Nuclease Activity of 1,10-Phenanthroline–Copper: Kinetic Mechanism

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Received March 22, 1988

Abstract: The nuclease activity of 1,10-phenanthroline–copper ion (OP–Cu) proceeds by an obligatory, ordered mechanism, whether the reaction is potentiated by thiol or superoxide. Freely diffusing 1,10-phenanthroline–cupric ion is reduced to the cuprous complex (eq 1a), which binds reversibly to the DNA (eq 1b). The noncovalently bound complex is then oxidized by hydrogen peroxide to generate the copper–oxo species directly responsible for strand scission (eq 1c). 1,10-Phenanthroline–cupric

$$(OP)_2Cu^{2+} \overset{1e^-}{\leftrightarrow} (OP)_2Cu^{+} \quad (1a)$$

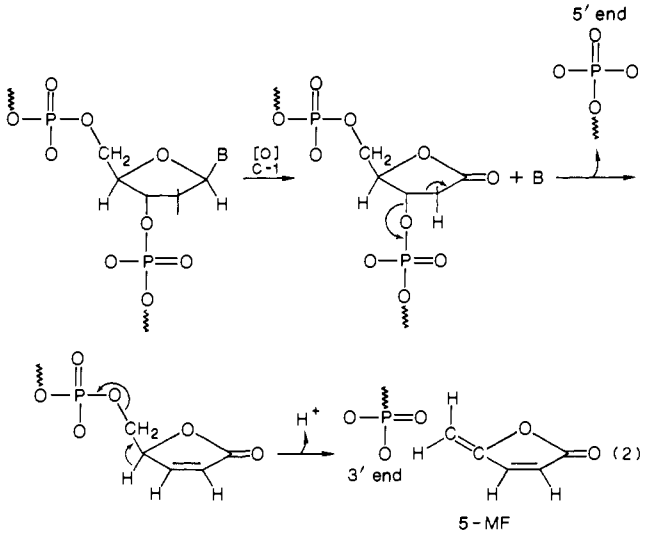
$$DNA + (OP)_2Cu^{+} \leftrightarrow DNA\text{-}(OP)_2Cu^{+} \quad (1b)$$

$$DNA\text{-}(OP)_2Cu^{+} \xrightarrow{H_2O_2} \text{nicked products} \quad (1c)$$

ion bound to DNA cannot be on the main reaction pathway because its reduction is not fast enough to be a kinetically competent step in the overall reaction. The tetrahedral cuprous complex is therefore the binding species responsible for the sequence-dependent reactivity of the nuclease. Cleavage patterns produced by the copper complexes of substituted 1,10-phenanthrolines are consistent with the nonintercalative binding of the tetrahedral complex in the minor groove. A model is proposed for this interaction.

The 2:1 1,10-phenanthroline–cuprous complex [$(OP)_2Cu^+$], with H_2O_2 as a coreactant, is a chemical nuclease that nicks DNA by a reaction mechanism that is sensitive to the conformation of the nucleic acid.[1] It cleaves B-DNA 3 times more efficiently than A-DNA, while Z-DNA and noncomplementary single-stranded DNA are not degraded under similar conditions.[2] With B-DNA, it exhibits sequence-dependent reactivity although scission can be observed at any of the four deoxynucleotides. This specificity can be explained by the initial formation of an obligatory, noncovalent complex between $(OP)_2Cu^+$ and DNA. A one-electron oxidation of the cuprous complex by H_2O_2 then produces the copper–oxo species, on the surface of the DNA, that is directly responsible for the oxidative damage observed.[3-5] Conformational sensitivity is not consistent with a mechanism that involves generating reactive intermediates remote from the DNA.

The primary products of the oxidative attack on B-DNA, and presumably on other secondary structures, are free bases, 5′- and 3′-phosphomonoester termini, and 5-methylene-2-furanone.[6,7] The reaction mechanism summarized in eq 2 accounts for the stable,



(2)

isolated products as well as an observed, transient intermediate on the 3′ end of the fragment produced.[6,7] Since the mechanism proposes that oxidative attack is initiated at the C-1 hydrogen of the deoxyribose moiety, which faces the minor groove, the coordination complex must bind and attack DNA from this structural domain. The sequence-dependent reactivity of $(OP)_2Cu^+$ therefore reflects the binding affinity of the coordination complex for a given sequence as well as the accessibility of the reactive copper–oxo species to the C-1 hydrogen.

Although the reaction is funneled through a noncovalent intermediate composed of the 1,10-phenanthroline–cuprous complex and DNA, its kinetics of formation are not known. Two alternatives are possible (eq 3). In the first, the 2:1 1,10-

$$\begin{array}{ccc} (OP)_2Cu^{2+} + DNA & \overset{c}{\rightleftharpoons} & (OP)_2Cu^{2+}\text{-}DNA \\ \Big\uparrow{\scriptstyle a}\ 1e^- & & \Big\uparrow{\scriptstyle d}\ 1e^- \\ (OP)_2Cu^{+} + DNA & \overset{b}{\rightleftharpoons} & (OP)_2Cu^{+}\text{-}DNA \xrightarrow[e]{H_2O_2} \text{products} \end{array} \quad (3)$$

phenanthroline–cuprous complex is formed in solution and diffuses to the minor groove where it is oxidized by H_2O_2. In the second case, it is the cupric complex that binds to the minor groove and then is reduced on the DNA surface before reacting with the H_2O_2. Since equilibrium dialysis and viscometry have demonstrated that both cupric and cuprous complexes of phenanthroline and its derivatives have affinity for DNA,[8] either pathway to the reactive complex is feasible.

