# 1,10-Phenanthroline-Linked *Escherichia coli* Trp Repressor as a Site-Specific Scission Reagent. Metal Ion Requirement

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*Received September 2, 1992*

The *Escherichia coli* Trp repressor has been converted into an efficient and site-specific nuclease by alkylating a cysteine residue, inserted by site-directed mutagenesis, with 5-iodoacetamido-1,10-phenanthroline. This derivatized mutant Trp repressor delivers and directs the chemical nucleolytic activity of 1,10-phenanthroline-copper to the minor groove, where it can cleave operator fragments of DNA via an oxidative pathway in the presence of cupric ion, thiol, and hydrogen peroxide. The metal ion dependence of this site-specific nuclease has been examined. Cu(II) is the most effective metal ion in directing scission in the presence of thiol and molecular oxygen. Although Fe(II) can be used in place of Cu(II), the efficiency of cleavage decreases 2–3-fold. Pb(II), Hg(II), and Mn(II), in the presence of 2,9-dimethyl-1,10-phenanthroline and deferoxamine (cuprous- and ferric-specific chelators, respectively), also support scission in the presence of thiol and hydrogen peroxide although their effect is just above background. No evidence of scission by a hydrolytic mechanism was observed. Non-redox active metals such as cadmium, zinc, europium, and terbium did not promote scission even in mixed ligand systems where exogenous nucleophiles such as ethanolamine, 2-pyridinealdoxime, and *N*-(2-hydroxylethyl)ethylenediamine were added.

## Introduction

The design of site-specific DNA scission agents is a challenging and active area of bioorganic chemistry.[1-3] The general approach has been to link a DNA recognition element with a reactive chemical functionality capable of cleaving the phosphodiester backbone of the nucleic acid.[4] Presently, redox active coordination complexes provide the most convenient approach for accomplishing strand scission on a chemically useful time scale.[5] The photochemical generation of singlet oxygen provides an alternative, but less widely used, solution of the chemical problem of strand scission.[6] Although endonucleases involved in DNA repair use elimination reactions to cleave the phosphodiester backbone at abasic sites,[7] most naturally occurring nucleases accomplish strand scission by hydrolysis. Despite ongoing attempts, coordination complexes which efficiently catalyze the scission of the phosphodiester backbone by a hydrolytic pathway efficiently are not available.[8,9] Rates of RNA hydrolysis by 2,2′-bipyridine-copper ion are modest and do not presently approach those needed to create an effective scission reagent.[10,11]

Our laboratory has recently reported the conversion of the *Escherichia coli* Trp repressor into an efficient and site-specific DNA scission reagent using site-directed mutagenesis and chemical modification.[12] Specifically, glutamate-49 has been converted into a cysteine residue which in turn has been alkylated with 5-iodoacetamido-1,10-phenanthroline. In the presence of thiol, hydrogen peroxide and cupric ion, this protein-ligand conjugate (Trp repressor E49C-OP) cleaves three operators, which are regulated by the wild type protein, by means of an oxidative pathway using the chemical nuclease activity of 1,10-phenanthroline-copper.[13] The wild type Trp repressor, after reaction with 5-iodoacetamido-1,10-phenanthroline, was used as a control in a gel retardation/cleavage experiment and exhibited no scission. The lambda phage cro protein has similarly been converted into a site-specific DNA scission agent by this combination of genetic and chemical methodologies.[14,15]

Trp repressor E49C-OP is remarkable in its reactivity because it makes two to three nicks on each strand and is very efficient in accomplishing double-stranded breaks. It makes double-stranded breaks with a first order rate constant of $3.8 \times 10^{-2}$ $min^{-1}$ and single-stranded nicks with a rate constant of 0.18 $min^{-1}$. Given a dissociation constant of $5 \times 10^{-10}$ M,[16] the second order rate constant for double-stranded breaks and single-stranded nicks are $3.8 \times 10^{-2}$ $min^{-1}/5 \times 10^{-10}$ M or $7.6 \times 10^{7}$ $min^{-1}$ $M^{-1}$ and 0.18 $min^{-1}/5 \times 10^{-10}$ M or $3.6 \times 10^{8}$ $min^{-1}$ $M^{-1}$.[10] Sequence position 49 was selected as the site for 1,10-phenanthroline attachment because it is (a) in a rigid region of the protein structure near the protein's C-2 dyad axis;[17,18] and (b) adjacent to the minor groove, the locus of the C-1 hydrogen of the deoxyribose, the initial site of attack by the oxidative chemical nuclease activity of 1,10-phenanthroline–copper (OP–Cu).[19,20]

