# Articles

## Copper(II) Macrocycles Cleave Single-Stranded and Double-Stranded DNA under Both Aerobic and Anaerobic Conditions

**Eric L. Hegg and Judith N. Burstyn***

Department of Chemistry, University of Wisconsin, Madison, Wisconsin 53706

*Received April 9, 1996*[⊗]

The mechanism of copper(II) 1,4,7-triazacyclononane dichloride [Cu([9]aneN$_3$)Cl$_2$]-catalyzed hydrolysis of activated phosphodiesters has been established. We now report that Cu([9]aneN$_3$)Cl$_2$ is also capable of cleaving both single-stranded and double-stranded DNA at near-physiological pH and temperature. Degradation of both single-stranded and double-stranded DNA was revealed by gel electrophoresis and quantitated via fluorimaging of ethidium bromide-stained gels. Single-stranded M13 DNA incubated with Cu([9]aneN$_3$)Cl$_2$ is efficiently and nonspecifically degraded. Supercoiled plasmid DNA (form I) incubated with Cu([9]aneN$_3$)Cl$_2$ is nicked to relaxed circular DNA (form II) and then more slowly degraded to form III (linear). A related complex, [Cu(*i*-Pr$_3$[9]aneN$_3$)(OH$_2$)(CF$_3$SO$_3$)]CF$_3$SO$_3$, displays a marked increase in DNA cleavage activity relative to the parent Cu([9]aneN$_3$)Cl$_2$. Interestingly, there appear to be at least two different mechanisms of DNA degradation: an $O_2$-dependent pathway and an $O_2$-independent pathway. This is one of only a few well-defined metal complexes demonstrated to cleave DNA in the absence of $O_2$ or some other oxidant.

Phosphodiesters form the backbone of DNA and are ubiquitous in biological systems. Hydrolysis of DNA is an important enzymatic reaction, but one that is exceedingly difficult to mimic in the laboratory due to the stability of DNA toward hydrolysis.[1] This stability is a result of the repulsion between the negatively charged phosphodiester backbone and potential nucleophiles.[2] Consequently, much of the literature on phosphodiester degradation by small molecules has focused on either hydrolysis of activated substrates or oxidative degradation of DNA. A number of oxidative cleavage reagents have been utilized with great success for DNA footprinting,[3] locating base mismatches and loop regions,[4] locating conformational variations in DNA,[5,6] and as chemotherapeutic agents.[7] Because these oxidative cleavage agents require activation by either light or an oxidant, the full therapeutic potential of these compounds cannot be realized. One advantage of hydrolytic cleavage agents is that they do not require coreactants and therefore could be more useful in drug design. Also, because oxidative cleavage agents tend to produce diffusible free radicals and products that are not amenable to further enzymatic manipulation, the use of these reagents has been limited in the field of molecular biology. Hydrolytic agents do not suffer from these shortcomings. Finally, the manner in which enzymes utilize metals to activate phosphodiester bonds toward hydrolysis is not well-understood. Small metal complexes that promote the hydrolysis of DNA therefore could be useful not only in molecular biology and drug design but also in elucidating the precise role of metal ions in enzyme catalysis.

Many of the metal complexes known to cleave DNA employ oxidative mechanisms. One commonly used oxidative cleavage agent is Fe(EDTA)$^{2-}$.[3,8,9] In the presence of $H_2O_2$ or $O_2$ plus a reducing agent, Fe(EDTA)$^{2-}$ efficiently cleaves both single-stranded and double-stranded DNA. In this process hydroxyl radicals are produced via the Fenton reaction, which in turn abstract a hydrogen atom from the deoxyribose ring; rearrangement of the sugar ring results in strand scission and free base release. Bleomycin (BLM), an antitumor antibiotic, is a second cleavage agent that requires $Fe^{2+}$ and $H_2O_2$ or $O_2$ plus a reducing agent for activity, although BLM does not produce free hydroxyl radicals.[7,10,11] Another reagent that cleaves DNA via a similar mechanism is the bis(1,10-phenanthroline-$N^1$,$N^{10}$)copper(I) cation [(OP)$_2$Cu$^+$].[6,12] Like its Fe counterparts Fe(EDTA)$^{2-}$ and Fe-BLM, (OP)$_2$Cu$^+$ initiates DNA cleavage by H-atom abstrac-

[⊗] Abstract published in *Advance ACS Abstracts,* September 15, 1996.

(1) Radzicka, A.; Wolfenden, R. *Science* **1995**, *267*, 90−93.
(2) Westheimer, F. H. *Science* **1987**, *235*, 1173−1178.
(3) (a) Dixon, W. J.; Hayes, J. J.; Levin, J. R.; Weidner, M. F.; Dombroski, B. A.; Tullius, T. D. *Methods Enzymol.* **1991**, *208*, 380−403. (b) Pogozelski, W. K.; McNeese, T. J.; Tullius, T. D. *J. Am. Chem. Soc.* **1995**, *117*, 6428−6433.
(4) (a) Chen, X.; Burrows, C. J.; Rokita, S. E. *J. Am. Chem. Soc.* **1992**, *114*, 322−325. (b) Burrows, C. J.; Rokita, S. E. *Acc. Chem. Res.* **1994**, *27*, 295−301.
(5) Price, M. A.; Tullius, T. D. *Methods Enzymol.* **1992**, *212*, 194−219.
(6) (a) Pope, L. E.; Sigman, D. S. *Proc. Natl. Acad. Sci. U.S.A.* **1984**, *81*, 3−7. (b) Sigman, D. S. *Biochemistry* **1990**, *29*, 9097−9105.
(7) (a) Stubbe, J.; Kozarich, J. W. *Chem. Rev.* **1987**, *87*, 1107−1136. (b) Cohen, S. S. In *Bleomycin: Chemical, Biochemical, and Biological Aspects*; Hecht, S. M., Ed.; Springer-Verlag: New York, 1979; pp 267−286.
(8) (a) Hertzberg, R. P.; Dervan, P. B. *J. Am. Chem. Soc.* **1982**, *104*, 313−315. (b) Hertzberg, R. P.; Dervan, P. B. *Biochemistry* **1984**, *23*, 3934−3945. (c) Dervan, P. B. *Methods Enzymol.* **1991**, *208*, 497−515.
(9) (a) Lempers, E. L. M.; Bashkin, J. S.; Kostić, N. M. *Nucleic Acids Res.* **1993**, *21*, 1983−1990. (b) Iverson, B. L.; Shreder, K.; Morishima, T.; Rosingana, M.; Sessler, J. L. *J. Org. Chem.* **1995**, *60*, 6616−6620.
(10) (a) Rabow, L. E.; McGall, G. H.; Stubbe, J.; Kozarich, J. W. *J. Am. Chem. Soc.* **1990**, *112*, 3203−3208. (b) Worth, L., Jr.; Frank, B. L.; Christner, D. F.; Absalon, M. J.; Stubbe, J.; Kozarich, J. W. *Biochemistry* **1993**, *32*, 2601−2609. (c) Absalon, M. J.; Wu, W.; Kozarich, J. W.; Stubbe, J. *Biochemistry* **1995**, *34*, 2076−2086.
(11) (a) Hecht, S. M. *Acc. Chem. Res.* **1986**, *19*, 383−391. (b) Van Atta, R. B.; Long, E. C.; Hecht, S. M.; van der Marel, G. A.; van Boom, J. H. *J. Am. Chem. Soc.* **1989**, *111*, 2722−2724.
(12) (a) Sigman, D. S.; Graham, D. R.; D'Aurora, V.; Stern, A. M. *J. Biol. Chem.* **1979**, *254*, 12269−12272. (b) Sigman, D. S.; Kuwabara, M. D.; Chen, C. H. B.; Bruice, T. W. *Methods Enzymol.* **1991**, *208*, 414−433. (c) Pan, C. Q.; Landgraf, R.; Sigman, D. S. *Mol. Microbiol.* **1994**, *12*, 335−342.

S0020-1669(96)00384-9 CCC: $12.00 

tion and requires either $H_2O_2$ or $O_2$ plus a reducing agent for activity. Numerous other ligands have also been complexed with copper ions to promote the oxidative degradation of DNA. These ligands include 2,2′-bipyridine,[13] various hydroxamic acids,[14] the siderophore desferal,[15] the tripeptide Gly-Gly-His,[16] and bis-Lys.[17] Square-planar $Ni^{2+}$ complexes have also been used to oxidatively degrade DNA.[4,18,19] Finally, oxidative degradation of DNA was also realized by using assorted metal porphyrins in the presence of an oxidant,[20] as well as various photolytic[21−24] and electrolytic[25,26] cleavage agents.