In this paper, we demonstrate that there is an obligatory reaction pathway that proceeds via formation of the cuprous complex in solution (steps 3a and 3b), whether thiol or superoxide is the one-electron donor. Nonintercalative binding of the tetrahedral cuprous complex in the minor groove is the recognition event that

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determines the sequence-dependent specificity of this chemical nuclease activity.

Materials and Methods

Materials. Strains containing the 203-bp fragment of the *lac* operon on a pBR322 plasmid were a gift of Dr. Annick Spassky. Their construction has been described previously.[9] The following enzymes and reagents were obtained commercially and used without further purification: 1,10-phenanthroline (OP) and its derivatives (G. F. Smith Co.); 3-mercaptopropionic acid (MPA) and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) (Aldrich); T4 polynucleotide kinase (BRL); sonicated calf thymus DNA (Pharmacia); spermidine, dithiothreitol, and Tris (Sigma); acrylamide and bis(acrylamide) (Bio-Rad); [γ-^{32}P]ATP (Amersham); magnesium chloride and copper sulfate (Mallinckrodt).

Methods. (A) Cleavage of *lac* Fragment. To determine the sequence specificity of $(OP)_2Cu^+$-mediated reactions, the 203-bp fragment of the *lac* operon was excised from its pBR322 plasmid with *Eco*RI, dephosphorylated with calf intestine alkaline phosphatase, and labeled on the 5′ termini with T4 polynucleotide kinase and [γ-^{32}P]ATP. A 186-bp fragment, uniquely labeled on the template strand, was created by secondary restriction with *Pvu*II. Labeling on the nontemplate strand was accomplished by filling in the "sticky" ends with the Klenow fragment of DNA polymerase I in the presence of [α-^{32}P]ATP.[6,11]

The labeled fragment was redissolved in 50 mM Tris-OAc (pH 8.0) containing 10 mM $MgCl_2$ and 100 mM KCl prior to digestion. Thiol-mediated digestions were initiated by the addition of MPA to a final concentration of 5.8 mM and were then incubated at 37 °C for 3 min. Cleavages mediated by γ-radiation were initiated by placing the samples in a ^{60}Co source and exposing them to 10 000 rads. Unless specified otherwise 200 μM OP and 45 μM $CuSO_4$ were present in the reaction mixtures.

(B) OP/Cu Concentration Dependence. To demonstrate that the digestion patterns of the coordination complex were independent of concentration, 25 000 cpm (Cerenkov) of the 5′-labeled UV5 186-bp fragment in 80 μL 50 mM Tris-HCl, pH 8.0, at room temperature was digested by the addition of 10 μL of a range of OP/Cu stock solutions and 10 μL of 58 mM mercaptopropionic acid. The concentrations of and reaction times for the OP/Cu stocks were as follows: 1000 μM OP, 225 μM $CuSO_4$, 0.5 min; 700 μM OP, 158 μM $CuSO_4$, 2.5 min; 400 μM OP, 90 μM $CuSO_4$, 5.0 min; 100 μM OP, 23 μM $CuSO_4$, 20 min.

All cleavage reactions were quenched by the addition of 10 mL of 28 mM 2,9-dimethyl-OP. Samples were ethanol precipitated, resuspended in 80% formamide, 0.1% (w/v) bromphenol blue, and 0.1% xylene cyanol, and loaded onto 10% sequencing gels.[11] The sequencing gels were run at 45W for 3 h. Gels were exposed to X-ray film for 3 days at −20 °C.

(C) Rates of Thiol Oxidation. The copper complexes of OP and its derivatives were tested for their ability to oxidize MPA by the method of Ellman.[12] An aliquot of 2 mM OP (or more of its derivatives) and 0.2 mM $CuSO_4$ was diluted 1:100 in a 1 mM solution of MPA buffered with 50 mM Tris-HCl (pH 7.5) and allowed to oxidize the thiol. At 1-min intervals, aliquots (30 μL) were removed and added to 970 μL of 0.257 mM dithiobis(2-nitrobenzoic acid) (DTNB). The reduction of DTNB was allowed to go to completion, and the level of 4-mercapto-2-nitrobenzoic acid (TNB) produced was assayed by absorbance at 412 nm (extinction coefficient = 1.36×10^4 M^{-1} cm^{-1}). An initial rate for the thiol oxidation was then calculated.

(D) Inhibition of $(OP)_2Cu^{2+}$ Reduction by DNA. The reduction of the $(OP)_2Cu^{2+}$ complex to the cuprous form in the presence of MPA can be followed spectrophotometrically. The cuprous complex has a strong absorbance at 425 nm (extinction coefficient = 7250 cm^{-1} M^{-1}) while the cupric complex does not. Thus, once MPA is added (final concentration, 3 mM) to a solution of 200 μM OP, 45 μM $CuSO_4$, and 20 mM Tris-HCl (pH 8.0), the initial rate of absorbance increase can then be measured. Sonicated, phenol-extracted calf thymus DNA was added, at the final concentrations indicated, to a solution of 200 μM OP and 45 μM $CuSO_4$ and preincubated at 22 °C for 3 min before the addition of MPA. The initial rate of the absorbance increase after the addition of MPA was recorded on a Gilford 250 recording spectrophotometer.