In initial studies of the chemical nuclease activity of 1,10-phenanthroline–copper, thiol and hydrogen peroxide were shown

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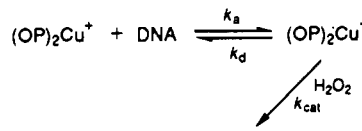
**Scheme I**




to be essential reactants in the chemical nuclease activity in addition to 1,10-phenanthroline and copper.[21] For the untargeted nuclease activity, the reactivity was specific for copper and tolerated little modification of the phenanthroline structure.[21] For example, 2,2′,2″-terpyridine and 2,2′-bipyridine, in combination with copper, thiol, and hydrogen peroxide, did not cleave DNA under the conditions where the 1,10-phenanthroline system was active. Since the 1,10-phenanthroline–copper reaction proceeds via the formation of an intermediate complex[21,22] (Scheme I) ($K_d = k_d/k_a$), it was not possible to identify the reason for their lack of reactivity. It could be due to the instability of the noncovalent intermediate and/or the inefficiency of the oxidative step.

With Trp E49C-OP, the reactivity of different metal ions can be systematically studied because the same intermediate complexes will be formed in all cases. Since the complex between Trp E49C-OP and the DNA recognition sequence will be independent of metal ion, it is possible to focus on the underlying chemistry. In this paper, we address the relative efficiencies of the oxidative chemistry and explore the possibility of metal ion catalyzed hydrolysis of the phosphodiester backbone.

## Experimental Section

**Materials.** 5-Iodoacetamido-1,10-phenanthroline was prepared as previously described.[23] The following reagents were obtained commercially: 1,10-phenanthroline (OP) and 2,9-dimethyl-1,10-phenanthroline (GFS, Columbus, OH), 3-mercaptopropionic acid and deferoxamine (Aldrich), cupric sulfate pentahydrate and Tris (Sigma).

The isolation, purification, and labeling of a restriction fragment containing the *E. coli aroH* operator has been previously described.[13]

**Preparation of Trp Repressor E49C-OP.** The gene for the wild type Trp repressor protein sequence was excised from pRPG 47[24] and placed into M13 mp18 for mutagenesis. Oligonucleotide directed mutagenesis was performed as described by Kunkel et al.[25] The gene for the mutant protein (Trp repressor E49C) was expressed using the same vector as the wild type. Cells transformed with vector were grown in LB (1% tryptone, 1% NaCl, 0.5% yeast extract) pelleted, resuspended in 1 mL of buffer containing 25 mM Tris, pH 8, 10 mM EDTA, 50 mM glucose, and 0.1 mM β-mercaptoethanol, and sonicated. After recentrifugation, the supernatant was subjected to streptomycin sulfate at a final concentration of 0.5% for 10 min at 85 °C. After centrifugation, 100 μL was diluted to 1 mL with resuspension buffer lacking thiol. 20 μL of 50 mM 5-iodoacetamido-1,10-phenanthroline was added at 0 °C and the reaction was incubated for 1 h. 10 μL of 3-mercaptopropionic acid was added and the reaction was continued for an additional 30 min. Glycerol was added to a final concentration of 10% and aliquots of the derivatized protein were stored at −80 °C.

