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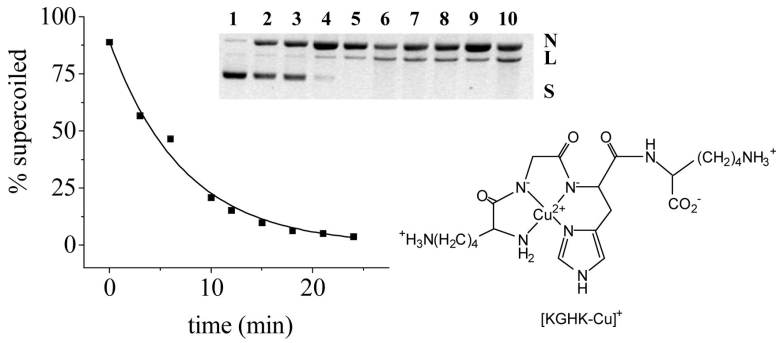

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DNA Cleavage by Copper−ATCUN Complexes. Factors Influencing Cleavage Mechanism and Linearization of dsDNA

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Abstract: The reactivity of two [peptide−Cu] complexes ([GGH−Cu]$^-$ and [KGHK−Cu]$^+$) toward DNA cleavage has been quantitatively investigated. Neither complex promoted hydrolytic cleavage, but efficient oxidative cleavage was observed in the presence of a mild reducing agent (ascorbate) and dioxygen. Studies with scavengers of ROS confirmed hydrogen peroxide to be an obligatory diffusible intermediate. While oxidative cleavage of DNA was observed for Cu^{2+}(aq) under the conditions used, the kinetics of cleavage and reaction products/pathway were distinct from those displayed by [peptide−Cu] complexes. DNA cleavage chemistry is mediated by the H_2O-dependent pathway following C-4′H abstraction from the minor groove. Such a cleavage path also provides a ready explanation for the linearization reaction promoted by [KGHK−Cu]$^+$. Kinetic activities and reaction pathways are compared to published results on other chemical nucleases. Both [peptide−Cu] complexes were found to display second-order kinetics, with rate constants $k_2 \sim 39$ and 93 $M^{-1}\,s^{-1}$ for [GGH−Cu]$^-$ and [KGHK−Cu]$^+$, respectively. Neither complex displayed enzyme-like saturation behavior, consistent with the relatively low binding affinity and residence time expected for association with dsDNA, and the absence of a prereaction complex. However, the intrinsic activity of each is superior to other catalyst systems, as determined from relative k_2 or k_{cat}/K_m values. Linearization of DNA was observed for [KGHK−Cu]$^+$ relative to [GGH−Cu]$^-$, consistent with the increased positive charge and longer residency time on dsDNA.

Introduction

DNA is a naturally stable polymer with an estimated half-life toward spontaneous hydrolysis of ∼130 000 years under physiological conditions.[1] Studies of DNA cleavage agents are fueled by recognition of their potential application in biotechnology and as therapeutic agents. A variety of metal complexes has been explored with the aim of cleaving DNA efficiently by either hydrolytic[2−13] or oxidative pathways.[12,14] Recent efforts in our laboratory have focused on copper complexes of aminoglycosides.[12,13] These catalytic agents demonstrated outstanding cleavage activity with multiple turnover behavior and mediated both hydrolytic and oxidative cleavage of DNA,[12,13] the latter by C-4′H abstraction that also resulted in linearization of dsDNA. An important realization from this work was the very obvious correlation between the binding affinity of a chemical nuclease and its DNA cleavage efficiency.

To further develop highly efficient chemical nucleases of practical value, we sought to explore other families of ligands that were readily available, tunable, and facile to evaluate. The ATCUN (amino terminal $\underline{Cu}^{2+}$- and $\underline{Ni}^{2+}$-binding) peptide motif occurs naturally in certain species of albumins[15] and has been demonstrated to bind Cu^{2+} ($K_D \sim 1.18 \times 10^{-16}$ M)[16] and Ni^{2+} ($K_D \sim 10^{-16}$ M)[17] with high affinity. The physiological function of albumin is to bind and transport divalent copper and certain other transition metals in the blood, which also suggests the [peptide−Cu^{2+}] motif to have very little cytotoxicity. NMR, EPR, visible spectroscopy, and X-ray crystallography are consistent with the structural motifs shown in Scheme 1 for GGH (GlyGlyHis) and KGHK (LysGlyHisLys), with the divalent copper ion coordinated in a square planar configuration by the α-NH_2-terminal nitrogen, the two intervening peptide nitrogens, and the imidazole nitrogen on the His at position 3.[15,16,18,19]

(1) Radzicka, A.; Wolfenden, R. *Science* **1995**, *267*, 90−93.
(2) Pitié, M.; Burrows, C. J.; Meunier, B. *Nucleic Acids Res.* **2000**, *28*, 4856−4864.
(3) Tawa, R.; Gao, D.; Takami, M.; Imakura, Y.; Lee, K.-H.; Sakurai, H. *Bioorg. Med. Chem.* **1998**, *6*, 1003−1008.
(4) Branum, M. E.; Tipton, A. K.; Zhu, S.; Que, L. J. *J. Am. Chem. Soc.* **2001**, *123*, 1898−1904.
(5) Francois, J.-C.; Saison-Behmoaras, T.; Barbier, C.; Chassignol, M.; Thuong, N. T.; Héléne, C. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 9702−9706.
(6) Dixon, N. E.; Geue, R. J.; Lambert, J. N.; Moghaddas, S.; Pearce, D. A.; Sargeson, A. M. *Chem. Commun.* **1996**, 1287−1288.
(7) Veal, J. M.; Merchant, K.; Rill, R. L. *Nucleic Acids Res.* **1991**, *19*, 3383−3388.
(8) Rammo, J.; Hettich, R.; Roigk, A.; Schneider, H.-J. *Chem. Commun.* **1996**, 105−107.
(9) Fitzsimons, M. P.; Barton, J. K. *J. Am. Chem. Soc.* **1997**, *119*, 3379−3380.
(10) Hettich, R.; Schneider, H.-J. *J. Am. Chem. Soc.* **1997**, *119*, 5638−5647.
(11) Bales, B. C.; Pitié, M.; Meunier, B.; Greenberg, M. A. *J. Am. Chem. Soc.* **2002**, *124*, 9062−9063.
(12) Cowan, J. A. *Curr. Opin. Chem. Biol.* **2001**, *5*, 634−642.
(13) Sreedhara, A.; Freed, J. D.; Cowan, J. A. *J. Am. Chem. Soc.* **2000**, *122*, 8814−8824.
(14) Detmer, C. A. I.; Pamatong, F. V.; Bocarsly, J. R. *Inorg. Chem.* **1996**, *35*, 6292−6298.
(15) Harford, C.; Sarkar, B. *Acc. Chem. Res.* **1997**, *30*, 123−130.
(16) Lau, S.-J.; Kruck, T. P. A.; Sarkar, B. *J. Biol. Chem.* **1974**, *249*, 5878−5884.
(17) Long, E. C. *Acc. Chem. Res.* **1999**, *32*, 827−836.