(E) Generation of Binding Model. Computer simulation of (5-phenyl-OP)$_2$Cu$^+$ complex binding to the dodecamer CGCGAATTCGCG was performed on a VAX 1170 computer (DEC) using an Evans and Sutherland PS 300 picture system and the GRAMPS molecular modeling program. The coordinates for the dodecamer structure were based on X-ray crystallographic data previously reported,[13] while those of the 5-phenylphenanthroline–cuprous complex were generated from idealized values. Once the complex had been appropriately positioned with respect to the DNA, the coordinates were transferred to the RMS (Raster Molecular Surfaces) program,[14] and a space-filling model was generated.

Results

Mechanisms of Potentiation. The nuclease activity of 1,10-phenanthroline–copper can be potentiated by thiol, superoxide generators such as xanthine–xanthine oxidase,[15] and NADH in the presence of the H_2O_2.[16] The latter two methods rely on superoxide for the in situ reduction of the 1,10-phenanthroline–cupric complex. The potentiation by thiol under aerobic conditions proceeds by the following reaction pathway:

$$2(OP)_2Cu^{2+} + 2RS^- \rightarrow R\text{-}S\text{-}S\text{-}R + 2(OP)_2Cu^+ \quad (4a)$$

$$2(OP)_2Cu^+ + 2O_2 \rightarrow 2O_2^{\bullet-} + 2(OP)_2Cu^{2+} \quad (4b)$$

$$2O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2 + O_2 \quad (4c)$$

Steady-state ^{60}Co radiation was used for generating superoxide because a chemical method, such as xanthine–xanthine oxidase, might introduce unanticipated complexity in a mechanistic study. The following processes probably account for the ^{60}Co-dependent superoxide generation:

$$\gamma + H_2O \rightarrow e^- + H_2O^+ \quad (5a)$$

$$H_2O^+ \rightarrow H^+ + OH^\bullet \quad (5b)$$

$$e^- + O_2 \rightarrow O_2^{\bullet-} \quad (5c)$$

$$e^- + (OP)_2Cu^{2+} \rightarrow (OP)_2Cu^+ \quad (5d)$$

$$R\text{-}CH_2OH\ (Tris) + OH^\bullet \rightarrow RC^\bullet HOH + H_2O \quad (5e)$$

$$RC^\bullet HOH + O_2 \rightarrow RCHO + O_2^{\bullet-} + H^+ \quad (5f)$$

$$(OP)_2Cu^{2+} + O_2^{\bullet-} \rightarrow (OP)_2Cu^+ + O_2 \quad (5g)$$

$$2O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2 + O_2 \quad (5h)$$

The solvated electron in eq 5c and 5d serves as a reductant for both molecular oxygen and 2:1 1,10-phenanthroline–cupric ion while the hydroxyl radical in eq 5e could also serve as a source of superoxide anion via reaction with the Tris buffer. Since in this reaction mechanism the reduction of $(OP)_2Cu^{2+}$ is mediated by $O_2^{\bullet-}$, the cleavage is inhibited by anaerobiosis and superoxide dismutase. The absolute requirement for diffusible H_2O_2, previously shown for the thiol-mediated reaction,[1,4] is also demonstrated for the superoxide-mediated reaction by the abolition of the reaction by catalase.

Cleavage Pattern Is Independent of Potentiation Method. All methods of potentiation can be interpreted in terms of the formation of the 2:1 1,10-phenanthroline–cuprous complex and hydrogen peroxide. If there is an obligatory pathway for OP–Cu-mediated DNA cleavage, then the sequence-dependent reactivity should be independent of the source of reducing equivalents or the pathway used to generate them. The nuclease efficiency at any sequence position will thus be solely a function of the binding affinity of one oxidation state of the coordination complex. Comparison of the digestion of the 186 base pair fragment of the *lac* UV-5 control region, potentiated by steady-state ^{60}Co radiation and thiol under aerobic conditions, indicates that comparable patterns of products are obtained (Figure 1).

Oxidation State of Complex upon Binding. The similarity of the sequence-dependent reactivity, regardless of the source of reducing equivalents, suggests that a unique productive reaction

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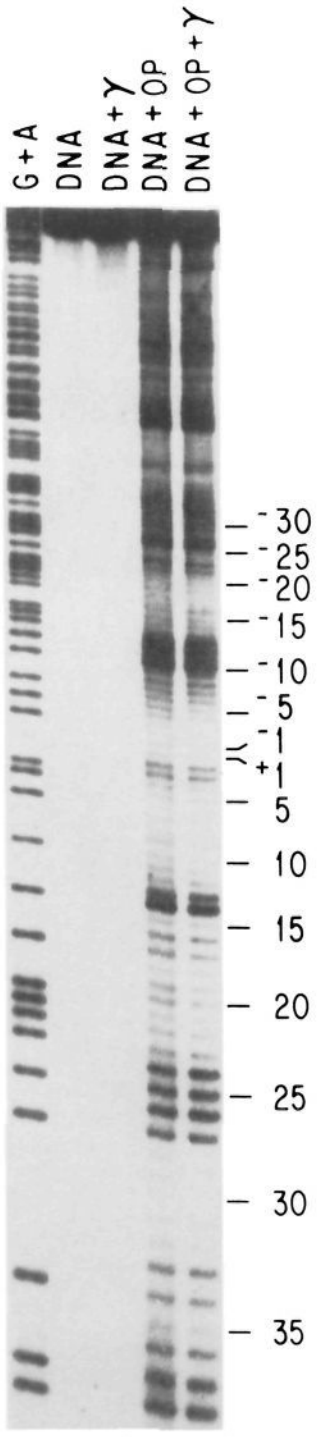