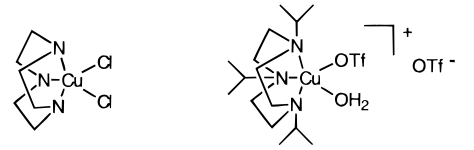
Because hydrolysis of DNA is so difficult compared to oxidative degradation of DNA, most studies of hydrolytic reactions utilize activated phosphodiesters. Substitutionally inert tetraamine complexes of $Co^{3+}$ [1,27,28] and $Ir^{3+}$ [29] exhibit an impressive ability to hydrolyze activated phosphodiesters and have been useful in determining the mechanism of metal-promoted hydrolysis, but they lack catalytic turnover. Various lanthanides, either as free ions[30] or as macrocyclic complexes,[31] have been used to catalytically hydrolyze activated phosphate esters. The coordination chemistry of lanthanides, however, is not as well-studied as that of transition metals, and many lanthanide complexes decompose in water due to their extreme lability.[32,33] Furthermore, because the coordination number of lanthanide complexes can be difficult to determine, the detailed mechanism of lanthanide-promoted hydrolysis of phosphodiesters is still unknown. Substitutionally labile amine complexes of $Cu^{2+}$ [13,34,35] and $Zn^{2+}$,[13,36] although usually slower hydrolytic agents than lanthanide complexes, provide both catalytic turnover and stable, well-characterized metal complexes. Recently, hydrolysis of phosphodiesters by dinuclear $Zn^{2+}$,[37,38] $Cu^{2+}$,[37] $Ni^{2+}$,[37] and $Co^{3+}$ [39] complexes has also proven to be promising, and there is currently much interest in developing dinuclear cleavage agents. Most of the examples mentioned here, however, with the exception of the hydrolysis of dimethyl phosphate,[27b] utilized substrates that were either activated toward hydrolysis or contained a metal-binding site on the phosphodiester itself. Hydrolysis of DNA itself has remained elusive.

Given the challenges of hydrolyzing DNA, it is perhaps remarkable that there are a few examples in the literature. It was reported that $(Ru(DIP)_2macro)^{n+}$, where $Ru(DIP)_2$ binds to DNA via intercalation and macro is a chelating ligand, hydrolyzes DNA in the presence of certain divalent metal cations.[40] Lanthanum and cerium ions and their complexes have been reported to hydrolyze dinucleotides, short oligonucleotides, and supercoiled plasmid DNA.[41,42] Reactions with $Ce^{3+}$ seem to require oxygen whereas reactions with $Ce^{4+}$ do not, ostensibly because $Ce^{3+}$ is oxidized to $Ce^{4+}$ and it is $Ce^{4+}$ that is the active species. Finally, it was recently reported that a diiron species rapidly hydrolyzes supercoiled DNA in the presence of $H_2O_2$ or $O_2$ plus a reducing agent;[43] these are conditions under which iron species typically oxidatively degrade DNA, but the products observed were consistent with a hydrolytic mechanism.

In this paper we report the discovery that the synthetic peptidase $Cu([9]aneN_3)Cl_2$ also cleaves DNA. We previously


(13) De Rosch, M. A.; Trogler, W. C. *Inorg. Chem.* **1990**, *29*, 2409−2416.
(14) (a) Hashimoto, S.; Yamashita, R.; Nakamura, Y. *Chem. Lett.* **1992**, 1639−1642. (b) Hashimoto, S.; Nakamura, Y. *J. Chem. Soc., Chem. Commun.* **1995**, 1413−1414.
(15) (a) Joshi, R. R.; Likhite, S. M.; Kumar, R. K.; Ganesh, K. N. *Biochim. Biophys. Acta* **1994**, *1199*, 285−292. (b) Joshi, R. R.; Ganesh, K. N. *Proc. Indian Acad. Sci. (Chem. Sci.)* **1994**, *106*, 1089−1108.
(16) (a) Shullenberger, D. F.; Long, E. C. *Bioorg. Med. Chem. Lett.* **1993**, *3*, 333−336. (b) Shullenberger, D. F.; Eason, P. D.; Long, E. C. *J. Am. Chem. Soc.* **1993**, *115*, 11038−11039.
(17) (a) Ranganathan, D.; Mishra, R. K.; Patel, B. K.; Vaish, N. K. *Proc. Indian Acad. Sci. (Chem. Sci.)* **1994**, *106*, 1071−1088.
(18) (a) Morrow, J. R.; Kolasa, K. A. *Inorg. Chim. Acta* **1992**, *195*, 245−248. (b) Mack, D. P.; Dervan, P. B. *J. Am. Chem. Soc.* **1990**, *112*, 4604−4606. (c) Mack, D. P.; Dervan, P. B. *Biochemistry* **1992**, *31*, 9399−9405.
(19) (a) Chen, X.; Rokita, S. E.; Burrows, C. J. *J. Am. Chem. Soc.* **1991**, *113*, 5884−5886. (b) Muller, J. G.; Chen, X.; Dadiz, A. C.; Rokita, S. E.; Burrows, C. J. *J. Am. Chem. Soc.* **1992**, *114*, 6407−6411.
(20) (a) Le Doan, T.; Perrouault, L.; Helene, C.; Chassignol, M.; Thuong, N. T. *Biochemistry* **1986**, *25*, 6736−6739. (b) Groves, J. T.; Marla, S. S. *J. Am. Chem. Soc.* **1995**, *117*, 9578−9579. (c) Ward, B.; Skorobogaty, A.; Dabrowiak, J. C. *Biochemistry* **1986**, *25*, 6875−6883.
(21) (a) Nielsen, P. E.; Jeppesen, C.; Buchardt, O. *FEBS Lett.* **1988**, *235*, 122−124. (b) Nielsen, P. E.; Møllegaard, N. E.; Jeppesen, C. *Nucl. Acids Res.* **1990**, *18*, 3847−3851. (c) Nielsen, P. E.; Hiort, C.; Sönnichsen, S. H.; Buchardt, O.; Dahl, O.; Nordèn, B. *J. Am. Chem. Soc.* **1992**, *114*, 4967−4975.
(22) Kalsbeck, W. A.; Grover, N.; Thorp, H. H. *Angew. Chem., Int. Ed. Engl.* **1991**, *30*, 1517−1518.
(23) (a) Sitlani, A.; Long, E. C.; Pyle, A. M.; Barton, J. K. *J. Am. Chem. Soc.* **1992**, *114*, 2303−2312. (b) Chow, C. S.; Barton, J. K. *Methods Enzymol.* **1992**, *212*, 219−242.
(24) Barton, J. K.; Raphael, A. L. *J. Am. Chem. Soc.* **1984**, *106*, 2466−2468.
(25) (a) Grover, N.; Gupta, N.; Singh, P.; Thorp, H. H. *Inorg. Chem.* **1992**, *31*, 2014−2020. (b) Neyhart, G. A.; Grover, N.; Smith, S. R.; Kalsbeck, W. A.; Fairley, T. A.; Cory, M.; Thorp, H. H. *J. Am. Chem. Soc.* **1993**, *115*, 4423−4428. (c) Cheng, C. C.; Goll, J. G.; Neyhart, G. A.; Welch, T. W.; Singh, P.; Thorp, H. H. *J. Am. Chem. Soc.* **1995**, *117*, 2970−2980.
(26) Johnston, D. H.; Cheng, C. C.; Campbell, K. J.; Thorp, H. H. *Inorg. Chem.* **1994**, *33*, 6388−6390.
(27) (a) Chin, J.; Zou, X. *J. Am. Chem. Soc.* **1988**, *110*, 223−225. (b) Kim, J. H.; Chin, J. *J. Am. Chem. Soc.* **1992**, *114*, 9792−9795.
(28) Hendry, P.; Sargeson, A. M. *Inorg. Chem.* **1990**, *29*, 92−97.
(29) Hendry, P.; Sargeson, A. M. *J. Am. Chem. Soc.* **1989**, *111*, 2521−2527.
(30) (a) Breslow, R.; Zhang, B. *J. Am. Chem. Soc.* **1994**, *116*, 7893−7894. (b) Takeda, N.; Irisawa, M.; Komiyama, M. *J. Chem. Soc., Chem. Commun.* **1994**, 2773−2774. (c) Schneider, H. J.; Rammo, J.; Hettich, R. *Angew. Chem., Int. Ed. Engl.* **1993**, *32*, 1716−1719. (d) Takasaki, B. K.; Chin, J. *J. Am. Chem. Soc.* **1995**, *117*, 8582−8585.
(31) (a) Hay, R. W.; Govan, N. *J. Chem. Soc., Chem. Commun.* **1990**, 714−715. (b) Oh, S. J.; Song, K. H.; Park, J. W. *J. Chem. Soc., Chem. Commun.* **1995**, 575−576.
(32) *Scandium, Yttrium & the Lanthanides: Inorganic & Coordination Chemistry*; King, R. B., Ed.; Encyclopedia of Inorganic Chemistry; John Wiley & Sons: New York, 1994; Vol. 7, Chapter 3.
(33) (a) Fenton, D. E.; Vigato, P. A. *Chem. Soc. Rev.* **1988**, *17*, 69−90. (b) Morrow, J. R.; Buttrey, L. A.; Shelton, V. M.; Berback, K. A. *J. Am. Chem. Soc.* **1992**, *114*, 1903−1905. (c) Amin, S.; Morrow, J. R.; Lake, C. H.; Churchill, M. R. *Angew. Chem., Int. Ed. Engl.* **1994**, *33*, 773−775.
(34) (a) Young, M. J.; Wahnon, D.; Hynes, R. C.; Chin, J. *J. Am. Chem. Soc.* **1995**, *117*, 9441−9447. (b) Morrow, J. R.; Trogler, W. C. *Inorg. Chem.* **1988**, *27*, 3387−3394. (c) Kövári, E.; Heitker, J.; Krämer, R. *J. Chem. Soc., Chem. Commun.* **1995**, 1205−1206.
(35) (a) Burstyn, J. N.; Deal, K. A. *Inorg. Chem.* **1993**, *32*, 3585−3586. (b) Deal, K. A.; Burstyn, J. N. *Inorg. Chem.* **1996**, *35*, 2792−2798. (c) Deal, K. A.; Hengge, A. C.; Burstyn, J. N. *J. Am. Chem. Soc.* **1996**, *118*, 1713−1718.
(36) (a) Browne, K. A.; Bruice, T. C. *J. Am. Chem. Soc.* **1992**, *114*, 4951−4958. (b) Koike, T.; Kimura, E. *J. Am. Chem. Soc.* **1991**, *113*, 8935−8941. (c) Hikichi, S.; Tanaka, M.; Moro-oka, Y.; Kitajima, N. *J. Chem. Soc., Chem. Commun.* **1992**, 814−815.
(37) Kesicki, E. A.; DeRosch, M. A.; Freeman, L. H.; Walton, C. L.; Harvey, D. F.; Trogler, W. C. *Inorg. Chem.* **1993**, *32*, 5851−5867.
(38) Chapman, W. H., Jr.; Breslow, R. *J. Am. Chem. Soc.* **1995**, *117*, 5462−5469.
(39) Wahnon, D.; Lebuis, A. M.; Chin, J. *Angew. Chem., Int. Ed. Engl.* **1995**, *34*, 2412−2414.
(40) Basile, L. A.; Raphael, A. L.; Barton, J. K. *J. Am. Chem. Soc.* **1987**, *109*, 7550−7551.
(41) (a) Rammo, J.; Hettich, R.; Roigk, A.; Schneider, H. J. *J. Chem. Soc., Chem. Commun.* **1996**, 105−107. (b) Takasaki, B. K.; Chin, J. *J. Am. Chem. Soc.* **1994**, *116*, 1121−1122.
(42) (a) Komiyama, M.; Shiiba, T.; Kodama, T.; Takeda, N.; Sumaoka, J.; Yashiro, M. *Chem. Lett.* **1994**, 1025−1028. (b) Komiyama, M.; Takeda, N.; Shiiba, T.; Takahashi, Y.; Matsumoto, Y.; Yashiro, M. *Nucleosides Nucleotides* **1994**, *13*, 1297−1309. (c) Komiyama, M.; Takeda, N.; Takahashi, Y.; Uchida, H.; Shiiba, T.; Kodama, T.; Yashiro, M. *J. Chem. Soc., Perkin Trans. 2* **1995**, 269−274.
(43) Schnaith, L. M. T.; Hanson, R. S.; Que, L., Jr. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 569−573.

