_2](NO_3)_4 \cdot 5H_2O$ (**1**) was first observed while surveying oxidative cleavage of a variety of nucleic acid structures. Incubation of the hairpin-forming oligonucleotide, **Ωa** (Figure 1A), with MPA for 15 min at ambient temperature induced specific strand scission directed at $A_{22}$.[18] Cleavage at $A_{22}$ represented 78% of the total cleavage at 0.5 $\mu$M complex **1** concentration, but use of higher concentrations of **1** yielded lower selectivity due to a nonspecific background of cleavage at all residues. The predicted secondary structure of **Ωa** indicates that position $A_{22}$ is situated in the 3′ single-stranded overhang of the hairpin at the junction with the double-helical stem region. The hairpin structure of **Ωa** and the location of $A_{22}$ in a single-stranded region were confirmed by chemical modification with $CoCl_2$ and KBr with oxone and $KMnO_4$.[35−39] To begin exploring the generality of this target selection, a frayed duplex **Ia** (Figure 2) was next subjected to reaction with **1**. This new structure

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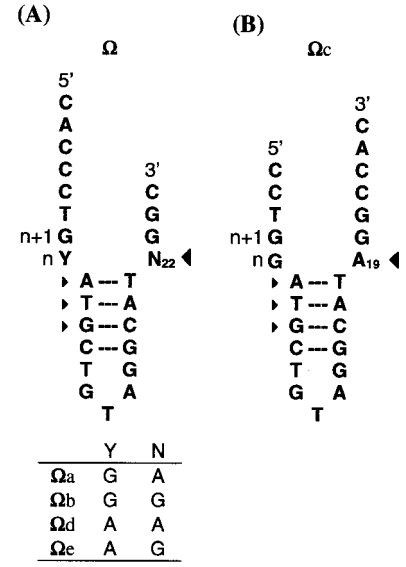
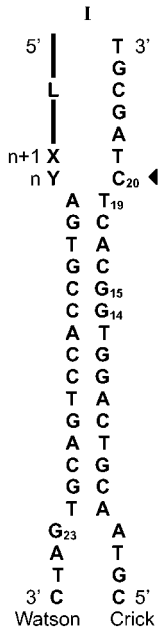



| | Y | N |
|---|---|---|
| Ωa | G | A |
| Ωb | G | G |
| Ωd | A | A |
| Ωe | A | G |

***Figure 1.*** Structures and sequences of the hairpin, **Ω**: (A) **Ωa**, **Ωb**, **Ωd**, **Ωe** and (B) **Ωc**.




| | X | Y | L[a] |
|---|---|---|---|
| Ia | G | A | TGCTC |
| Ib | – | – | – |
| Ic | – | p[b] | – |
| Id | – | A | – |
| Ie | G | A | – |
| If | A | A | – |
| Ig | T | A | – |
| Ih | C | A | – |
| Ii | G | T | – |

[a]Sequence of 5' overhang beyond X,Y
[b]p = 5'-phosphoryl

***Figure 2.*** Structures and sequences of the frayed duplex, **I**.

presented a duplex region flanked by 3′ and 5′ overhangs on each strand and provided two junctions at which reaction could occur with the potential cleavage sites occupied by $G_{23}$ and $C_{20}$ (Figure 2). Reaction of **Ia** under the standard conditions with 0.5 $\mu$M **1** produced 1% total cleavage on the Crick strand (shown on the right in Figure 2) with 80% of that directed at $C_{20}$ (Figure 3, lane 4). A 10-fold increase in complex concentration to 5 $\mu$M **1** increased the total cleavage of **Ia** to 23%, but selectivity for $C_{20}$ decreased to 50% of total fragment products (Figure 3, lane 6). This is due to a general increase of reaction at all sites, but most particularly at $G_{14}$ and $G_{15}$. Such a dependence of selectivity on the concentration of **1** is consistent with the results

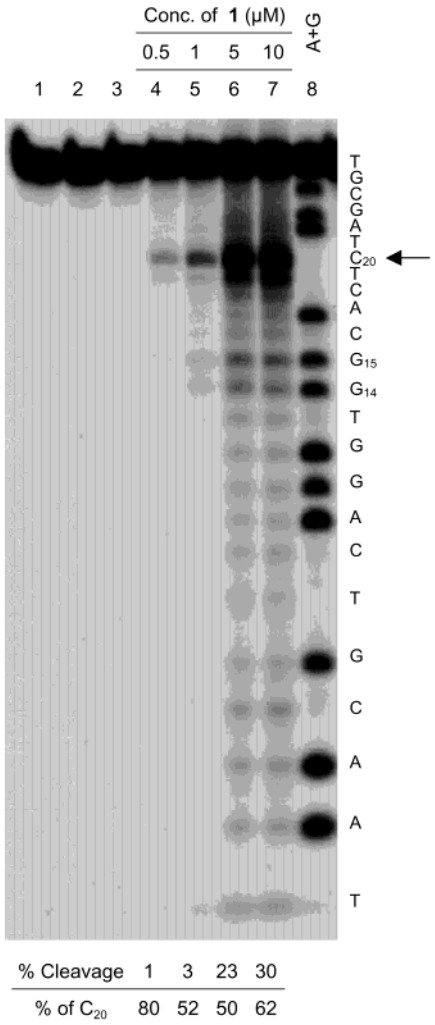



***Figure 3.*** Autoradiogram of a 20% polyacrylamide denaturing gel (7 M urea) showing cleavage products of 5′-$^{32}$P-labeled **Ia** (Crick strand, 100 nM) incubated with **1** and MPA for 15 min in phosphate buffer (10 mM, pH 6.8) at ambient temperature. Lane 1: **Ia**. Lane 2: **Ia** with 10 $\mu$M **1**. Lane 3: **Ia** with 5 mM MPA. Lanes 4−7: 0.5, 1, 5, and 10 $\mu$M **1** with 5 mM MPA and **Ia**. Lane 8: A+G sequencing lane.

of **Ωa**, which also showed a decrease in cleavage selectivity from 78% to 66% after the same 10-fold increase in concentration of **1** due to an increase in background cleavage at the other bases.[18] Alternative $^{32}$P-labeling of the Watson strand (shown on the left in Figure 2) produced 1% total cleavage resulting from low levels of sequence-neutral cleavage in the presence of 1 $\mu$M **1** (Figure 4, lane 5). Further increases in the concentration of **1** to 5 and 10 $\mu$M produced 1.5-fold less total cleavage than observed on the Crick strand (Figure 4, lanes 6 and 7). No more than 10% of the total cleavage was directed at a given nucleotide, including $G_{23}$ where specific strand scission might have been expected. This result indicated that the necessary attributes for selective cleavage were not present at this alternative junction. The background reaction on both strands of **Ia** was similar in its intensity and lack of specificity. The relative efficiency of nonspecific reaction is similar for the Watson and Crick strands, but the added specific reaction on the Crick strand enhances it in apparent overall reaction.