Figure 1. Comparison of thiol- and γ-radiation-potentiated cleavage patterns. A 5′-^{32}P end-labeled 186 base pair fragment of the *lac* operon was cleaved with 200 μM OP/45 μM $CuSO_4$ in reactions that were potentiated either by MPA (DNA + OP) or ^{60}Co irradiation (DNA + OP + γ). Control lanes (DNA and DNA + γ) show the *lac* fragment in the absence of $(OP)_2Cu^+$ while all other conditions remain identical. A Maxam–Gilbert G>A calibration lane is shown in the leftmost lane.

pathway does exist but does not distinguish whether the DNA-bound 1,10-phenanthroline–cuprous complex is formed by the reduction of DNA-bound $(OP)_2Cu^{2+}$ (eq 3c and 3d) or by the binding to DNA of $(OP)_2Cu^+$ which has been produced by the reduction of $(OP)_2Cu^{2+}$ in solution (eq 3a and 3b). Both mechanisms are possible since equilibrium dialyses have indicated that both the planar cupric complex and the tetrahedral cuprous complex (as the 2,9-dimethyl-1,10-phenanthroline–cuprous complex) bind to the DNA.[8] Viscometry studies have indicated that

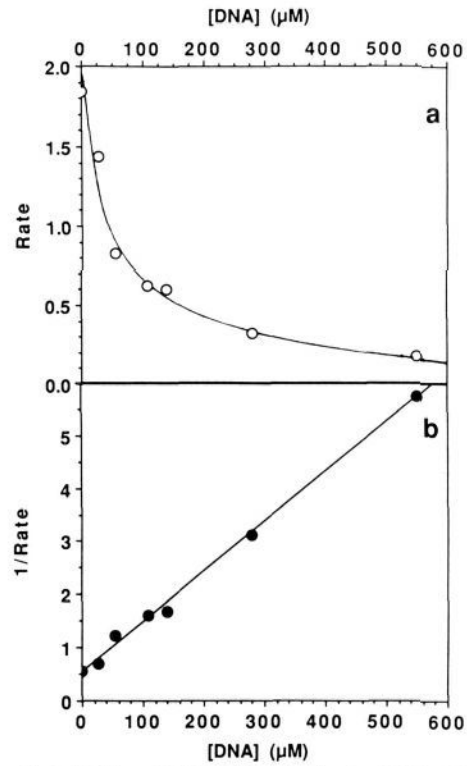

Figure 2. (a) Inhibition of $(OP)_2Cu^{2+}$ reduction by DNA. The initial rate of formation of $(OP)_2Cu^+$ was measured spectrophotometrically at 425 nm in the presence of increasing amounts of calf thymus DNA. The DNA was incubated with a $(OP)_2Cu^{2+}$ (200 μM OP; 45 μM $CuSO_4$) solution for 3 min at 22 °C before the $(OP)_2Cu^{2+}$ was reduced by the addition of MPA. The mean of three determinations of the initial rate of absorbance increase (ΔAU/min) [indicating formation of $(OP)_2Cu^+$] is plotted. (b) Reciprocal plot of the inhibition of $(OP)_2Cu^{2+}$ reduction by DNA. The data of panel a were plotted according to eq 6. The data are linear with the form $1/v = (0.52 + 9.4 \times 10^{-3})$[DNA] and have a correlation coefficient of 0.996.

the cupric complex binds by an intercalative mechanism whereas the cuprous complex does not.[8]

Direct evidence that the common reaction pathway is through the reduction of the diffusible cupric complex has been provided by kinetic studies of the formation of the cuprous complex in the presence of superoxide and thiol. Czapski and Goldstein have previously studied the kinetics of reduction by the 1,10-phenanthroline–copper complex using superoxide generated by pulsed radiolysis.[17] They observed that calf thymus DNA inhibited the reduction of the cupric complex. Using a kinetic analysis, they found that the dissociation constant of $(OP)_2Cu^{2+}$ from calf thymus DNA at pH 7.0 is 2.7×10^{-4} M whereas the dissociation constant of the cuprous complex from DNA is 5×10^{-6} M. They further determined that the second-order rate constant for the reduction of the free cupric complex is diffusion controlled (1.9×10^9 M^{-1} s^{-1}), whereas the rate constant for the DNA bound coordination complex is too slow to be measured.