**Gel Retardation and Purification of the Trp Repressor E49C-OP-*aroH* Complex.** Gel retardation was used to purify Trp repressor E49C-OP from other 1,10-phenanthroline labeled proteins present in the bacterial extract, excess 5-iodoacetamido-1,10-phenanthroline from the modification reaction, and other contaminants. To block DNA scission potentiated by trace levels of copper and iron in the reaction mixture, EDTA at a final concentration of 0.1 mM was included in the preliminary incubation mixture of the gel retardation assay. For gel retardation, a 10% nondenaturing polyacrylamide gel described by Carey[16] was used. For each binding reaction, 50 000–100 000 cpm of operator fragment was dissolved in a buffer containing 100 mM Tris, pH 7.5, 200 mM KCl, 6 mM $MgCl_2$, 11 mM L-tryptophan, and 0.1 mM poly (dI-dC) in a final volume of 10 μL. Trp repressor (200 nM) was added and the reaction was incubated for 10 min at 25 °C. After adding 5 μL of 40% sucrose/0.01% bromophenol blue, the entire reaction was then loaded on the polyacrylamide gel. The gel was run at 300 V for 5 min and at 200 V for 4 h with recirculation of the 10 mM $NaH_2PO_4$, pH 6/0.5 mM L-Trp buffer. The gel was then exposed to film and the protein–DNA complexes localized. The gel matrix containing the complexes was excised with a razor blade.

**Scission Reaction.** All reactions were carried out on Trp repressor-DNA complexes within the gel matrix. They were incubated in 200 μL of a 2X scission buffer containing 20 mM $NaH_2PO_4$, pH 8, 1 mM L-Trp and 1 mM $MgCl_2$. When the copper-dependent DNA cleavage was examined, 10 μM of copper sulfate was added along with 3 mM $H_2O_2$ and 3 mM 3-mercaptopropionic acid or an alternative reducing agent (e.g. ascorbic acid). The reaction mixture is incubated for 20 mins at 25 °C. To quench the reaction, 2,9-dimethyl OP was added to a final concentration of 2 mM.

When the reaction with iron was studied, 2,9-dimethyl-1,10-phenanthroline is added to a final concentration of 0.1 mM to block copper dependent cleavage. Both 2,9-dimethyl-1,10-phenanthroline (0.1 mM) and deferoxamine (0.25 mM) were added to eliminate the copper and iron dependent cleavage reactions when the reactivity of other metal ions was examined.

**Analysis of Products.** The gel slice was crushed into very small pieces and 330 μL of PAGE elution buffer (1M ammonium acetate, pH 7, 1 mM EDTA) was added. The Eppendorf tube was placed in a 37 °C heat block overnight in order to elute the reaction products from the gel slice. The following day, the eluted products were ethanol precipitated, washed with 70% ethanol, and dried in a Speed-Vac.

For the analysis of single-stranded nicks, the products were dissolved in 15 μL of a denaturing loading buffer (80% deionized formamide, 0.1% bromophenol blue, 0.1% xylene cyanol, and 1 mM EDTA). The reaction products were electrophoresed on a 10% denaturing gel (10% polyacrylamide, 1X TBE, and 7 M urea). The gel was run at 55 watts for 2 h after loading. Autoradiography was performed overnight at −80 °C with an intensifying screen.

For analysis of the double-stranded breaks, the products were dissolved in 15 μL 40% sucrose and 0.1% bromophenol blue and analyzed on an 8% non-denaturing polyacrylamide gel.

## Results

### Copper-Dependent Scission by Trp Repressor E49C-OP.

Since the untargeted chemical nuclease activity was specific for copper ion, this metal ion had always been used in scission reactions of 1,10-phenanthroline directed by a targeting ligand. Although the chemistry of scission has been established for the untargeted nuclease and involves oxidative attack on the C-1 hydrogen of the deoxyribose moiety, a detailed characterization of products of site-specific scission reactions is experimentally challenging because they necessarily generate restricted amounts of chemical product. Two criteria which have justified extrapolating the reaction mechanism of untargeted scission to that of targeted scission are (a) the formation of 3′-phosphomonoester termini and (b) the stagger of the scission sites on the two strands in the 3′-direction diagnostic of minor groove attack. The formation of 3′-phosphomonoester termini was confirmed by migration similar to products generated by a Maxam–Gilbert chemical sequencing reaction and a micrococcal nuclease digest (yielding 3′ phosphates) and different from a DNase I enzymatic digestion (yielding 5′ phosphates).