Subsequent piperidine treatment of **Ia** oxidized by **1** and MPA resulted in an increase in strand scission of both strands, but no significant change in the cleavage pattern.[39] The increased

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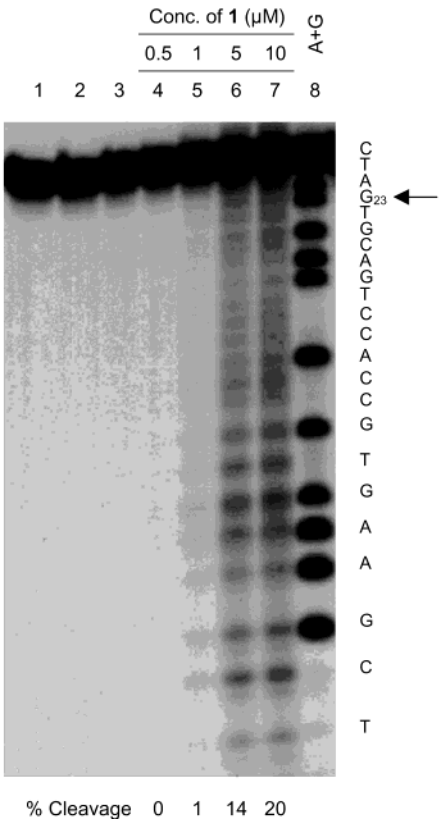


***Figure 4.*** Autoradiogram of a 20% polyacrylamide denaturing gel (7 M urea) showing cleavage products of 5′-$^{32}$P-labeled **Ia** (Watson strand,100 nM) incubated with **1** and MPA for 15 min in phosphate buffer (10 mM, pH 6.8) at ambient temperature. Lane 1: **Ia**. Lane 2: **Ia** with 10 $\mu$M **1**. Lane 3: **Ia** with 5 mM MPA. Lanes 4−7: 0.5, 1, 5, and 10 $\mu$M **1** with 5 mM MPA and **Ia**. Lane 8: A+G sequencing lane.

amount of cleavage following piperidine treatment might indicate that **1** can facilitate oxidation of both the nucleobases and the deoxyribose moieties. However, this piperidine labile fraction may also result only from additional types of sugar oxidation that do not produce spontaneous strand scission. Selective cleavage at $C_{20}$ was observed prior to piperidine treatment and therefore was likely a result of sugar and not nucleobase oxidation at the cytosine.

Specific reaction of **1** could be due to an intrinsic tendency for oxidation of $C_{20}$. To test this theory, each Watson and Crick strand of **I**a was reacted independently with **1** in the absence of its respective complement. The Watson strand produced 1% total cleavage in the presence of **1** (1 $\mu$M), similar to its reactivity as a frayed duplex structure in **Ia**.[39] No selectivity is observed for any of the nucleotides in this single strand. When the Crick strand was single stranded, total cleavage at 1 $\mu$M **1** was 2.5% (Figure 5, lane 5). At the higher complex concentrations, cleavage of the single Crick strand is greater than that of the duplex (Figure 5, lanes 6 and 7). Binding by the complex in the major or minor grooves of the duplex should produce differences in the overall cleavage of single- vs double-stranded DNA with a preference for reaction with the duplex. The appearance of low-level sequence/structure neutral cleavage of both **Ia** and the respective single-stranded sequences suggests that **1** either interacts equally or not at all with most of the strand. In contrast, $[Cu(OP)_2]^{2+}$, which is known to bind in the minor groove of duplex DNA, does not effect cleavage of single-stranded DNA.[40]

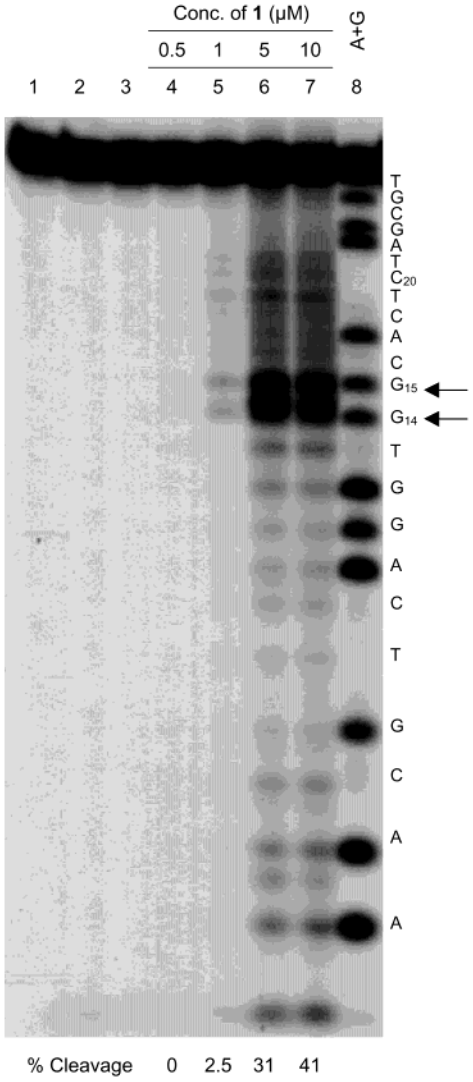


***Figure 5.*** Autoradiogram of a 20% polyacrylamide denaturing gel (7 M urea) showing cleavage products of single-stranded 5′-$^{32}$P-labeled **Ia** (Crick strand, 100 nM) incubated with **1** and MPA for 15 min in phosphate buffer (10 mM, pH 6.8) at ambient temperature. Lane 1: **Ia**. Lane 2: **Ia** with 10 $\mu$M **1**. Lane 3: **Ia** with 5 mM MPA. Lanes 4−7: 0.5, 1, 5, and 10 $\mu$M **1** with 5 mM MPA and **Ia**. Lane 8: A+G sequencing lane.

The lack of selectivity observed at $C_{20}$ in single-stranded DNA suggests that this site is not inherently reactive. Treatment of the single strands with piperidine also does not indicate a preference for reaction at $C_{20}$ or any particular nucleobase. An interesting feature of the single-stranded Crick strand of **Ia** is its enhanced reactivity at $G_{15}$ and $G_{14}$ (Figure 5). Selective reaction at these two nucleotides, which accounts for a combined 35 $\pm$ 4% of the total cleavage at 1, 5, and 10 $\mu$M **1**, led to higher than expected cleavage of the strand. This result could be due to the enhanced oxidizability of guanine[3] or preferential chelation of the two guanines by **1** due to an unknown structure not detected by standard folding programs.

The specific cleavage reaction facilitated by **1** typically requires adjacent double-helical and single-stranded portions of DNA and therefore involves more than structural recognition of either single- or double-stranded DNA. Initial observation of specific strand scission at two sites ($A_{22}$ of **Ωa** and $C_{20}$ of

(40) Marshall, L. E.; Graham, D. R.; Reich, K. A.; Sigman, D. S. *Biochemistry* **1981**, *20*, 244−250.

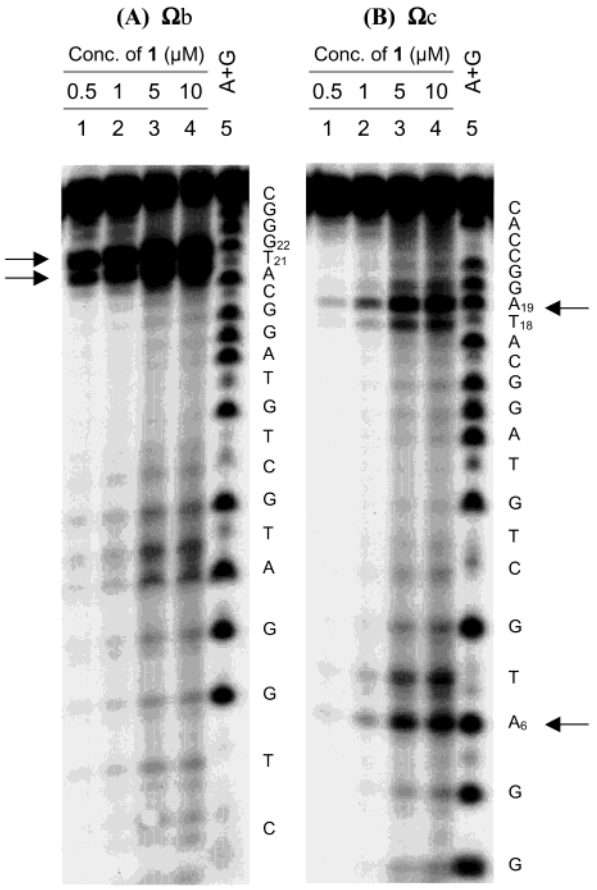


***Figure 6.*** Autoradiogram of a 20% polyacrylamide denaturing gel (7 M urea) showing cleavage products of 5′-$^{32}$P-labeled **Ωb** (100 nM) and **Ωc** (100 nM) incubated with **1** and MPA for 15 min in phosphate buffer (10 mM, pH 6.8) at ambient temperature. (A) Lanes 1−4: **Ωb** and 5 mM MPA with 0.5, 1, 5, and 10 $\mu$M **1**. Lane 5: A+G sequencing lane. (B) Lanes 1−4: **Ωc** and 5 mM MPA with 0.5, 1, 5, and 10 $\mu$M **1**. Lane 5: A+G sequencing lane.