demonstrated that Cu([9]aneN$_3$)Cl$_2$ hydrolyzes proteins[44] as well as activated phosphodiesters.[35] Herein we demonstrate that Cu([9]aneN$_3$)Cl$_2$ and a related analogue, [Cu(*i*-Pr$_3$[9]aneN$_3$)(OH$_2$)(OTf)]OTf (OTf = $CF_3SO_3^-$), are capable of cleaving both single-stranded and double-stranded DNA. DNA degradation proceeds by at least two different mechanisms: one that is $O_2$-dependent and a second that is $O_2$-independent. Significantly, these are two of only a few well-defined metal complexes that have demonstrated their ability to cleave DNA in the absence of $O_2$ or some other oxidant. Furthermore, Cu([9]aneN$_3$)Cl$_2$ is the first metal complex shown to cleave both DNA and proteins in the absence of an oxidant.




**Cu([9]aneN$_3$)Cl$_2$** **[Cu(*i*-Pr$_3$[9]aneN$_3$)(OH$_2$)(OTf)]OTf**

## Experimental Section

**Chemicals and Reagents.** The biological buffer *N*-(2-hydroxyethyl)piperazine-*N*′-ethanesulfonic acid (HEPES), ethylenediaminetetraacetic acid disodium salt (EDTA), DL-dithiothreitol (DTT), boric acid, ethidium bromide, ampicillin, chloramphenicol, and type III-A agarose were all purchased from Sigma. Methanol, sodium hydroxide, and tris(hydroxymethyl)aminomethane (Tris base) were purchased from Fisher. Cesium chloride was obtained from Gallard-Schlesinger Industries. Dimethyl sulfoxide (DMSO) was purchased from Aldrich, and glacial acetic acid was purchased from EM Science. Argon and molecular oxygen were obtained from Liquid Carbonic. All chemicals were used without further purification.

The metal complex Cu([9]aneN$_3$)Cl$_2$ was prepared as previously described.[35b] [Cu(*i*-Pr$_3$[9]aneN$_3$)(OH$_2$)(OTf)]OTf was synthesized according to the literature procedure[45] and was provided to us as a gift from Professor William Tolman (University of Minnesota). All solutions were prepared by using water purified by passage through a Millipore purification system and sterilized by autoclave. Reaction solutions were prepared according to standard sterile techniques.

**Preparation of Single-Stranded M13 DNA.** Wild-type ss-M13 DNA was prepared and isolated according to standard protocols.[46] The *Escherichia coli* cell line used for infection was DH5αF′. The purity of the M13 DNA was confirmed via both agarose gel electrophoresis and UV spectroscopy by determining the ratio of absorbance at 260 nm to the absorbance at 280 nm. The concentration of DNA was determined from the absorbance at 260 nm (1.0 $OD_{260}$ = 40 μg/mL). Aqueous M13 stock solutions (1.0 mg/mL) were stored frozen at −20 °C until ready for use.

**Preparation of Supercoiled DNA.** Plasmid pBluescript II ks(−) was purchased from Stratagene Cloning Systems. The DNA was transformed into JM109 bacterial cell lines via electroporation, and the transformed bacteria were cultured in medium containing ampicillin and chloramphenicol. The supercoiled DNA was harvested according to standard procedures[46] and purified via three cycles of centrifugation in a CsCl gradient. The DNA was further purified via ethanol precipitation and stored as a pellet until just prior to use. The DNA was then dissolved in an appropriate volume of sterile water. The purity and concentration of the DNA were determined by agarose gel electrophoresis and UV spectroscopy as described for single-stranded M13 DNA by using the extinction coefficient appropriate for double-stranded DNA (1.0 $OD_{260}$ = 50 μg/mL).

**Cleavage of Single-Stranded DNA.** Solutions (100 μL total volume) containing 0.05 mg/mL single-stranded M13 DNA (23 nM in M13 or 0.15 mM in phosphate units) were incubated in sterile 0.5 mL reaction tubes with varying concentrations of Cu([9]aneN$_3$)Cl$_2$ at 50 °C. The pH was maintained at 7.88 ± 0.07 with 40 mM HEPES buffer as determined by an Orion Research digital pH meter (Model 611) equipped with a Model 8103 semimicro Ross combination pH electrode. The pH was measured at each time point using separate but similar solutions; the volumes were 500 μL and the samples lacked single-stranded DNA. At the appropriate times, the reactions were quenched via ethanol precipitation. The DNA pellets were then redissolved in10 μL of water and stored at −80 °C until analysis by gel electrophoresis.