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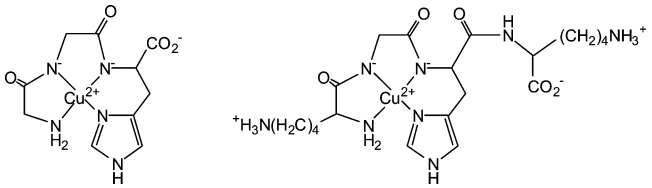
Scheme 1. Structural Representation of [GGH−Cu]$^-$ and [KGHK−Cu]$^+$, Consistent with Prior Spectroscopic Characterization[16]



The GGH sequence is representative of the natural ATCUN peptide motif; however, any peptide that satisfies these criteria demonstrates copper binding. Recent research has shown that the ATCUN peptide motif may have more physiological relevance than merely serving as a metal transport site. The ATCUN peptide sequence in human sperm protamine P2a in metal-exposed individuals may provide a potential mechanism for carcinogenesis or birth defects in offspring,[20] while a [GGH−Cu]/ascorbate system was found to kill Ehrlich ascites tumor cells in vitro[21] and was shown to cleave DNA under certain conditions.[22,23] However, such DNA cleavage chemistry has not been critically addressed under physiological conditions, nor systematically and quantitatively evaluated.

Previous studies of Ni^{2+} and Co^{3+} complexes of GGH-derived ATCUN peptides have focused on investigations of their DNA recognition, binding, and modification properties.[24,25] DNA cleavage has been demonstrated with coreactants such as $KHSO_5$, MMPP, H_2O_2,[26−28] or even ambient O_2 and light activation in the case of [peptide−Co^{3+}] complexes.[29] High-resolution denaturing polyacrylamide gel analysis of cleavage products suggested that [peptide−Ni^{2+}] with coreactants causes DNA cleavage through a mechanism involving C-4′H oxidation.[28] [Peptide−Ni^{2+}] complexes, in concert with appropriate coreactants, have also demonstrated selective RNA modification and cleavage of both $tRNA^{phe}$ and the TAR RNA of HIV-1.[30]

In this paper, we report a rigorous investigation of the chemical nuclease activity of [GGH−Cu]$^-$ and [KGHK−Cu]$^+$ complexes toward double-stranded DNA. Several prior reports have noted the potential of the former for catalysis of DNA cleavage chemistry, but the details and scope of this earlier work is limited.[22,23] Herein, we present a detailed account of the kinetics and mode of cleavage of representative copper complexes of ATCUN peptides under hydrolytic and oxidative conditions. Our results indicate that both [GGH−Cu]$^-$ and [KGHK−Cu]$^+$ promote DNA cleavage quite effectively under physiological conditions by oxidative pathways, but no cleavage is observed under hydrolytic conditions. Linearization of plasmid DNA is also observed in the case of [KGHK−Cu]$^+$. Comprehensive kinetic and product analyses of the DNA cleavage reactions by both [GGH−Cu]$^-$ and [KGHK−Cu]$^+$ complexes suggest C-4′H abstraction to initiate the DNA cleavage pathway.

Materials and Methods

General Materials. The GGH tripeptide and KGHK tetrapeptide were purchased from Bachem Co. and used without further purification. Unless stated, all other reagents used in this research were obtained from Sigma Chemical Co.

Synthesis and Characterization. A solution of 1 mM peptide in 10 mM Tris buffer (pH = 7.4) was mixed with 0.8 mM $CuCl_2$ in 10 mM Tris buffer solution in 1:1 v/v ratio. This yielded a 1.25:1 peptide/copper ratio, and so there was effectively no free Cu (II) ion in the cleavage reaction mixture. The resulting solution was stirred at room temperature for ∼30 min, resulting in a light reddish-purple solution that displayed a UV−vis spectrum similar to that observed previously.[16] Samples were maintained at 4 °C, and concentrations and stabilities of the [GGH−Cu]$^-$ and [KGHK−Cu]$^+$ complexes were routinely verified prior to use.

DNA Cleavage Studies. Plasmid pUC19 (2686 bp) was purchased from New England Biolabs Inc. The plasmid was transformed into DH5α competent cells and amplified,[13] and pure plasmid was isolated using QIAGEN protocols. Fresh plasmid DNA (over 90−95% supercoiled) was prepared before each experiment to avoid contamination by any other form of plasmid DNA. In general, DNA cleavage experiments were performed with 50 μM base pair concentration of pUC 19, 25 μM Cu−peptide, and 250 μM ascorbate in 10 mM Tris buffer, pH 7.4, at 37 °C. A control reaction was carried out using the same conditions as the cleavage reaction but lacking the Cu−peptide complex. The reactions were quenched with a loading buffer containing 0.5 M EDTA.[31]

Electrophoresis. Agarose gel electrophoresis (0.8%) containing ethidium bromide was performed under standard conditions.[31] DNA samples were run on horizontal gels in 1× TAE buffer for 90 min at 120 mV.

DNA Cleavage Quantitation. Quantitation of closed circular, nicked, and linear DNA was made by densitometric analysis of ethidium bromide containing agarose gels. Quantitation was performed by fluorescence imaging by use of a Gel-Doc 1000 (BioRad) and data analysis with Multianalysis software (version 1.1) provided by the manufacturer using the volume quantitation method. In all cases, background fluorescence was subtracted by reference to a lane containing no DNA. A correction factor of 1.47 was used for supercoiled DNA since the ability of ethidium bromide to intercalate into supercoiled DNA (form I) decreased relative to nicked (form II) and linear DNA (form III). The fraction of each form of DNA was calculated by dividing the intensity of each band by the total intensities of all the bands in the lane. All results were obtained from experiments that were performed at least in triplicate.

Investigation of DNA Linearization. Following cleavage, the fraction of full-length linear DNA, f(III), is related to the number, $n2$, of double-stranded breaks per molecule given by the first term of a Poisson distribution (eq 1).[32]

$$f(\mathrm{III}) = n2\,\exp(-n2) \qquad (1)$$

The sum of single-stranded, $n1$, and double-stranded, $n2$, breaks per

(18) Camerman, N.; Camerman, A.; Sarkar, B. *Can. J. Chem.* **1975**, *54*, 1309−1316.
(19) Conato, C.; Kozlowski, H.; Mlynnarz, P.; Pulidori, F.; Remelli, M. *Polyhedron* **2002**, *21*, 1469−1474.
(20) Bal, W.; Jezowska-Bojezuk, M.; Kasprzak, K. S. *Proc. Am. Assoc. Cancer* **1996**, *37*, 131.
(21) Kimoto, E.; Tanaka, H.; Gyotoku, J.; Morishige, F.; Pauling, L. *Cancer Res.* **1983**, *43*, 824−828.
(22) Mack, D. P.; Iverson, B. L.; Dervan, P. B. *J. Am. Chem. Soc.* **1988**, *110*, 7572−7574.
(23) Chiou, S.-H. *J. Biochem.* **1983**, *94*, 1259−1267.
(24) Liang, Q.; Eason, P. D.; Long, E. C. *J. Am. Chem. Soc.* **1995**, *117*, 9625−31.
(25) Huang, X.; Pieczko, M. E.; Long, E. C. *Biochemistry* **1999**, *38*, 2160−2166.
(26) Mack, D. P.; Dervan, P. B. *J. Am. Chem. Soc.* **1990**, *112*, 4604−4606.
(27) Mack, D. P.; Dervan, P. B. *Biochemistry* **1992**, *31*, 9399−9405.
(28) Liang, Q.; Ananias, D. C.; Long, E. C. *J. Am. Chem. Soc.* **1998**, *120*, 248−257.
(29) Long, E. C.; Claussen, C. A. In *Small Molecule DNA and RNA Binders*; Demeunynck, M., Bailly, C., Wilson, W. D., Eds.; Wiley-VCH: Weinheim, Germany, 2004; Vol. 1, pp 88−125.
(30) Brittain, I. J.; Huang, X.; Long, E. C. *Biochemistry* **1998**, *37*, 12113−12120.
(31) Sambrook, J.; Fritsch, E. F.; Maniatis, T. *Molecular Cloning*; Cold Spring Harbor Laboratory Press: New York, 1989.
(32) Povirk, L. F.; Wubker, W.; Kohnlein, W.; Hutchinson, F. *Nucleic Acids Res.* **1977**, *4*, 3573−3579.