**Thiol Requirement.** Prior to examining the reactivity of other metal ions in this system, it was essential to examine in greater detail the range of conditions which led to copper-dependent scission. To this end, 3-mercaptopropionic acid (MPA), which has been used to activate the chemical nuclease because it is a poor chelating agent, was substituted by an identical concentration (i.e. 3 mM) of other reducing agents including ascorbic acid, dithiothreitol and β-mercaptoethanol. Ascorbic acid activated

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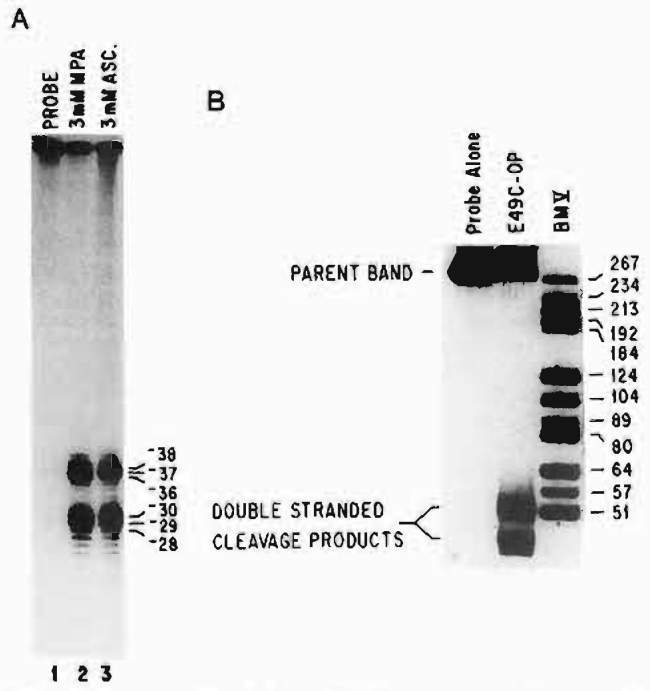


**Figure 1.** Scission of 3′-Labeled *aroH* by the Copper Complex of *E. coli* Trp Repressor E49C-OP Activated by Different Reducing Agents. (A). Single-stranded scission: lane 1, 3′-*aroH* DNA; lane 2, 3 mM 3-mercaptopropionic acid; lane 3, 3 mM ascorbic acid. Following isolation of the complex of Trp repressor E49C-OP with *aroH* by gel retardation, a slice of the acrylamide matrix was incubated with cupric ion and 3 mM hydrogen peroxide for 10 min and the products separated on a sequencing gel. (B). Double-stranded scission: lane 1, 3′-*aroH* DNA; lane 2, scission of the DNA by E49C-OP; lane 3, Hae III digest of pBR322 used as a calibration for the double-stranded breaks.