**Cleavage of Supercoiled DNA.** Solutions (50 μL total volume) containing 0.05 mg/mL pBluescript II ks(−) supercoiled DNA (25 nM in supercoiled DNA or 0.15 mM in phosphate units) were incubated in sterile 0.5 mL reaction vials with varying concentrations of Cu([9]aneN$_3$)Cl$_2$ or [Cu(*i*-Pr$_3$[9]aneN$_3$)(OH$_2$)(OTf)]OTf at 50 °C. The pH was maintained at 7.80 ± 0.06 with 40 mM HEPES buffer as described earlier. The pH was monitored by using separate but similar solutions; the volumes were 500 μL and the samples did not contain any supercoiled DNA. At the appropriate times, the reactions were quenched via ethanol precipitation. The DNA pellets were resuspended in 10 μL of 0.5× TBE buffer[47a] and stored at 4 °C until analysis by gel electrophoresis.

**Anaerobic Reactions.** Deoxygenated water was prepared by either four or five freeze−pump−thaw cycles. Before the final two cycles, the water was equilibrated with argon to aid in the deoxygenation process. The deoxygenated water was stored under an argon atmosphere prior to use. All anaerobic stock solutions were prepared in a nitrogen-filled glove bag using the deoxygenated water. Reaction mixtures were prepared in the glove bag by the addition of the appropriate volumes of stock solutions to the reaction tubes. The reactions were initiated by quick centrifugation, transferred to an argon-filled vacuum desiccator, and incubated in the sealed desiccator at 50 °C. All reactions were quenched by ethanol precipitation; no effort was made to keep the reactions anaerobic during this procedure. All other conditions were the same as those listed for individual aerobic cleavage reactions.

**Reactions under a Pure $O_2$ Atmosphere.** Oxygenated water was prepared in a manner identical to that used for the deoxygenated water described in the anaerobic procedure, except that the water was equilibrated with pure molecular oxygen instead of argon. Reaction mixtures were quickly prepared on the benchtop open to the atmosphere (less than 5 min), transferred to an oxygen-filled vacuum desiccator, and incubated in the sealed desiccator under pure $O_2$ at 50 °C. All other conditions were the same as those listed for aerobic cleavage reactions of single-stranded M13 DNA.

**Oxidative Cleavage of DNA by Fe(EDTA)$^{2-}$/DTT/$O_2$.** Supercoiled pBluescript II ks(−) DNA (0.05 mg/mL) or single-stranded M13 DNA (0.05 mg/mL) was incubated with 10 mM DTT and 0.1 mM Fe(EDTA)$^{2-}$ for 60 min at 50 °C. All other reaction conditions were the same as those listed earlier for the degradation of DNA by Cu([9]aneN$_3$)Cl$_2$. Reactions were quenched via ethanol precipitation and analyzed by agarose gel electrophoresis.

**Reactions in the Presence of Radical Scavengers.** Supercoiled pBluescript II ks(−) DNA (0.05 mg/mL) or single-stranded M13 DNA (0.05 mg/mL) was incubated with 1.0 mM Cu([9]aneN$_3$)Cl$_2$ at 50 °C under both aerobic and anaerobic conditions. The pH was maintained as described earlier for the individual cleavage reactions. Reaction mixtures contained either no radical scavenger, 0.4 M DMSO, or 2.5 M methanol. These concentrations of DMSO and methanol completely inhibited the oxidative cleavage of DNA by Fe(EDTA)$^{2-}$/DTT/$O_2$. Reactions were quenched after 24 h via ethanol precipitation and analyzed by agarose gel electrophoresis.

**Oxidative Cleavage of DNA by Cu([9]aneN$_3$)Cl$_2$/DTT/$O_2$.** Single-stranded M13 DNA (0.05 mg/mL) was incubated with 3.0 mM DTT and 0.25 mM Cu([9]aneN$_3$)Cl$_2$ under aerobic conditions for 24 h at 50 °C. Reactions were performed in both the presence and absence of the radical scavengers DMSO and MeOH, as described earlier. All

(44) Hegg, E. L.; Burstyn, J. N. *J. Am. Chem. Soc.* **1995**, *117*, 7015−7016.
(45) Halfen, J. A.; Mahapatra, S.; Wilkinson, E. C.; Gengenbach, A. J.; Young, V. G., Jr.; Que, Jr., L.; Tolman, W. B. *J. Am. Chem. Soc.* **1996**, *118*, 763−776.
(46) Sambrook, J.; Fritsch, E. F.; Maniatis, T. In *Molecular Cloning: A Laboratory Manual*, 2nd ed.; Ford, N., Ed.; Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY, 1989.

(47) (a) 0.5× TBE buffer is 22.5 mM Tris−borate and 0.5 mM EDTA. (b) 1× TAE buffer is 40 mM Tris−acetate and 1.0 mM EDTA.

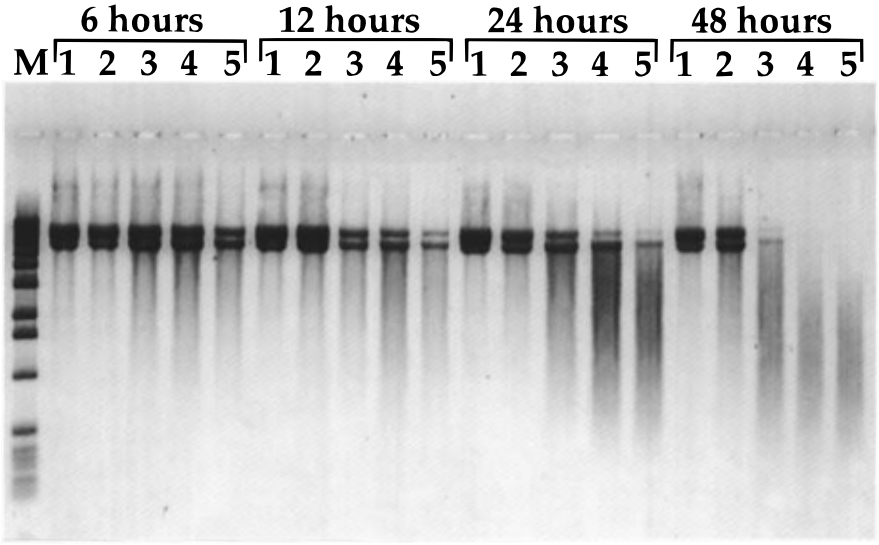



**Figure 1.** M13 ss-DNA (0.05 mg/mL) was incubated aerobically with Cu([9]aneN$_3$)Cl$_2$ for 6, 12, 24, or 48 h at pH 7.88 ± 0.07 in 40 mM HEPES at 50 °C. Lane M: molecular weight marker. Lane 1: control, no metal complex. Lane 2: 0.25 mM Cu([9]aneN$_3$)Cl$_2$. Lane 3: 0.50 mM Cu([9]aneN$_3$)Cl$_2$. Lane 4: 0.75 mM Cu([9]aneN$_3$)Cl$_2$. Lane 5: 1.0 mM Cu([9]aneN$_3$)Cl$_2$. The two bands observed are the result of different conformers of the M13 ss-DNA.

other reaction conditions were the same as those for the aerobic cleavage of single-stranded M13 DNA by Cu([9]aneN$_3$)Cl$_2$.

**Product Analysis and Quantitation.** The extent of degradation was determined via densitometric analysis of ethidium bromide-stained agarose gels. Electrophoresis was performed by using a PNA-200 horizontal gel electrophoresis apparatus from Pharmacia LKB Biotechnology. Gels of single-stranded M13 contained 2.5 $\mu$g of DNA per lane, while those of supercoiled DNA contained 0.33 $\mu$g/lane. The 0.8% agarose gels were run at 120 V for 2−3 h in either 1× TAE or 0.5× TBE buffer.[47] Following electrophoresis, the gels were stained by incubation for 30 min in buffer containing ethidium bromide at a concentration of 1 $\mu$g/mL. The gels were then destained in water for approximately 20 min before being imaged on a Molecular Dynamics FluorImager 575 equipped with a 610 nm long pass filter.