moleculue ($n1 + n2$) was determined from the fraction f(I) of supercoiled DNA remaining after treatment with the [peptide−Cu] reagent.[32]

$$f(\mathrm{I}) = \exp[-(n1 + n2)] \quad (2)$$

The Freifelder−Trumbo relation (eq 2) shows that the number of double-stranded breaks expected from coincidences of random single-stranded breaks is less than 0.01 per molecule, ($n1/n2$) > 100.[33] Consequently, from comparison of the ratio of $n1$ and $n2$ ($n1/n2$) relative to 100, one can determine if the linearization of DNA resulted from random or nonrandom cleavage. In these studies, both $n1$ and $n2$ were calculated by use of eqs 1 and 2.

Characterization and Quantitation of Reactive Oxygen Species. DNA was incubated with [peptide−Cu] complexes and ascorbate under the same aerobic conditions as described perviously with the addition of either 100 mM DMSO (as a hydroxyl radical scavenger),[34] or 10 mM NaN_3 (as a singlet oxygen scavenger),[13] or 300 units of SOD (superoxide dismutase, as a superoxide scavenger),[13,14] or 2.5 mg/mL catalase (10 000−40 000 units/mg, as a peroxide scavenger).[23] Control reactions were carried out in the absence of the [ACTUN−Cu] complex. The production of hydroxyl radicals was detected and quantitated by following the degradation of the reporter dye, rhodamine B (∼15 μM),[13,14] in the presence of either 300 μM Cu^{2+}(aq), [GGH−Cu]$^-$, or [KGHK−Cu]$^+$. The reaction (in 100 mM Tris buffer, pH = 7.4) was activated by the addition of either ascorbate or H_2O_2 (to 3 mM). The degradation of the dye was spectrophotometrically monitored at 552 nm, $\epsilon = 1.07 \times 10^5$ M^{-1} cm^{-1}.[13] The time-dependent change in concentration of rhodamine B is a direct measure of the production of hydroxyl radicals in the reaction mixture. Parallel experiments were performed to follow the formation of hydroxyl radicals in the presence of Cu^{2+}(aq) or [peptide−Cu] complexes and in the absence of ascorbate and H_2O_2 (the first 20 min).

HPLC Analysis of Product Release following DNA Cleavage. Analysis and quantitation of products released during the [KGHK−Cu]$^+$/ascorbate DNA cleavage reactions were achieved by HPLC analysis. A 500 μM solution of calf thymus DNA (base pair concentration) from Amersham Biosciences was treated with 200 μM [KGHK−Cu]$^+$ complex and a 10-fold molar excess of ascorbate (2 mM) in 100 mM Tris buffer, pH 7.4 at 37 °C for 2 h. Subsequently, the reaction mixture was loaded onto a VYDAC monomeric C-18 reverse phase column equilibrated with 0.1 M NH_4OAc, pH 6.8 (solvent A). Products were eluted with an acetonitrile (solvent B) gradient (0−10 min, 100% A; 10−25 min, 0−0.6% B in A; 25−40 min, 0.6−2% B in A; 40−50 min, 2−10% B in A), monitored at 254 nm. The same reaction mixture was heated at 85 °C for 30 min, loaded onto the C-18 column, and eluted with the same profile to evaluate the formation of 5-methylene furanone (5-MF) and furaldehyde.[2] Authentic 5-MF was synthesized and purified[35] and was characterized by mass spectrometry (mass = 96)[36] and UV−vis spectroscopy (with λ_{max} = 260 nm).[35] The column was independently calibrated with authentic materials, four nucleic acid bases, 5-MF (C-1′H abstraction product), and furaldehyde (furfural, a C-5′H abstraction product) to confirm the identity of the signature products from cleavage reactions. Standard calibration plots of peak areas versus concentration were prepared for each base, and for 5-MF, to quantitate the products released.

TBA (Thiobarbituric Acid) Assay. The standard thiobarbituric acid (TBA) assay was performed by heating the DNA cleavage reaction mixture in 0.6% TBA to 95 °C for 20 min.[37,38] The resulting pink solution shows a λ_{max} ∼532 nm, corresponding to the adduct formed by reaction of TBA with malondialdehyde. A calibration curve was obtained by using 0.1−10 μM malondialdehyde as a standard.

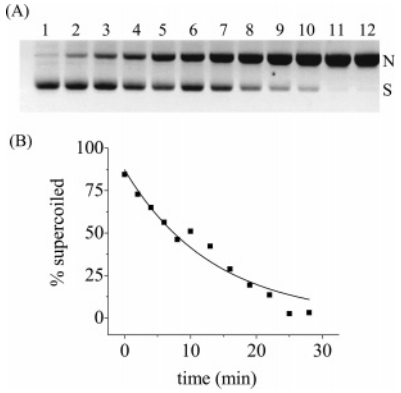


Figure 1. Cleavage activity of [GGH−Cu]$^-$ monitored by 0.8% agarose gel electrophoresis, where [DNA] = 50 μM, [[GGH−Cu]$^-$] = 25 μM, and [ascorbate] = 250 μM. Time course measured in 10 mM Tris buffer, pH = 7.4, 37 °C, showing the disappearance of supercoiled DNA (S) at **(1)** 0 min, **(2)** 2 min, **(3)** 4 min, **(4)** 6 min, **(5)** 8 min, **(6)** 10 min, **(7)** 13 min, **(8)** 16 min, **(9)** 19 min, **(10)** 22 min, **(11)** 25 min, and **(12)** 28 min. (A) Gel image showing nicked (N) and supercoiled (S) DNA. (B) Reaction curve, showing a pseudo-first-order kinetic profile ($R^2 = 0.952$), k_{obs} ∼0.07 min^{-1}.