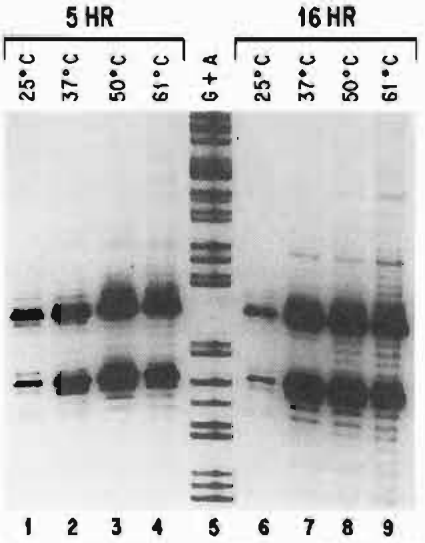


**Figure 2.** Scission of 3′-Labeled *AroH* within the Gel Slice without Added Thiol and Hydrogen Peroxide. Following isolation of the Trp repressor E49C-OP-*aroH* complex by a gel retardation assay, the slice of the acrylamide matrix was incubated for 16 h. Single-stranded scission was assayed using a denaturing sequencing gel. Key: lanes 1 and 6, scission at 25 °C after 5 h (lane 1) or 16 h (lane 6); lanes 2 and 7, scission at 37 °C after 5 h (lane 2) or 16 h (lane 7); lanes 3 and 8, scission at 50 °C after 5 h (lane 3) or 16 h (lane 8); lanes 4 and 9, scission at 61 °C after 5 h (lane 4) or 16 h (lane 8); lane 5, Maxam–Gilbert G+A chemical sequencing lane.

scission to a comparable extent as MPA while dithiothreitol and β-mercaptoethanol afforded little or no scission (Figure 1A). The reason for the lack of activity of the latter two agents was not investigated further but possibly it was due to their sequestration of cupric ion since they are both effective bidendate ligands.[26] The single- and double-stranded scission of *aroH* activated by MPA are compared in Figures 1A,1B.

As a control experiment in our investigations of the reducing agent requirement in this targeted scission reaction, the rates of scission in the acrylamide gel slice were examined when thiol and hydrogen peroxide were omitted. Surprisingly, efficient DNA scission was observed after a 24 h incubation. The scission products migrated at the same rate as those produced in a 10 min incubation with MPA and hydrogen peroxide. Analysis of the 3′-termini indicated that they were 3′-phosphomonoesters. Since this slow 24 h reaction can be completely inhibited by the addition of catalase, these products must have been caused by a slow oxidative reaction in which the reducing environment of the gel matrix reduces the cupric ion to cuprous ion which in turn is oxidized by molecular oxygen to generate hydrogen peroxide. In the absence of added reducing agent and hydrogen peroxide, the generation of cuprous ion and hydrogen peroxide are definitively rate limiting but the underlying chemistry of the cleavage reaction remains unchanged. The single-stranded scission products observed after a 5 or 16 h incubation in the absence of reducing agents are presented in Figure 2.

**Iron-Dependent Scission.** The 3:1 1,10-phenanthroline–iron complex does not cleave DNA under the conditions where 1,10-phenanthroline–copper completely digests DNA. Two possible factors may contribute to this lack of reactivity. The first is that the 3:1 complex is octahedral in contrast to the 2:1 1,10-phenanthroline–cuprous complex. Unlike the tetrahedral 2:1 1,10-phenanthroline–cuprous complex which binds in the minor groove, octahedral complexes of 1,10-phenanthroline bind in the major groove.[27] The second is that the 3:1 1,10-phenanthroline–ferrous complex is more oxidatively stable than 1,10-phenanthroline–copper.[28] For example, the 1,10-phenanthroline–cuprous complex is oxidized within the time of mixing in an aerobic environment whereas the 3:1 1,10-phenanthroline–ferrous complex is not. However, neither of these considerations are relevant to the ability of iron to activate the scission reaction of Trp repressor E49C-OP.

To test their competence to activate scission, ferrous and ferric ions were added to gel slices containing the Trp repressor E49C-OP-*aroH* complex. 2,9-Dimethyl-1,10-phenanthroline was included in all incubation mixtures to block any reaction due to adventitious copper.[21] The addition of thiol and hydrogen peroxide activated the oxidative scission by iron. The products of the single- and double-stranded scission were very similar to those of the copper dependent reaction (Figure 3). The iron dependent reaction is the same whether ferrous or ferric ion are initially added as would be expected for a reaction involving redox-cycling. Analysis of the 3′ ends indicate the predominant formation of phosphomonoester termini suggestive of a reaction pathway involving oxidative attack on the C-1 hydrogen. Ascorbic acid did not result in as high an efficiency with respect to site-specific cleavage and, in addition, contributed a significant background in the presence of hydrogen peroxide.