The extent of DNA degradation was determined by using the volume quantitation method in ImageQuaNT version 4.1. In all cases, background fluorescence was determined by quantitation of a lane in the gel containing no DNA. For reactions involving supercoiled DNA, a correction factor of 1.42 was utilized to account for the decreased ability of ethidium bromide to intercalate into form I DNA versus forms II and III.[48] The relative amounts of the different forms of DNA were determined by dividing the fluorescence intensity for a particular band by the sum of the fluorescence intensities for each band in that lane. The total percent cleaved was calculated as [% form II + 2(% form III)] since there must be at least two cleavage events to go from supercoiled to linear DNA. In the single-stranded DNA cleavage reactions, the percent cleaved is simply the intensity of the smear divided by the total fluorescence intensity in that lane. The bar graphs depict the percent cleaved above the control reactions. All experiments were performed at least in triplicate. The error bars in the graphs denote the standard deviations that were determined by using the formula for a small number of data points.

## Results

**Cleavage of Single-Stranded DNA.** Cu([9]aneN$_3$)Cl$_2$ cleaves single-stranded DNA at near-physiological pH (Figure 1). After 48 h of incubation at the higher metal concentrations, the single-stranded DNA is completely degraded. Significantly, the DNA cleavage appears to be nonspecific; Cu([9]aneN$_3$)Cl$_2$ cleaves the single-stranded DNA into numerous different fragments, which results in a smear on the agarose gel. The two bands

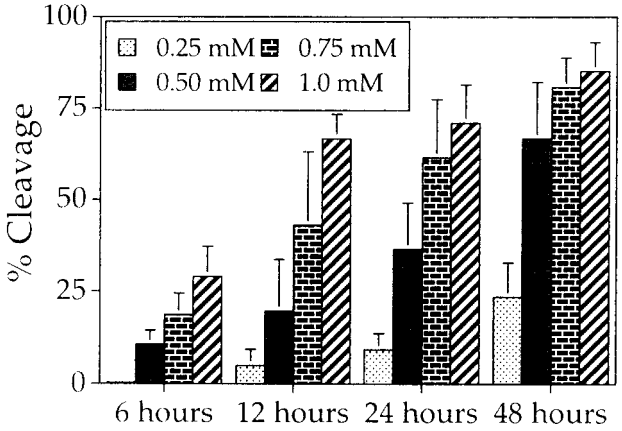



**Figure 2.** Extent of aerobic cleavage of M13 ss-DNA by Cu([9]aneN$_3$)Cl$_2$ determined via fluorimaging. The reaction conditions are described in Figure 1. The graph was created with the data from four separate experiments, and the error bars are the standard deviations calculated by using the formula for a small number of data points. The extent of cleavage was normalized to control reactions.

observed for the intact single-stranded M13 are probably due to different conformers that are separated during gel electrophoresis; only a single band is observed when the buffer is changed from TBE to TAE. The extent of cleavage was quantitated by staining the gel with ethidium bromide and measuring the fluorescence intensity of each band as described in the Experimental Section. The results were corrected for the degraded DNA observed in the control reactions. Significantly, the degree of cleavage in the controls did not change over time, indicating that the degraded single-stranded M13 was present in the stock solution and was not the result of incubation with buffer. Figure 2 clearly demonstrates that the extent of degradation is dependent on both metal complex concentration and time of incubation. From these results, it is concluded that Cu([9]aneN$_3$)Cl$_2$ is the reagent responsible for the degradation of DNA.

**Cleavage of Double-Stranded DNA.** Cu([9]aneN$_3$)Cl$_2$ is also capable of cleaving double-stranded DNA at near-physiological pH. When supercoiled pBluescript DNA was incubated with Cu([9]aneN$_3$)Cl$_2$, the supercoiled DNA was degraded from form I (supercoiled) to form II (relaxed circular) and then more slowly to form III (linear). The results are presented in Figure

(48) Haidle, C. W.; Lloyd, R. S.; Robberson, D. L. In *Bleomycin: Chemical, Biochemical, and Biological Aspects*; Hecht, S. M., Ed.; Springer-Verlag: New York, 1979; pp 222−243.

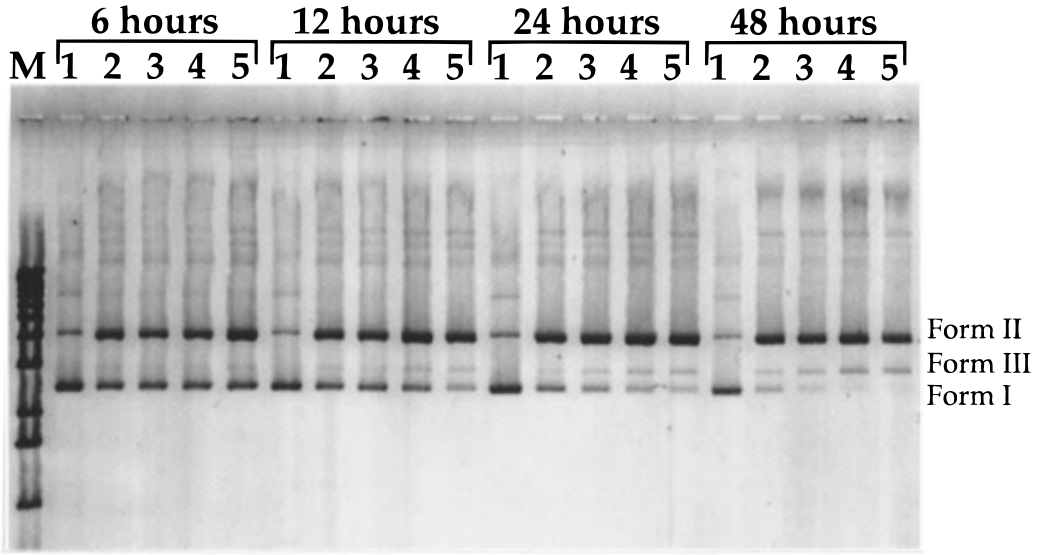



**Figure 3.** Supercoiled ds-DNA (0.05 mg/mL) incubated aerobically with Cu([9]aneN$_3$)Cl$_2$ for 6, 12, 24, or 48 h at pH 7.80 ± 0.06 in 40 mM HEPES at 50 °C. Lane M: molecular weight marker. Lane 1: control, no metal complex. Lane 2: 0.25 mM Cu([9]aneN$_3$)Cl$_2$. Lane 3: 0.50 mM Cu([9]aneN$_3$)Cl$_2$. Lane 4: 0.75 mM Cu([9]aneN$_3$)Cl$_2$. Lane 5: 1.0 mM Cu([9]aneN$_3$)Cl$_2$.

**Table 1.** Aerobic Cleavage of Supercoiled pBluescript II ks(−) by Cu([9]aneN$_3$)Cl$_2$

| incubation time (h) | [Cu([9]aneN$_3$)Cl$_2$] (mM) | DNA % form[a] | | |
|---|---|---|---|---|
| | | supercoiled[b] | nicked | linear |
| 6 | control | 80 ± 4 | 20 ± 1 | 0 ± 0 |
| | 0.25 | 51 ± 17 | 49 ± 17 | 0 ± 0 |
| | 0.50 | 47 ± 10 | 52 ± 10 | 1 ± 2 |
| | 0.75 | 39 ± 6 | 60 ± 5 | 1 ± 2 |
| | 1.0 | 37 ± 9 | 61 ± 6 | 3 ± 3 |
| 12 | control | 79 ± 2 | 21 ± 2 | 0 ± 0 |
| | 0.25 | 44 ± 14 | 56 ± 14 | 0 ± 0 |
| | 0.50 | 38 ± 14 | 61 ± 13 | 1 ± 2 |
| | 0.75 | 26 ± 8 | 71 ± 6 | 3 ± 2 |
| | 1.0 | 27 ± 13 | 70 ± 11 | 2 ± 2 |
| 24 | control | 73 ± 12 | 27 ± 12 | 0 ± 0 |
| | 0.25 | 26 ± 12 | 73 ± 11 | 1 ± 2 |
| | 0.50 | 12 ± 2 | 84 ± 1 | 4 ± 2 |
| | 0.75 | 11 ± 4 | 83 ± 2 | 6 ± 3 |
| | 1.0 | 12 ± 4 | 84 ± 4 | 5 ± 2 |
| 48 | control | 73 ± 7 | 27 ± 7 | 0 ± 0 |
| | 0.25 | 14 ± 11 | 80 ± 7 | 6 ± 5 |
| | 0.50 | 5 ± 4 | 84 ± 5 | 11 ± 9 |
| | 0.75 | 2 ± 2 | 82 ± 11 | 15 ± 11 |
| | 1.0 | 3 ± 2 | 82 ± 9 | 15 ± 10 |