**Ia**) suggested that **1** may preferentially recognize junctions between single- and double-stranded DNA. However, the absence of a similar reaction at $G_{23}$ of **I**a demonstrated that a further requirement, possibly derived from the sequence surrounding the junction, may also control selectivity and scission. To test this possibility, the nucleotide sequence proximal to the specific cleavage site was systematically varied.

**Nucleotide Sequence Dependence for Selective Strand Cleavage.** Since cleavage appeared selective for a nucleotide position rather than a specific nucleobase, reaction dependent on **1** was thought to be completely independent of the nucleobase at the target site. To test this possibility, the adenine at the cleavage site in **Ωa** was replaced with a guanine affording **Ωb** (Figure 1A). Reaction of **Ωb** with **1** resulted in an increase in total cleavage relative to that of **Ωa** at all concentrations of **1** (Figure 6A). Selective strand scission occurred primarily at $G_{22}$ and $T_{21}$, both of which are near the junction. Reaction at $G_{22}$ was more intense, representing 60% of the total cleavage at 0.5 $\mu$M **1** (Figure 6A, lane 1). An additional 25% of the total cleavage was directed at $T_{21}$ (Table 1) at each concentration tested. Reaction at $G_{22}$ and $T_{21}$ together resulted in specific cleavage events accounting for 85% of the total cleavage in **Ωb** at 0.5 $\mu$M **1**. This compares favorably with the amount of cleavage directed at $A_{22}$ in **Ωa** (78%). Exchange of adenine for guanine at the cleavage site of **Ωb** led to the targeting of two

***Table 1.*** Cleavage of the Hairpin (**Ω**) Constructs

| | | concn of **1** | | |
|---|---|---|---|---|
| | | 0.5 $\mu$M | 1 $\mu$M | 5 $\mu$M |
| **Ωa** | total cleavage | 4 | 9 | 40 |
| | % at $A_{22}$ | 78 | 72 | 66 |
| **Ωb** | total cleavage | 16 | 34 | 70 |
| | % at $G_{22}$ | 60 | 53 | 40 |
| | % at $T_{21}$ | 25 | 25 | 28 |
| **Ωc** | total cleavage | 2 | 7 | 36 |
| | % at $A_{19}$ | 40 | 24 | 23 |
| | % at $T_{18}$ | 14 | 8 | 10 |
| | % at $A_6$ | 19 | 17 | 14 |
| **Ωd** | total cleavage | <1 | 2 | 56 |
| | % at $A_{22}$ | na | 85 | 71 |
| **Ωe** | total cleavage | 1 | 9 | 67 |
| | % at $G_{22}$ | 60 | 63 | 45 |
| | % at $T_{21}$ | 25 | 21 | 20 |
| | % at $A_{20}$ | 10 | 8 | 9 |

nucleotides, but the primary locus of cleavage, $G_{22}$, was still immediately 3′ to the stem region of the hairpin. This result suggests that the nucleobase of the reaction site appears to be neither the target of reaction nor a necessary element of recognition.

The variables that might possibly affect the selective reactivity are local sequence, the unique structure of nucleotide residues around the junction, or proximity to the frayed 3′ termini. This last possibility was explored through circular permutation of the hairpin, which involved transfer of three bases from the 5′ end of **Ωa** to its 3′ end so that $A_{22}$ of **Ωa** became $A_{19}$ of **Ωc** (Figure 1B). Due to the ability of copper to coordinate to phosphate oxygens[41] in addition to nucleobase nitrogens, this change was made to explore a proximity effect resulting from the nearness of the 3′ terminus and helical junction of **Ωa**. Cleavage of **Ωc** with **1** targeted $A_{19}$ in **Ωc** (Figure 6B, lane 2). Reaction at this site appears to rule out an interaction of **1** with the 3′ end of the oligonucleotide. The lower selectivity of cleavage at $A_{19}$ in **Ωc** is linked to an increase in the cleavage of $A_6$, which forms part of the stem of **Ωc** (Table 1). Otherwise, nonspecific background cleavage mediated by **1** is comparable for the two hairpin structures. The differences in the cleavage patterns likely reflect slight variations in the structures of **Ωa** and **Ωc** that affect the binding and recognition of **1** and allow for reaction at both a paired and unpaired base in the helix junction. Since the reaction selectivity was relatively independent of the target nucleotide, the local sequence and structure near the junction may then control scission.

**Role of Guanine in the *n*+1 Position of the 5′ Overhang.** A comparison of the hairpin **Ωa** and its derivatives, **Ωb** and **Ωc** (Figure 1A,B), with the frayed duplex **Ia** (Figure 2) shows no similarities in the sequences of the 3′ overhangs. Review of the *n*+1 positions of the hairpins and the frayed duplex structures reveals that all the sequences for which specific cleavage was observed possessed a guanine in the *n*+1 position of the 5′ overhang. The 5′ sequence opposite $C_{20}$ on **Ia** was first completely truncated to give duplex **Ib** and **Ic** (Figure 2). Reaction of **Ib** in the presence of **1** resulted in a loss of selectivity for $C_{20}$.[39] Therefore, at least some portion of the 5′ overhang is necessary in the recognition and reaction process. The deleted sequence presented numerous coordination sites on the phosphate backbone and the nucleobases for transition metal

(41) Sigel, H. *Chem. Soc. Rev.* **1993**, *22*, 255−267.

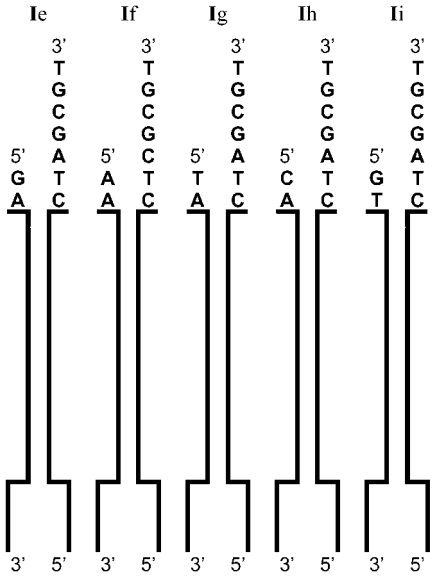


***Figure 7.*** Structures of **Ie**−**i** exhibiting 5′ truncated overhangs.

ions in general and copper more specifically.[42,43] Coordination of phosphate compounds to Cu(II) in a related ligand environment has previously been observed[44] and therefore a phosphoryl group was added back to the truncated sequence yielding **Ic** (Figure 2) in an attempt to test the importance of this specific ligand. Reaction of the 5′ phosphorylated and truncated sequence **Ic** with **1** produced only nonspecific background cleavage. No recovery of reaction at $C_{20}$ was apparent.[39] Consequently, complex **1** does not likely interact directly with this phosphate. Alternatively, nucleobases surrounding the junction on the 5′ overhang opposite the cleavage site may be responsible for directing **1**. However, restoration of a single nonhelical adenine in the *n* position of the 5′ overhang (**Id**, Figure 2) was not sufficient to induce specific cleavage.[39] Further extension of the truncated sequence to include a nonhelical guanine in the *n*+1 position of the 5′ overhang (Figure 7) restored the selective strand scission at $C_{20}$ of **Ie**, although at a lower level than observed with **Ia** (Figure 8A). Truncation of the 5′ overhang also produced an increase in cleavage at $G_{14}$ and $G_{15}$. These results may indicate reduced stability of **Ie** as a duplex relative to **Ia** thereby changing the equilibrium of DNA structures in the reaction mixture.