**Reactivity of Other Redox-Active Metal Ions.** The reactivity of Pb(II), Hg(II), and Mn(II), was also examined in the presence of thiol and hydrogen peroxide. To assay their effectiveness in activating the scission reaction, deferoxamine and 2,9-dimethyl-1,10-phenanthroline were added to the gel slice containing the DNA–protein complex in order to suppress the reaction of trace levels of copper and iron. Since the concentration of the Trp

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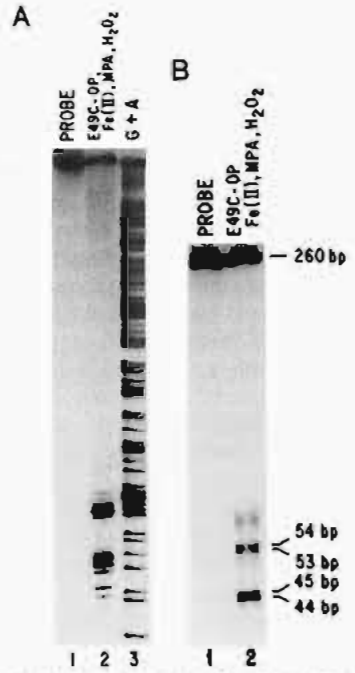


**Figure 3.** Scission of 3′-Labeled *aroH* by Trp Repressor E49C-OP Activated by $Fe^{+2}$. (A). Trp repressor E49C-OP-*aroH* complex isolated by gel retardation and incubated with $Fe^{+2}$ in the presence of 3 mM 3-mercaptopropionic acid and 3 mM $H_2O_2$ for 3.5 h. Analysis of single-stranded nicks: lane 1, probe alone; lane 2, scission by E49C-OP complexed with $Fe^{+2}$; lane 3, Maxam–Gilbert G+A chemical sequencing lane. (B). Double-stranded scission: lane 1, 3′-*aroH* DNA; lane 2, scission of the DNA by E49C-OP.

repressor E49C-OP-DNA complex is in the $10^{-9}$–$10^{-8}$ M range, incorporating metal ion specific chelating agents in the assay mixture provides a useful, but not necessarily definitive, approach for eliminating spurious reactions of contaminating metal ions. It is not practically possible to reduce contaminating levels of metal ion to a level where they may not interfere with the experiment. Our results indicate that Pb(II), Hg(II), and Mn(II) weakly support oxidative scission although the reactions promoted by these metal ions are just above background. The reactivity of cobalt and nickel were also examined. Neither of these metals promoted scission in the presence of thiol and hydrogen peroxide. In the case of the nickel, the reactant monoperoxyphthalic acid was added in place of thiol and peroxide as an oxygen donor to activate scission.[29] No scission was observed in this case either.

A complex artifact that is difficult to exclude and may account for the lack of reaction for cobalt and nickel in comparison to the marginal reactivity of Pb(II), Hg(II) and Mn(II) relates to intrinsic affinity of 1,10-phenanthroline for the different metal ions examined. The stability constants for the formation of the 1:1 1,10-phenanthroline–metal ion complexes are 4.65, 3.7, and 3.5 for Pb(II), Hg(II), and Mn(II), respectively, while those for Fe(II) and Cu(II) are 5.86 and 8.82, respectively.[30] The corresponding constants for the phenanthroline complexes of Co(II) and Ni(II) are 7.02 and 8.0.[30] Given the higher affinity of 1,10-phenanthroline for Co(II) and Ni(II), it is possible that they are the only metal ions which can completely block the binding of Cu(II) and Fe(II). Even in the presence of 0.1 mM 2,9-dimethyl-1,10-phenanthroline and 0.25 mM deferoxamine, trace amounts of OP–Cu and OP–Fe may form despite the availability of 10 μM of Hg(II), Pb(II), or Mn(II). Therefore, it is possible that the Fe(II) and Cu(II) dependent reactions are definitively blocked only in the presence of Co(II) and Ni(II) and that Fe(II) and Cu(II) are the only two redox-active metal ions which can support scission.