[a] All reactions were performed in triplicate and the standard deviations are shown. The reactions were run at pH 7.80 ± 0.06 (40 mM HEPES) at 50 °C with varying concentrationsof Cu([9]aneN$_3$)Cl$_2$ for up to 48 h. [b] The fluorescence intensities were corrected for the decreased ability of ethidium bromide to intercalate into supercoiled (form I) DNA.[48]

3. After 48 h at higher metal concentrations, Cu([9]aneN$_3$)Cl$_2$ completely degraded the supercoiled DNA to forms II and III. A single cut or nick on a strand of supercoiled DNA relaxes the supercoiling and leads to form II. A second cut on the complementary strand, within approximately 12 base pairs[25a,48,49] of the original cut site, linearizes the DNA to form III. Therefore, Cu([9]aneN$_3$)Cl$_2$ must have cut the DNA at least twice to convert it from form I DNA to form III DNA. The extent of DNA cleavage again was quantitated via fluorimaging (Table 1). Fluorescence intensities were corrected for the different quantum yields of forms I, II, and III as described in the Experimental Section.[48] To directly compare these results

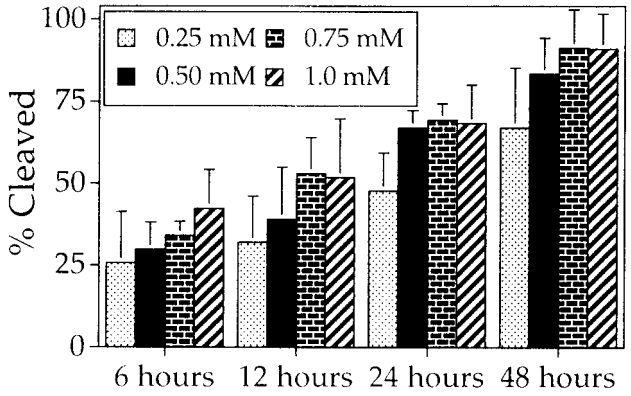



**Figure 4.** Extent of aerobic cleavage of supercoiled ds-DNA by Cu([9]aneN$_3$)Cl$_2$ determined via fluorimaging according to the following formula: total % cleaved = [(% form II) + 2(% form III)]. The reaction conditions are described in Figure 3. This graph was created from the data from three separate experiments, and the error bars are standard deviations calculated by using the formula for a small number of data points. Fluorescence intensities were corrected for the decreased ability of ethidium bromide to intercalate into form I DNA.[48] The results are normalized to control reactions.

with those obtained by using M13 as the substrate, the percent of DNA cleaved was also calculated. In this case, however, it was necessary to account for products that required at least two different cleavage events (i.e., form III DNA). Therefore, the following formula was utilized to construct Figure 4:

$$\text{total \% cleaved} = [(\text{\% form II}) + 2(\text{\% form III})]$$

It should be noted that if the double-stranded cleavage events are random single-stranded cuts that serendipitously occur within approximately 12 base pairs[25a,48,49] of another single-stranded cut site on the opposite strand, then the percent cleaved is actually much higher.

**Anaerobic Cleavage of DNA.** To probe the effects of molecular oxygen on the degradation of DNA by Cu([9]aneN$_3$)Cl$_2$, reactions were also performed under rigorously anaerobic conditions. Cu([9]aneN$_3$)Cl$_2$ still effectively cleaves single-stranded M13 DNA, although the rate of the reaction is significantly decreased when compared to that of the aerobic reaction (Figure 5). As can be seen by comparing Figures 5 and 2, the extent of cleavage decreased by approximately 30%. Supercoiled DNA is also cleaved by Cu([9]aneN$_3$)Cl$_2$ in the

(49) (a) Basile, L. A.; Barton, J. K. *J. Am. Chem. Soc.* **1987**, *109*, 7548−7550. (b) Kishikawa, H.; Jiang, Y. P.; Goodisman, J.; Dabrowiak, J. C. *J. Am.Chem. Soc.* **1991**, *113*, 5434−5440.

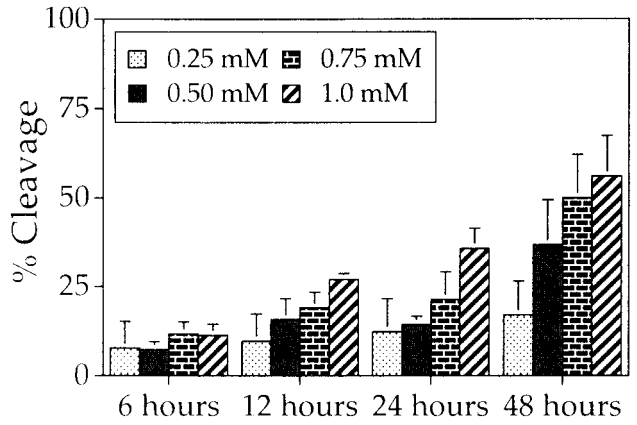



**Figure 5.** Extent of anaerobic cleavage of M13 ss-DNA by Cu([9]-aneN$_3$)Cl$_2$ determined via fluorimaging. The reaction conditions are described in Figure 1. The graph was created with the data from three separate experiments, and the error bars are the standard deviations calculated by using the formula for a small number of data points. The extent of cleavage was normalized to control reactions.

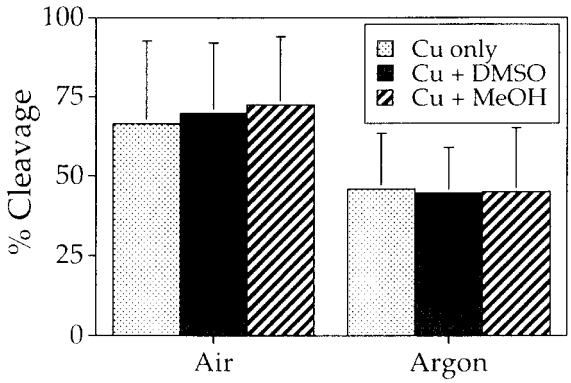



**Figure 6.** Extent of cleavage of M13 ss-DNA by Cu([9]aneN$_3$)Cl$_2$ in the presence of radical scavengers determined via fluorimaging. M13 ss-DNA (0.05 mg/mL) was incubated with 1.0 mM Cu([9]aneN$_3$)Cl$_2$ at pH 7.88 ± 0.07 (40 mM HEPES) and 50 °C for 24 h under both aerobic and anaerobic conditions. Reaction mixtures contained either no radical scavenger, 0.4 M DMSO, or 2.5 M methanol. The graph was created with the data from four separate experiments, and the error bars are the standard deviations calculated by using the formula for a small number of data points. The results are normalized to control reactions.

absence of $O_2$. As in the case of single-stranded DNA, however, the extent of degradation once again decreased by approximately 30% when compared to that of the aerobic reaction (data not shown). It should be noted, however, that to ensure complete deoxygenation of water is difficult, and since the experiments utilize low concentrations of substrate (approximately 25 nm in DNA or 0.15 mM in phosphate units), residual $O_2$ could potentially account for a significant amount of cleavage in the "anaerobic" experiments. The likelihood of residual $O_2$-dependent cleavage in the anaerobic reactions was assessed by using the oxidative cleaving system Fe(EDTA)$^{2-}$/DTT. In the presence of $O_2$, this system rapidly degrades both single-stranded and supercoiled DNA by producing diffusible hydroxyl radicals via the Fenton reaction. Under the anaerobic conditions described in the Experimental Section, however, DNA incubated with Fe(EDTA)$^{2-}$/DTT for over 24 h did not exhibit any degradation. Thus, we conclude that the cleavage of DNA by Cu([9]aneN$_3$)Cl$_2$ in anaerobic experiments is oxygen-independent.

To make certain that the decrease in the extent of DNA cleavage was due to the absence of $O_2$ and not some other atmospheric gas, such as $CO_2$, reactions were also performed under a pure $O_2$ atmosphere. The single-stranded M13 DNA was degraded equally regardless of whether incubation with Cu([9]aneN$_3$)Cl$_2$ occurred under aerobic conditions or a pure $O_2$ atmosphere (data not shown). This result is consistent with the diminished DNA degradation resulting from the loss of $O_2$ under anaerobic conditions. Furthermore, since performing the reaction in pure $O_2$ does not increase the extent of DNA cleavage compared to aerobic conditions, $O_2$ does not appear to be the limiting reagent in the $O_2$-dependent pathway.