To test the necessity of guanine versus other nucleobases in the *n*+1 position, analogous DNA substrates substituted with adenine, thymine, and cytosine (duplexes **If**, **Ig**, and **Ih**, Figure 7) were tested with **1**. None of these resulting derivatives were capable of mediating specific cleavage at $C_{20}$.[39] If the data are normalized to $G_{15}$, then the reaction at $C_{20}$ in **Ia** is between 10- and 20-fold more efficient than in the truncated sequences with adenine, thymine, or cytosine at the *n*+1 position. An equivalent comparison of reaction at $C_{20}$ in **Ia** and the minimal target of **Ie** shows that reaction at $C_{20}$ is only 1.5-fold more efficient. The basal level of nonspecific background cleavage was once again the same for all the frayed duplexes. *The presence of guanine in the n+1 position of the 5′ overhang adjacent to the junction appears to be an absolute and minimal sequence requirement for recognition by* $[Cu_3^{II}(\mathbf{L})(H_2O)_3(NO_3)_2](NO_3)_2 \cdot 5H_2O$ ***(1)***.

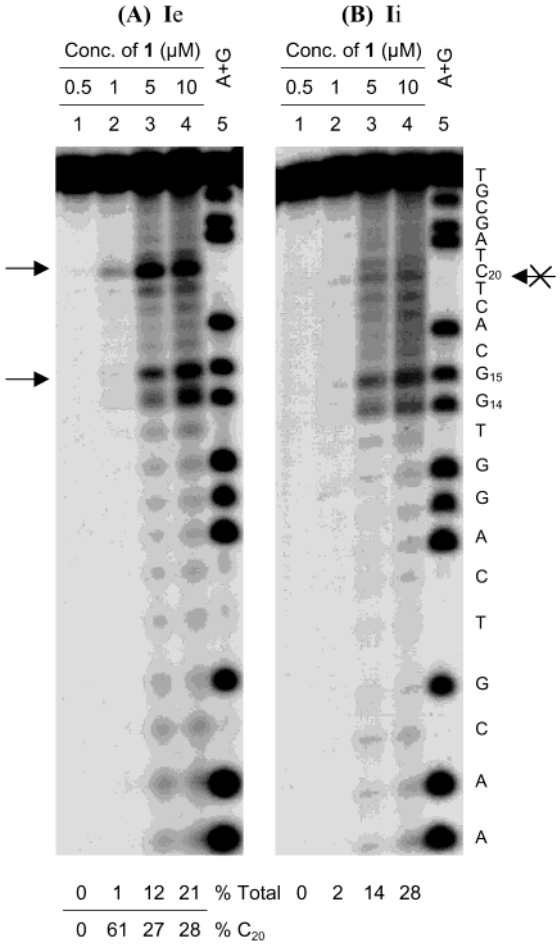


***Figure 8.*** Autoradiogram of a 20% polyacrylamide denaturing gel (7 M urea) showing cleavage products of 5′-$^{32}$P-labeled **Ie** (Crick strand,100 nM) and **Ii** (Crick strand, 100 nM) incubated with **1** and MPA for 15 min in phosphate buffer (10 mM, pH 6.8) at ambient temperature. (A) Lanes 1−4: **Ie** and 5 mM MPA with 0.5, 1, 5, and 10 $\mu$M **1**. Lane 5: A+G sequencing lane. (B) Lanes 1−4: **Ii** and 5 mM MPA with 0.5, 1, 5, and 10 $\mu$M **1**. Lane 5: A+G sequencing lane.

The dependence on guanine at the helical junction and considerable precedent for transition metal coordination to nucleobases available in the literature suggests recognition of either a structure dependent on guanine or direct coordination to the guanine in the *n*+1 position. Of the potential coordination sites,[42,45] the N7 of guanine exhibits the highest affinity for binding to transition metal complexes.[46] In double-helical DNA, however, this position is situated within the major groove and is only partially accessible. For this reason, interactions between **1** and the N7 are more likely to occur in a region of DNA that is single stranded. Whether guanine is necessary to maintain a unique oligonucleotide fold or a metal coordination site, it does not appear to participate in or be a target of reaction. Instead, this guanine most likely serves to anchor or localize the trinuclear complex for its ultimate scission of the adjacent strand.

(42) Lippard, S. J.; Berg, J. M. *Principles of Bioinorganic Chemistry*; University Science Books: Mill Valley, CA, 1994.
(43) Kazakov, S. A. In *Bioorganic Chemistry*: *Nucleic Acids*; Hecht, S. M., Ed.; Oxford University Press: New York, 1996; pp 244−287.
(44) Young, M. J.; Wahnon, D.; Hynes, R. C.; Chin, J. *J. Am. Chem. Soc.* **1995**, *117*, 9441−9447.
(45) Sigel, H. In *Metal-DNA Chemistry*; Tullius, T. D., Ed.; American Chemical Society: Washington, DC, 1989; pp 159−204.
(46) Martin, R. B. *Acc. Chem. Res.* **1985**, *18*, 32−38.

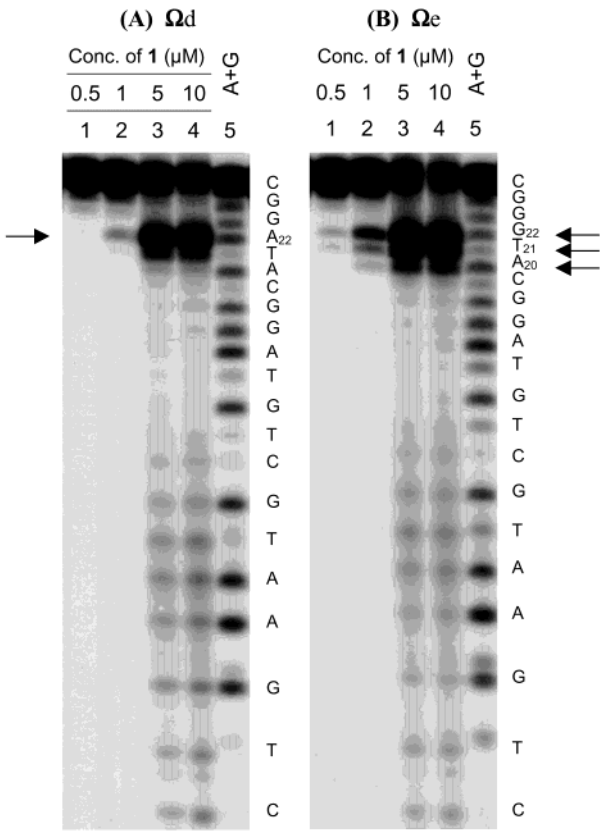


***Figure 9.*** Autoradiogram of a 20% polyacrylamide denaturing gel (7 M urea) showing cleavage products of 5′-$^{32}$P-labeled **Ωd** (100 nM) and **Ωe** (100 nM) incubated with **1** and MPA for 15 min in phosphate buffer (10 mM, pH 6.8) at ambient temperature. (A) Lanes 1−4: **Ωd** and 5 mM MPA with 0.5, 1, 5, and 10 $\mu$M **1**. Lane 5: A+G sequencing lane. (B) Lanes 1−4: **Ωe** and 5 mM MPA with 0.5, 1, 5, and 10 $\mu$M **1**. Lane 5: A+G sequencing lane.