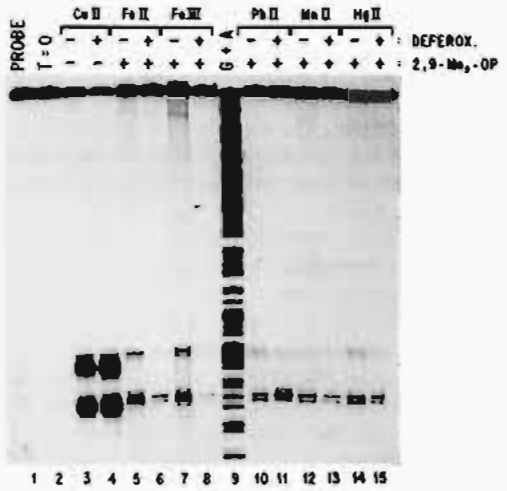


**Figure 4.** Comparison of Redox-Active Metal Ions in the Scission of 3′-Labeled *AroH* by Trp Repressor E49C-OP. Assay of Pb(II), Mn(II), and Hg(II) required the inclusion of deferoxamine (deferox) and 2,9-dimethyl 1,10-phenanthroline (2,9-$Me_2$-OP) in order to block the iron- and copper-dependent scission. The presence of deferox and 2,9-$Me_2$-OP is indicated by a + in the appropriate row; their absence is indicated by a –. Components of the incubation mixture for the polyacrylamide gel slice containing the Trp repressor E49C-OP complex include 10 mM $NaH_2PO_4$, pH 8, 0.5 mM L-Trp, and 0.5 mM $MgCl_2$ and 10 μM of the designated metal.

**Nonredox-Active Metal Ions.** Cadmium, zinc, europium and terbidium were added to the Trp repressor E49C-OP-*aroH* complex in the presence of both deferoxamine and 2,9-dimethyl-1,10-phenanthroline. No scission was observed. Moreover neither ethanolamine, 2-pyridinealdoxime,[31,32] nor *N*-(2-hydroxylethyl)ethylenediamine[33] promoted scission in the presence of copper, cadmium or zinc even though these ligands could coordinate to the metal ions simultaneously with Trp repressor E49C-OP and contribute a metal bound nucleophile. The metal ions thallium and cerium were also ineffective in promoting scission under any reaction conditions.

## Discussion

Binding affinity and reaction specificity are two important factors which will influence the efficiency in the design of site-specific scission reagents. The high efficiency and limited number of scission sites of the copper-dependent oxidative scission reaction of Trp repressor E49C-OP suggested the metal ion is rigidly constrained in this chimeric reagent. Since the covalent modification is at a sequence position that does not contribute directly to the DNA–protein interaction, Trp repressor E49C-OP is an interesting construct to test metal ion specificity. The different cations are unlikely to make a significant impact on the DNA binding affinity of the conjugated protein. Scission of the phosphodiester bond by a nucleophilic rather than an oxidative mechanism might be possible in this system because of the precise orientation of the 1,10-phenanthroline relative to the phosphodiester backbone.

Copper ion is the most effective metal ion for promoting both the untargeted and targeted DNA scission reactions involving 1,10-phenanthroline. One important difference between the untargeted and targeted reaction is that the former requires two moles of OP per cupric ion while the latter requires one mole of OP per metal ion.[34] Two phenanthrolines per copper are probably essential in the untargeted reaction in order to generate a hydrophobic cation with modest affinity (dissociation constant,

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$10^{-5}$ M) for DNA.[35,36] For Trp repressor E49C-OP and other examples of targeted scission, the carrier ligand provides the principal binding affinity essential for holding the OP–Cu close to the nucleic acid surface. It is not known if water or buffer (e.g. tris) molecules complete the coordination sphere of the metal ion. The fact that only one phenanthroline has been attached to the Trp repressor has been verified using [$^3$H]-1,10-phenanthroline of known specific activity.