Results

Preparation and Stability of Copper−ATCUN Peptide Complexes. Coordination of Cu^{2+} to the tri- and tetra-peptides GGH and KGHK (Scheme 1), respectively, was demonstrated by UV−vis spectral analysis. A maximal absorbance was observed at 525 nm, and extinction coefficients were determined as ϵ_{525} ∼108 M^{-1} cm^{-1} for [GGH−Cu]$^-$ and ∼85 M^{-1} cm^{-1} for [KGHK−Cu]$^+$, respectively. Both are consistent with prior observations for these complexes.[16,19]

The stabilities of [peptide−Cu] complexes have been well-documented in the literature (ranging from $K_D = 1.18 \times 10^{-16}$ M for [GGH−Cu]$^{-16}$ to $K_D = 1.0 \times 10^{-19}$ M for [KGHK−Cu]$^+$).[16,19] Under anaerobic reducing conditions (ascorbate addition), both [GGH−Cu]$^-$ and [KGHK−Cu]$^+$ were reduced, with a loss of the 525 nm absorption band. Following exposure to dioxygen, both the cupric oxidation state and the 525 nm absorbance were recovered. However, as detailed later, the copper ion appears to remain coordinated to the peptide ligand in the presence of ascorbate under the turnover conditions used in the DNA cleavage studies.

DNA Cleavage by [GGH−Cu]$^-$and [KGHK−Cu]$^+$ Complexes. [GGH−Cu]$^-$ was found to mediate the rapid degradation of supercoiled (form I) plasmid DNA to produce nicked (form II) plasmid DNA at concentrations as low as 10 μM in the presence of ascorbate but not under hydrolytic conditions (lacking ascorbate). The spontaneous hydrolysis of DNA was not observed under these conditions. Consistent with previous observations, neither ascorbate nor [GGH−Cu]$^-$ alone demonstrated any apparent cleavage activity.[23] Subsequently, the DNA cleavage chemistry of [KGHK−Cu]$^+$ was evaluated, with good activity levels evident even at 10 μM concentration, and was also observed to degrade DNA more rapidly than [GGH−Cu]$^-$ (Figures1 and 2). Linear DNA was formed in the [KGHK−Cu]$^+$ cleavage samples (Figure 2), requiring two breaks on opposing strands within 10 bp of each other, whereas linear DNA was not found in samples following treatment with [GGH−Cu]$^-$. A smear pattern was observed for DNA treated

(33) Freifelder, D. *Biopolymers* **1969**, *7*, 681−693.
(34) Hegg, E. L.; Burstyn, J. N. *Inorg. Chem.* **1996**, *35*, 7474−7481.
(35) Shaw, E. A *J. Am. Chem. Soc.* **1946**, *68*, 2510−2513.
(36) Meijler, M. M.; Zelenko, O.; Sigman, D. S. *J. Am. Chem. Soc.* **1997**, *119*, 1135−1136.
(37) Patwardhan, A.; Cowan, J. A. *Chem. Commun.* **2001**, 1490−1491.
(38) Giloni, L.; Takeshita, M.; Johnson, F.; Iden, C.; Grollman, A. P. *J. Biol. Chem.* **1981**, *256*, 8608−8615.

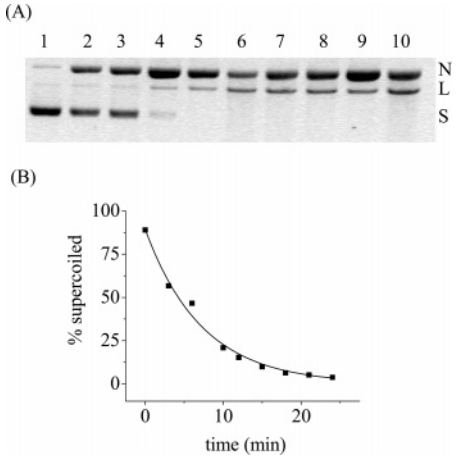


Figure 2. Cleavage activity of $[KGHK-Cu]^+$ monitored by 0.8% agarose gel electrophoresis, where [DNA] = 50 μM, $[[KGHK-Cu]^+]$ = 25 μM, and [ascorbate] = 250 μM. Time course measured in 10 mM Tris buffer, pH = 7.4, 37 °C, showing the disappearance of supercoiled DNA (S), **(1)** 0 min, **(2)** 3 min, **(3)** 6 min, **(4)** 9 min, **(5)** 12 min, **(6)** 15 min, **(7)** 18 min, **(8)** 21 min, **(9)** 24 min, and **(10)** 27 min (DNA began to smear). (A) Gel image showing nicked (N), linear (L), and supercoiled (S) DNA. (B) Reaction curve, showing a pseudo-first-order kinetic profile (R^2 = 0.99), k_{obs} ~0.14 min^{-1}.

with Cu^{2+}(aq) and ascorbate, indicating a random pattern of scission reaction, which is consistent with prior observations.[23]

Kinetic Parameters for $[GGH-Cu]^-$ and $[KGHK-Cu]^+$ Promoted DNA Cleavage. Kinetic parameters underlying the DNA cleavage chemistry of $[GGH-Cu]^-$ and $[KGHK-Cu]^+$ were determined by following the time-dependence of the reaction under pseudo-first-order conditions ([DNA] = 50 μM, $[[GGH-Cu]^-]$ = 25 μM, and [ascorbate] = 250 μM). The loss of supercoiled DNA and increased levels of nicked and linear DNA were quantitated following gel electrophoresis as described in the Materials and Methods. Typical results from this set of experiments are shown in Figures 1 and 2. Both $[GGH-Cu]^-$ and $[KGHK-Cu]^+$ were found to mediate the cleavage of one strand of dsDNA in the initial stage of the reaction. Quantitative production of nicked plasmid DNA (form II) was observed within 28 min for $[GGH-Cu]^-$ (Figure 1A) and within 6 min for $[KGHK-Cu]^+$ (Figure 2A, although no linear DNA was formed during this initial period). A slower subsequent nicking was then promoted by $[KGHK-Cu]^+$ that resulted in formation of linear DNA (form III).

The reaction profile for the $[GGH-Cu]^-$ mediated reaction displayed approximately pseudo-first-order kinetic behavior (Figure 1B), with k_{obs} ~0.07 min^{-1} and R^2 = 0.95. The reaction profile for DNA treated with $[KGHK-Cu]^+$ was also fitted to a pseudo-first-order reaction (R^2 = 0.99) with k_{obs} ~0.14 min^{-1} (Figure 2B). For the gel shown in Figure 2A, the last point in lane 10 was omitted from the graph in Figure 2B since the DNA had already begun to smear as a result of multiple cleavage events.

The influence of added ascorbate was maximal when the ratio of [ascorbate]/$[GGH-Cu]^-$ was greater or equal to 10:1, and so a ratio of at least 10:1 was typically used to satisfy the need for pseudo-first-order conditions in the kinetic analysis. Both ascorbate and H_2O_2 showed similar behavior in DNA cleavage reactions, although H_2O_2 was slightly more active than ascorbate. Neither ascorbate nor H_2O_2 alone, at 250 μM concentration, showed any background DNA cleavage.

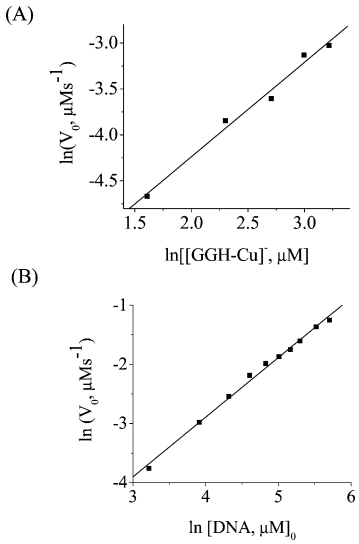


Figure 3. (A) Dependence of ln V_0 vs ln$[[GGH-Cu]^-]$. Fitting to eq 5, where $k_{obs} = k_2$[DNA], yielded k_2 ~39 M^{-1} s^{-1} and m ~1. (B) The dependence of ln V_0 vs ln$[DNA]_0$ was similarly fitted, where $k_{obs} = k_2$[complex], yielded k_2 ~39 M^{-1} s^{-1} and m ~1.