Our kinetic studies of the reduction of $(OP)_2Cu^{2+}$ by thiol in the presence of increasing concentrations of DNA are consistent with these observations. As has been observed with superoxide, DNA inhibits the reduction of $(OP)_2Cu^{2+}$ by thiol (Figure 2a). If the rate of reduction of DNA-bound $(OP)_2Cu^{2+}$ $[(OP)_2Cu^{2+}{}_{DNA}]$ is assumed to be negligible, the initial rate of reduction of diffusible $(OP)_2Cu^{2+}$ $[(OP)_2Cu^{2+}{}_{free}]$ can be written as

$$v = k[(OP)_2Cu^{2+}{}_{free}][\text{thiol}]$$

If K is the dissociation constant of $(OP)_2Cu^{2+}{}_{DNA}$ and is defined as

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$$K = \frac{[(OP)_2Cu^{2+}][DNA]}{[(OP)_2Cu^{2+}{}_{DNA}]}$$

and $[(OP)_2Cu^{2+}{}_{free}]_{total}$ is the total concentration of coordination complex in the absence of DNA, then

$$\frac{1}{v} = \frac{1}{k[(OP)_2Cu^{2+}{}_{free}]_{total}} + \frac{[DNA]}{Kk[(OP)_2Cu^{2+}{}_{free}]_{total}} \quad (6)$$

The plot of the data according to eq 6 (Figure 2b) permits K to be determined by dividing the intercept by the slope. Its value, 56 μM, compares favorably to that of 270 μM cited above. This result indicates that step 3d, the reduction of the DNA-bound cupric complex by either thiol or superoxide, is too slow to participate in the cleavage chemistry. It is possible that the planar $(OP)_2Cu^{2+}$ ion, which binds via an intercalative mechanism,[8] is not sterically accessible to either superoxide or thiol. Alternatively, rearrangement to the tetrahedral cuprous complex required for cleavage may not be possible while it is bound to the DNA template. Independent of the origin of the kinetic barrier for reduction of the bound cupric complex, these results indicate that the obligatory kinetic mechanism is reduction of the free cupric complex to the cuprous form, followed by its diffusion to the minor groove.

Since the $(OP)_2Cu^{2+}$ complex also binds to DNA, the digestion patterns produced by the nuclease may be influenced by the presence of this intercalated species. This possibility can be excluded by monitoring the digestion pattern as a function of the concentration of the coordination complex. The same pattern of hypersensitive and hyposensitive bands is obtained if the scission chemistry is carried out in the presence of 5 mM MPA with 200 μM OP and 45 μM Cu^{2+} or with 10 μM OP and 2 μM Cu^{2+}. Thus, the sequence-dependent reactivity of the nuclease is a reflection of intrinsic structural features of the substrate DNA and not alterations to that structure induced by the binding of excess, nonreactive 1,10-phenanthroline–copper complexes.

Cleavage by Copper Complexes of 5-Substituted 1,10-Phenanthrolines. Knowledge of the oxidation state required for binding imposes many constraints on the mode of binding that the complex can tolerate and still generate scission. Space-filling models of the tetrahedral 2:1 1,10-phenanthroline–cuprous complex in the minor groove reveal that substituents at the 5- or 6-positions of the phenanthroline can be accommodated without displacing the copper ion from close proximity to the C-1 hydrogen, its primary site of attack (Figure 4). In contrast, substituents at these positions should influence the binding of the planar cupric complex, especially if it binds by an intercalative mechanism as viscometry experiments indicate. Thus, cleavage by the copper complexes of 5-substituted phenanthrolines should not be dependent on the size of the substituted group, if the cuprous complex is formed in solution and then diffuses to a minor groove binding site.

To explore this prediction, a series of 5-substituted phenanthroline derivatives were used to cleave the same 186 base pair fragment of the *lac* operon used above. Digestion of the fragment, either labeled on the template strand (Figure 3) or labeled on the nontemplate strand (not shown), produces a pattern that remains remarkably constant for nearly all of the 1,10-phenanthroline derivatives tested. The digestion patterns of the copper complexes of 5-methyl-, 5-(glycylamido), 5-nitro-, or 5-bromo-1,10-phenanthroline all preserve the characteristic specificity whether the reaction is potentiated by thiol or by superoxide. A similar pattern is also generated by the 5-phenyl phenanthroline–copper complex, although the relative intensity of the preferred sites of reactivity (e.g., sequence positions −13 through −10) has decreased slightly relative to the background. When the equivalent 5- and 6-positions on the phenanthroline ring are substituted to form 5,6-dimethyl-1,10-phenanthroline, its copper complex also generates the same digestion pattern as that of 1,10-phenanthroline. These data confirm a size-independent tolerance to substitution at the 5- and 6-positions of the phenanthroline ring.

In contrast to the above results, substitution of a succinylamido moiety in the 5-position dramatically reduced the nucleolytic