**Cleavage Reactions in the Presence of Radical Scavengers.** Single-stranded M13 DNA was incubated with Cu([9]aneN$_3$)-Cl$_2$ in the presence of either 0.4 M DMSO or 2.5 M MeOH as the radical scavenger. Regardless of whether the reaction was performed aerobically or anaerobically, neither the addition of DMSO nor the addition of MeOH had any effect on the cleavage of the single-stranded M13 (Figure 6). Under identical conditions, however, these radical scavengers completely inhibited the oxidative degradation of DNA by Fe(EDTA)$^{2-}$/DTT. The presence of DMSO or MeOH as radical scavenger also had no effect on reactions when supercoiled DNA was used as the substrate (data not shown). These observations suggest that the cleavages of single-stranded and double-stranded DNA are occurring via a similar mechanism. While these results do not rule out the possibility of an oxidative mechanism for the cleavage of DNA by Cu([9]aneN$_3$)Cl$_2$, they do suggest that diffusible free radicals are not involved in either the $O_2$-dependent or the $O_2$-independent mechanism.

**Oxidative Cleavage of DNA by Cu([9]aneN$_3$)Cl$_2$/DTT/O$_2$.** To ascertain whether adventitious reducing agents present in the reaction mixtures could account for the increased DNA degradation in the presence of $O_2$, reactions were performed in which a reducing agent was deliberately added to the solution (data not shown). When single-stranded M13 DNA was incubated with Cu([9]aneN$_3$)Cl$_2$ under aerobic conditions, the extent of DNA degradation increased substantially in the presence of DTT. After 24 h of incubation in the presence of 3.0 mM DTT and 0.25 mM Cu([9]aneN$_3$)Cl$_2$, the DNA was completely degraded. By comparison, incubation in the presence of 0.25 mM Cu([9]aneN$_3$)Cl$_2$ alone leaves a significant portion of the DNA intact (Figure 1). These results suggest that small amounts of adventitious reducing agents could be responsible for the increased DNA cleavage by Cu([9]aneN$_3$)-Cl$_2$ observed under aerobic conditions. Significantly, neither DMSO nor MeOH inhibited this reductant-induced oxidative cleavage reaction, suggesting that, once again, a diffusible free radical is not involved in the mechanism. Control experiments in the presence of DTT but in the absence of Cu([9]aneN$_3$)Cl$_2$ demonstrated that the metal complex was required for this reductant-induced oxidative cleavage to occur; therefore, the reaction is mediated by Cu([9]aneN$_3$)Cl$_2$ and not by residual metal ions present in the water.

**Comparison of Different Metal Complexes.** Endeavoring to find a more efficient cleavage agent, we also tested the efficiency of [Cu(*i*-Pr$_3$[9]aneN$_3$)(OH$_2$)(OTf)]OTf at promoting the degradation of supercoiled DNA. We selected this metal complex on the basis of its ability to hydrolyze the activated phosphodiester BNPP; we have demonstrated that [Cu(*i*-Pr$_3$[9]-aneN$_3$)(OH$_2$)(OTf)]OTf hydrolyzes BNPP much more rapidly than does Cu([9]aneN$_3$)Cl$_2$.[50] Supercoiled plasmid DNA was incubated anaerobically[51] with either Cu([9]aneN$_3$)Cl$_2$ or [Cu-

(50) Hegg, E. L.; Mortimore, S. H.; Cheung, C. L.; Huyett, J. E.; Burstyn, J. N. University of Wisconsin—Madison, manuscript in preparation.

(51) [Cu(*i*-Pr$_3$[9]aneN$_3$)(OH$_2$)(OTf)]OTf forms an insoluble $\mu$-carbonato dimer in the presence of atmospheric $CO_2$[52] and therefore it was necessary to exclude $CO_2$ from the reaction mixtures.

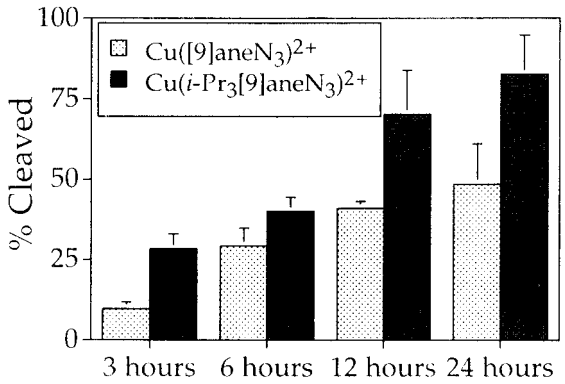



**Figure 7.** Extent of anaerobic cleavage of supercoiled ds-DNA determined via fluorimaging according to the following formula: total % cleaved = [(% form II) + 2(% form III)]. Supercoiled ds-DNA was incubated anaerobically with 1.0 mM Cu([9]aneN$_3$)Cl$_2$ or 1.0 mM [Cu(*i*-Pr$_3$[9]aneN$_3$)(OH$_2$)(OTf)]OTf for 3, 6, 12, or 24 h at pH 7.80 ± 0.06 (40 mM HEPES) and 50 °C. This graph was created with the data from three separate experiments, and the error bars are standard deviations calculated by using the formula for a small number of data points. Fluorescence intensities were corrected for the decreased ability of ethidium bromide to intercalate into form I DNA.[48] The results are normalized to control reactions.

(*i*-Pr$_3$[9]aneN$_3$)(OH$_2$)(OTf)]OTf for varying lengths of time. The amount of cleavage in each reaction was determined, and a comparison of the relative efficiencies of anaerobic DNA degradation is presented in Figure 7. It is quite evident from this graph that [Cu(*i*-Pr$_3$[9]aneN$_3$)(OH$_2$)(OTf)]OTf is more proficient than Cu([9]aneN$_3$)Cl$_2$ at cleaving DNA anaerobically.

## Discussion

We previously demonstrated that Cu([9]aneN$_3$)Cl$_2$ catalytically hydrolyzes activated phosphodiesters.[35] The mechanism established for this reaction reveals that Cu([9]aneN$_3$)$^{2+}$ is in a monomer−dimer equilibrium, with the dimer being the strongly favored species but the monomer being the precursor to the active catalyst. The pH versus rate profile indicates that deprotonation of one of the two coordinated waters (p$K_{a1}$ = 7.3) must occur to form the active catalyst. The substrate binds to the monomer with displacement of $H_2O$, followed by an intramolecular attack of a metal-bound hydroxide resulting in substrate hydrolysis. Loss of the hydrolyzed substrate regenerates the active catalyst. Having elucidated the mechanism of catalytic hydrolysis of activated phosphodiesters by Cu([9]aneN$_3$)Cl$_2$, we wanted to determine whether Cu([9]aneN$_3$)Cl$_2$ was also capable of cleaving unactivated phosphodiesters, or more specifically, whether Cu([9]aneN$_3$)Cl$_2$ could cleave DNA.

In this paper, we establish that Cu([9]aneN$_3$)Cl$_2$ is capable of cleaving both single-stranded and double-stranded DNA. When single-stranded M13 is incubated with Cu([9]aneN$_3$)Cl$_2$ at near-physiological pH and temperature, the single-stranded DNA is nonspecifically degraded, producing a myriad of fragments. Double-stranded DNA is also a substrate for Cu([9]aneN$_3$)Cl$_2$. Supercoiled plasmid DNA (form I) incubated with Cu([9]aneN$_3$)Cl$_2$ at near-physiological pH and temperature is nicked to relaxed circular DNA (form II). Cu([9]aneN$_3$)Cl$_2$ more slowly degrades form II DNA to form III (linear). The extent of DNA degradation depends on both the metal complex concentration and the time of incubation, regardless of whether the substrate is single-stranded or double-stranded DNA. In both cases, anaerobic reactions exhibit an approximately 30% decrease in the extent of DNA degradation relative to aerobic reactions at the same metal complex concentration. The presence of radical scavengers, however, has no effect on the cleavage reactions. Control experiments have unambiguously determined that it is the metal complex and not the buffer that is responsible for the cleavage activity under both aerobic and anaerobic conditions.