Rate Law for $[GGH-Cu]^-$ and $[KGHK-Cu]^+$ Promoted Cleavage of dsDNA. The dependence of cleavage activity on catalyst concentration showed no saturation behavior indicative of enzyme-like behavior. Consequently, the reaction was considered in terms of the standard rate law shown in eq 3. The initial rate (V_0) corresponding to less than 5% conversion was considered as a function of [complex] (eqs 4 and 5).[39]

$$\text{rate} = V = \text{d[DNA]/d}t = k_{obs}[\text{complex}]_t^m \qquad (3)$$

where $k_{obs} = k_n$[DNA]

$$t = 0,\ V_0 = k_{obs}[\text{complex}]_0^m \qquad (4)$$

and

$$\ln V_0 = m \ln[\text{complex}]_0 + \ln k_{obs} \qquad (5)$$

A plot of ln V_0 versus ln$[[GGH-Cu]^-]_0$ at a constant DNA concentration produced a straight line relationship (R^2 = 0.99) (Figure 3A). The value of m ~1 demonstrated the reaction to be first order with respect to the $[GGH-Cu]^-$ complex. We have already demonstrated the reaction to be first order with respect to DNA (Figure 1B), and so the overall reaction is second-order with $k_n = k_2$ ~39 M^{-1} s^{-1}.

A plot of ln V_0 versus ln$[DNA]_0$ at constant $[GGH-Cu]^-$ concentration (Figure 3B) yielded a value of m ~1 for DNA, again consistent with a first-order reaction for DNA, as shown in Figure 1B. With the same calculation method, a k_2 ~39 M^{-1} s^{-1} was obtained, consistent with the k_2 (39 M^{-1} s^{-1}) obtained by plotting ln V_0 versus ln$[[GGH-Cu]^-]_0$. Similarly, k_2 ~93 M^{-1} s^{-1} was obtained for DNA cleavage by $[KGHK-Cu]^+$ (Figure 4).

Investigation of DNA Linearization by $[KGHK-Cu]^+$. The complex $[KGHK-Cu]^+$ was observed to produce a well-defined electrophoresis band for linear DNA following limited reaction with supercoiled DNA (Figure 2). A linear control (form

(39) Connors, K. A. *Chemical Kinetics: The Study of Reaction Rates in Solution*; VCH Publishers: New York, 1990.

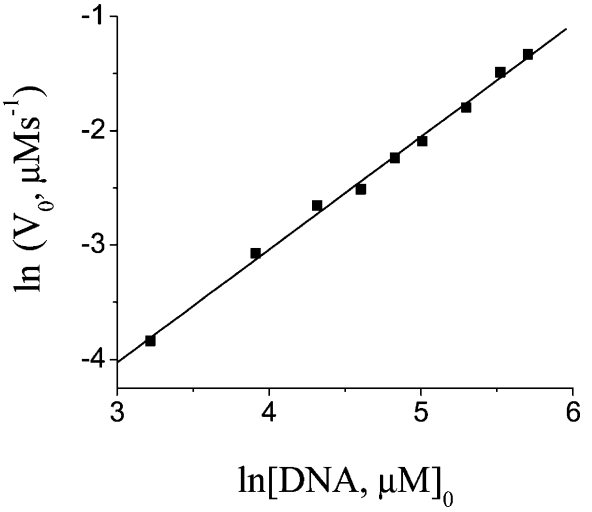


Figure 4. Dependence of ln V_0 vs ln[DNA]$_0$, where $k_{obs} = k_2$[complex], yielded $k_2 \sim 93$ M^{-1} s^{-1} and $m \sim 1$.

III) was prepared by digesting plasmid DNA with either *Eco*RI or *Bam*HI restriction endonucleases. To determine if the observed linearization arose from random or nonrandom DNA cleavage, a standard statistical test was applied.[32,33] This test assumes a Poisson distribution of strands cuts with calculation of the average number of dsDNA breaks per molecule, $n2$, from the fraction of linear DNA following strand scission. The total average number of ssDNA and dsDNA breaks ($n1 + n2$) was calculated from the fraction of uncleaved supercoiled DNA.

The Freifelder−Trumbo relationship indicates that more than 100 ssDNA breaks are required to obtain one dsDNA break under completely random conditions. In our experiments, the ratio of $n1/n2 = 14.6 \pm 2.9$, which is $\ll 100$, suggesting a nonrandom cleavage path by [KGHK−Cu]$^+$ to efficiently form linear DNA.

Other Transition Metals. A number of other transition metal ions (Ni^{2+}, Fe^{2+}, and Co^{2+}) were observed to form complexes with GGH. For Mn^{2+} and Zn^{2+}, which showed no significant new absorption features, we compared the DNA cleavage capacities of mixtures of the metal ions with GGH relative to the free metal ions. No significant enhancement of cleavage was observed for the latter two (Supporting Information), suggesting that they either did not form complexes with GGH or that the resulting complexes were inactive. The results of kinetic studies of DNA cleavage (the formation of nicked DNA) by other transition metal complexes under oxidative conditions are summarized in Table 1 and demonstrate [GGH−Cu]$^-$ and [KGHK−Cu]$^+$ (in the presence of ascorbate) to possess the highest cleavage capacities (Supporting Information).

In the presence of ascorbate, the solvated ions Cu^{2+}(aq), Fe^{2+}(aq), and Ni^{2+}(aq) each showed DNA cleavage (in order of decreasing activity), while Co^{2+} (aq) showed no activity. Both Cu^{2+}(aq) and Fe^{2+}(aq) mediated cleavage in a fashion that was faster and more random relative to the corresponding complexes with ATCUN peptides. At the concentrations used, free metal ions demonstrated no DNA cleavage chemistry in the absence of ascorbate.

Involvement of Reactive Oxygen Species and Netropsin Inhibition. Experiments with scavenging agents were carried out to identify intermediate reactive oxygen species that might form (including hydroxyl radical, superoxide, singlet oxygen, and hydrogen peroxide) in the DNA cleavage reaction promoted by [peptide−Cu]/ascorbate.[13,14,23,34] Neither NaN_3 nor SOD was observed to inhibit the DNA cleavage reaction, while DMSO partially inhibited the reaction (Supporting Information). Furthermore, catalase was found to completely repress the DNA cleavage reactivity of [peptide−Cu] complexes (Supporting Information).