**Role of a Purine in the *n* Position of the 5′ Overhang.** The presence of a guanine in the *n*+1 position of the 5′ overhang is not the only factor determining reaction at the helix/coil junction. Use of additional target sequences suggested that a purine must also be present in the *n* position of the 5′ overhang for selective strand scission (Figures 1 and 2). The importance of this criteria was illustrated when a structure containing a nonhelical guanine and thymine in the *n*+1 and *n* positions (**Ii**, Figure 7), respectively, was reacted with **1**. Regardless of the concentration of **1**, the amount of background cleavage of **Ii** was comparable to that observed on other constructs that lacked the necessary sequence elements for specific strand scission and reaction at $C_{20}$ was dramatically suppressed (Figure 8B). Less than 1% of the total cleavage was directed at the target $C_{20}$, suggesting a requirement for a purine at the *n* position of the 5′ overhang.

The role of purine in the *n* position in modulating reaction selectivity was assessed by replacing the guanines of **Ωa** and **Ωb** with adenine. This nucleobase was determined to affect targeting by **1** to a lesser degree than the base occupying the cleavage site. When the cleavage site is occupied by guanine, strand scission is no longer limited to just one nucleotide target. In contrast, adenine seems to direct **1** more precisely at a single base near the junction on the 3′ strand. Hairpin **Ωd**, which possesses an adenine both at the cleavage site and in the *n* position of the 5′ overhang, supported highly specific cleavage

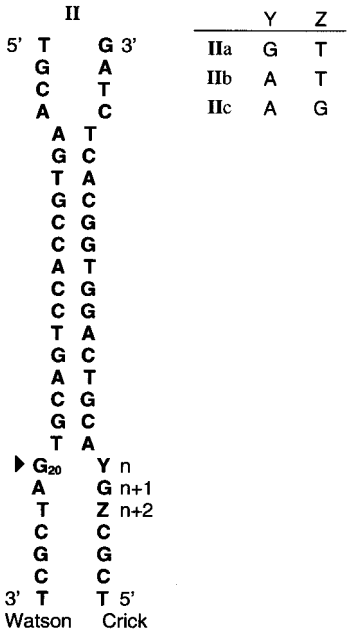


***Figure 10.*** Structure and sequences of the frayed duplex, **II**.

at $A_{22}$ and limited reaction at adjacent stem and single-stranded portions (Figure 9A, Table 1). Scission at $A_{22}$ accounted for 85% of the total cleavage with 1 $\mu$M **1**. In contrast, placement of a guanine at the cleavage site with an adenine at position *n* (**Ωe**) led to cleavage at three adjacent sites, $G_{22}$ and the preceding nucleotides $T_{21}$ and $A_{20}$ (Figure 9B). When cleavage at these three nucleotides is added together they account for 92% of the total cleavage at 1 $\mu$M **1**. These results are consistent with the differential specificity of **1** with **Ωa** vs **Ωb** for which the adenine at the cleavage site was exchanged with guanine resulting in target expansion from $G_{22}$[18] to include $G_{22}$ and $T_{21}$ (Figure 6 and Table 1). A guanine at the target locus regardless of the identity of the purine in the *n* position may offer more than one possible conformation or ligand for binding of the complex leading to less targeted strand scission. Similar competition was previously observed with distamycin conjugates of EDTA·Fe(II), which were attributed to multiple binding positions of the drug.[47]

**Engineering a Frayed Duplex for Specific Strand Scission.** To observe specific strand scission of DNA by **1**, the target appeared to exhibit a minimum of three prerequisites. First, a junction between single- and double-stranded regions must be present; second, guanine is required at the *n*+1 position of the 5′ overhang; and third, a purine is required at the *n* position (Figures 1 and 2). The predictive nature of these requirements was next tested by redesigning an unreactive junction of **Ia** to include the necessary sequence elements (**IIa**, Figure 10). According to the proposed rules for specific reactivity, the frayed end containing $G_{23}$ of the Watson strand of **Ia** (Figure 2) did not previously exhibit selective cleavage because it lacked the necessary sequence on the 5′ overhang. To induce specific strand scission the nucleotide in the *n*+1 position on the 5′ overhang of the Crick strand of **Ia** was modified from a thymine to a guanine. Additionally, the nucleotide in the *n* position was similarly changed from an adenine to a guanine. The overhangs near the junction were also lengthened merely to increase the

(47) Taylor, J. S.; Schultz, P. G.; Dervan, P. B. *Tetrahedron* **1984**, *40*, 457−465.

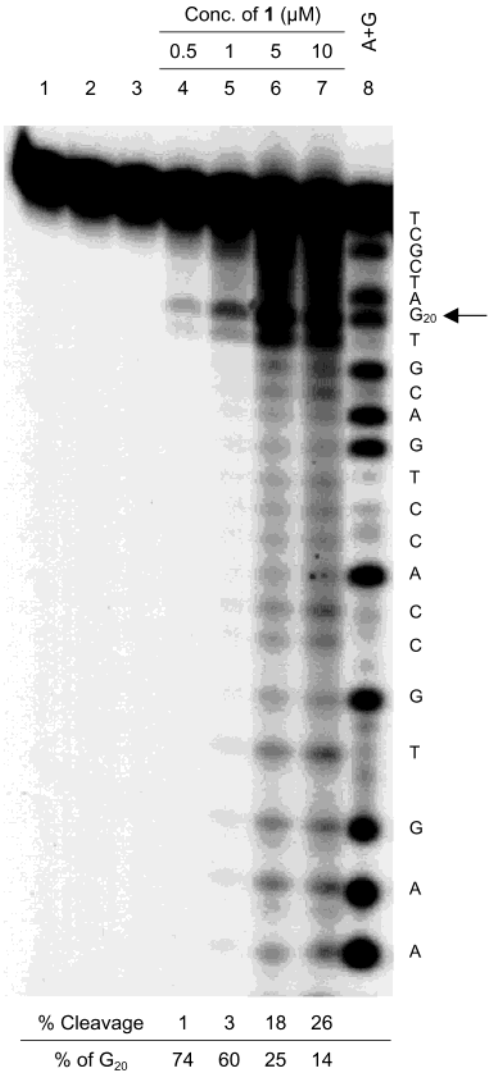


***Figure 11.*** Autoradiogram of a 20% polyacrylamide denaturing gel (7 M urea) showing cleavage products of 5′-$^{32}$P-labeled **IIa** (Crick strand, 100 nM) incubated with **1** and MPA for 15 min in phosphate buffer (10 mM, pH 6.8) at ambient temperature. Lane 1: **IIa**. Lane 2: **IIa** with 10 $\mu$M **1**. Lane 3: **IIa** with 5 mM MPA. Lanes 4−7: 0.5, 1, 5, and 10 $\mu$M **1** with 5 mM MPA and **IIa**. Lane 8: A+G sequencing lane.

resolution of scission products by using a sequencing gel with 5′-labeled oligonucleotides. Insertion of a guanine in both the $n+1$ and $n$ positions of the 5′ overhang of **IIa** resulted in specific strand scission at $G_{20}$ of the Watson strand corresponding to $G_{23}$ of **Ia** (Figure 11). The percentage of total cleavage was comparable to that of the Crick strand of **Ia**, although specific strand scission appeared to target both $G_{20}$ and $T_{19}$. Scission over a two-residue region is typical for adjacent sequences containing guanine at both the $n$ and $n+1$ positions. In both cases, the primary site of cleavage is at the first unpaired target and the secondary cleavage site occurs at the adjacent base-paired nucleotide.