Ascorbic acid and 3-mercaptopropionic acid are efficient reducing agents in activating the scission reaction. 3-Mercaptopropionic acid seems superior to ascorbic acid because background cleavage is less. The reaction of Trp repressor E49C-OP-Cu is so robust that if the protein-DNA complex is incubated in the gel slice without added thiol overnight, strong scission is observed. The cleavage reaction is this case must be activated from organic components within the gel matrix. The inhibition of the reaction by catalase and the generation of 3′-phosphorylated ends suggest that this reaction is oxidative and not hydrolytic.

Iron, which is ineffective in promoting the untargeted DNA scission reaction by 1,10-phenanthroline, can potentiate the reaction by Trp repressor E49C-OP. The reaction is one-third to one-half as efficient as the copper-dependent reaction. The product distribution and the chemical ends generated are indistinguishable from those produced in the copper-promoted reaction. The oxidized product of the deoxyribose, presumably 5-methylenefuranone, has not been isolated for either the copper- or iron-dependent reaction since it is not produced in sufficient amounts for analysis.

The ability of other redox-active metal ions to support the DNA cleavage reaction has not been unambiguously resolved. Pb(II), Hg(II), and Mn(II) exhibit marginal activity when assayed but cobalt and nickel give no detectable scission. Despite the fact that the reactions were carried out in the presence of 2,9-dimethyl-1,10-phenanthroline and deferoxamine, the possible interference of trace levels of copper and iron in the cleavage reactions cannot be rigorously excluded in the reactions with Hg(II), Pb(II), and Mn(II).

None of the metal ions, including copper and iron, cause DNA cleavage in the absence of thiol and hydrogen peroxide in solution. There is therefore no evidence for any hydrolytic cleavage despite the efficiency of the oxidative reaction. Moreover, zinc and cadmium, which are redox inactive, also fail to cause any detectable scission. Terbium, europium, thallium, and cerium are unable to promote scission under any reaction conditions. As noted above, the reaction potentiated by copper ion after a 12 h incubation in the acrylamide gel is inhibited by catalase and must be promoted by reducing equivalents present in the organic matrix of the acrylamide gel.

Previous studies have indicated that a nucleophile within or adjacent to the coordination sphere of a metal ion can be activated by the cation and that reactions within these mixed ligand systems can be more efficient than a metal ion catalyzed hydrolysis reaction in which a metal ion bound hydroxyl ion attacks an electrophilic center. For example, *N*-(hydroxyethyl)ethylenediamine in the presence of zinc is rapidly acylated by *p*-nitrophenylpicolinate but not by *p*-nitrophenylisopicolinate in a rate that exceeds the zinc ion catalyzed hydrolysis.[33] 2-Pyridinealdoxime[31] is rapidly acylated by 8-hydroxyquinoline acetate in the presence of zinc ion; this ligand can also be phosphorylated in a mixed ligand system[32] in the presence of zinc ion and phosphorylimidazole. Zinc ion can catalyze the phosphorylation of 1,10-phenanthroline-2-carbinol by ATP in a reaction which proceeds through an obligatory mixed complex intermediate.[37] Since hydrolysis could not be readily observed with Trp repressor E49C-OP, mixed ligand systems were prepared using ethanolamine, 2-pyridinealdoxime and *N*-(hydroxyethyl)ethylenediamine with several metal ions. No directed DNA scission could be observed in these cases. New approaches to accomplish phosphodiester backbone scission by nonoxidative mechanisms are required.

The efficiency of the copper and iron potentiated oxidative scission by Trp repressor E49C-OP is so remarkable that trace levels of these metals cause measurable amounts of cleavage of the target DNA during a gel retardation assay unless EDTA is included in the running buffer. This background cleavage provides a chemical model for the mutagenesis of DNA that may occur in nature by the binding of adventitious metal ions to protein–DNA complexes.

**Acknowledgment.** This research was supported by USHPS GM 21199 and Office of Naval Research Grant N-00014-86-K0524. A.M. was a postdoctoral fellow of the Lucille P. Markey Foundation in the Department of Biological Chemistry, UCLA, 1990–1991, and of the Bank of America-Giannini Foundation, 1991–1992.

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