The production of hydroxyl radicals was quantitated by use of a standard rhodamine B assay,[13,14] which provides a target with a high reactivity ($\sim 10^9$ M^{-1} s^{-1})[14] for hydroxyl radical as well as the advantage of direct quantitation by electronic spectroscopy (Supporting Information). Rhodamine B was not degraded by either Cu^{2+}(aq) or [peptide−Cu] complexes alone over the experimental time frame used in these studies, consistent with the lack of hydroxyl radical production under these conditions. Moreover, optical spectra of 15 μM rhodamine B with the 300 μM [peptide−Cu] complex were identical to those obtained for the dye in the absence of the copper complex, indicating that there is no direct inner-sphere coordination between rhodamine B and the copper ion. Following the addition of ascorbate or H_2O_2, any hydroxyl radicals formed are trapped by reaction with rhodamine B over the time frame of the experiments. In the presence of DNA and following the addition of either ascorbate or H_2O_2, the formation of diffusible hydroxyl radicals was detected only for Cu^{2+}(aq). Neither [KGHK−Cu]$^+$ nor [GGH−Cu]$^-$ showed evidence for the release of diffusible hydroxyl radicals in the presence of an excess of substrate DNA.

Netropsin (a minor groove inhibitor)[41] was observed to partially inhibit the cleavage of supercoiled plasmid DNA (Supporting Information), while it completely inhibited the cleavage of calf thymus DNA by [peptide−Cu]/ascorbate. This observation is consistent with a prior report by Long and coworkers that demonstrated competitive inhibition of [peptide−Ni^{2+}]-promoted oxidative cleavage of DNA.[24]

Characterization of Product Release following Oxidative Cleavage. To quantitate the extent of C-1′H abstraction relative to total base release, the C-1′H abstraction signature product, 5-methylene furanone (5-MF), was synthesized for comparison, purified, and characterized by mass spectrometry ($m/z = 119$, corresponding to 5-MFNa^+ (119 = 96 (5-MF) + 23 (Na^+)) and UV spectrometry ($\lambda_{max} = 260$ nm).[35] Subsequently, 5-MF and other authentic controls were used to calibrate the C-18 column for HPLC analysis (Supporting Information). Following a 2 h reaction,[2] the reaction mixture was heated to 85 °C for 30 min, and an additional peak corresponding to 5-MF appeared in the HPLC profile (Supporting Information), but no evidence for furaldehyde production (furfural, the unique product of C-5′H abstraction[2,42]) was found. For each mole of 5-MF generated by C-1′H abstraction, one mole of base was released.[36] Consequently, the concentration of base released through the C-1′H abstraction pathway is equivalent to the concentration of 5-MF and was found to constitute only ~20% of total released base products. The HPLC profiles (Supporting Information) demonstrate that heat treatment is essential for the release of 5-MF, confirming the recent observations that C-1′H abstraction leads to the formation of abasic sites without strand

(40) Ragunathan, K. G.; Schneider, H.-J. *Angew. Chem., Int. Ed. Engl.* **1996**, *35*, 1219−1221.
(41) Bailly, C.; Chaires, J. B. *Bioconjugate Chem.* **1998**, *9*, 513−538.
(42) Pogozelski, W. K.; Tullius, T. D. *Chem. Rev.* **1998**, *98*, 1089−1107.

Table 1. Summary of Second-Order Kinetic Rate Constants Determined for a Variety of Metal-Complex Mediated DNA Cleavage Reactions[a]

complex[b]	k_2 (M^{-1} min^{-1})	solution conditions	type of cleavage	ref
[ionophore−Pr_2]$^{6+}$	52[c]	10 mM EPPS buffer, pH = 7.0, 37 °C	hydrolytic	40
[HXTA−Ce_2]$^{3+}$	840	10 mM Tris buffer, pH = 8.0, 37 °C	hydrolytic (ssDNA)	4
[HXTA−Ce_2]$^{3+}$	78	20 mM Tris buffer, pH = 8.0, 37 °C	hydrolytic (dsDNA)	4
[cyclen−Co]$^{(4-5)+}$	13 to 90[c]	10 mM EPPS buffer, pH = 7.0, 37 °C	hydrolytic	10
[tamen−Co]$^{3+}$	3	20 mM Tris buffer, pH = 7.6, 37 °C	hydrolytic	6
Eu^{3+}(aq)	108[c]	10 mM EPPS buffer, pH = 7.0, 37 °C	hydrolytic	8
[ionophore−Eu]$^{3+}$	64[c]	10 mM EPPS buffer, pH = 7.0, 37 °C	hydrolytic	8
Rh^{3+}(phi)$_2$bpy′−peptide (Zn^{2+})	300	20 mM Na-borate buffer, pH = 6.0, 37 °C	hydrolytic	9
[neamine−Cu]$^{4+}$	7690[c]	10 mM HEPES buffer, pH = 7.3, 37 °C	hydrolytic	13
[ionophore−Cu]$^{+}$	201	20 mM phosphate buffer, pH = 7.0	oxidative ssDNA cleavage with ascorbate and peroxide	14
[ionophore−Cu]$^{+}$	333	20 mM phosphate buffer, pH = 7.0	oxidative dsDNA cleavage with ascorbate and peroxide	14
[GGH−Ni]$^{-}$	480	10 mM Tris buffer, pH = 7.4	oxidative with ascorbate	this paper
[KGHK−Ni]$^{+}$	1320	10 mM Tris buffer, pH = 7.4	oxidative with ascorbate	this paper
[GGH−Cu]$^{-}$	2340	10 mM Tris buffer, pH = 7.4	oxidative with ascorbate	this paper
[KGHK−Cu]$^{+}$	5580	10 mM Tris buffer, pH = 7.4	oxidative with ascorbate	this paper
[GGH−Ni]$^{-}$	204	20 mM Na-cacodylate buffer, pH = 7.5	oxidative with ascorbate	this paper
[KGHK−Ni]$^{+}$	258	20 mM Na-cacodylate buffer, pH = 7.5	oxidative with ascorbate	this paper
[GGH−Cu]$^{-}$	2400	20 mM Na-cacodylate buffer, pH = 7.5	oxidative with ascorbate	this paper
[KGHK−Cu]$^{+}$	5760	20 mM Na-cacodylate buffer, pH = 7.5	oxidative with ascorbate	this paper
[GGH−Ni]$^{-}$	2820	20 mM Na-cacodylate buffer, pH = 7.5	oxidative with $KHSO_5$	this paper
[KGHK−Ni]$^{+}$	3150	20 mM Na-cacodylate buffer, pH = 7.5	oxidative with $KHSO_5$	this paper
[GGH−Cu]$^{-}$	11640	20 mM Na-cacodylate buffer, pH = 7.5	oxidative with $KHSO_5$	this paper
[KGHK−Cu]$^{+}$	13700	20 mM Na-cacodylate buffer, pH = 7.5	oxidative with $KHSO_5$	this paper

[a] ssDNA stands for single-stranded DNA, and dsDNA stands for double-stranded DNA. [b] With the exception of one entry, the charge shown outside of the parentheses is the overall charge of the complex cation, including contributions from both metal ion and ligand. [c] In the case of the enzyme-like cleavage reactions, the number listed here is k_{cat}/K_m.