Reaction might additionally have been expected after insertion of an adenine rather than a guanine at the $n$ position of the 5′ overhang. However, reaction of **IIb** and **IIc**, both possessing a guanine in the $n+1$ position and an adenine in the $n$ position of the 5′ overhang, resulted in a surprising lack of cleavage at $G_{20}$.[39] Although the structure of the duplex and sequence on the 5′ overhang conform to the basic requirements for recognition and specific strand scission, reactivity could be hindered by formation of tandem G·A mismatches between the 5′ and 3′ overhangs of these frayed duplex structures. The extent of helical structure in these sequences was assessed by comparing the thermal melting temperatures of the reactive target **IIa** and the unreactive derivatives **IIb** and **IIc**. As anticipated, the $T_m$ of **IIa** was 38 °C, whereas the $T_m$ temperatures of **IIb** and **IIc** were 44 and 41 °C.[39] Although one G·A mismatch at the terminus or within a helix is destabilizing, adjacent G·A, A·G mismatches confer a surprising stability through purine−purine stacking interactions.[48,49] When the G·A, A·G mismatch is flanked by a 5′ pyrimidine and a 3′ purine (5′-YGAR-3′, Y = pyrimidine and R = purine), the thermodynamic parameters are similar to those of a Watson−Crick base-paired duplex. This is reflected in the higher $T_m$ of **IIb**, which contained a 5′ thymine and 3′ adenine surrounding the G·A mismatch. In contrast, the mismatch in **IIc** is flanked by a 5′ guanine and 3′ adenine and appeared less stable than **IIb**.

A helix/coil junction is still present in **IIb** and **IIc**, but not in a comparable position to **IIa**. Consequently, the sequence extending beyond the helix is no longer appropriate for recognition and reaction by **1**. The sensitivity of **1** to weak hydrogen bonding and its inability to interrupt those interactions as illustrated by the lack of selective reaction of **IIb** and **IIc** may be beneficial in DNA footprinting experiments in which target disruption by a probe could produce misleading results. Once the source and nuances of recognition were elaborated, the reaction process by complex **1** and the thiol reductant could be examined.

**Strand Scission in the Presence of Different Reductants.** MPA was chosen as a convenient reductant of $[Cu_3^{II}(\mathbf{L})(H_2O)_3(NO_3)_2](NO_3)_4\cdot 5H_2O$ (**1**) to form its Cu(I) state, but this thiol is not unique in its ability to promote strand scission. Several commonly used biological reductants also support reaction of **1** with **Ia** under the standard conditions (5 mM reductant). The relative efficiency of the sulfur-containing reductants to promote the background and targeted cleavage of DNA is MPA > glutathione (GSH) ≫ dithiothreitol (DTT) > sodium dithionite. Although the amount of total cleavage varies significantly between the reductants from 13% with MPA to 6% with GSH, 3% with DTT and less than 1% with dithionite, there is no change in the relative selectivity for scission at $C_{20}$.[39]

A Cu(I) form of **1** can be expected to be generated through a thiol-mediated reduction of the initial Cu(II) complex leading to strand scission.[50] The similarity of the cleavage patterns produced in the presence of different thiols also indicates that the reactive intermediate is likely derived from the interaction of Cu(I) with a common component in each reaction mixture and is not a copper−thiol complex or a copper-derived thiol species such as thiyl radical.[51,52] Consequently, the reductant is not intimately involved in recognition of DNA or in formation of the ultimate oxidant. The observed differences in reactivity may be explained in part by the varying reduction potentials of the thiols tested. Depending on its reduction potential and the affinity of the thiol for coordination to copper, changes in the reductant could result in differing rates of oxidative cleavage of DNA.

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(51) John, D. C. A.; Douglas, K. T. *Biochem. J.* **1993**, *289*, 463−468.
(52) John, D. C. A.; Douglas, K. T. *Biochem. Biophys. Res. Commun.* **1989**, *165*, 1235−1242.

**Comparison of Thiol Reductants to Ascorbate.** Prior study of $[Cu(OP)_2]^{2+}$ demonstrated that both MPA and mercaptoethanol coordinate to copper in competition with the second phenanthroline ligand.[53] Spectroscopic detection of the reduction of $[Cu(OP)_2]^{2+}$ with MPA failed to yield a spectrum characteristic of the reduced complex, $[Cu(OP)_2]^{+}$. A new species was nevertheless observed following reduction by MPA and was assigned as a mixed phenanthroline−thiol−copper complex. This derivative appeared not to be involved in strand scission. An equivalent mixed complex was not detected when ascorbate was used to reduce $[Cu(OP)_2]^{2+}$. Although the overall rate of strand scission by $[Cu(OP)_2]^{2+}$ was higher in the presence of ascorbate, the cleavage pattern was identical with that observed with the thiol reductants, suggesting a common intermediate.[53] Relative to ascorbate, the rate of strand scission was slower in the presence of thiol reductants due to their competitive binding to copper effectively lowering the concentration of $[Cu(OP_2)]^{2+}$ in the reaction mixture.

To rule out a unique role of thiol-containing reductants in specific strand scission of **Ia** by **1**, ascorbate was used as an alternative reductant. At 5 mM ascorbate and 2.5 $\mu$M **1**, 29% of the Crick strand was cleaved.[39] Ascorbate was significantly more efficient than MPA (13% cleavage) and the concentration of **1** with ascorbate could be lowered to 0.25 $\mu$M to induce the same level of overall cleavage with MPA.[39] Given the 10-fold difference in complex concentration, DNA scission in the presence of ascorbate appears to be more efficient than that with sulfur-containing reductants. The selectivity of cleavage obtained at low concentrations of **1** with 5 mM ascorbate is comparable to that observed when higher concentrations of **1** are reacted with DNA in the presence of 5 mM MPA. The similarities in the relative amount and pattern of cleavage suggest that the Cu(I) form of **1** is the active species involved in both nonspecific and selective strand scission. The differences in the overall rates of cleavage in the presence of MPA or ascorbate may be due to competitive coordination of the thiol reductant to **1** that lowers the concentration of the active species in the reaction mixture.

**$O_2$ Dependence of Strand Scission.** The requirement of a reductant for reaction of $[Cu_3^{II}(\mathbf{L})(H_2O)_3(NO_3)_2](NO_3)_4\cdot 5H_2O$ (**1**) suggests that the intermediate responsible for strand scission derives from reaction of its Cu(I) species and dioxygen. To confirm the role of $O_2$, a standard reaction mixture containing 2.5 $\mu$M **1**, 5 mM MPA, and **Ia** was degassed with nitrogen to create an $O_2$-limited environment. Under these conditions, total cleavage decreased 10-fold relative to a standard air-saturated reaction.[39] Conversely, when an equivalent reaction mixture was saturated with dioxygen, total consumption of **Ia** increased by approximately 50%.[39] In either case, selectivity of strand scission at $C_{20}$ remained at 55 ± 3% of the total reaction. Involvement of an $O_2$-derived intermediate is consistent with the mechanism proposed for $[Cu(OP)_2]^{2+}$ and certain other transition metal complexes that mediate oxidative cleavage of nucleic acids.[8] Most of these complexes follow a Fenton-type mechanism wherein the reduced form of the metal interacts with dioxygen to produce a reactive oxygen species (i.e. superoxide, peroxide, hydroxyl radical) that is capable of abstracting a hydrogen atom from the deoxyribose moiety of a nucleotide residue. The reaction is nonspecific when this species is freely diffusible, but becomes successively more selective when the intermediate is held by metal coordination and localization to particular recognition sites.