Interestingly, the results obtained from the anaerobic reactions suggest that there are at least two different mechanisms of DNA cleavage: an $O_2$-dependent pathway and an $O_2$-independent pathway. The $O_2$-dependent pathway presumably involves oxidative degradation of the DNA; however, since the radical scavengers DMSO and MeOH do not affect the rate of the cleavage reaction, diffusible free radicals are not involved in the mechanism. These results rule out the possibility of Fenton-type chemistry analogous to the well-known oxidative cleavage system Fe(EDTA)$^{2-}$/DTT/$O_2$, in which $H_2O_2$ is generated *in situ* and reacts with Fe(EDTA)$^{2-}$ to form diffusible hydroxyl radicals.[3] That radical scavengers do not affect the rate of DNA degradation by Cu([9]aneN$_3$)Cl$_2$ does not, however, rule out the possibility of an oxidative cleavage mechanism. For example, the oxidative cleavage of DNA by (OP)$_2$Cu$^+$ is postulated to involve a copper-coordinated hydroxyl radical or a copper−oxene complex as the reactive species.[50] Since these species are effectively caged radicals, DNA degradation by (OP)$_2$Cu$^+$ is not significantly affected by the presence of radical scavengers.

Are there other similarities between the $O_2$-dependent reactions of Cu([9]aneN$_3$)Cl$_2$ and (OP)$_2$Cu$^+$? (OP)$_2$Cu$^+$ utilizes $H_2O_2$ as an oxidant. If $H_2O_2$ is not added directly to the reaction mixture, it can be generated *in situ* by oxidation of the Cu(I) complex by $O_2$.[6,12] However, reduction to Cu$^+$ should be more difficult in the presence of triazacyclononane than OP due to both electronic and geometric constraints. The $\pi$-acid character of an aromatic amine such as OP stabilizes the Cu$^+$ oxidation state, while the pure $\sigma$-donor character of an aliphatic amine such as triazacyclononane stabilizes the Cu$^{2+}$ oxidation state.[13] Furthermore, the constrained triazacyclononane ligand inhibits formation of the tetrahedral d$^{10}$ cuprous complex by maintaining facial coordination with N−Cu−N bond angles of less than 90°. The importance of geometric flexibility was observed in the (OP)$_2$Cu$^+$-mediated cleavage of DNA by Sigman et al. when they discovered that (OP)$_2$Cu$^+$ derivatives with substituents ortho to the chelating nitrogens were unable to cleave DNA.[6b] In this case, steric constraints prevented the formation of the square-planar d$^9$ cupric complex, and inhibition of the Cu(I)/Cu(II) redox cycle prevented *in situ* formation of $H_2O_2$. Nevertheless, Cu([9]aneN$_3$)$^+$ has been synthesized under anaerobic conditions in nonaqueous media,[52] and our results from experiments performed in the presence of excess DTT clearly demonstrate that Cu([9]aneN$_3$)Cl$_2$ is capable of oxidatively cleaving DNA in the presence of reducing agents.[53] Therefore, it is reasonable to conclude that the $O_2$-dependent pathway involves an oxidative mechanism mediated by Cu([9]aneN$_3$)Cl$_2$ and adventitious reducing agents.

Although the $O_2$-dependent reaction mechanism may be similar, Cu([9]aneN$_3$)Cl$_2$ is unlikely to interact with DNA in a manner analogous to (OP)$_2$Cu$^+$. (OP)$_2$Cu$^+$ binds to the minor groove of the DNA before initiating cleavage and therefore shows specificity for double-stranded over single-stranded DNA. (The binding presumably occurs initially via intercalation, although it is a nonintercalative mode of binding that is

(52) Halfen, J. A.; Schneider, J. L.; Tolman, W. B. University of Minnesota, unpublished results.

(53) Due to the disproportionation of Cu$^+$ in water, the oxidation potential for this complex cannot be measured under the reaction conditions utilized for DNA cleavage. In acetonitrile, however, a related complex, [Cu(*i*-Pr$_3$[9]aneN$_3$)(CH$_3$CN)]$^+$, undergoes quasi-reversible one-electron oxidation at $E_{1/2}$ = 0.36 V versus SCE.[45]

postulated to be important in cleavage.)[54] Thus, single-stranded DNA generally is not a substrate for $(OP)_2Cu^+$. Due to stable hairpin regions and other metastable tertiary structures in single-stranded M13, however, this substrate is cleaved by $(OP)_2Cu^+$.[12b] As expected, the extent of single-stranded M13 degradation decreases as the temperature increases and local structure is lost. At the elevated temperature at which we are performing our reactions, the extent to which single-stranded M13 retains this metastable structure is unknown. Nonetheless, Cu([9]aneN$_3$)Cl$_2$ does not appear to discriminate between single-stranded and double-stranded DNA.

One of the most interesting and remarkable aspects of the Cu([9]aneN$_3$)Cl$_2$-promoted cleavage of DNA is the amount of activity retained in the absence of $O_2$: approximately 70% of the cleavage activity is retained when the reactions are performed under rigorously anaerobic conditions. Cu([9]aneN$_3$)Cl$_2$ is one of only a few metal complexes shown to cleave DNA in the absence of $O_2$ or some other oxidant.[40,42] Because many oxidative DNA cleavage pathways produce products that are similar to the products of hydrolysis, however, it is very difficult to conclusively demonstrate that DNA cleavage is hydrolytic. Detailed product analysis must show not only that the products expected from hydrolysis are present but also that other products, which may be at very low concentrations, are absent. Hydrolysis of dinucleotides and short oligonucleotides by lanthanide ions and complexes has been demonstrated by direct product analysis via NMR, HPLC, and HPLC−mass spectrometry;[42] all of the observed products are consistent with a hydrolytic mechanism.

Unfortunately, neither dinucleotides nor oligonucleotides up to 30 nucleotides long (both single-stranded and hairpin structures) are cleaved very efficiently by Cu([9]aneN$_3$)Cl$_2$. Although cleavage of the oligonucleotides was observed via mass spectrometry, HPLC, and autoradiography of sequencing gels loaded with $^{32}$P-labeled DNA, the extent of cleavage was very low and detailed product analysis was not possible. (Interestingly, these experiements were performed aerobically, indicating that neither the $O_2$-dependent nor the $O_2$-independent reaction is very efficient with short oligonucleotides.) Significantly, [Cu(*i*-Pr$_3$[9]aneN$_3$)(OH$_2$)(OTf)]OTf cleaves DNA under anaerobic conditions nearly twice as fast as Cu([9]aneN$_3$)Cl$_2$. Future experiments will determine whether [Cu(*i*-Pr$_3$[9]aneN$_3$)(OH$_2$)(OTf)]OTf also cleaves short oligonucleotides and whether product analysis is feasible. Nevertheless, the fact that DNA cleavage occurs in the absence of $O_2$ or any other oxidant leaves open the possibility that Cu([9]aneN$_3$)Cl$_2$ cleaves DNA via a hydrolytic mechanism.

## Conclusion

In conclusion, we have demonstrated that, in addition to hydrolyzing activated phosphodiesters, Cu([9]aneN$_3$)Cl$_2$ also cleaves both single-stranded and double-stranded DNA. The rate of DNA degradation increases by a factor of 2 when the alkylated *i*-Pr$_3$[9]aneN$_3$ ligand is used. The cleavage reaction proceeds via at least two distinct mechanisms: an $O_2$-dependent pathway and an $O_2$-independent pathway. Significantly, these are two of only a few well-defined metal complexes that have been shown to cleave DNA in the absence of $O_2$ or some other oxidant. Furthermore, Cu([9]aneN$_3$)Cl$_2$ is the first metal complex shown to cleave both DNA and proteins in the absence of an oxidant.

**Acknowledgment.** The authors thank Dr. Richard Guilfoyle and Todd Francisco for their help in preparing single-stranded M13 DNA, Dr. Leslie Waite for her assistance in the preparation of supercoiled DNA, and Andrew Thiel for his assistance in fluorimaging. The authors also gratefully acknowledge Professor William Tolman, Jason Halfen, and Jamie Schneider at the University of Minnesota for supplying [Cu(*i*-Pr$_3$[9]aneN$_3$)(OH$_2$)(OTf)]OTf. Funding was provided by NIH 5T32GMO8349 (Biotechnology Training Grant for E.L.H.) and the University of Wisconsin Graduate School Research Committee.

IC960384N

(54) Sigman, D. S.; Mazumder, A.; Perrin, D. M. *Chem. Rev.* **1993**, *93*, 2295−2316.