Table 2. Quantitation of Nucleobase, Base Propenal, and 5-MF Release Following Cleavage of Calf Thymus DNA

	nucleo base release (μM)						
catalyst	C	G	T	A	total	base propenals (μM)	5-MF (μM)
[KGHK−Cu]$^{+}$	4.5	4.1	4.4	4.6	17.6	1.2	3.3
Cu^{2+}(aq)	8.8	8.4	9.1	9.2	35.5	1.4	0

cleavage.[37,43] The C-1′H site also exhibits a lower solvent accessibility than C-4′H.[42] In our case, an aliquot of the same reaction mixture that was loaded onto the HPLC column showed smeared DNA fragments when analyzed by agarose gel electrophoresis, consistent with DNA strand cleavage occurring simultaneously with base release. Base propenal is an alternative product that can be released during DNA cleavage following C-4′H (O_2) abstraction.[42] HPLC peaks with retention times in the range of 12.3−15.6 min were collected and identified as base propenals by accurate mass measurement. Production of base propenal was also monitored by use of the TBA assay and found to account for only ∼8% of total DNA cleavage observed under aerobic conditions. Consequently, C-4′H (H_2O) attack is the major pathway that results in base release, in marked contrast to our findings for Cu−aminoglycoside mediated cleavage.[37] All of the products released from the cleavage of calf thymus DNA by [KGHK−Cu]$^{+}$/ascorbate (without heating) are listed in Table 2 and support the conclusion that C-4′H abstraction is the predominant pathway for oxidative cleavage of dsDNA by [peptide−Cu] complexes.

Comparison of Cleavage Patterns Mediated by Free Cu^{2+}-(aq) and [Peptide-Cu^{2+}] Complexes. Under the conditions used, neither Cu^{2+}(aq) nor [peptide−Cu^{2+}] shows any DNA cleavage activity in the absence of reducing agents. However, Cu^{2+}(aq) does mediate DNA cleavage with added ascorbate, showing a smeared band in the agarose gel used to monitor the reaction, indicative of random cleavage and consistent with prior observations.[23] This contrasts with the activity of [peptide−Cu], which shows a more regulated cleavage pattern in the presence of ascorbate. In particular, [KGHK−Cu]$^{+}$/ascorbate was found to cleave DNA in a nonrandom manner with production of linear DNA. In the presence of ascorbate, Cu^{2+}-(aq) promoted the rapid degradation of BNPP, while [peptide−Cu] did not show any capacity to cleave BNPP under the same conditions.

Discussion

Factors Influencing Cleavage Efficiency and Reaction Mechanism. (1) Comparison of Rate Laws and Kinetic Efficiency. Table 1 summarizes the cleavage efficiencies for a large number of complexes that has been previously investigated under hydrolytic or oxidative conditions. This group of complexes can also be divided into two additional categories; namely, those that follow the second-order profile typical of homogeneous catalysts (Figures 3 and 4) and the zeroth order kinetic profile common to enzyme-like catalysts and those most frequently represented by Michaelis−Menten plots. A requirement for the latter class of catalyst is formation of a prereaction complex. To stabilize such a complex, the substrate must have a relatively high residence time on the catalyst, relative to the rate of reaction to form product. It is evident from Table 1 that those complexes that are expected to bind with reasonable affinity to DNA, either through electrostatic, hydrogen-bonding, or intercalative binding modes, demonstrate Michaelis−Menten kinetic behavior. None of these binding modes is particularly significant for the [peptide−Cu] complexes described in this paper, and so a typical second-order profile is both expected and observed (Figures 3 and 4). However, within this group, it is evident that relative cleavage activity is directly related to relative binding affinity.

In previous published work, we have discussed the importance of binding affinity for efficient catalytic cleavage. As expected, the higher net positive charge on the [KGHK−Cu]$^{+}$ complex

(43) Oyoshi, T.; Sugiyama, H. *J. Am. Chem. Soc.* **2000**, *122*, 6313−6314.

Table 3. Oxidative Cleavage Pathways for DNA

metal complex	coreactant	position of H abstraction	ref
Fe^{2+}−bleomycin	O_2	C-4	46
Cu^{2+}−aminoglycoside	ascorbate or H_2O_2	C-1 and C-4[a]	37
Ni^{2+}−peptide	$KHSO_5$, MMPP, or H_2O_2	C-4	28
Cu^{2+}− (1,10-phenanthroline)	thiol or H_2O_2	C-1, C-4, and C-5	43, 47
Cu^{2+}−peptide	ascorbate or H_2O_2	C-1 and C-4[a]	this paper

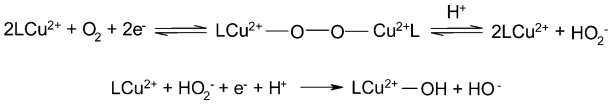
[a] In both cases, the C-1′ pathway was minor and only evident for plasmid DNA in the case of copper aminoglycoside mediated cleavage.

resulted in more effective cleavage relative to $[GGH-Cu]^-$. Moreover, the increased residency time for $[KGHK-Cu]^+$ allowed for more efficient multiple cleavage events during residency, resulting in linearization of DNA. This is of some significance from a practical standpoint inasmuch as double-stranded DNA cleavage cannot be repaired intracellularly, and so such molecules constitute potential drug candidates.

(2) Product Analysis of [Peptide−Cu] Promoted DNA Cleavage. HPLC analysis (Supporting Information) demonstrated base release to be the predominant reaction product. Quantitation of base release is documented in Table 2. Cytosine typically coelutes with ascorbate, and so cytosine release was monitored following complete ascorbate consumption. Although C-1′H, C-3′H, and C-4′H abstraction pathways can all potentially give rise to free base products,[42] the C-3′H abstraction pathway is rarely observed,[42,44] reflecting its limited accessibility and/or the low stability of the 3′-deoxyribosyl radical.[42] Moreover, C-3′H abstraction, which requires cleavage to be carried out through the major groove, is inconsistent with the finding that netropsin (a minor groove inhibitor)[41] completely inhibited the cleavage of calf thymus DNA by [peptide−Cu]/ascorbate. Accordingly, C-3′H abstraction does not appear to be involved in DNA cleavage by [peptide−Cu] complexes. Similarly, the C-2′H abstraction pathway is also rarely observed as a consequence of low accessibility and reactivity.[42] In fact, C-2′H abstraction has only been observed in gamma radiolysis of poly-(U) and photolysis of oligonucleotides containing halogenated uracil.[42,45]

Table 3 lists the pathways involved in oxidative DNA cleavage, with C-1′H, C-4′H, and C-5′H constituting the most commonly observed. However, C-1′H abstraction does not result in direct DNA strand breakage, unless heated to 85 °C for 30 min. Copper complexes of the ATCUN peptide appear to mediate oxidative cleavage of DNA via C-4′H abstraction as the major pathway, consistent with findings for [peptide−Ni]-promoted cleavage.[28] This preference for C-4′ H abstraction most likely reflects accessibility and the stereoproximity to the active center of [peptide−Cu]. The factors that influence the selection of a C-4′H (O_2) versus C-4′H (H_2O) cleavage paths are less clear; however, empirically we have noted that supercoiled plasmid DNA tends to yield products from a C-4′H (H_2O) degradation path, while more structurally ordered ds duplex DNA with a better defined B-conformation tend to follow a C-4′H (O_2) degradation path, possibly reflecting more facile access of water in the more open supercoiled conformation. Long and co-workers have noted a dependence of C-4′H (O_2) versus C-4′H (H_2O) cleavage paths with binding affinity,[28] although this would also be consistent with structural variations that promote distinct binding modes for each complex to DNA and impact both relative binding affinity and solvent access. Local depletion of dissolved oxygen levels is unlikely to be a factor inasmuch as similar reaction conditions were used for all of the studies reported here, as were earlier studies with copper aminoglycosides that also displayed distinct product ratios according to the nucleic acid substrate.[37]