$$M^{n+} + O_2 \rightarrow M^{n+1} + O_2^{-} \quad (1)$$

$$M^{n+} + O_2^{-} + 2H^{+} \rightarrow M^{n+1} + H_2O_2 \quad (2)$$

$$M^{n+} + H_2O_2 \rightarrow M^{n+1} + OH^{-} + {}^{\bullet}OH \quad (3)$$

$$(M^{n+} = Fe^{II} \text{ or } Cu^{II})$$

The ability of $[Cu_3^{II}(\mathbf{L})(H_2O)_3(NO_3)_2](NO_3)_4\cdot 5H_2O$ (**1**) to cause direct strand scission of DNA strongly suggests a cleavage mechanism involving hydrogen atom abstraction from the sugar. The limited profile of scission products formed by selective reaction argues against the intermediacy of a diffusible hydroxyl radical. This was further explored by the addition of known radical scavengers during cleavage of **Ia**. A decrease in the amount of selective and total cleavage would be expected if either process involved a diffusible radical species. If the intermediate remained "coordinated" to the metal center as proposed for $[Cu(OP)_2]^{2+}$ or was not a radical species, then addition of the scavengers would result in no change in the observed reaction. Strand scission of **Ia** by **1** in the presence or absence of 10 mM ethanol, D-mannitol, or *tert*-butyl alcohol exhibited no inhibition in total cleavage (11 ± 1%) and compares favorably with the reaction without radical quenching agent (12% degradation).[39] Specific strand scission at $C_{20}$ also remains relatively constant at 67 ± 3% of the total products detected. These results suggest that neither background nor specific cleavage proceed through a freely diffusible radical intermediate. This is consistent with earlier results obtained from the oxidation of pBR322 by **1** and MPA.[19] Piperidine treatment of the above reaction mixtures containing radical scavengers still resulted in an increase in overall strand scission.[39] However, there was no change in the intensity or pattern of cleavage relative to reaction in the absence of added quenching agent, suggesting that the species responsible for nucleobase oxidation is also not a diffusible radical.

Reaction of $[Cu(OP)_2]^{2+}$ with DNA also lacks a quenchable oxidative intermediate as shown by its insensitivity to the presence of 10 mM ethanol, mannitol, sodium formate, or potassium iodide.[40] In contrast, EDTA·Fe(II) has been shown by comparison with $\gamma$-radiolysis of water to mediate strand scission via generation of diffusible hydroxyl radicals in bulk solution.[54] Hydroxyl radical cleavage of DNA produced by the above methods shows no specificity for a particular sequence or nucleotide and is subject to quenching by standard radical scavengers. Tethering of EDTA·Fe(II) to a DNA recognition unit delivers its oxidizing intermediate to multiple contiguous positions near the site of complex interaction.[47,55] Specific strand scission by **1**, however, normally results in reaction directed at only one or two nucleotides. Both target specifity and lack of reactivity to H-atom donors demonstrate that the $O_2$-derived intermediate is not a freely diffusible radical species.

The exact nature of the ultimate oxidant responsible for cleavage of DNA by $[Cu_3^{II}(\mathbf{L})(H_2O)_3(NO_3)_2](NO_3)_4\cdot 5H_2O$ (**1**)

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will require additional experiments out of the scope of this report, although intermediates can be proposed through comparison with other DNA cleavage systems. The putative intermediate of $[Cu(OP)_2]^{2+}$ and MPA has been presented as a copper-bound "caged" radical species with the formula $[CuO]^+$ or $[CuOH]^{2+}$.[8,56] On the other hand, cupric hydroperoxo ($Cu^{II}$-OOH) or cupric superoxo ($Cu^{II}$-$O_2^-$) intermediates (or higher valent species derived from these) have been proposed as the active $O_2$-derived species for the copper-containing enzymes, dopamine $\beta$-hydroxylase and peptidyl glycine monooxygenase, that also effect hydrogen atom abstraction reactions.[57−59] In the absence of further chemical and spectroscopic insights, similar types of $O_2$-derived intermediates may be suggested for reaction of **1** and MPA with DNA in the presence of $O_2$.

**Sensitivity of Strand Scission to Electrostatics.** In aqueous solution, **1** is expected to be cationic with water or hydroxide occupying the vacant coordination sites[60] and therefore should experience a strong electrostatic attraction to the anionic phosphate backbone of DNA. While this effect does not lead to specific interaction of the complex with DNA, a general affinity would still enhance its local concentration around the substrate. Reaction of the hairpin **Ωa** with $[Cu_3^{II}(\mathbf{L})(H_2O)_3(NO_3)_2](NO_3)_4 \cdot 5H_2O$ (**1**) in 10 mM sodium phosphate buffer in the presence of 50 mM NaCl, $NaNO_3$, or $NaClO_4$ resulted in a decrease in the overall yield of strand scission relative to reaction in the absence of added salt[39] and is consistent with electrostatic shielding of the DNA. The presence of salt inhibits this interaction, effectively lowering the complex concentration, which leads to decreased strand scission. However, selectivity of cleavage continued to exhibit a distinct preference for $A_{22}$. By comparison, EDTA·Fe(II) does not interact with DNA due to its overall negative charge and shows no dependence on the ionic strength of the reaction mixture.[61] This result suggests that **1** accumulates near the surface of DNA.

The use of nitrate as the counterion in $[Cu_3^{II}(\mathbf{L})(H_2O)_3(NO_3)_2](NO_3)_4 \cdot 5H_2O$ (**1**) raised some concern regarding the ability of nitrate to act as an oxidant due to its thermodynamically favorable redox potential. However, this is not likely in this study since the extent and pattern of cleavage with additional nitrate was not different from that observed with excess perchlorate or chloride. The lack of correlation between reaction efficiency and the coordination strength of the anions suggests that although chloride may bind to copper and compete with the thiol reductant, DNA, or $O_2$, it is sufficiently labile in aqueous solution to not interfere with reactivity.

**Comparison of Multi- and Mononuclear Complexes.** The solid and solution properties of $[Cu_3^{II}(\mathbf{L})(H_2O)_3(NO_3)_2](NO_3)_4 \cdot 5H_2O$ (**1**) provide intriguing mechanistic possibilities for selective recognition and reaction of DNA. The X-ray structure of **1** reveals two copper ions, which are tridentate with respect to the ligand and are capable of coordinating two exogenous ligands, consistent with the tendency of Cu(II) to be pentacoordinate.[19] The ligand coordination derives from two pyridyl nitrogens and a tertiary amine. While the exogenous ligands in the solid state are water and nitrate, these sites may alternatively be occupied by hydroxide, substrate, or a reactive intermediate in solution. The third copper atom possesses tetradentate ligand coordination similar to that of the tridentate sites with an additional tertiary amine nitrogen in its coordination sphere. Tetradentate coordination leaves only one additional site on copper for coordination. The tri- and tetradentate coordination sites have the potential to exhibit very different reactivity with the reductant, substrate, and dioxygen, such that not all of the coppers within each complex would necessarily be involved in oxidative cleavage of DNA.

To better understand the role of the individual coppers in $[Cu_3^{II}(\mathbf{L})(H_2O)_3(NO_3)_2](NO_3)_4 \cdot 5H_2O$ (**1**), two mononuclear analogues of the complex were synthesized. One of these, $[Cu^{II}(\mathbf{L}')](NO_3)_2$ (**2**), was designed to model the tridentate sites, while the second compound, $[Cu^{II}(\mathbf{L}'')](NO_3)_2$ (**3**), was designed to model the tetradentate site. Equivalent copper ion concentrations were used for comparison of DNA cleavage by the mononuclear analogues and **1** in the presence of MPA under the standard reaction conditions. Less than 1% of the Crick strand of **Ia** was cleaved by **2** or **3** at every copper concentration tested (1.5 and 7.5 $\mu$M).[39] The failure of the mononuclear compounds to promote general cleavage of DNA may indicate that they do not react with dioxygen to generate the necessary intermediate. Conclusions about selective strand scission cannot be made in the absence of distinguishable reactivity. A level of cooperativity between the multiple metal ions of **1** therefore likely produces both nonspecific and specific cleavage of DNA, which cannot be mimicked by intermolecular derivatives.