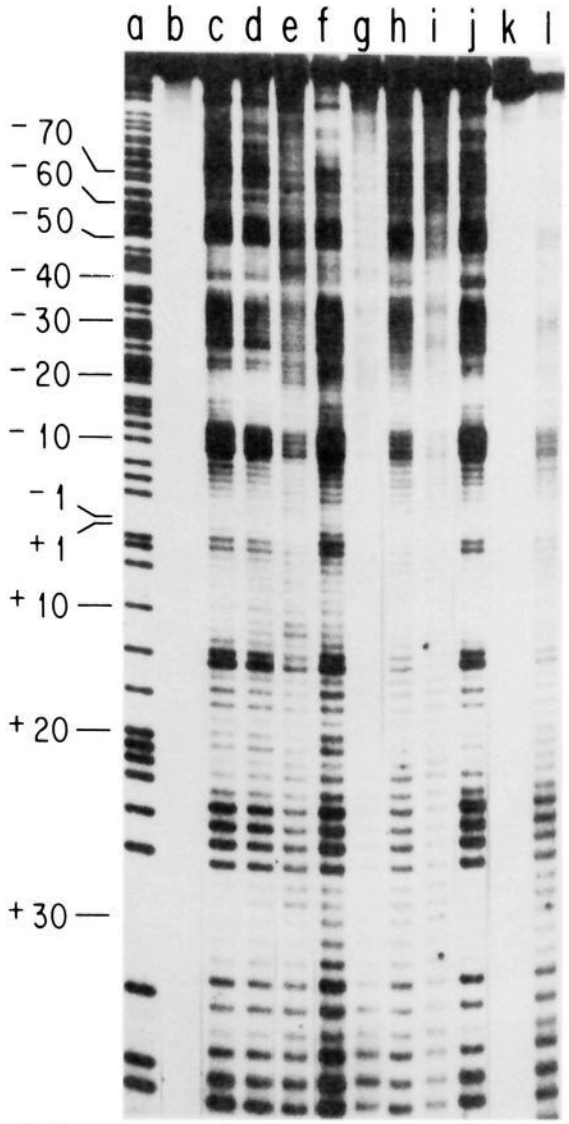

Figure 3. Cleavage pattern produced by copper complexes of substituted OP derivatives. Cleavage of a 186 base pair 5′-^{32}P end-labeled fragment of the *lac* operon was performed at a concentration of 200 μM 1,10-phenanthroline derivative in the presence of 45 μM $CuSO_4$ and 50 mM Tris-HCl (pH 8.0). All reactions were initiated with MPA and incubated for 3 min at 37 °C. Lanes: (a) Maxam–Gilbert G>A calibration; (b) control; (c) OP; (d) 5-methyl-OP; (e) 4,7-dimethyl-OP; (f) 5,6-dimethyl-OP; (g) 3,4,7,8-tetramethyl-OP; (h) 5-phenyl-OP; (i) 4,7-diphenyl-OP; (j) 5-Br-OP; (k) 5-(succinylamido)-OP; (l) 5-(glycylamido)-OP. The difference in the cleavage rates in lanes k and l is evident from comparison of undigested parent bands.

efficacy of the resulting copper complex (lane k, Figure 3). In view of the insensitivity of the reaction specificity to the size of a substituent in this position, the most likely reason for the difference in the nuclease activity of the succinylamido derivative is its net charge at the assay pH. Its cuprous complex is negatively charged at pH 8.0, while the copper complexes of the other derivatives are positive. Diminished rates of cleavage by the copper complexes of this derivative can therefore be attributed to the instability of the intermediate complex with DNA, due to the electrostatic repulsion between the negatively charged phosphodiester backbone and the 5-(succinylamido)-OP–cuprous complex.

The model of the (5-phenyl-OP)$_2Cu^+$–DNA complex (Figure 4) shows that the 3-, 4-, 7-, and 8-positions lie in the interior of the minor groove. Substituents at these positions should disrupt minor groove binding and decrease cleavage efficiency. For example, the 3,4,7,8-tetramethyl derivative has a greatly reduced rate of cleavage and does not produce the cleavage pattern

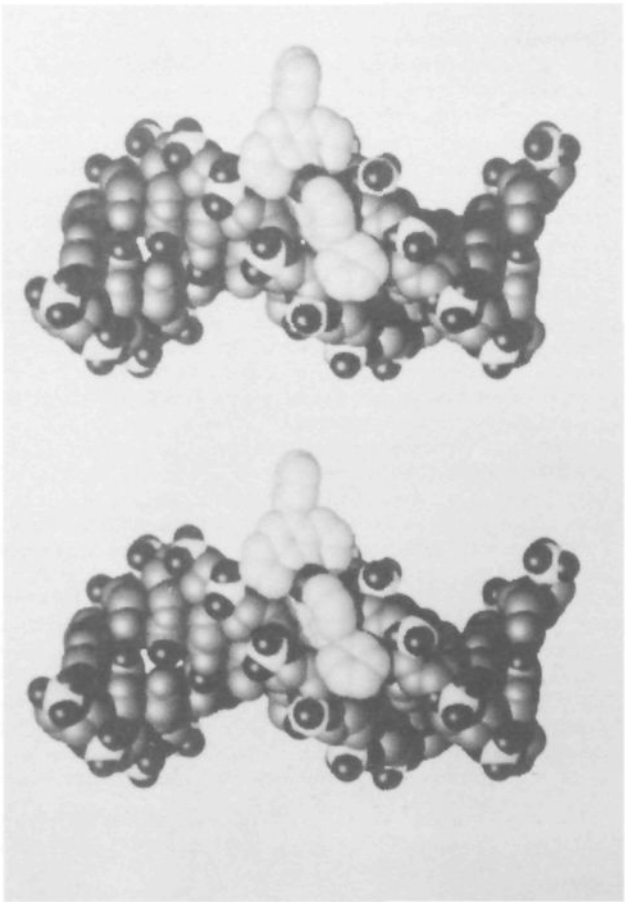

Figure 4. Model of 2:1 5-phenyl-1,10-phenanthroline–cuprous complex bound to DNA. The tetrahedral, cuprous complex of the 5-phenyl-1,10-phenanthroline derivative is shown bound within the minor groove of the dodecamer CGCGAATTCGCG. In this orientation, substituents at the 5- and 6-positions face away from the helix and thus do not interfere with minor groove contacts. Conversely, substituents at the 3- and 4-positions (equivalent to the 7- and 8-positions) lie at the base of the minor groove. The centrally coordinated copper ion lies adjacent to the deoxyribose moieties of either strand.