Scheme 2. [Peptide−Cu] Promoted Formation of ROS with Ascorbate/O_2



Mechanism of Oxidative Cleavage and Characterization of Intermediate ROS. Our experimental results suggest that neither singlet oxygen nor superoxide are active oxidants in the [peptide−Cu]/ascorbate DNA cleavage system. Hydrogen peroxide would appear to be an essential intermediate ROS for DNA cleavage; however, neither ascorbate/O_2 nor H_2O_2 alone promoted any significant DNA cleavage. Prior studies with other transition metal complexes have reported a need for both ascorbate and H_2O_2 to promote DNA cleavage.[14,48] However, in our hands, either H_2O_2 or ascorbate/O_2 alone are sufficient to effect dsDNA cleavage with $[peptide-Cu^{2+}]$. The results of the radical trapping experiment using rhodamine B are consistent with our prior findings for copper aminoglycoside mediated cleavage of DNA and the intermediacy of a ROS that is intimately bound to the copper center and is not released to solution. Such a model provides a rational explanation for the inability of scavenging systems to inhibit the reaction, with the exception of catalase that consumes the hydrogen peroxide formed from the reaction of reduced [peptide−Cu] with dioxygen. Two-electron reduction of O_2 (Scheme 2) forms diffusible H_2O_2 that subsequently follows Fenton-type chemistry to produce a copper-bound hydroxyl radical, the formal reactive species.

Catalyst Stability. Complexes of ATCUN peptides with divalent copper are generally stable in aqueous solution.[16] In a recent EPR study of $[peptide-Cu^{2+}]$ binding to DNA fibers, evidence for a minor loss of copper ion was noted, but only if the ATCUN peptide motif contained a carboxylate residue (Asp) in the position following His.[49] However, this is not the case for the complexes described here, while the presence of an excess of ligand (typically a 25% excess) further prevented dissociation.

(44) Sitlani, A.; Long, E. C.; Pyle, A. M.; Barton, J. K. *J. Am. Chem. Soc.* **1992**, *114*, 2303−2312.
(45) Sugiyama, H.; Tsutsumi, Y.; Fujimoto, K.; Saito, I. *J. Am. Chem. Soc.* **1993**, *115*, 4443−4448.
(46) Burger, R. M.; Berkowitz, A. R.; Peisach, J.; Horwitz, S. B. *J. Biol. Chem.* **1980**, *255*, 11832−11838.
(47) Kuwabara, M.; Yoon, C.; Goyne, T.; Thederahn, T.; Sigman, D. S. *Biochemistry* **1986**, *25*, 7401−7408.
(48) Pamatong, F. V.; Detmer, C. A., III; Bocarsly, J. R. *J. Am. Chem. Soc.* **1996**, *118*, 5339−5345.
(49) Nagane, R.; Koshigoe, T.; Chikira, M. *J. Inorg. Biochem.* **2003**, *93*, 204−212.

Prior studies of copper complexes of ATCUN peptides with free carboxylates at the C-terminus have also documented the formation of alkene peptides following oxidation via a Cu(III) intermediate.[50] Those results were obtained under strongly oxidizing conditions, requiring the presence of both H_2O_2 and ascorbic acid at acidic pH. The experiments described herein were carried out at pH 7.4 with only ascorbate present. While our proposed mechanism (Scheme 2) suggests the transient production of H_2O_2, it appears to be quickly converted to a hydroxyl radical prior to the formation of Cu(III). Moreover, electrochemical studies of the copper complexes used in this study show no evidence for the formation of Cu(III).

Accordingly, the copper complexes used in this study appear to be stable under the reaction conditions described. However, to provide a more definitive comparative study, further control experiments were carried out with Cu^{2+}(aq).

Comparison of DNA Cleavage Mediated by Free Cu^{2+}(aq)/Ascorbate Relative to [Peptide-Cu^{2+}]/Ascorbate. A series of control experiments was carried out to eliminate the possibility that [peptide−Cu^{2+}]-mediated cleavage of DNA was actually carried out by free copper ion following reductive release from the ATCUN peptide ligand. A detailed kinetic analysis for Cu^{2+}(aq)-mediated oxidative cleavage of DNA yielded $m = 1$ and a $k_2 \sim 2000\ M^{-1}\ s^{-1}$ after fitting to eq 5, which agrees well with our previous study that Cu^{2+}(aq)/ascorbate causes smeared DNA very quickly even at very low concentration. The inhibitors DMSO, NaN_3, SOD, and catalase were found to effect only partial inhibition of the cleavage reaction, which indicated that the reactive oxygen species involved in Cu^{2+}(aq)/ascorbate mediated cleavage may be a more complex mix than that found in [peptide−Cu]/ascorbate reactions. The complexity of the reaction, relative to that mediated by [peptide−Cu] complexes, is evident from the fact that k_2 for Cu^{2+}(aq)-mediated cleavage in the presence of ascorbate is $\sim 2000\ M^{-1}\ s^{-1}$, relative to only $93\ M^{-1}\ s^{-1}$ for $[KGHK-Cu]^+$/ascorbate mediated cleavage. Moreover, the total base release during DNA cleavage by the former was only modestly greater that that produced by the latter (Table 2), while the ratio of base release to base propenal formation was clearly distinct. Production of 5-MF was observed with [peptide−Cu] but not with Cu^{2+}(aq) (Table 2).

Factors Promoting Linearization of dsDNA. Efforts to find artificial nucleases with drug therapeutic value have been ongoing for several decades. The goal is to promote efficient and irreversible cleavage of DNA, such that double-stranded breakage is achieved instead of simply causing nicks on DNA, which are more readily repaired in vivo.[51] In our work, $[KGHK-Cu]^+$ mediates more efficient linearization of supercoiled (form I) DNA than previously reported, using significantly lower complex concentrations combined with faster reactions.[14,48] We have demonstrated that this linearization arises from nonrandom cleavage and consistent with the relatively higher binding affinity of $[KGHK-Cu]^+$ relative to $[GGH-Cu]^-$, consistent with the two positive charges on the lysine side-chains. Double-stranded cleavage also requires proper orientation of $[KGHK-Cu]^+$ in the DNA minor groove, usually close to C-4′H, consistent with the cleavage pathway elucidated in our research. Future efforts will be directed toward optimizing the key structural and mechanistic parameters highlighted by these studies.

Acknowledgment. This work was supported by the National Institutes of Health (GM63740).

Supporting Information Available: Plots of the relative DNA cleavage activities of other [GGH−transition metal] complexes, netropsin inhibition of plasmid DNA cleavage for both $[GGH-Cu]^-$ and $[KGHK-Cu]^+$, HPLC product profiles for the [peptide−Cu] promoted DNA cleavage reaction, and tables of HPLC product retention times and the effect of ROS scavengers on the DNA cleavage reaction promoted by [peptide−Cu]/ascorbate. This material is available free of charge via the Internet at http://pubs.acs.org.

JA0503985

(50) Burke, S. K.; Xu, Y.; Margerum, D. W. *Inorg. Chem.* **2003**, *42*, 5807−5817.

(51) Povirk, L. F. *Mutat. Res.* **1991**, *257*, 127−143.