**Comparison of 1 to $Cu^{2+}$.** Facile ligand exchange by Cu(II) presents the possibility of significant quantities of free copper in the reaction mixture. To address this, $Cu(NO_3)_2 \cdot 2.5H_2O$ (1.5 and 7.5 $\mu$M) and MPA (5 mM) were incubated with **Ia**. Less than 1% of the oligonucleotide was cleaved under the standard reaction conditions at either concentration.[62] The strand scission observed in the presence of $[Cu_3^{II}(\mathbf{L})(H_2O)_3(NO_3)_2](NO_3)_4 \cdot 5H_2O$ (**1**) was thus not due to free copper as demonstrated by the lack of significant reactivity with $Cu(NO_3)_2 \cdot 2.5H_2O$. The inability of aqueous Cu(II) to mediate general strand scission also precluded observation of selective cleavage. This conclusion further supports the earlier suggestion that the metal ions of the multinuclear complex act synergistically.

**Comparison of 1 with $[Cu(OP)_2]^{2+}$.** Comparisons of DNA cleavage were also made between $[Cu(OP)_2]^{2+}$ and $[Cu_3^{II}(\mathbf{L})(H_2O)_3(NO_3)_2](NO_3)_4 \cdot 5H_2O$ (**1**) under the standard reaction conditions. Since the 1,10-phenanthroline−copper complex is also mononuclear, it was tested at complex concentrations three times greater than those of **1**. Extensive cleavage of the frayed duplex **Ia** was observed in the presence of 1.5 and 7.5 $\mu$M $[Cu(OP)_2]^{2+}$ and 5 mM MPA, yielding nearly complete degradation of the parent oligonucleotide at the higher concentration.[62] At the lower concentration, the phenanthroline−copper complex demonstrated a preference for oxidation of double-helical 5′ GT sequences consistent with prior reports.[12,13] The amount of cleavage on the Watson and Crick strands of **Ia** was equivalent, in contrast to the results obtained with **1**. Cleavage of the Crick strand of **Ia** by **1** was 1.5-fold greater than that

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observed on the Watson strand because of the specific strand scission event at $C_{20}$ of the Crick strand. Selective cleavage at $C_{20}$ does not occur in reaction of **Ia** with $[Cu(OP)_2]^{2+}$ and MPA. The multinuclear complex **1** then possesses a unique ability to recognize novel DNA structures that is dependent on the nature of its coordination properties.

**Proposed Model of Reactivity and Recognition.** The advantage of a multinuclear complex lies in its potential capacity to form a reactive intermediate like peroxide, hydroperoxide, or hydroxyl radical on the surface of DNA without aid of a second equivalent of the complex. In contrast, oxidative cleavage of DNA by $[Cu(OP)_2]^{2+}$ effectively requires 3 equiv of complex to cleave one strand of the duplex. The reaction is initiated by reduction of $[Cu(OP)_2]^{2+}$ to $[Cu(OP)_2]^{+}$, which then reacts with dioxygen in solution, returning the starting compound and superoxide anion.[63] Spontaneous dismutation of two molecules of superoxide yields dioxygen and hydrogen peroxide which can then react with another equivalent of the reduced complex yielding a nondiffusible radical species. Strand scission results after the reduced complex, $[Cu(OP)_2]^{+}$, binds in the minor groove of the double-helical DNA. Kinetic measurements of 1,10-phenanthroline−copper demonstrated that strand scission is a bimolecular process requiring 2 equiv of the complex.[64] Such a mechanism suggests intermolecular formation of a dimeric copper−peroxo species. Since 1 equiv of $[Cu(OP)_2]^{+}$ is thought to bind in the minor groove of DNA, formation of a dimer requires that the other equivalent remain unbound and free in solution. In the event that all available complex binds to DNA, then the reactive intermediate may not be generated. A similar process may occur with the mononuclear complexes **2** and **3** and could help to explain their inability to cleave DNA. Mononuclear copper complexes with pyridylmethyl ligands are known to form intermolecular $\mu$-peroxo $Cu-O_2^{2-}-Cu$ dimers in reactions with dioxygen.[20,26,65] The addition of another (i.e. second) copper to form a dinuclear complex significantly lowers the entropic barrier and reduces the time-scale for formation of the intramolecular bridging peroxo species.[26]

A synergy between metal ions in $[Cu_3^{II}(\mathbf{L})(H_2O)_3(NO_3)_2](NO_3)_4 \cdot 5H_2O$ (**1**) likely supports an intramolecular $O_2$-derived intermediate that is produced through the reduction of dioxygen by the Cu(I) form of **1**. General strand scission of DNA is observed due to proximal generation of the intermediate as a result of nonspecific electrostatic interactions between the complex and DNA. Selective strand scission may involve the same reactive intermediate as well as a selective association at junctions between single- and double-stranded regions of DNA that possess the appropriate purine sequence on the 5′ overhang. A mechanism can be proposed for specific cleavage, wherein one copper recognizes or identifies a sequence-structure motif or guanine itself at the $n+1$ position of the 5′ overhang. The other two coppers remain in close proximity to deliver the reactive intermediate or its precursor to either the coordinated copper prior to strand scission or directly to the deoxyribose sugar. This mechanism consequently predicts cooperativity between at least two of the three metals in the trinuclear complex to aid in recognition of the substrate, formation of the $O_2$-derived intermediate, and delivery of the reactive species responsible for selective strand scission to the proper site.

## Summary and Conclusions

Reaction of DNA induced by $[Cu_3^{II}(\mathbf{L})(H_2O)(NO_3)_2](NO_3)_4 \cdot 5H_2O$ (**1**) presages broad potential for multinuclear complexes to both coordinate selectively to target structures of DNA and promote their modification in a manner that might otherwise require involvement of multiple mononuclear complexes. Multinuclearity in the complex (i.e. the presence of two or three proximate copper ions) facilitates formation of the intermediate, but also appears to be important in recognition of the cleavage site within DNA. Through examination of a series of oligonucleotide targets, specific strand scission by **1** has now been shown to require a junction between single- and double-stranded DNA and the presence of guanine at the $n+1$ (i.e. second unpaired) position of the 5′ overhang. Either adenine or guanine can occupy the $n$ position (i.e. next to the base-paired region) of that same strand, but both the $n+1$ and the $n$ positions must meet the necessary requirements for the reaction to occur.

Complexes exhibiting such a high level of specificity as $[Cu_3^{II}(\mathbf{L})(H_2O)_3(NO_3)_2](NO_3)_4 \cdot 5H_2O$ (**1**) may be potentially useful both in vitro and in vivo as probes of nonclassical nucleic acid structures. Complex **1**, in particular, may be useful in vivo where its simple requirements of a reductant and dioxygen for reactivity can be easily met. The sensitivity of **1** to weak hydrogen bonding may also allow for its use in probing structures formed by redundant sequences such as those associated with triplet repeats and telomeres in which noncanonical base pairs are thought to form. The ability of $[Cu_3^{II}(\mathbf{L})(H_2O)_3(NO_3)_2](NO_3)_4 \cdot 5H_2O$ (**1**) to selectively modify other DNA and RNA structures containing guanine and guanine/adenine containing loops is currently under investigation.

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**Supporting Information Available:** Autoradiograms of cleavage by **1** of the hairpin **Ωa**; piperidine treated **Ia**; frayed duplexes **Ib**, **Ic**, **Id**, **If**, **Ig**, and **Ih**; frayed duplexes **IIb** and **IIc**; **Ia** with 50 mM salt, dithionite, ascorbate, standard radical scavengers, and variable $O_2$ concentrations; autoradiograms of the chemical modification of **Ωa** and **Ia**; and plots of $OD_{260}$ vs temperature to determine $T_m$ values (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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