characteristic of unsubstituted $(OP)_2Cu^+$. Although the copper complex of 4-phenyl-OP generates a cleavage pattern comparable to that of $(OP)_2Cu^+$, the rate is significantly reduced (data not shown). A productive complex could possibly be achieved by positioning the unsubstituted, equivalent 7-position of the phenanthroline ring in the interior of the minor groove. This binding mode is eliminated if both the 4- and the 7-positions are substituted, as in the case of 4,7-diphenyl-1,10-phenanthroline. Its copper complex has a greatly reduced rate of cleavage and generates a cleavage pattern markedly different from that of $(OP)_2Cu^+$ (Figure 3, lane i). The degree of scission is approximately equivalent at all sequence positions; the characteristic $(OP)_2Cu^+$ hypersensitive sites are not produced.

Redox Activity of the Copper Complexes of Substituted Phenanthrolines Influences the Nuclease Activity. The nuclease activity of phenanthroline complexes is not only a function of the binding of their tetrahedral complexes to DNA but also a measure of their ability to participate in the redox chemistry. For example, the 2,9-dimethyl-1,10-phenanthroline–cuprous complex binds to DNA but lacks any nucleolytic activity due to its stability to oxidation.[18] The complex is constrained to a tetrahedral geometry by steric constraints imposed by the ortho substituents. The copper complexes of other ortho-substituted phenanthrolines are similarly inactive as nucleases.

One reflection of the ability of a coordination complex to carry out an oxidative cycle is its catalysis of the oxidation of thiol by molecular oxygen. This assay, which measures both the reduction and reoxidation of the copper complexes, proceeds by a superoxide intermediate as indicated in eq 4. The data summarized in Table I demonstrate that phenanthrolines substituted in the 5-position are efficient catalysts of thiol oxidation but those substituted in the 2-position, as well as 4,7-diphenyl-phenanthroline, are inefficient catalysts. The hydrogen peroxide generated in the reoxidation is an essential coreactant for the nucleolytic activity (eq 4).

Table I. Half-Lives for the Oxidation of 3-Mercaptopropionic Acid by Copper Complexes of Substituted Phenanthrolines[a]

derivative	half-life (min)
OP	3.13 ± 0.48
5-phenyl-OP	6.43 ± 0.61
5-nitro-OP	13.01 ± 5.8
5,6-dimethyl-OP	3.87 ± 0.02
5-Br-OP	4.95 ± 0.09
5-methyl-OP	2.68 ± 0.09
5-(glycylamido)-OP	16.60 ± 6.3
5-(succinylamido)-OP	10.90 ± 4.3
4,7-dimethyl-OP	4.31 ± 0.62
3,4,7,8-tetramethyl-OP	2.41 ± 0.04
4,7-diphenyl-OP	59.60 ± 12.3
2,9-dimethyl-OP	∞
2-(carboxylic acid)-OP	∞

[a] 200 μM OP (or one of its derivatives) and 45 μM $CuSO_4$ were allowed to oxidize a 1 mM solution of MPA. At 1-min intervals aliquots were used to reduce a solution of DTNB, and the production of TNB was followed spectrophotometrically. The half-life of the thiol oxidation was then calculated from triplicate values of the initial rates of oxidation.

Discussion

The nucleolytic activity of the 2:1 1,10-phenanthroline–copper complex proceeds by an ordered kinetic pathway that is independent of the 1-electron reductant used to potentiate it. The cuprous complex, one of the essential coreactants, is formed in solution by the reduction of the cupric ion complex, either by superoxide or by thiol, and then diffuses to the surface of the nucleic acid. Sequence-dependent cutting is therefore due to the site-specific binding of the tetrahedral cuprous complex in the minor groove.

Hydrogen peroxide, the second essential coreactant, then oxidizes the cuprous complex on the surface of the DNA to form the oxidative species that is directly responsible for the cleavage. Although the products of the oxidation of the deoxyribose moiety have been identified (eq 2), the precise chemical nature of the species responsible for the oxidative attack on the C-1 carbon is not yet known. Recent mechanistic studies have suggested that the 1,10-phenanthroline–cuprous complex with hydrogen peroxide is not an efficient generator of diffusible hydroxyl radicals.[19] Therefore, a copper–oxo species may be the oxidant which initially attacks the deoxyribose moiety.

The evidence that the coordination complex attacks the deoxyribose from a binding site within the minor groove is based on studies of the detailed chemical mechanism[7] as well as the footprinting of ligand–DNA complexes of known structure.[6] For example, the antibiotic netropsin complexes with the central AATT sequence of the self-complementary dodecamer CGCGAATTCGCG entirely within the minor groove and has been shown to completely protect that central sequence from nucleolytic attack by $(OP)_2Cu^+$.[6] In contrast, when the complex between the restriction endonuclease *Eco*RI and the same dodecamer is digested, there is no protection from cleavage because all of the protein's nucleic acid contacts occur within the major groove.[6]

The precise structural features of the minor groove which determine the sequence-specific binding of the tetrahedral cuprous complex are not yet known. However, the tolerance of the nu-

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cleolytic activity to substitution in the 5-position of the phenanthroline ring, with methyl, phenyl, bromo, nitro, glycylamido and acetylamido functional groups, can be readily accommodated within a minor groove binding model. A model of the tetrahedral 5-phenyl phenanthroline–cuprous complex bound to DNA, as shown in Figure 4, indicates that substituents on the 5-position are exposed to the aqueous environment and therefore are unlikely to interfere with the binding. In contrast, intercalative binding, such as that preferred by the square-planar cupric complex,[8] would be subject to steric interference by bulky substituents at this position. Examination of the molecular model indicates that the stability of the DNA–1,10-phenanthroline–cuprous complex will be substantially influenced by substituents at the 3-, 4-, 7-, and 8-positions because they are in close contact with the floor of the minor groove.

Our experimental results indicate that substitution at the 3- and 8-positions with methyl groups drastically destabilizes the intermediate complexes, whereas substitution with methyl groups at the 4- and 7-positions is tolerable. For example, the 3,4,7,8-tetramethyl derivative is ineffective in DNA scission (Figure 3, lane g), even though its copper complex efficiently catalyzes the oxidation of thiol by molecular oxygen (Table I). In contrast, the copper complex of the 4,7-dimethyl derivative, which is also an efficient catalyst of thiol oxidation, shows no significant differences in the cleavage pattern and only a slight dimunition in rate relative to the unsubstituted ligand (Figure 3, lane e). However, substitution of larger phenyl groups at the 4- and 7-positions produces a complex which is markedly less specific than that of OP, or any other derivative tested (Figure 3, lane i). Possibly the binding of the 4,7-diphenyl-OP–copper complex to the minor groove is so unfavorable that the coordination complex binds in the major groove as has been suggested for the octahedral complexes prepared from this ligand with ruthenium, cobalt, and zinc ions.[20] In this case, the chemistry of the cleavage reaction would be fundamentally different. The possibility that these effects are mediated by hydrophobicity, and not simple steric hindrance, is not supported by the cleavage rate of the 5,6-dimethyl-OP–Cu complex, which is slightly higher than that of the 5-methyl-OP–Cu complex and comparable, if not faster, then that of $(OP)_2Cu^+$ itself (Figure 3, lane f).

The experimental results reported here have had two important impacts on our continuing studies of the nuclease activity of 1,10-phenanthroline–copper ion. Superoxide-potentiated cleavage by ^{60}Co of the nucleolytic activity has permitted the isolation of 5-methylene-2-furanone as the primary product of the deoxyribose oxidation.[7] The product, a Michael acceptor, could not be isolated with thiol-activated cleavage because it would have reacted with the sulfhydryls. Since no difference has been observed in the kinetic mechanism or the sequence-dependent reactivity with either thiol or superoxide potentiating reaction, the deoxyribose chemistry proven with superoxide activation clearly applies to the thiol-activated reaction. Second, the nucleolytic activity of 1,10-phenanthroline–copper has now been targeted with oligonucleotides and DNA binding proteins as carriers. The chemistry of the attachment has involved linking the 1,10-phenanthroline via a 5-(glycylamido) linker in the case of oligonucleotides and via a 5-(iodoacetamido) group in the case of proteins.[21,22] Although the original reason for the choice of the 5-position was experimental convenience, the strategy is desirable because it permits the phenanthroline moiety to bind within the minor groove with a minimum of steric interference.

The kinetic mechanism presented here is consistent with our initial observation that the conversion of DNA from a supercoiled to a linear form, by $(OP)_2Cu^+$, proceeds via nicked circles.[4] Double-stranded scission of DNA by 1,10-phenanthroline–copper must be accomplished by two successive single-stranded nicks. After the first strand is nicked, the coordination complex dissociates from the minor groove of the DNA. A second $(OP)_2Cu^+$ complex then binds near this nick and cleaves on the opposite strand to create a double-stranded break. Double-stranded scission has been observed with the semisynthetic nuclease generated by chemically linking 1,10-phenanthroline–copper to the *Escherichia coli trp* repressor.[22] These double-stranded breaks may be possible because of the slow off-rate of the DNA binding protein as well as the accessibility of the copper ion to thiol in solution.

Acknowledgment. We thank Thomas Goyne for useful discussions. This research was supported by USPHS 21199 and ONR NOO14-86K-0574.

Registry No. $(OP)_2Cu^{1+}$, 17378-82-4; (5-phenyl-$OP)_2Cu^{1+}$, 120685-64-5; (5-nitro-$OP)_2Cu^{1+}$, 59751-73-4; (5,6-dimethyl-$OP)_2Cu^{1+}$, 17378-88-0; (5-Br-$OP)_2Cu^{1+}$, 120685-65-6; (5-methyl-$OP)_2Cu^{1+}$, 17702-23-7; (5-glycylamido-$OP)_2Cu^{1+}$, 120685-66-7; (5-succinylamido-$OP)_2Cu^{1+}$, 120685-68-9; (4,7-dimethyl-$OP)_2Cu^{1+}$, 17702-27-1; (3,4,7,8-tetramethyl-$OP)_2Cu^{1+}$, 120685-67-8; (4,7-diphenyl-$OP)_2Cu^{1+}$, 62320-24-5; (2,9-dimethyl-$OP)_2Cu^{1+}$, 21710-12-3; (2-carboxylic acid-$OP)_2Cu^{1+}$, 120685-69-0; MPA, 107-96-0; H_2O_2, 7722-84-1; $O^{\bullet}_2$, 11062-77-4; CGCGAATTCGCG, 77889-82-8; deoxyribonuclease, 9003-98-9.

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